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Original article

# GmDREB6, a soybean transcription factor, notably affects the transcription of the *NtP5CS* and *NtCLC* genes in transgenic tobacco under salt stress conditions

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ABSTRACT

Soil is contaminated with salinity, which inhibits plant growth and development and reduces crop yields. The *DREB* (dehydration responsive element binding protein) gene responds to salt stresses through enhanced transcriptional expression and activation of genes involved in plant salinity resistance. In this study, we present the results of the analysis of the expression of the *GmDREB6* transgene, a gene that encodes the soybean DREB6 transcription factor, regulating the transcription of the *NtP5CS* and *NtCLC* genes in transgenic tobacco under salt stress conditions. The transcription of *GmDREB6, NtP5CS*, and *NtCLC* in transgenic tobacco lines was confirmed by qRT-PCR. Under salt stress conditions, the *GmDREB6* gene transcription levels in the transgenic tobacco lines L1 and L9 had increased from 2.40- to 3.22- fold compared with the condition without salinity treatment. Two transgenic lines, L1 and L9, had transcription levels of the P5CS gene that had increased from 1.24- to 3.60- fold compared with WT plants. For the NtCLC gene, under salt stress conditions, the transgenic lines had transcription levels that had increased by 3.65–4.54 (fold) compared with WT plants ($P < 0.05$). The L1-transgenic tobacco line showed simultaneous expression of both the *GmDREB6* transgene and two intrinsic genes, the *NtP5CS* and *NtCLC* genes. This study demonstrated that expression of the *GmDREB6* gene from soybean increases the transcription levels of the *NtP5CS* and *NtCLC* genes in transgenic tobacco plants under salt stress conditions. The analysis results have suggested that the *GmDREB6* gene is a potential candidate for improving the salt tolerance of plants, opening up research and development opportunities for salt stress-tolerant crops to respond to climate change and the rise in sea levels.


## 1. Introduction

Salinity is an abiotic stress that inhibits growth and development and reduces the yield of soybean plants (Chen et al., 2018). Due to climate change, sea levels have risen, and salinity intrusion has increased in many countries around the world. The encroachment of seawater into the soil, the use of low-quality irrigation water, heavy use of chemical fertilizers, and year-round intensive farming accelerate the accumulation of salt, causing soil salinity (Shrivastava and Kumar, 2015). The adverse effects of salt stress can be observed at the cellular, organ, and whole plant levels at the osmotic (early/short-term) and ionic (late/long-term) phases. High salinity has negative effects on biological processes in plants, such as disruption of osmotic and ionic equilibrium, protein synthesis, photosynthesis, energy, and lipid metabolism (Muchate et al., 2016). Plants' ability to resist salt stress is related to complex physiological processes, metabolic pathways, and the expression of

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related genes (Gupta and Huang, 2014). Plants living under salinity stress showed common symptoms or physiological responses due to the accumulation of toxic levels of ions ($Na^+$, $Cl^-$, and $SO_2^-$) in their cells, leading to nutritional and osmotic imbalances and dehydration (Shu et al., 2017; Rehman et al., 2019). Furthermore, reactive oxygen species (ROS), such as anionic superoxide ($\bullet O_2^-$), hydroxyl radicals ($\bullet OH^-$), and hydrogen peroxide ($H_2O_2$), accumulate in salt-stressed plants (Jan et al., 2018; Ahmad et al., 2019). Ultimately, the plant cell wall can be disrupted, causing the plant cell to die (Shu et al., 2017; Rehman et al., 2019). Osmotic stress is related to the accumulation of $Na^+$ and $Cl^-$ ions in the soil solution to toxic levels, at which these ions interfere with other essential elements, such as calcium and potassium (Hussain et al., 2013). Schubert et al. (2009) demonstrated that cell wall expansion inhibits cell growth during the early stages of salt stress. Active cell wall expansins loosen the cell wall, regulating cell elongation at pH < 5 (Uddin et al., 2013). The osmosis process is primarily met with the accumulation of solutes under drought and salinity conditions to lower the water potential without reducing the actual water content (Serraj and Sinclair, 2002). Soluble sugars, proline, betaine glycine, organic acids, and trehalose sugars are some of the main osmotic agents. Kaya et al. (2010) reported that proline accumulation was increased in salt-stressed maize plants. Furthermore, the genes encoding proline and arginine synthesis enzymes and several other genes may have important roles in salt tolerance in *S. chilense* (Kashyap et al., 2020). The proline biosynthesis pathway in plants involves the participation of two main enzymes: Δ'-pyrroline-5-carboxylate reductase (P5CR) and Δ'-pyrroline-5-carboxylate synthetase (P5CS) (Delauney and Verma, 1993). P5CS overexpression in soybean increased the proline content in transgenic plants, resulting in a transgenic line with high salt tolerance (Nguyen et al., 2019). Proline accumulation is one of the main causes of increased osmotic pressure, thereby increasing the plant's water-holding capacity (Zhang et al., 2015). The first plant *CLC* genes were cloned in tobacco (Lurin et al., 1996) and *Arabidopsis* (Hechenberger et al., 1996); to date, most information on real system *CLC* genes and protein objects has been accumulated (Zifarelli and Pusch, 2010). In soybean, Li et al. (2006) reported that the *CLC* gene *GmClC-1* is 78% homologous with the *AtClC*-a gene in *Arabidopsis*. The CLC protein is detected when the plant receives signals from NaCl stress and drought stress. Expression of this protein increased $Cl^-$ transfer from the cytoplasm to the vacuole and induced NaCl tolerance to tobacco BY-2 cells (Li et al., 2006).

Several reports on the application of farming techniques to reduce salinity stress in soybeans have shown very little success (Kim et al., 2015; Liu et al., 2016; Shu et al., 2017). However, some of the studies on the association of several important genes with the ability to counteract salinity stress in soybeans are the basis for the application of advances in biotechnology to the creation of transgenic crops with good growth and development under abiotic stress conditions.

Several soybean genes have been described to be responsive to abiotic stresses at the transcriptional level, including a group of genes encoding the transcription factor DREB (dehydration responsive element binding protein). The AP2 domain of common DREB proteins is between 58 and 59 amino acids and includes several amino acids related to the dehydration responsive element (DRE) or GCC box. DREB contains *trans*-acting factors, which bind with the *cis*-acting elements of the promoter to trigger transcription of the target genes under stress signals in plants (Agarwal et al., 2006). Studies on DREB have all confirmed that the DREB protein is a transcription factor that actively participates in intolerance to adverse external conditions, such as drought, salinity, and cold, without the need for the influence of ABA (Agarwal et al., 2006). The activated *DREB* gene increases the transcription of target genes whose products are directed against adverse factors in the environment. The *GmDREB1* gene has the function of tolerance to heat, drought, salinity, and cold (Jiang et al., 2014). GmDREB2 has been confirmed to increase the drought and salt tolerance of transgenic plants (Zhang et al., 2013; Dao et al., 2015; Pham et al., 2020). GmDREB3 enhanced cold, drought, and salt tolerance in transgenic Arabidopsis (Chen et al., 2009); GmDREB5 has been linked to salt tolerance (Rahmawati et al., 2019). The DREB protein may improve tolerance to abiotic stress in plants by modulating the expression of downstream genes involved in environmental stress tolerance (Jiang et al., 2014). In this context, our study aimed to analyse the expression of the GmDREB6 transgene from soybean and to clarify the effect of GmDREB6 expression in enhancing the transcription levels of the *P5CS* and *CLC* genes above those of genetically modified tobacco plants under high-salinity conditions.

## 2. Materials and methods

### 2.1. Materials

Leaf fragments of the tobacco plants (*Nicotiana tabacum*) in vitro, cultivar K326, were used as transgene receiving material. The *Agrobacterium tumefaciens* strain containing the plant expression vector of the *GmDREB6* gene (*pBI121_GmDREB6*) from soybean (Fig. 1) was transformed into tobacco leaf tissue (Nguyen et al., 2019). *GmDREB6* transgenic tobacco lines obtained from in vitro selection were used to analyse the expression levels of the *GmDREB6, NtP5CS,* and *NtCLC* genes in transgenic tobacco under salt stress.

## 3. Methods

### 3.1. Genetic transformation of the GmDREB6 gene into tobacco and confirmation of transcription of the GmDREB6 transgene

Genetic transformation of the *GmDREB6* gene into tobacco leaf tissue mediated by *A. tumefaciens* was performed as described by Topping (1998), including multiple shoot regeneration, rooting and selection, in vitro propagation, and create transgenic tobacco lines. The leaf pieces were submerged into the *A. tumefaciens* suspension for 30 min; then, explants were transferred to a co-cultivation medium for 3 days in the dark. The transformed samples were regenerated on MS medium was added with BAP, 500 mg $L^{-1}$ cefotaxime, and 50 mg $L^{-1}$ kanamycin. The transformed samples were multi-bud regenerated in the shoot induction medium (basic MS, and 1.0 mg $L^{-1}$ BAP, and 30 g $L^{-1}$ sucrose, and 9.0 g $L^{-1}$ agar) in 4 weeks. After the next 4 weeks, good growing shoots were transferred to rooting medium (basic MS, 1.0 g $L^{-1}$ MES, 30 g $L^{-1}$ sucrose, 9.0 g $L^{-1}$ agar, and 100 ml $L^{-1}$ coconut water), and the addition IBA (0.5 mg $L^{-1}$), and kanamycin (50 mg $L^{-1}$) in 5 weeks to form plants with sufficient roots, stem, and leaves. The plantlets with good growth were planted in pots containing a mixture, included soil, rice husk biochar, coir with a ratio of 2:1:2, and the surviving plants were cared for in greenhouse conditions.

Analysis of transgenic tobacco plants using RT-PCR. Total RNA was extracted from tobacco leaves using TRIzol® LS reagent according to the company's instructions (Ambion - Life Technology Corporation, USA). The quality of total RNA was tested on a 1% agarose gel. Good-quality RNA was used to synthesize cDNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). PCR was performed to confirm the presence of the mRNA, which is the copy of the GmDREB6 transgene in the transgenic soybean plants, with the PCR primers DREB6-F/DREB6-R (Table 1). PCR was performed using a final volume of 20 μL and contained 0.5 μL of each primer (10 pmol/μL), 12.5 ml of 2× Master mix, 2.0 μL of

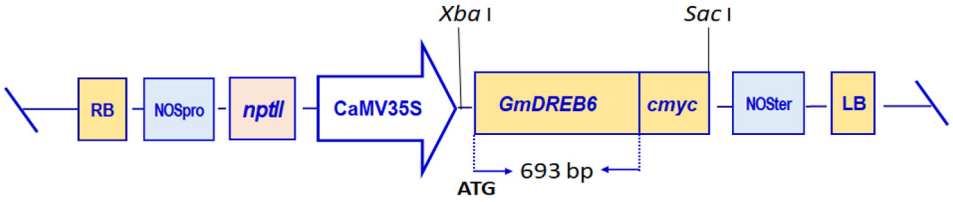


**Fig. 1.** Diagram of the *pBI121_GmDREB6* vector used for *Agrobacterium*-mediated transformation (Nguyen et al., 2019). *LB* and *RB*: left and right T-DNA border; *nptII:* neomycin-phospho-transferase II; CaMV35S: cauliflower mosaic virus 35S promoter; *GmDREB6_cmyc:* The soybean *DREB6* gene attaches a nucleotide sequence that encodes the peptide cmyc. *Xba* I, and *Sac* I: The restriction enzymes used for cloning.

**Table 1**
List of primers used in PCR and qRT-PCR.

| Primers | Nucleotide sequence (5′-3′) | Temperature of applying primers (°C) |
|---|---|---|
| *GmREB6-F/GmDREB6-R* | ATGGTCATGGAAGAATCTAACCCA | 58 |
| | TTAATTATGATTCCCATAGA | 58 |
| qRT-DREB-F/qRT-DREB6-R | TAATGAAGGCAAGCACCCTAC | 60 |
| | CGTCGGCTAATTCTGGGAAA | 60 |
| qRT-P5CS-F/qRT-P5CS-R | TGCTCGTGAGATGGCAGTTGC | 60 |
| | AGCCTGTTGAGCAGCAACCAC | 60 |
| qRT-CLC-F/qRT-CLC-R | CTTGGAGGCCTTCTCGGAAGC | 60 |
| | AGCCTACAGGACATGGTGTGC | 60 |
| qRT-ActN-F/qRT-ActN-R | GATCTTGCTGGTCGTGATCTT | 60 |
| | GTCTCCAACTCTTGCTCATAGTC | 60 |

GmDREB6, a soybean transcription factor, notably affects the transcription of the *NtP5CS* and *NtCLC* genes in transgenic tobacco under salt stress conditions

cDNA (500 ng/mL), and 4.5 μL of water. The PCR protocol, set by the conditions of the thermocycler, consisted of the following steps: initial denaturation at 95 °C for 5 min, followed by 35 cycles of temperature cycling at 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s, and final elongation at 72 °C for 10 min. The PCR products were excised from a 1.0% agarose gel and were purified using a gel extraction kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.

### 3.2. Salt treatment

The experiment was carried out in a growing room with a temperature maintained at 25 °C and a humidity of 80% with a morning and evening cycle of 16 am/8 pm. Wild-type (WT) and relatively homogeneous transgenic tobacco plants (GmDREB6) were grown in vitro in pots of 115 × 85 × 105 mm containing a mixture of Tribat compost, including organic soil, alluvial soil, porous mixture, activated carbon, and microorganisms. The experimental plants were watered with 50 ml of 200 mM NaCl daily for 3 weeks. Control plants were watered with equal amounts of $H_2O$. Leaf samples were collected on the last day of the experiment for gene expression analysis.

### 3.3. Analysis of gene expression using real-time quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed at a volume of 20 μL using 10 U of SYBR Premix Ex Taq II, 0.75 μL of 10 mM forward primer, 0.75 μL of 10 mM reverse primer (Table 1), deionized, DNase-free water, and 1 μL of cDNA. The steps to perform the amplification reaction included holding at 95 °C for 3 min, performing the amplification for 40 cycles in steps of 95 °C for 10 s, applying primers at 60 °C for 20 s, and extending at 72 °C for 20 s. The reactions were repeated 3 times. The results were synthesized and analysed using Q-Rex version 1.0.0 software (QIAGEN, Germany), and Livak's -ΔΔ CT method was used to analyse gene expression data (Livak and Schmittgen, 2001).

## 4. Results

### 4.1. A. tumefaciens-mediated transformation of the 35S_GmDREB6_cmyc construct and transcriptional analysis in tobacco

The structural transformation of the *GmDREB6* transgene occurred through *A. tumefaciens* infection of tobacco leaves. After 3 transformations, with 90 samples in the experimental group, 356 shoots were obtained, and through antibiotic selection, 278 shoots were obtained. In the RM rooting medium, 182 root shoots and 154 plants were selected for conversion to pots, and as a result, 85 plants survived under the net house conditions. The control group of 30 undenatured leaf samples regenerated in an antibiotic-free medium produced 116 shoots, 87 root shoots, and 30 plants transferred into pots under greenhouse conditions. Transgenic and nontransgenic plants were used to analyse the expression levels of the *GmDREB6, NtP5CS*, and *NtCLC* genes.

Nine lines of transgenic tobacco were selected for *GmDREB6* transgene expression analysis by RT-PCR. The results of testing RT-PCR products by electrophoresis analysis, shown in Fig. 2, revealed that only 6 out of 8 transgenic tobacco plants had DNA bands with a size of approximately 0.7 kb, corresponding to the

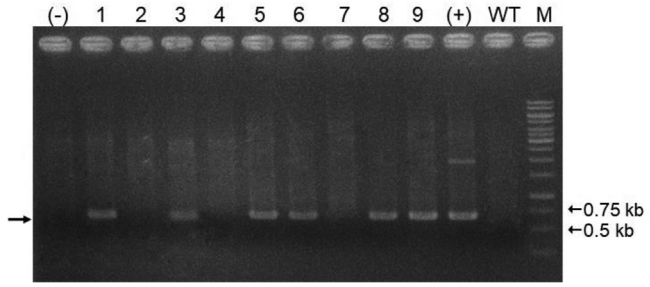


**Fig. 2.** Electrophoresis results confirmed the presence of the *GmDREB6* cDNA in the transgenic and nontransgenic tobacco plants by RT-PCR. M: DNA marker; (+): plasmid *pBI121_GmDREB6*; (−) $H_2O$; WT: nontransgenic tobacco plants. The electrophoresis lanes from 1 to 9 are the T0 generation transgenic plants.


**Fig. 3.** Morphology of *GmDREB6* transgenic tobacco plants and nontransgenic plants under $H_2O$ and NaCl irrigation in the plant growth cabinet after 3 weeks. A: watering with 50 ml of $H_2O$ daily; B: watering with 50 ml of 200 mM NaCl daily. L1, L3, L9: *GmDREB6* transgenic tobacco lines; WT: nontransgenic tobacco plants.

size of the *GmDREB6* gene, corresponding to plants in electrophoresis lanes 1, 3, 5, 6, 8, and 9. Transgenic lines 1, 3, 5, 6, 8, and 9 are denoted L1, L3, L5, L6, L8, and L9, respectively.

Nontransgenic lines and three RT-PCR-positive *GmDREB6* transgenic lines (L1, L3, L9) with normal growth and development and uniform morphology were selected for salt stress treatment in a plant growth cabinet under high-salinity conditions. Watering with 50 ml of 200 mM NaCl was performed daily for 3 weeks, and then gene expression levels were then analysed by real-time RT-PCR. The controls were *GmDREB6* transgenic lines and non-transgenic plants irrigated with 50 ml of $H_2O$ daily (Fig. 3).

### *4.2. Analysis of the expression levels of the GmDREB6, NtP5CS, and NtCLC genes in response to salt stress in transgenic tobacco lines*

The L1, L3, and L9 transgenic tobacco lines were used to analyse the expression levels of the *GmDREB6*, *NtP5CS*, and *NtCLC* genes by qRT-PCR to determine the role of the *GmDREB6* gene from soybean in the expression of the *NtP5CS* and *NtCLC* genes of tobacco.

Under normal conditions, the *GmDREB6* transgenic gene of the transgenic lines was overexpressed, while expression was not observed in the nontransgenic (WT) plants. This result confirmed that the *GmDREB6* transgene was incorporated into the tobacco genome and that transcription of the *GmDREB6* gene was performed. Under salinity treatment, the *GmDREB6* gene expression level in transgenic tobacco lines increased compared with that under normal conditions (Fig. 4A). In particular, transgenic lines L1 and L9 exhibited expression levels of the *GmDREB6* gene that had increased 2.40 fold (L1) to 3.22 fold (L9) compared with the condition of no saline treatment. Thus, these results indicated that the expression of the *GmDREB6* gene was directly related to the response to salt stress in tobacco plants.

Under salt conditions, the *P5CS* gene encoding the P5CS enzyme and the *CLC* gene encoding the vacuolar $Cl^-$ transport protein have also been shown to play roles in enhancing plant tolerance (Yamada et al., 2005; Zhang et al., 2014; Wei et al., 2019). In this study, for the *NtP5CS* gene, the results in Fig. 4B show that, under the condition of non-NaCl treatment, there was no difference in the transcriptional levels between transgenic tobacco lines com-

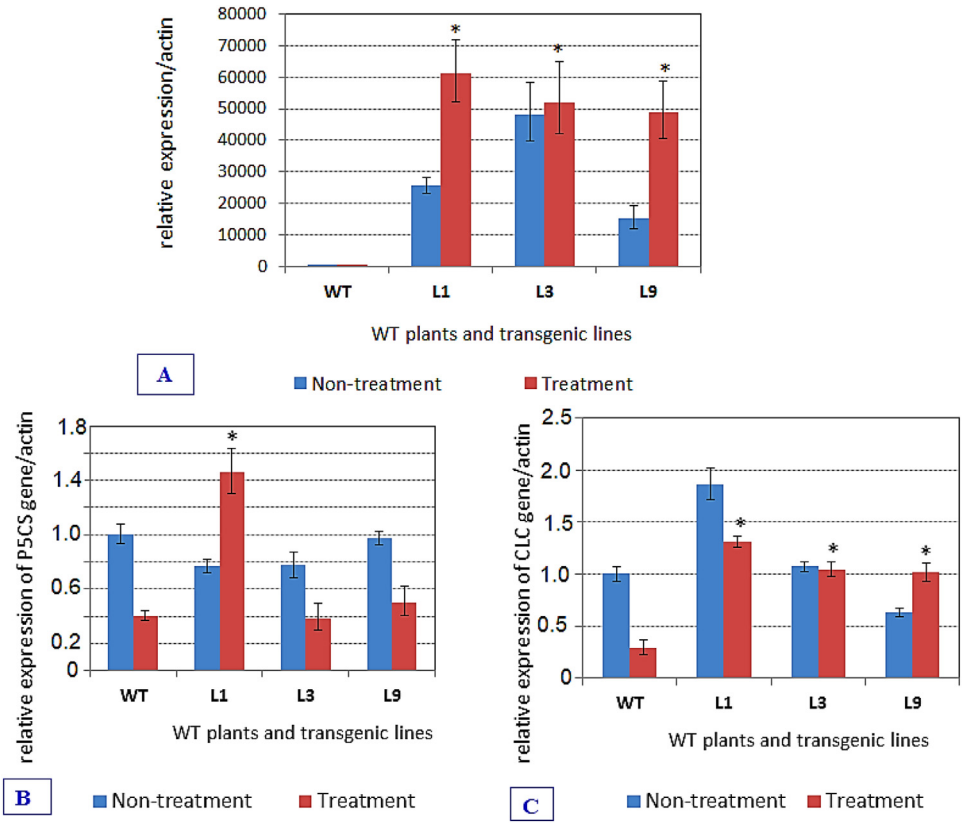


**Fig. 4.** Analysis of the expression levels of the *GmDREB6, NtP5CS,* and *NtCLC* genes of transgenic tobacco lines under salt stress by qRT-PCR; A: Expression of the *GmDREB6* gene under salt stress; B: expression of the *NtP5CS* gene (HM854026.1) under salt stress; C: expression of the *NtCLC* gene (NM_001325489.1) under salt stress; Actin: reference gene; WT: nontransgenic tobacco plants; L1, L3, and L9: transgenic tobacco lines. A (*) sign on each column represents a statistically significant difference ($P < 0.05$) compared with WT plants.

pared with WT plants. However, under salt stress, in transgenic lines L1 and L9, the transcriptional level of the *P5CS* gene increased from 1.24- to 3.60-fold compared with that in the WT plants. Among the 3 transgenic tobacco lines, the L1 line had a 1.9-fold increase in the transcriptional level of the *P5CS* gene compared with the untreated line, while the L3 and L9 lines did not show this change in expression (Fig. 4B). For the *NtCLC* gene, in the absence of treatment, only the L1 transgenic tobacco line had a higher transcription level than WT plants, while under the NaCl treatment, the transgenic lines all had increased transcription levels, ranging from 3.65- to 4.54-fold compared with WT plants ($P < 0.05$) (Fig. 4C).

Morphological observations of transgenic tobacco lines and WT plants also showed normal development of transgenic tobacco lines under salt stress, while WT plants showed yellowing and stopped growing.

## 5. Discussion

Seawater encroachment causing soil salinity is increasing in many coastal countries; thus, crop improvement oriented towards increasing salt tolerance is of particular current concern. Enhancement of the gene expression of transcription factors was performed to evaluate the effects on downstream abiotic stress tolerance genes. DREBs belong to the ethylene transcription factor (ERF) family of factors that bind to DRE/CRT factors in the promoter regions of a large number of genes involved in abiotic stress signalling in plants. In recent years, considerable progress has been made in clarifying the regulatory role of DREBs in crop responses to abiotic stresses. The *DREB* gene has been introduced to some transgenic plants that have exhibited increased tolerance to a variety of abiotic stresses (Khan, 2011). Previous studies have also demonstrated that the DREB subfamily plays an important role in the plant's salt tolerance response via the ABA-independent pathway. The enhanced expression of the *DREB* gene has triggered the expression of other tolerant response genes in abiotic stress conditions (Khan, 2011; Morran et al., 2011; Sarkar et al., 2014). *GmDREB6* in the *DREB* gene subfamily of soybean has been demonstrated to increase proline accumulation and to enhance salt tolerance in genetically modified soybean (Nguyen et al., 2019). In the results of our study on transgenic tobacco plants under the conditions of watering with 50 ml of 200 mM NaCl daily for 3 weeks, the transgenic tobacco lines exhibited increases in the transcription levels of the *GmDREB6* gene ranging from 108.03% to 321.95% compared with the transgenic plants under normal watering conditions ($P < 0.05$). This result confirmed the strong expression response of the *GmDREB6* gene when transgenic tobacco plants received the salt stress signal from the environment.

The *GmDREB6* gene in the soybean genome (gene ID 100101914) is located on chromosome 5. The coding region of a *GmDREB6* gene, which is 693 bp in size, encodes the DREB6 protein, which is 230 amino acids in length. The DREB6 protein contains the 59-aa AP2 region and an 11-aa DNA-binding having domain (.rg.r.r. w.k... e.r ..... r.w.t.) (Liu et al., 2007). The promoter for GmDREB6 contains the *cis*-elements GT-1 and DRE (DRE (−1113), GT-1 (−133, −1398, −1488, −1560, −1993), and the transcription factor DREB6 can be linked to the GT or DRE domain in the promoter regions of functional genes. The *cis*-elements in the promoter of the soybean *GmP5CS* gene are GT-1 (−56, −1243, 1641) and GCC (−1899) (Zhang et al., 2013). Nguyen et al. (2019) demonstrated that overexpression of the *GmDREB6* gene enhanced the transcriptional activity of the *GmP5CS* gene in transgenic soybean plants. In this study, the results of the analysis of *GmDREB6* gene expression on transgenic tobacco plants showed that when tobacco plants received signals of salinity stress from the environment, the transcription level of the *GmDREB6* gene increased to 321.95% compared with that in plants not treated with NaCl.

Proline is an osmotic agent, and a high content of proline in the cell will increase the cell's ability to retain water. In tobacco and plants in general, there are two important enzymes, P5CS and P5CR, that are involved in the synthesis of proline in cells. Overexpression of the *NtP5CS* gene will increase the concentration of the P5CS enzyme in tobacco, increase proline synthesis and accumulation, and lead to an increase in osmotic pressure, thereby increasing the plant's water-holding capacity (Zhang et al., 2015). The chloride channel (CLC) protein is an important anionic carrier that exists in bacteria, yeasts, plants, and animals (Sun et al., 2018). $Cl^-$ ions are important for several biological processes in cells, such as membrane depolarization, cell volume regulation, resistance to salt stress, and metal tolerance. It is speculated that the CLC protein may be involved in $Cl^-$ transport through intracellular compartments (Zifarelli and Pusch, 2010). Expression of the CLC protein increased the transfer of $Cl^-$ from the cytoplasm to the vacuole and created tolerance to NaCl in the cells (Li et al., 2006).

Upon investigating the transcription of the *NtP5CS* and *NtCLC* genes, we found that the L1 transgenic tobacco line had a significant increase in expression of the *NtP5CS* gene compared with the nontransgenic plants and compared with the two transgenic lines, L3. and L9. At the same time, the L1 transgenic line was also shown to have increased expression of the *NtCLC* gene compared with WT plants. While the L3 and L9 transgenic tobacco lines only recorded enhanced responses to *NtCLC* expression under salt conditions, the L1 transgenic line recorded enhancements in the simultaneous expression of the *GmDREB6* transgene and the two *NtP5CS and NtCLC* genes of tobacco plants (Fig. 4B, C). These results revealed the role of the *GmDREB6* gene from soybean relative to the expression of the *NtP5CS* and *NtCLC* genes in tobacco. The results of *GmDREB6* gene role analysis showed that salt stress tolerance in crops could be improved by controlling the expression of DREBs; however, further evaluation of the role of *GmDREB6* is also needed to identify other functional genes in response to salt stress.

## 6. Conclusion

This study demonstrated that overexpression of the *GmDREB6* gene in soybeans under saline stress conditions increases the transcription levels of the *NtP5CS* and *NtCLC* genes in transgenic tobacco plants. The transcription of *GmDREB6, NtP5CS,* and *NtCLC* in transgenic tobacco lines was confirmed by qRT-PCR. Under salt stress conditions, transgenic tobacco lines L1 and L9 had transcription levels of the *GmDREB6* gene increased from 2.40- to 3.22-fold compared with the condition without salinity treatment. For two transgenic lines, L1 and L9, the level of transcription of the *NtP5CS* gene increased from 1.24- to 3.60-fold ($P < 0.05$). For the *NtCLC* gene, under salt stress conditions, transgenic lines all had transcription levels that increased by 3.65- to 4.54-fold compared with WT plants ($P < 0.05$). The enhancements of simultaneous expression in both a *GmDREB6* transgene and two intrinsic genes, *NtP5CS* and *NtCLC*, were confirmed in L1 transgenic tobacco lines. The results of the expression analysis of the *GmDREB6, NtP5CS,* and *NtCLC* genes suggested that the *GmDREB6* gene is a potential candidate for use to improve the salt tolerance of plants, opening research directions for the development of crops tolerating salt stress amid climate change and increasing sea levels.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author contributions

P.T.D, Y.T.H.N., and M.H.C. conceived and designed the experiments; P.V., P.T.D., Y.T.H.N., N.H.N., T.T.M.L., and T.Q.T. performed the experiments; P.V., N.T.T.P, H.Q.N., T.Q.T., L.T.N.N., P.T.D., Y.T.H.N. performed data analyses; Y.T.H.N., L.T.N.N, H.Q.N., and H.M.C. prepared the manuscript and performed the proofreading. All authors approved the manuscript.

## References

Agarwal, P.K., Agarwal, P., Reddy, M.K., Sopory, S.K., 2006. Role of dreb transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. 25 (12), 1263–1274. https://doi.org/10.1007/s00299-006-0204-8.

Ahmad, P., Ahanger, M.A., Alam, P., Alyemeni, M.N., Wijaya, L., Ali, S., Ashraf, M., 2019. Silicon (si) supplementation alleviates nacl toxicity in mung bean [vigna radiata (l.) wilczek] through the modifications of physio-biochemical attributes and key antioxidant enzymes. J. Plant Growth Reg. 38 (1), 70–82. https://doi.org/10.1007/s00344-018-9810-2.

Chen, H., Liu, X., Zhang, H., Yuan, X., Gu, H., Cui, X., Chen, X., 2018. Advances in salinity tolerance of soybean: Genetic diversity, heredity, and gene identifcation contribute to improving salinity tolerance. J. Integr. Agric. 17 (10), 2215–2221. https://doi.org/10.1016/S2095-3119(17)61864-1.

Chen, M., Xu, Z., Xia, L., Li, L., Cheng, X., Dong, J., Wang, Q., Ma, Y., 2009. Cold-induced modulation and functional analyses of the dre-binding transcription factor gene, gmdreb3, in soybean (glycine max l.). J. Exp. Bot. 60 (1), 121–135. https://doi.org/10.1093/jxb/ern269.

Dao, X.T., Ho, M.T., Vu, T.T.T., Le, V.S., Chu, H.M., 2015. Cloning and overexpression of gmdreb2 gene from a vietnamese drought-resistant soybean variety. Braz. Arch. Biol. Technol. 58 (5), 651–657. https://doi.org/10.1590/S1516-89132015050170.

Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plant. Plant J. 4, 215–223. https://doi.org/10.1046/j.1365-313X.1993.04020215.x.

Gupta, B., Huang, B., 2014. Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. Int. J. Genom. 2014. https://doi.org/10.1155/2014/701596. Article ID 701596 (18 pages).

Hechenberger, M., Schwappach, B., Fischer, W.N., Frommer, W.B., Jentsch, T.J., Steinmeyer, K., 1996. A family of putative chloride channels from arabidopsis and functional complementation of a yeast strain with a clc gene disruption. J. Biol. Chem. 271 (52), 33632–33638. https://doi.org/10.1074/jbc.271.52.33632.

Hussain, M., Park, H.W., Farooq, M., Jabran, H., Lee, D.J., 2013. Morphological and physiological basis of salt resistance in different rice genotypes. Int. J. Agric. Biol. 15, 113–118.

Jan, S., Alyemeni, M.N., Wijaya, L., Alam, P., Siddique, K.H., Ahmad, P., 2018. Interactive effect of 24-epibrassinolide and silicon alleviates cadmium stress via the modulation of antioxidant defense and glyoxalase systems and macronutrient content in pisum sativum l. Seedlings. BMC Plant Biol. 18 (1), 146. https://doi.org/10.1186/s12870-018-1359-5.

Jiang, Q., Hu, Z., Zhang, H., Ma, Y., 2014. Overexpression of gmdreb1 improves salt tolerance in transgenic wheat and leaf protein response to high salinity. Crop J. 2 (2-3), 120–131. https://doi.org/10.1016/j.cj.2014.02.003.

Kashyap, S.P., Prasanna, H.C., Nishi, K., Pallavi, M., Singh, B., 2020. Understanding salt tolerance mechanism using transcriptome profiling and de novo assembly of wild tomato solanum chilense. Sci. Rep. 10. https://doi.org/10.1038/s41598-020-72474-w. Article number: 15835.

Kaya, C., Tuna, A.L., Okant, A.M., 2010. Effect of foliar applied kinetin and indole acetic acid on maize plants grown under saline conditions. Turk. J. Agric. For. 34, 529–538. https://doi.org/10.3906/tar-0906-173.

Khan, M.S., 2011. The role of dreb transcription factors in abiotic stress tolerance of plants. Biotechnol. Biotechnol. Equip. 25 (3), 2433–2442. https://doi.org/10.5504/BBEQ.2011.0072.

Kim, Y.H., Hwang, S.J., Waqas, M., Khan, A.L., Lee, J.H., Lee, J.D., Nguyen, H.T., Lee, I.J., 2015. Comparative analysis of endogenous hormones level in two soybean (glycine max l.) lines differing in waterlogging tolerance. Front. Plant Sci. 6, 714. https://doi.org/10.3389/fpls.2015.00714.

Li, W.I.F., Wong, F.L., Tsai, S.N., Phang, T.H., Shao, G., Lam, H.M., 2006. Tonoplast-located gmclc1 and gmnhx1 from soybean enhance nacl tolerance in transgenic bright yellow (by)-2 cells. Plant Cell Environ. 29 (6), 1122–1137. https://doi.org/10.1111/pce:2006.29.issue-610.1111/j.1365-3040.2005.01487.x.

Liu, Y., Yu, L., Qu, Y., Chen, J., Liu, X., Hong, H., Liu, Z., Chang, R., Gilliham, M., Qiu, L., Guan, R., 2016. Gmsalt3, which confers improved soybean salt tolerance in the field, increases leaf cl- exclusion prior to na+ exclusion but does not improve early vigor under salinity. Front. Plant Sci. 7, 1485. https://doi.org/10.3389/fpls.2016.01485.

Liu, Y.W., Chen, M., Xu, Z.S., Zh, G.Y., Li, L.C., Ma, Y.Z., 2007. Glycine max dehydration-responsive element binding protein 6 mrna. complete cds. GenBank EF551166.1. https://www.ncbi.nlm.nih.gov/nuccore/146552061.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2-δδct method. Methods Mol. Biol. 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Lurin, C., Geelen, D., Barbier-Brygoo, H., Guern, J., Maurel, C., 1996. Cloning and functional expression of a plant voltage-dependent chloride channel. Plant Cell 8 (4), 701–711. https://doi.org/10.1105/tpc.8.4.701.

Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Shirley, N., Langridge, P., Lopato, S., 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of dreb/cbf factors. Plant Biotechnol. J. 9 (2), 230–249. https://doi.org/10.1111/j.1467-7652.2010.00547.x.

Muchate, N.S., Nikalje, G.C., Rajurkar, N.S., Suprasanna, P., Nikam, T.D., 2016. Plant salt stress: Adaptive responses, tolerance mechanism and bioengineering for salt tolerance. Bot. Rev. 82 (4), 371–406. https://doi.org/10.1007/s12229-016-9173-y.

Nguyen, H.Q., Vu, T.K.L., Nguyen, T.N.L., Pham, T.T.N., Nguyen, T.H.Y., Le, V.S., Chu, H.M., 2019. Overexpression of the gmdreb6 gene enhances proline accumulation and salt tolerance in genetically modified soybean plants. Sci. Rep. 9, 19663. https://doi.org/10.1038/s41598-019-55895-0.

Pham, T.T.N., Nguyen, H.Q., Nguyen, T.N.L., Dao, X.T., Sy, D.T., Le, V.S., Chu, H.M., 2020. Overexpression of the gmdreb2 gene increases proline accumulation and tolerance to drought stress in soybean plants. AJCS 14 (03), 495–503. https://doi.org/10.21475/ajcs10.21475/ajcs.20.14.0410.21475/ajcs.20.14.03.p2173.

Rahmawati, N., Rosmayati Delvian, D., Mohammad, B., Hirosuke, O., 2019. Evaluation of resistance improvement of soybean (glycine max (l) merr.) against salinity using mass selection and gene expression of salinity tolerant. In: Proceedings of the International Conference on Natural Resources and Technology (ICONART), pp. 30–37. https://doi.org/10.5220/0008387500300037.

Rehman, S., Abbas, G., Shahid, M., Saqib, M., Farooq, A.B.U., Hussain, M., Murtaza, B., Amjad, M., Naeem, M.A., Farooq, A., 2019. Effect of salinity on cadmium tolerance, ionic homeostasis and oxidative stress responses in conocarpus exposed to cadmium stress: implications for phytoremediation. Ecotoxicol. Environ. Saf. 171, 146–153. https://doi.org/10.1016/j.ecoenv.2018.12.077.

Sarkar, T., Thankappan, R., Kumar, A., Mishra, G.P., Dobaria, J.R., Zhang, J.S., 2014. Heterologous expression of the atdreb1a gene in transgenic peanut-conferred tolerance to drought and salinity stresses. PLoS ONE 9 (12), e110507. https://doi.org/10.1371/journal.pone.0110507.

Schubert, S., Neubert, A., Schierholt, A., Sümer, A., Zörb, C., 2009. Development of salt resistant maize hybrids: the combination of physiological strategies using conventional breeding methods. Plant. Sci. 177 (3), 196–202. https://doi.org/10.1016/j.plantsci.2009.05.011.

Serraj, R., Sinclair, T.R., 2002. Osmolyte accumulation: can it really help increase crop yield under drought conditions. Plant Cell Environ 25, 333–341. https://doi.org/10.1046/j.1365-3040.2002.00754.x.

Shrivastava, P., Kumar, R., 2015. Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. Saudi J. Biol. Sci. 22 (2), 123–131. https://doi.org/10.1016/j.sjbs.2014.12.001.

Shu, K., Qi, Y., Chen, F., Meng, Y., Luo, X., Shuai, H., Zhou, W., Ding, J., Du, J., Liu, J., Yang, F., Wang, Q., Liu, W., Yong, T., Wang, X., Feng, Y., Yang, W., 2017. Salt stress represses soybean seed germination by negatively regulating ga biosynthesis while positively mediating aba biosynthesis. Front. Plant Sci. 8, 1372. https://doi.org/10.3389/fpls.2017.01372.

Sun, H., Shen, L., Qin, Y., Liu, X., Hao, K., Li, Y., Wang, J., Yang, J., Wang, F., 2018. Clc-nt1 affects potato virus y infection via regulation of endoplasmic reticulum luminal ph. New Phytol. 220 (2), 539–552. https://doi.org/10.1111/nph.15310.

Topping, J.F., 1998. Tobacco transformation. Methods Mol. Biol. 81, 365–372. https://doi.org/10.1385/0-89603-385-6:365.

Uddin, M.N., Hanstein, S., Leubner, R., Schubert, S., 2013. Leaf cell-wall components as influenced in the first phase of salt stress in three maize (zea mays l.) hybrids differing in salt resistance. J. Agron. Crop Sci. 199 (6), 405–415. https://doi.org/10.1111/jac.12031.

Wei, P., Che, B., Shen, L., Cui, Y., Wu, S., Cheng, C., Liu, F., Li, M.W., Yu, B., Lam, H.M., 2019. Identification and functional characterization of the chloride channel gene, gsclc-c2 from wild soybean. BMC Plant Biol. 19 (1), 1–15. https://doi.org/10.1186/s12870-019-1732-z.

Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi-Shinozaki, K., Shinozaki, K., Yoshiba, Y., 2005. Effects of free proline accumulation in petunias under drought stress. J. Exp. Bot. 56 (417), 1975–1981. https://doi.org/10.1093/jxb/eri195.

Zhang, G.C., Zhu, W.L., Gai, J.Y., Zhu, Y.L., Yang, L.F., 2015. Enhanced salt tolerance of transgenic vegetable soybeans resulting from overexpression of a novel δ1-pyrroline-5-carboxylate synthetase gene from solanum torvum swartz. Hort. Envir. and Bio. 56 (1), 94–104. https://doi.org/10.1007/s13580-015-0084-3.

Zhang, X., Tang, W., Liu, J., Liu, Y., 2014. Co-expression of rice osp5cs1 and osp5cs2 genes in transgenic tobacco resulted in elevated proline biosynthesis and enhanced abiotic stress tolerance. Chinese J. Appl. Environ. Biol. 20 (4), 717–722. https://doi.org/10.3724/SP.J.1145.2014.03010.

Zhang, X.X., Tang, Y.J., Ma, Q.B., Yang, C.y., Mu, Y.H., Suo, H.C., Luo, L.H., Nian, H., Zhang, J., 2013. Osdreb2a, a rice transcription factor, significantly affects salt

tolerance in transgenic soybean. PLoS ONE 8 (12), e83011. https://doi.org/10.1371/journal.pone.0083011.

Zifarelli, G., Pusch, M., 2010. Clc transport proteins in plants. FEBS Lett. 584, 2122–2127. https://doi.org/10.1016/j.febslet.2009.12.042.