# Proton gradient regulator 5 of *Gossypium arboreum* enhances salt-stress tolerance in *Gossypium hirsutum*

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**Cotton is the most important cash and fibrous crop, and is grown in more than 50 countries of the world. Cotton crop yield is seriously affected by soil salinity. This deleterious effect can be reduced by genetic modification in stress-susceptible cotton plants. Salt stress tolerant gene *gaPGR5* (proton gradient regulator 5) was isolated from *Gossypium arboreum* and transformed into the stress-susceptible cotton cultivar (*G. hirsutum*). The *gaPGR5* gene was cloned into pCAMBIA-1301 vector and transformed in young embryos by *Agrobacterium*-mediated method. Plant *GUS* gene was used as reporter gene that showed blue colouration during histochemical assay. Molecular analysis of transgenic plants was done up to $T_2$ generation. Selection of salt-tolerant transgenic plants was done by salt-stress (NaCl) treatment with different concentrations in a hydroponic culture. Transgene expression in salt-tolerant transgenic plants was evaluated through quantitative real-time PCR. Maximum transgene expression was recorded in those plants which were tolerant to higher salt concentration (175 mM NaCl) and vice versa. The plants which give higher transgene expression against salt stress are valuable for cultivation in salt-affected areas.**

**Keywords:** *Gossypium arboreum*, *Gossypium hirsutum*, proton gradient regulator, salt-stress tolerance.

COTTON is a vital cash crop throughout the world. It is also an integral part of the economic development of Pakistan. There are four main cotton-producing countries in the world and Pakistan is one of them[1]. Drought and salinity are prevalent in different areas and may cause severe salinity in more than 50% of all arid land by 2050 (ref. 2). Cotton is the main source to get good quality fibre and oil. The growth and yield of cotton, especially at germination and emergence stages, are repressed in highly salt-affected soil[3].

Asiatic desi cotton has a huge potential to grow under different stress conditions, like biotic and abiotic. *Gossypium arboreum* L. and *G. herbaceum* L. are diploid species, while *G. barbadense* L. and *G. hirsutum* L. are tetraploid species in ordinary agricultural practice. The cotton diploid species contribute to only 2% of the world cotton. These cotton species are the main source of vital biotic and abiotic tolerant genes with more fibre and agronomic characters. They are also helpful in the study of the *Gossypium* genome which is responsible for several biotic and abiotic stresses using advanced molecular biology techniques[4,5].

The small *PGR5* (proton gradient regulation 5) gene is found in the nucleus and encodes a protein which is important for a pathway in *A. thaliana* and *Synechocystis* ps. PCC 6803. The pathway is called FQR-dependent CET pathway[6–8]. Extra accumulation of this gene under specific conditions in thylakoid membranes increases the action of PSI cyclic electron transport (CET) and decreases the development of chloroplast which ultimately restrains plant growth[9]. It does not have any metal-binding motifs. It is stable in mutant background lacking PSII, PSI, cytochrome b6/f complex or ATPase. This shows that *PGR5* is not a component of any of these main complexes[10]. Thylakoid membrane contains the *PGR5* gene[9]. It works as the powerhouse of secondary transport systems in plants that produce ion fluxes under stress conditions[11]. The *gaPGR5* genes upregulated during salt stress in *G. arboreum*[12].

The present study is aimed to overcome salinity stress on cotton crop by transforming locally isolated salt stress-tolerant gene (*gaPGR5*) in salt-susceptible species *G. hirsutum* (var-MNH-786) using the *Agrobacterium*-mediated transformation method. The transgenic plants transformed with *gaPGR5* showed tolerance against salt stress in hydroponic solution. The results obtained will be helpful to resolve soil salinity-related issues for cotton growth worldwide, and in particular Pakistan.

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## Materials and methods

### *Identification and isolation of salt stress-tolerant gaPGR5 gene*

Desi cotton (*G. arboreum*) plants were subjected to 200 mM NaCl stress in hydroponic culture for 72 h. The cDNA was synthesized using total RNA isolated from cotton plants. Differential display PCR was performed with random (anchored and arbitrary) primers. Initially, the *PGR5* fragment was identified at transcript level and then full-length gene sequence was obtained using Invitrogen Gene-Racer kit (Cat# L1500-01). The *gaPGR5* gene was characterized against different abiotic stresses (drought, salt, low and high temperature) before cloning into a plant expression vector[12].

### *Co-cultivation of plant embryos and bacterial culture*

Seeds of wild type cotton, *G. hirsutum* of cultivar MNH-786 were collected from Central Cotton Research Institute, Multan, Pakistan. The recombinant plasmid vector was cloned into bacterial *Escherichia coli* cells (strain TOP10) and the bacteria *Agrobacterium tumefaciens* (strain LBA4404) was propagated to get transformants.

### *Vector construction*

A binary vector, pCAMBIA-1301 was used for cloning of *gaPGR5* in a plant expression vector. This binary vector contains a hygromycin resistance gene and kanamycin for plant and bacterial selection respectively. It has a complete functional *gusA* gene which works as a reporter gene (Figure 1). A *gaPGR5* cDNA fragment was obtained from *G. arboreum*. PCR was performed using restriction sites in forward P-NcoF: (5-CATGCCATGGATGGCTATTTCAATTTCGGC-3) and reverse primer P-BglR: (5-TCTCGGAGATCTTCATGCAAGGAATCCAAGC-3) of *Nco*I and *Bgl*II respectively. PCR reaction was performed in final reaction volume of 25 µl having 0.5 µg DNA template, 0.2 mM of each dNTP, 0.5 µM of each primer, 1× buffer, 1.5 mM $MgCl_2$ and 1U *Taq* DNA polymerase under conditions as given by Kiani *et al.*[13], with little modification of primer annealing at 60°C. Restriction enzymes, *Nco*I and *Bgl*II were used to the presence of insert into the plasmid DNA. Sequencing was carried out to confirm orientation of the gene region in the final plant expression vector.

### *Transformation of gaPGR5 in salt stress-sensitive cotton cultivar*

The *G. hirsutum* cultivar MNH-786 embryos were used for transformation of the desired gene by *Agrobacterium*-mediated transformation method following the protocol developed by Kiani *et al.*[13] with little changes, while the control was transformed plasmid without any desired gene.

### *Histochemical localization of GUS gene*

This test was performed to study the transient expression of *GUS* gene as described by Kiani *et al.*[13]. Gus solution was prepared using 100 mM $NaH_2PO_4$, 10 mM EDTA, 25 mg/l X-gluc, 50% methanol and 0.1% Triton X-100 (pH 8.0). Gus solution was kept away from sunlight. The embryos were placed in Gus solution in a 1.5 ml tube after co-cultivation (72 h) with *Agrobacterium*. The embryo-containing solution was maintained at 37°C overnight and then observed under a light microscope to observe blue spots.

### *Molecular analysis of transformants*

The integration of *gaPGR5* in $T_0$ transgenic plants was confirmed by PCR using gene-specific primers, while for negative control wild-type plants were used. DNA was extracted from 12-week-old putative transgenic plants using the method of Saha *et al.*[14], with slight changes. PCR was performed for the detection of *gaPGR5* by forward primer (5′-ACCCATCAAGCTTTACAACCAT-3′) and reverse primer (5′-TGGTAGCAGAAGTACAGTGAAGG-3′). The PCR reaction was programmed according to Shahid *et al.*[12].

### *Treatment of transgenic plants with salt stress*

The 12-week-old putative transgenic and non-transgenic plants at growing vegetative stage were treated with salt (NaCl). When transgenic plants were initially stabilized in the simple hydroponic culture solution and gained 5–6 leaves, they were gradually exposed for 24–72 h to different concentrations of salt (NaCl), i.e. 50, 100, 125, 150, 175 and 200 mM.

### *Quantitative real-time PCR analysis*

To compare the expression of *gaPGR5* gene in transformed plants under normal and salt-stressed conditions, qPCR was performed. Plants with an empty vector (having a plasmid with no transgene) were used as negative control. Total RNA from leaves of selected plants was isolated according to the method of Jaakola *et al.*[15] and cDNA was synthesized by hexamer primers using First-Strand Synthesis kit (Thermo Scientific). Real-time PCR reaction with 20 ng cDNA was carried out using 2× Master-mix kit (Thermo Scientific; Cat# K1622). Data were normalized using *GAPDH* as the reference gene. The

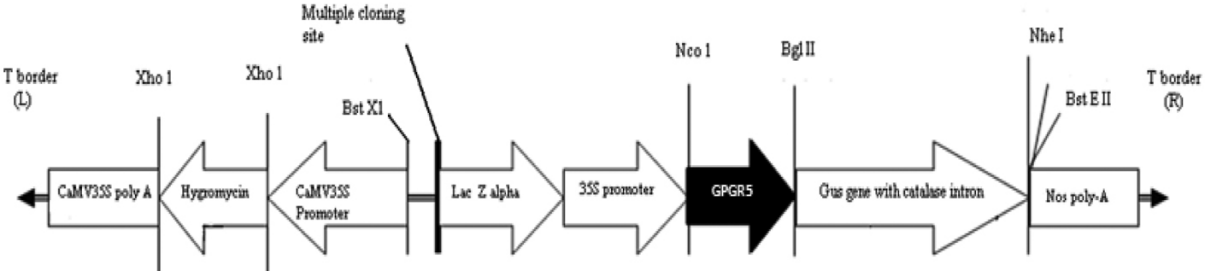


**Figure 1.** Diagrammatic representation of plant expression vector pCAMBIA-1301 showing transfer DNA for plant transformation. L, Left border; R, Right border; *gaPGR5* 281 bp ORF of *Gossypium arboreum* proton gradient regulator gene.

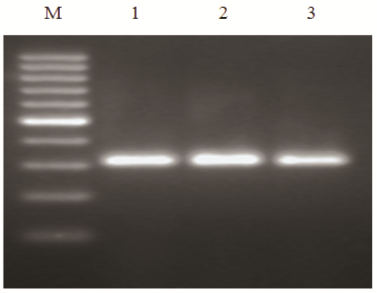


**Figure 2.** Amplified product of *gaPGR5* gene using cDNA. Lane M, DNA ladder 100 bp and lanes 1–3, *gaPGR5* cDNA fragments.

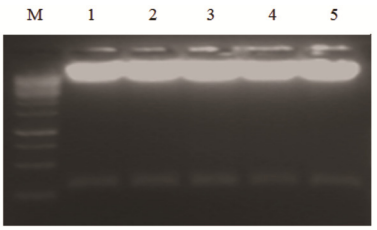


**Figure 3.** Digestion of *gaPGR5* positive clones through *Nco*I and *Bgl*II. Lane M, 1 kb DNA ladder and lanes 1–5, positive clones.

PCR reaction was conducted according to Shahid *et al.*[12]. The PCR cycles were elaborated up to 40 cycles. Analysis of gene expression was done using SDS V3.1 software (Applied Biosys Inc, USA). In order to avoid any bias in the results, all PCR reactions were carried out in triplicate.

### *Generation data of salt-tolerant transgenic plants*

The plants showing tolerance against highest salt stress (175 mM NaCl) were further studied for three generations in order to obtain true salt-tolerant transgenic plants. The plants that gave higher transgene expression in qPCR after exposure to higher salt stress conditions in hydroponic culture were shifted to field conditions in normal soil to obtain seeds. The seeds obtained were allowed to germinate initially in pots containing sand and at the five-leaf stage they were shifted to simple hydroponic culture. When the young plants became stable, they were exposed to 175 mM NaCl stress for 72 h. Leaves of the plants surviving after three days were chosen to isolate total RNA. After cDNA synthesis, qPCR was performed as mentioned earlier.

## Results and discussion

More than 800 million hectares (m ha) of land is affected by salt all around the world. It is either affected by Salinity, i.e. 397 m ha or other related conditions of salinity like sodicity, i.e. 434 m ha. About 45 m ha is affected with salt out of the present 230 m ha of irrigated land, i.e. 20% of the total[16]. High salinity has significantly affected the growth and lint yield in cotton[3].

To overcome salinity problems in salt-susceptible cotton plants, a full-length gene, *gaPGR5* of size 302 bp was amplified using cDNA as tempelate (Figure 2). RNA for cDNA was isolated from salt stress-tolerant cotton leaf tissues. It was then cloned in plant expression binary vector (pCAMBIA-1301). The vector was transformed in *E. coli* (TOP10) strain and random selected colonies were used for plasmid DNA isolation and confirmation of gene through gene-specific PCR. Further confirmation of the presence of insert in the plasmid was made with restriction enzymes *Nco*1 and *Bgl*II, which gave similar range of fragment size as that with PCR of cDNA (Figure 3). The final orientation of the desired gene in the vector was confirmed through sequencing. The pairwise alignment algorithm programs of NCBI BLAST were used to analyse sequence. The result of an inserted sequence with 100% homology to the original *gaPGR5* sequence was studied (JQ861978). Twelve bacterial colonies were confirmed with PCR using gene-specific primers after electroporation.

GUS assay was used to study the transient expression of *GUS* gene after *Agrobacterium* transformation. The presence of *GUS* gene into transformed embryos compared with control embryos (non-transgenic) was confirmed with the appearance of blue colour on the embryo after incubation with X-gluc solution (Figure 4 *a* and *b*).

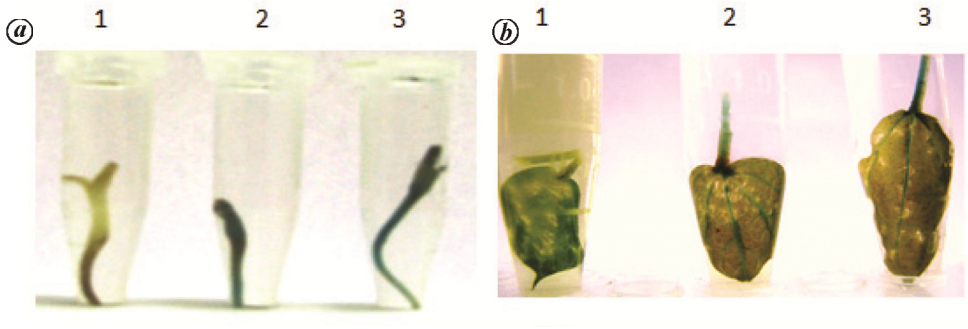


**Figure 4** ***a***, ***b***. Immuno-histochemical GUS assay for putative transforming embryos and plants leaves accomplished by *Agrobacterium*-mediated transformation. Sample 1, Negative control; Samples 2, 3, Positive embryos.

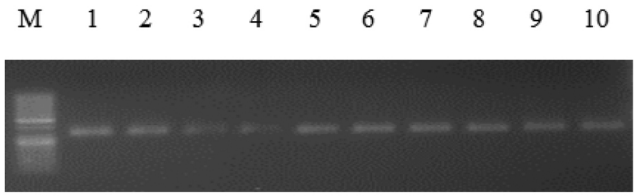


**Figure 5.** Expression of amplified *gaPGR5* gene of transformed plants in the form of DNA bands. Lane M, 50 bp DNA ladder and lanes 1–10, Positive plants.

More than 1200 mature cotton embryos were used. The 125 putative transgenic plants were obtained after eight weeks of selection on 50 mg/ml hygromycin. Among 71 surviving plants, only 10 had successfully acclimatized in hydroponic culture. In general, efficiency of transformation was observed to be 0.83% – same as that reported by Smith *et al.*[17] and higher than that of Majeed *et al.*[18] (0.60%); this may be due to species-specific transformation. All the 10 putative transformants were analysed and integration of the desired gene was confirmed by PCR (Figure 5). All the transformants were observed to grow well and had normal phenotype.

The results of salt stress application showed that transgenic plants 1–5, carrying the *gaPGR5* gene were more tolerant to salt stress compared to control plant, i.e. non-transgenic (Figure 6 *a*). The non-transformed plants showed drooping after 24 h of salt stress at a concentration of 50 mM. On the other hand, transgenic plants 1–5 showed drooping at 100, 125, 150, 175 and 200 mM respectively.

The level of expression of transgenes was customized by real-time qPCR study. Only five out of ten transgenic plants could revive after being subjected to salt stress. The transgenic plants exhibited changeable expression levels of transgenes. A significant expression of the gene was detected in real-time PCR in transformed plants under salt stress compared to control plants. Among all transformed plants, plant-1 exhibited expression, i.e. 0.2-fold at 100 mM salt stress, while plant 4 exhibited the highest expression, i.e. 2.1-fold at 175 mM salt stress. Plants 2, 3 and 5 showed 0.4-, 2- and 1.7-fold expression at 125, 150, and 200 mM salt stress respectively (Figure 6 *b*).

The seeds were collected from plants 4 and 5 showing good expression at high salt stress condition. To obtain generation ($T_1$) data, seeds of transgenic plants were sown in two lines, one for plant-4 seeds and the other for plant-5 seeds and having a third line for the control plants. At seedling stages 175 mM salt stress was applied to plants of both lines in hydroponic culture[19,20]. Some plants were killed in both lines. The salt stress-tolerating plants were used for expression study through quantitative real-time PCR, while also using control plants. Stress-tolerating plants were grown till seed stage. The seeds of the plants showing good expression against salt stress were collected and grown as $T_2$ generation for confirmation of stability of gene in the plant genome[21].

Seeds of transgenic plants were sown in two lines in the soil with the third line for control plants (20 plants in each line). Two salt concentrations (0 and 150 mM) were applied for all three lines at 4–5 leaves stage. The growth of transgenic and non-transgenic plants was observed in terms of plant height and root length at different salt concentrations. The plant height and root length were same in transgenic and control plants at 0 mM salt concentration, while transgenic plants of both lines showed greater height and root length compared to control plants at 150 mM salt concerntration (Figure 7). The fresh and dry shoot weights were calculated for both categories of plants. They were the same in transgenic and non-transgenic plants at 0 mM NaCl concentration, but at 150 mM concentration the fresh and dry shoot weights were observed to be nearly double in transgenic lines compared to control (Figure 8). The root has an important role in salt stress tolerance, which ultimately leads to drought stress. No effect on root weight was observed in 0 mM salt stress, whereas a clear-cut difference was observed in the transgenic and non-transgenic plants at 150 mM salt concentration. As previously discussed, the length of the roots, increased in transgenic plants and thus fresh and dry root weights of

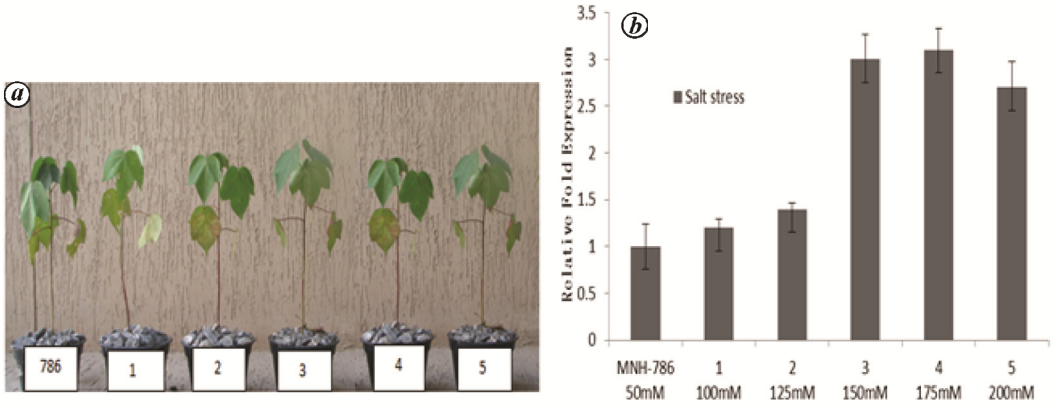


**Figure 6** ***a***, ***b***. Growth and expression analysis of *gaPGR5* among non-transformants and transformants at various concentrations of NaCl: non-transforming plant present drooping and stunted growth at 50 mM of salt stress (NaCl) concentration, while transgenic plants (1–5) of *gaPGR5* are healthy at different salt (NaCl) concentrations, applied from 50, 100, 125, 150, 175 to 200 mM steadily. Standard deviation (±SD) of three replicates as indicated using error bars.

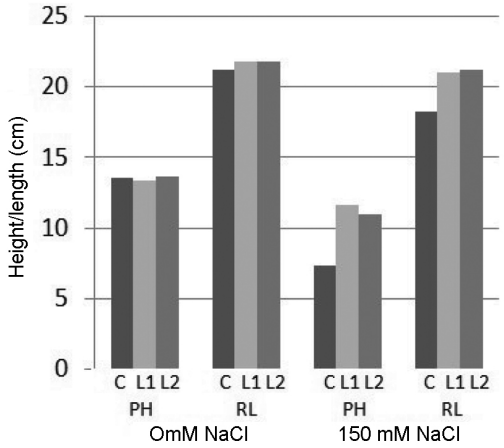


**Figure 7.** Evaluation of plant height and root length in transgenic and non-transgenic cotton plants at different salt stress (NaCl) concentrations. PH, Plant height RL, Root length.

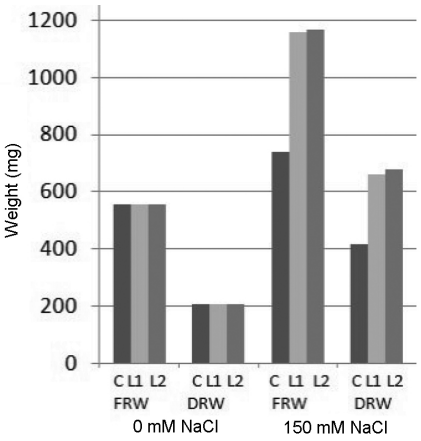


**Figure 9.** Comparison of fresh root weight and dry root weight in transgenic and non-transgenic cotton plants at different salt-stress (NaCl) concentrations. FRW, Fresh root weight; DRW, Dry root weight.

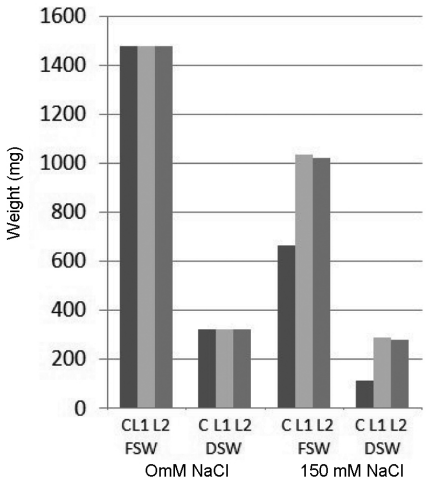


**Figure 8.** Comparison of fresh shoot weight and dry shoot weight in transgenic and non-transgenic cotton plants at different salt stress (NaCl) concentrations.

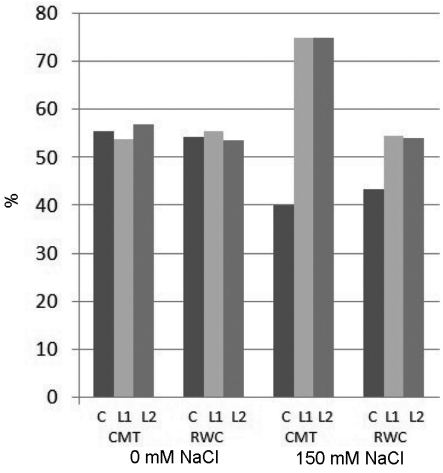


**Figure 10.** Comparison of cell membrane thermostability and relative water content in transgenic and non-transgenic cotton plants at different salt stress (NaCl) concentrations. CMT, Cell membrane thermostability; RWC, Relative water content.

**Table 1.** Mean performance of control and salt stress-tolerant/transgenic plants for morphological characters

| Morphological characters | NaCl (mM) | Negative control (MNH-786) | Line-1 (*gaPGR5*) | Line-2 (*gaPGR5*) |
|---|---|---|---|---|
| Plant height (cm) | 0 | 13.567a | 13.335b | 13.688a |
| Root length (cm) | | 21.219a | 21.780b | 21.870a |
| Fresh shoot weight (mg) | | 1478.9a | 1477.9 | 1475.8b |
| Fresh root weight (mg) | | 553.37a | 553.23a | 552.99a |
| Dry shoot weight (mg) | | 323.63b | 322.95b | 322.90b |
| Dry root weight (mg) | | 204.30b | 203.99b | 204.05a |
| Root–shoot ratio | | 0.560b | 0.560b | 0.561b |
| Plant height (cm) | 175 | 7.348b | 11.666b | 10.969b |
| Root length (cm) | | 18.276b | 21.068a | 21.250 |
| Fresh shoot weight (mg) | | 666.9a | 1032.7a | 1020.9b |
| Fresh root weight (mg) | | 735.80a | 1154.7a | 1165.0b |
| Dry shoot weight (mg) | | 115.90b | 289.15a | 278.8a |
| Dry root weight (mg) | | 412.65b | 657.43a | 674.22a |
| Root–shoot ratio | | 0.566a | 0.846b | 0.845a |

**Table 2.** Mean performances of control and salt stress-tolerant/transgenic plants for physiological characters

| Physiological attributes (%) | NaCl (mM) | Control (MNH-786) | Line-1 (*gaPGR5*) | Line-2 (*gaPGR5*) |
|---|---|---|---|---|
| Cell membrane thermostability | 0 | 55.36b | 53.70 | 56.99 |
| Relative water content | | 54.23a | 55.40 | 53.60 |
| Cell membrane thermostability | 175 | 40.08 | 75.09 | 74.90 |
| Relative water content | | 43.37 | 54.61 | 53.94 |

transgenic plant lines were observed to be 1.5 times more than control (non-transgenic plants) (Figure 9 and Table 1). The cell membrane thermostability (CMT) and relative water content (RWC) percentage were observed in control and both transgenic lines at 0 and 150 mM salt stress concentration. At high salt concentration, control plants reduced their CMT and RWC, while transgenic plants of both lines showed enhanced CMT while maintaining RWC compared to no stress condition (Figure 10 and Table 2). The root-to-shoot ratio was same in transgenic plants and control at 0 mM salt stress, while it was 1.5 times more at 150 mM concentration (Table 1)[22].

Ten plants from each line were sacrificed for morphological and physiological data generation. The remaining plants were grown till flowering and cotton-boll formation. It was observed that the number and size of bolls for transgenic and control plants were approximately the same. The quality and strength of fibre needs to be studied. The *gaPGR5* gene was stable in the transgenic plant genome and helped increase salt stress tolerance. These transgenic cultivars might be grown effectively in salt-affected lands and thus annual cotton yield may be enhanced.

*Conflict of interest:* The authors declare that there is no conflict of interest regarding this research work.

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