

## Review

# Genetic improvement of cotton tolerance to salinity stress

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**Soil salinity is a great threat to cotton production worldwide. Plant adaptation to environmental stresses involves the expression of specific stress-related genes. Consequently, engineering genes that protect and maintain the function and structure of cellular components can enhance tolerance to salinity stress. Engineered cotton plants have been reported to perform much better than their wilt plants either in greenhouse or field conditions under salinity stress. However, engineered cotton with improved salt-tolerance is still far behind the requirements of commercial production due to its limited salinity tolerance or poor agronomic performance. This review highlights recent advances in genetic improvement, particularly molecular breeding for salinity tolerance of cotton. It is suggested that future research should focus on the development of specific cotton cultivars with high salt tolerance through a combination of traditional breeding and molecular technology.**

**Key words:** Cotton, salinity tolerance, genetic improvement, molecular breeding.

## INTRODUCTION

Soil salinity has been a major concern to global agriculture throughout human history (Lobell et al., 2007). Currently over 800 million hectares of land throughout the world are salt-affected (FAO, 2005). This is over 6% of the world's total land area (Munns, 2005). In recent times, it has become even more prevalent as the intensity of land use increases globally (Egamberdieva et al., 2010). Cotton is an important cash crop worldwide. Although it is classified as one of the most salt-tolerant major crops and considered a pioneer crop in reclamation of saline soils (Maas, 1990), its growth and development as well as yield and fiber quality are negatively affected by excessive salts in the soil (Maas and Hoffman, 1977; Qadir and Shams, 1997; Higbie et al., 2010). Soil salinity are usually expressed by ECe (electrical conductivity of a saturated-paste extract), and ECes around 7.7, 12, and 17 dS m<sup>-1</sup> are referred to as low, moderate and high salinity level, respectively (Chen et al., 2010; Maas and Grattan, 1999).

In general, soil salinity delays and reduces germination and emergence, decreases cotton shoot growth, and finally leads to reduced seed cotton yield and fiber quality characteristics at moderate to high salinity levels (Khorsandi and Anagholi, 2009). However, the cotton plant has a complete self-protection system from salinity (Ashraf, 2002). Under salt stress, the protection system within a cotton plant can be activated to enhance salt tolerance. Salt tolerance can be improved through chemical priming or genetic breeding. Over the last thirty years, studies have been conducted on the response of cotton yields on saline soils and/or irrigated with saline water. Progress has been made in all aspects of soil salinity-cotton plant. Understanding how plants respond to salinity can play a major role in stabilizing crop performance under saline conditions and in the protection of natural vegetation. Adequate management techniques and plant genetic breeding are the tools to improve resource use efficiency (including water) by plants.

With the development of molecular biology and technology, comparative genomics and functional genomics, the transgenic technology of important crops has become an important area of research. Since cotton is the most suitable major crop grown in saline soils, the

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adoption of transgenic technology to improve its salt tolerance is one of the research goals in recent years. A great number of responsive salt-tolerant genes have been identified and cloned from cotton. And a number of transgenic cotton lines have been reported to have improved salt tolerance even in field conditions. Since plant response to salinity and salinity tolerance have been previewed (Ashraf, 2002; Ahmad et al., 2002; Gorham et al., 2009; Pessaraki, 2001). The present review covers research advances in genetic breeding, with a focus on molecular improvement of salinity tolerance of cotton.

### **DIRECT SCREENING OR SELECTION FOR SALT TOLERANCE**

The use of cotton varieties tolerant to higher soil salinity levels is one of the approaches to control salinity stress effects on cotton. Therefore, identification and screening of salt-tolerant cotton cultivars or germplasm is of great importance (Ashraf, 1994). There are two common screening methods for salt tolerance, one being the seedling-based, solution culture method, the other being plant yield-based, soil culture method (Khan et al., 1995). By comparing the two methods, Akhtar et al. (2010) concluded that solution culture based on seedling was as accurate as but more simple than soil culture. However, identification and evaluation of salt tolerant plants based on plant biomass or yield, whether in laboratory or field conditions, is a cumbersome work.

Therefore, a number of physiological indicators have been recommended for identification and evaluation of salt tolerance. The  $K^+/Na^+$  ratio has been used as a successful criterion for selection of salt tolerance in cotton (Akhtar et al., 2010). Munis et al. (2010) indicated that  $Na^+$  and  $K^+$  content, decrease in leaf area, seed germination and seedling growth were the best indicators of salinity for cotton cultivar selection, but proline and chlorophyll concentrations were not useful for accurate assessment of salinity tolerance. Sexton and Gerard (1982) measured the emergence force exerted by germinating cotton seedlings using a transducer. Increasing salinity reduced the emergence force and increased the time required to develop the maximum force. Gorham et al. (2009) suggested that such tests could be used to determine the salt tolerance of emerging seedlings. Germination of pollen grains has also been used to screen for salt tolerance (Shen et al., 1997).

Salt tolerance varies with cotton genotypes. Some wild upland cotton and wild species have relatively strong salt tolerance (Ye and Liu, 1998). Development of salt-tolerant cotton varieties was initially attempted through direct selection from the existing cotton varieties, strains, and germplasms. After extensive research, however, it was considered that the probability of success by direct selection or screening was very low, particularly in the current commercial cotton varieties (Ye and Liu, 1998).

Zhang et al. (2010) examined 47 upland cotton accessions, including 23 salinity-tolerant and 24 salinity-sensitive cotton materials using 88 simple sequence repeat (SSR) markers. They found a total of 338 alleles at 88 SSR loci with an average of 3.841 alleles per locus; 333 of these alleles were detected in salinity tolerant germplasm and 312 alleles in salinity sensitive germplasm. Mean polymorphism information content (PIC), the average effective number of alleles ( $N_e$ ) and the average genotype diversity index ( $H'$ ) were 0.613, 2.929 and 1.083 in the salinity tolerant germplasm, and 0.605, 2.883 and 1.071 in the salinity sensitive germplasm. The similarity coefficients were similar between salinity tolerant and salinity sensitive germplasms. They varied from 0.530 to 0.979 in salinity tolerant germplasm, with a wider range than in salinity tolerant germplasm. The varieties were clustered into one major group and two small groups. The high genetic similarity coefficients observed in Chinese salinity-tolerant germplasm indicated narrow pedigrees within the group (Zhang et al., 2010). Therefore, little success has been made in developing considerably salt-tolerant varieties with the conventional methods due to narrow germplasm resources available and the complexity of tolerance mechanisms in cotton. Nevertheless, such work is also important, because it not only deepens our understanding of the salt-tolerant cotton resources, and also is the most direct and reliable method to look for salt-tolerant cotton germplasm.

### **MOLECULAR BREEDING FOR SALT TOLERANCE**

During the 1980s and 90s, the use of tissue culture to screen salt-tolerant mutants held great promise (Das et al., 1990, 1992; Misra et al., 1990, 1995). It was once considered an effective way to develop salt-tolerant varieties and lines directly (Das et al., 1990, 1992; Zhang et al., 1995; Misra et al., 1990, 1995). However, studies on salt-tolerance screening stagnated only at the salt-tolerant callus stage (Das et al., 1990, 1992; Misra et al., 1990, 1995), and did not yield somatic embryos and plantlets with increased salt tolerance. This is due to the great difficulty in plant regeneration in tissue culture under salinity stress. The regenerative capacity of callus may be decreased or even lost, and the embryogenic would also be transformed to non-embryogenic callus due to salt stress. Thus, even if the salt-tolerant cell line or salt-tolerant callus was obtained, it is still difficult to obtain salt-tolerant plantlets.

At the same time, direct introduction of exogenous DNA technology also received great attention for salt-tolerance breeding of cotton. It was reported that exogenous dogbane (*Apocynum venetum*) DNA with salt resistance were introduced into the cotton ovaries after self pollination by pollen tube pathway (Shen et al., 1995).

The second generation ( $D_2$ ) was screened for salt

tolerance by salinity stress in saline soil. Two salt-tolerant strains 91-11 and 91-5 were identified. However, further studies especially on application of the strains have never been reported. The direct introduction of exogenous DNA has not been widely recognized. Although there is limited progress in genetic improvement of salinity tolerance of cotton through screening of salt-tolerant mutants and direct introduction of exogenous DNA technology, a great number of responsive salt-tolerant genes have been cloned, and a number of transgenic cotton lines have been reported to have improved salt tolerance even in field conditions.

### Responsive genes of salt tolerance

A number of genes are involved in regulation of plant response to salinity stress in cotton. Rodriguez-Urbea et al. (2011) identified 720 salt-responsive genes in an intra-specific backcross population of cotton, of which 695 were down-regulated and only 25 were up-regulated in the salt tolerant bulk. Gene ontology of annotated genes revealed that at least some of the identified salt responsive transcripts belong to pathways known to be associated with salt stress, including osmolyte and lipid metabolism, cell wall structure, and membrane synthesis. Rodriguez-Urbea et al. (2011) suggested that further analysis of these salt-responsive genes might assist in laying a foundation for molecular manipulation in development of new cotton cultivars with improved salt tolerance.

A cotton cDNA clone, *GhNHX1*, which showed high sequence identity with plant vacuolar-type  $\text{Na}^+/\text{H}^+$  antiporters was isolated via differential hybridization in response to salinity stress in cotton seedlings by Wu et al. (2004). Northern blot analysis showed that mRNA accumulation of *GhNHX1* in cotton seedlings was strongly induced by salt stress. *GhNHX1* activity in a mutant for yeast tonoplast  $\text{Na}^+/\text{H}^+$  antiporter showed function complementation, thereby proving that the antiporter is in the vacuolar membrane. Transgenic tobacco plants over-expressing *GhNHX1* had higher salt tolerance than the wild-type plants.

Xue et al. (2009) isolated a cDNA clone (*GhMT3a*) encoding a 64-amino acid type 3 metallothionein protein from cotton (*Gossypium hirsutum*). Northern blot analysis indicated that mRNA accumulation of *GhMT3a* was up-regulated by high salinity. Transgenic tobacco (*Nicotiana tabacum*) plants over-expressing *GhMT3a* showed increased tolerance against abiotic stresses compared with wild-type plants. *GhMT3a* could function as an effective scavenger of reactive oxygen species (ROS) and its expression could be regulated by abiotic stresses through ROS signaling (Xue et al., 2009). A cotton dehydration responsive element binding protein gene, *GhDREB*, was isolated from the cDNA library of cotton cv. Simian 3 (Gao et al., 2009). RNA blot analysis showed

that the *GhDREB* gene was induced in cotton seedlings by drought, high salt and cold stresses. Functional analysis indicated that the transgenic *GhDREB* wheat plants had improved tolerance of high salt stresses through accumulating higher levels of soluble sugar and chlorophyll in leaves after stress treatments (Gao et al., 2009). Wei et al. (2009) isolated two cDNAs (*Gh14-3-3b* and *Gh14-3-3c*) encoding putative 14-3-3 proteins from cotton cDNA libraries. The *Gh14-3-3b* gene encodes a protein of 268 amino acids, while the *Gh14-3-3c* gene encodes a protein of 261 amino acids. The expression of these *Gh14-3-3* genes in roots was significantly up regulated by salt treatments, suggesting their involvement in the signaling pathways in response to salt stress in cotton (Wei et al., 2009).

Fasciclin-like arabinogalactan proteins (FLAs), a subclass of arabinogalactan proteins (AGPs), are usually involved in cell development in plants. Huang et al. (2008) isolated 19 *GhFLA* genes (cDNAs) from cotton (*G. hirsutum* L), of which 15 were predicted to be glycosylphosphatidylinositol anchored to the plasma membranes. Expressions of the *GhFLA* genes are in response to NaCl stress (Huang et al., 2008). Zinc finger proteins are a super-family involved in many aspects of plant growth and development as well as stress tolerance. A cDNA clone designated *G. hirsutum* zinc finger protein 1 (*GhZFP1*), which encodes a novel CCCH-type zinc finger protein, was isolated from a salt-induced cotton cDNA library. Over-expression of *GhZFP1* in transgenic tobacco enhanced tolerance of salt stress (Guo et al. 2009). Li et al. (2010) identified two genes encoding Di19-like Cys2/His2 zinc-finger proteins in cotton. GFP fluorescence assay showed that *GhDi19-1* and *GhDi19-2* are two nuclear-localized proteins. Quantitative RT-PCR and Northern blot analyses revealed that mRNA accumulation in both *GhDi19-1* and *GhDi19-2* was significantly promoted by salinity. Over-expression of *GhDi19-1* and *GhDi19-2* in Arabidopsis resulted in seedlings exhibiting hypersensitivity to high salinity. Seed germination and seedling growth of the transgenic Arabidopsis were dramatically inhibited by salinity and ABA, compared with the wild type. Both *GhDi19-1* and *GhDi19-2* may be involved in response to salt/drought stress and ABA signaling during early stages of plant development (Li et al., 2010).

Huang and Liu (2006) found that the cotton dehydration-responsive element (DRE) binding protein 1 (*GhDBP1*) could function as an active transcriptional repressor for DRE-mediated gene expression. Huang and Liu (2006) also showed that *GhDBP1*'s transcriptional repression domain is located at the C-terminus, and is known as an ERF-associated amphiphilic repression (EAR)-motif. Furthermore, the amino acid residues, aspartic acid (D), leucine (L), asparagine (N) and proline (P) are conserved in the EAR-motif, and are necessary for repression through mutational analysis. Transgenic *Arabidopsis* plants over-

expressing *GhDBP1* were more sensitive to high salinity stress and appeared to down-regulate the expression levels of the stress-induced effector genes (Dong et al., 2010).

### Transgenic cotton plants with improved salt tolerance

Enhancement of solute concentration in the vacuoles of plant cells (osmotic adjustment) is believed to be an important way to enhance salt tolerance in plants. Increasing the vacuolar osmotic pressure with the concomitant decrease in water potential would favor water movement from soil into plant root cells. Two approaches have been used to increase solute contents in plant vacuoles (Pasapula et al., 2011). The first approach involves increasing the activity of a vacuolar sodium / proton ( $\text{Na}^+/\text{H}^+$ ) antiporter that mediates the exchange of cytosolic  $\text{Na}^+$  for vacuolar  $\text{H}^+$ . The second approach involves increasing the activity of an  $\text{H}^+$  pump on the vacuolar membrane to move more  $\text{H}^+$  into the vacuoles, therefore generating a higher proton electrochemical gradient ( $\text{DIH}^+$ ) that can be used to energize secondary transporters, including vacuolar  $\text{Na}^+/\text{H}^+$  antiporters. Both approaches enhance  $\text{Na}^+$  accumulation in the vacuoles and reduce the potential of  $\text{Na}^+$  toxicity in the cytoplasm, leading to higher salt tolerance in transgenic plants. A transgenic cotton plant was developed to over-express *AtNHX1*, an *Arabidopsis* vacuolar  $\text{Na}^+/\text{H}^+$  antiporter by He et al. (2005, 2007). Cotton plants with *AtNHX1* had more biomass and produced more fibers when grown in the presence of high  $\text{NaCl}$ . Over-expression of both of the tonoplast  $\text{Na}^+/\text{H}^+$  antiporters increases sodium transfer into vacuoles, which leads to higher vacuolar salt concentration and therefore higher salt tolerance.

Similarly, cotton plants over-expressing *TsVp*, an  $\text{H}^+$ -PPase gene cloned from *Thellungiella halophila*, transports more  $\text{Na}^+$  into vacuoles and significantly enhanced stomatal conductance, photosynthetic rate, root and shoot dry matter accumulation, and finally the salt tolerance of cotton (Lv et al., 2008). The *Arabidopsis* *AVP1* gene encodes a vacuolar pyrophosphatase that functions as a proton pump on the vacuolar membrane. Pasapula et al. (2011) reported that increased expression of the vacuolar membrane-bound  $\text{H}^+$  pump can indeed lead to increased salt tolerance in transgenic cotton, and that *AVP1*-expressing cotton produces more fiber under field conditions (Pasapula et al., 2011). It is believed that the sequestering of  $\text{Na}^+$  in the vacuoles confers two advantages: reduced toxic levels of  $\text{Na}^+$  in cytosol, and increased osmotic potential of the vacuole and therefore a more negative water potential that aids water uptake by the cells and water retention under high salt conditions (Lubbers et al., 2007). Glycine betaine is an osmoprotectant that plays an important role and accumulates

rapidly in many plants during salinity or drought stress (Ashraf and Foolad, 2007). Choline monoxygenase (CMO) is a major catalyst in the synthesis of glycine betaine. A *CMO* gene (*AhCMO*) cloned from *Atriplex hortensis* was introduced into cotton (*G. hirsutum* L.) via *Agrobacterium* mediation by Zhang et al. (2007). Transgenic cotton over-expressing *AhCMO* was more tolerant to salt stress due to elevated accumulation of glycine betaine, which provided greater protection of the cell membrane and photosynthetic capacity than in non-transgenic cotton. Seed cotton yield of the transgenic plants was lower under normal conditions, but was significantly higher than that of non-transgenic plants under salt-stressed field conditions. The results indicate that over-expression of *AhCMO* in cotton enhanced salt stress tolerance (Zhang et al., 2009).

Annexins belong to a multigene family of  $\text{Ca}^{2+}$  dependent phospholipid and cytoskeleton binding proteins. They have been shown to be up-regulated under various stress conditions. Divya et al. (2010) generated transgenic cotton plants expressing mustard annexin (*AnnBj1*), which showed enhanced tolerance of different abiotic stress treatments like sodium chloride, mannitol, polyethylene glycol and hydrogen peroxide. The tolerance to these treatments was associated with decreased hydrogen peroxide levels and enhanced total peroxidase activity, enhanced content of osmoprotectants—proline and sucrose in transgenic plants (Divya et al., 2010). Based on the aforesaid reports, salt tolerance of cotton can be significantly improved using transgenic technology (Table 1). However, field studies have also indicated that the effect of single-gene transformation is still relatively limited (Zhang et al., 2009) because salt-induced response is the result of multi-channel and multi-gene coordination. Therefore, it is often difficult to get a long-term effective salt tolerance through transformation of a functional gene alone. Yield and quality improvement in cotton not only depends on its salt tolerance, but also on a number of other characteristics.

### SUMMARY

Salt damage to cotton plants in saline soils is mainly due to ion toxicity, osmotic stress and nutrient imbalance. Although the development of salt-tolerant cotton is not an easy job due to the complexity of the tolerance mechanisms, narrow germplasm resource, lack of selection criteria and variation in salt responses at different developmental stages (Ashraf et al., 2008), some progress has still been made in salt-tolerant improvement, particularly through molecular breeding, and has shown a bright prospect. Using and/or regulating transgene effects on the plant responses to salinity has shown much success and will continue to increase our understanding of the complexity of the physiological

**Table 1.** Examples of transgenic cotton with improved salt tolerance.

Gene	Source	Function	Performance	Reference
<i>AtNHX1</i>	<i>Arabidopsis</i>	Encoding a vacuolar Na <sup>+</sup> /H <sup>+</sup> antiporter that increases sodium uptake in vacuoles, leading to increased vacuolar solute concentration	Improved salt tolerance as well as increased plant biomass and fiber in greenhouse and field under salinity stress	He et al. 2005, 2007
<i>TsVP</i>	<i>Thellungiella halophila</i>	A H <sup>+</sup> -PPase gene that causes accumulation of Na <sup>+</sup> and Cl <sup>-</sup> in vacuoles.	Improved salt tolerance as well as increased plant biomass in greenhouse under salinity stress	Lv et al. 2008
<i>AVP1</i>	<i>Arabidopsis</i>	Encoding a vacuolar pyrophosphatase that functions as a proton pump on the vacuolar membrane	Improved tolerance of both drought and salt stresses in greenhouse, and increased fibre yield under dry-land conditions in the field	Pasapula et al. 2011
<i>AhCMO</i>	<i>Atriplex hortensis</i>	Encoding Choline monooxygenase (CMO) that increases synthesis of glycine betaine	Improved salt tolerance as well as increased plant biomass in greenhouse and yield in field.	Zhang et al. 2007, 2009
<i>AnnBj1</i>	<i>Mustard</i>	Ca <sup>2+</sup> dependent, phospholipid and cytoskeleton binding protein	Improved tolerance as indicated by enhanced fresh weight, relative water content, and dry weight	Divya et al. 2010

pathways in cotton. Improvements in all areas of molecular breeding are almost certain, but the most effective improvements will come from exploiting our improved understanding of the genetic architecture (Lubbers et al., 2007).

Transgenic cotton with improved salt-tolerance is currently far behind the requirements of commercial production. This is because on the one hand, the salt tolerance has not yet reached the expectation for cotton growing in saline fields, and on the other hand, strains of these salt-tolerant materials have relatively poor agronomic traits. Therefore, future studies should focus on deep exploitation of salt-tolerant genes, effective transformation and development of new salt-tolerant cotton varieties. Developing transgenic cotton varieties with both high salt-tolerance and

other well-integrated-traits is the key element of cotton improvement.

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