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 *gapGPR5* (proton gradient regulator 5) was isolated from *Gossypium arboreum* and transformed into the stress-susceptible cotton cultivar (*G. hirsutum*). The *gapGPR5* 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 T2 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. 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.

Asian 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.

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. 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. 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. Thylakoid membrane contains the *PGR5* gene. It works as the powerhouse of secondary transport systems in plants that produce ion fluxes under stress conditions. The *gapGPR5* genes upregulated during salt stress in *G. arboreum*.

The present study is aimed to overcome salinity stress on cotton crop by transforming locally isolated salt stress-tolerant gene (*gapGPR5*) in salt-susceptible species *G. hirsutum* (var-MNH-786) using the *Agrobacterium*-mediated transformation method. The transgenic plants transformed with *gapGPR5* 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.

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 (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-Nco: (5-CATGCCATGGTCTCAGAAGTACAGTGAAGG) and reverse primer P-Bgl: (5-TCATGCAAGGAATCCAAGC) of NcoI and BglII respectively. PCR reaction was performed in final reaction volume of 25 μl having 0.5 μg DNA template, 0.2 mM of each dNTP, 1.5 mM MgCl2 and 1U Taq DNA polymerase under conditions as given by Kiani et al., with little modification of primer annealing at 60°C. Restriction enzymes, NcoI and BglII 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. 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. Gus solution was prepared using 100 mM NaH2PO4, 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 T0 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., with slight changes. PCR was performed for the detection of gaPGR5 by forward primer (5′-ACCCATCAAGCTTCTACATTTACATTTACAT-3′) and reverse primer (5′-TGTTAGCAGAAGTACAGTGAAGG-3′). The PCR reaction was programmed according to Shahid et al.

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., 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
PCR reaction was conducted according to Shahid et al.\textsuperscript{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\textsuperscript{16}. High salinity has significantly affected the growth and lint yield in cotton\textsuperscript{3}.

To overcome salinity problems in salt-susceptible cotton plants, a full-length gene, gaPGR5 of size 302 bp was amplified using cDNA as template (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 \textit{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 \textit{NcoI} and \textit{BglII}, 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 \textit{GUS} gene after \textit{Agrobacterium} transformation. The presence of \textit{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\textit{a} and \textit{b}).
Figure 4a, 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 6a). 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 6b).

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 concentration (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
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.

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.

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.

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


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

<table>
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<tr>
<th>Morphological characters</th>
<th>NaCl (mM)</th>
<th>Negative control (MNH-786)</th>
<th>Line-1 (gaPGR5)</th>
<th>Line-2 (gaPGR5)</th>
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<tr>
<td>Plant height (cm)</td>
<td>0</td>
<td>13.567a</td>
<td>13.353b</td>
<td>13.688a</td>
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<td>Root length (cm)</td>
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<td>21.219a</td>
<td>21.780b</td>
<td>21.870a</td>
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<td>Fresh shoot weight (mg)</td>
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<td>1477.9</td>
<td>1475.8b</td>
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<td>Fresh root weight (mg)</td>
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<td>553.37a</td>
<td>553.23a</td>
<td>552.99a</td>
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<td>Dry shoot weight (mg)</td>
<td></td>
<td>323.63b</td>
<td>322.95b</td>
<td>322.90b</td>
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<td>Dry root weight (mg)</td>
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<td>204.30b</td>
<td>203.99b</td>
<td>204.05a</td>
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<td>Root-shoot ratio</td>
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<td>0.560b</td>
<td>0.560b</td>
<td>0.561b</td>
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<tr>
<td>Plant height (cm)</td>
<td>175</td>
<td>7.348b</td>
<td>11.666b</td>
<td>10.969b</td>
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<td>Root length (cm)</td>
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<td>18.276b</td>
<td>21.068a</td>
<td>21.250</td>
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<tr>
<td>Fresh shoot weight (mg)</td>
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<td>666.9a</td>
<td>1032.7a</td>
<td>1020.9b</td>
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<td>Fresh root weight (mg)</td>
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<td>1154.7a</td>
<td>1165.0b</td>
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<td>Dry shoot weight (mg)</td>
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<td>115.90b</td>
<td>289.15a</td>
<td>278.8a</td>
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<td>Dry root weight (mg)</td>
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<td>412.65b</td>
<td>657.43a</td>
<td>674.22a</td>
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<tr>
<td>Root-shoot ratio</td>
<td></td>
<td>0.566a</td>
<td>0.846b</td>
<td>0.845a</td>
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Table 2. Mean performances of control and salt stress-tolerant/transgenic plants for physiological characters

<table>
<thead>
<tr>
<th>Physiological attributes (%)</th>
<th>NaCl (mM)</th>
<th>Control (MNH-786)</th>
<th>Line-1 (gaPGR5)</th>
<th>Line-2 (gaPGR5)</th>
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<tbody>
<tr>
<td>Cell membrane thermostability</td>
<td>0</td>
<td>55.36b</td>
<td>53.70</td>
<td>56.99</td>
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<td>Relative water content</td>
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<td>54.23a</td>
<td>55.40</td>
<td>53.60</td>
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<tr>
<td>Cell membrane thermostability</td>
<td>175</td>
<td>40.08</td>
<td>75.09</td>
<td>74.90</td>
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<tr>
<td>Relative water content</td>
<td></td>
<td>43.37</td>
<td>54.61</td>
<td>53.94</td>
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