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STUDY ON *GmDREB6* GENE EXPRESSION TO IMPROVE SALT TOLERANCE IN TRANSGENIC PLANTS

Major: Genetics
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SUMMARY OF BIOLOGY DOCTORAL THESIS

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1. The thesis will be presented in front of the doctoral thesis council at University of Education – Thai Nguyen University
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The thesis can be found at**:**

2. National Library of Vietnam
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THE PUBLISHED ARTICLES RELATED TO THE CURRENT THESIS

1. Tan Quang Tu, Phutthakone Vaciaxa, Thu Thi Mai Lo, Nhung Hong Nguyen, Nhan Thi Thanh Pham, Quan Huu Nguyen, Phat Tien Do, Lan Thi Ngoc Nguyen, Yen Thi Hai Nguyen, Mau Hoang Chu (2021), "GmDREB6, a soybean transcription factor, notably affects the transcription of the *NtP5CS* and *NtCLC* genes in transgenic tobacco under saltstress conditions", *Saudi Journal of Biological Sciences* 28(12), pp.7175-7181. https://doi.org/10.1016/j.sjbs.2021.08.018. (SCIE, Q1, IF: 4,219)

2. Phutthakone Vaciaxa, Tran Thi Hong, Pham Thi Thanh Nhan, Vu Thi Thu Thuy, Chu Hoang Mau (2021), "Study on *Agrobacterium tumefaciens*-mediated transformation of *GmDREB6* gene in Vietnamese soybean cultivar ĐT22", *TNU Journal of Science and Technology* 226(01), pp. 57 – 64.

3. Nguyen Thi Ngoc Lan, Phutthakone Vaciaxa, Nguyen Thanh Chung, Nguyen Huu Quan, Pham Thi Thanh Nhan, Vu Thi Thu Thuy, Chu Hoang Mau (2021), "Characteristics and phylogeny of *DREB* gene subfamily
in soybeans [*Glycine max* (L.) Meril]", *Việt Nam Journal of science and Technology* 63 (2), pp.60-64.

4. Thi Ngoc Lan Nguyen, Phutthakone Vaciaxa, Thi Mai Thu Lo, Thi Hai Yen Nguyen, Thi Thanh Nhan Pham, Van Son Le, Hoang Mau Chu (2019), "Design of Construct Carrying GmDREB6 to Enhance Soybean Gene Expression Related to Abiotic Stress Response", *European Journal of Engineering Research and Science* 4 (6), pp. 135-139.

INTRODUCTION

1. Essence of the thesis

Soybean [Glycine max (L.) Merrill (2n=40)] belongs to the Legume family (Fabaceae) is a crop that plays an important role in the structure of crops and human life in many countries around the world. world and in Vietnam. Soybean not only has economic and nutritional value but also plays an important role in improving soil fertility and sustainable use of arable land resources.

Soybean is considered a crop sensitive to abiotic adverse factors and belongs to the group of plants with poor tolerance to drought and salinity. Drought and salinity are the most serious abiotic factors and can reduce soybean yield by about 40%, even up to 90%, and also reduce grain quality. Currently, due to global climate change, especially prolonged drought, sea-level rise encroaches on arable land, causing great damage to agricultural production in many countries, including Vietnam. Therefore, the solution to select and breed soybean varieties with drought and salt tolerance to cope with climate change is an urgent and topical issue in Viet Nam and the world.

The drought and salt tolerance characteristics of soybean are regulated by many genes. The product of each gene can be directly related to the expression of drought and salt tolerance, such as genes involved in proline synthesis, root elongation, or genes that regulate the tolerant gene pool. Studying the expression of genes that regulate transcription of abiotic stress tolerance genes is a promising approach in the strategy of developing soybean varieties with good tolerance to abiotic factors. biological factors, such as drought, salinity, dryness, heat, etc. Several genes encoding transcription factors in soybean have been described to respond to drought and salinity at transcriptional levels, including DREB (Dehydration responsive element) protein. binding proteins). DREB is a subfamily of the AP2/ERF (APETALA2/Ethylene-Responsive) transcription factor, which has a trans-acting pattern and can bind to cis sequences to activate target

gene expression in response to abiotic stress signals. learn. The soybean DREB subfamily consists of members identified in the genome and some translation products of *GmDREB* genes that have been confirmed to have drought and salt tolerance functions. However, a few members of the *DREB* gene subfamily have not been fully studied and their roles in drought and salt tolerance in soybean have not been fully investigated, including the *GmDREB6* gene.

Approach to applying transgenic technique encoding DREB transcription factor and clarifying the function of some *GmDREB* genes in soybean genome to improve genetic characteristics and create transgenic lines adapted to the conditions drought and salinity are of particular interest. Therefore, the gene encoding transcription factor DREB6 related to tolerance to abiotic stresses in general and tolerance to drought and salt, in particular, in particular, was selected as a transgene to improve drought and salt tolerance. of soybean plants. Stemming from the above reasons, we chose and conducted the thesis: "Study on *GmDREB6* gene expression to improve salt tolerance in transgenic plants".

2. Objectives of the thesis

2.1. Characterization of the *DREB* gene subfamily in the genome of soybean (Glycine max (L.) Merrill).

2.2. Expressed recombinant protein GmDREB6 and evaluated biological function of transgene *GmDREB6* in transgenic tobacco.

2.3. Structural transformation carried the transgene GmDREB6 into soybean and created transgenic soybean plants.

3. Contents of the thesis

3.1. Study on characteristics of *DREB* gene subfamily in soybean by Bioinformatics.

1) Using Bioinformatics to search for *DREB* gene sequences of soybean on NCBI databank.

2) Determine the number of *DREB* genes, position, and copy number of each *DREB* gene in the soybean genome.

3) Building a phylogenetic tree of the DREB subfamily in soybean.

3.2. Study on design of plant transgenic vector containing *GmDREB6* gene and analysis of *GmDREB6* gene expression in tobacco plants.

1) Researching information on soybean *DREB6* gene from NCBI data bank, artificial design and synthesis of *GmDREB6*.

2) Design a structural transgenic vector containing the *GmDREB6* gene.

3) Expression analysis of *GmDREB6* gene from soybean on tobacco at the transcript level.

3.3. Studying the expression levels of *GmDREB6, NtP5CS, NtCLC* genes in transgenic tobacco plants by Real-time qRT-PCR

1) Analysis of *GmDREB6* gene expression from soybean on transgenic tobacco plants.

2) Analysis of expression levels of *NtP5CS* and *NtCLC* genes in *GmDREB6* transgenic tobacco plants.

3.4. Structural transformation of GmDREB6 gene into soybean

1) Infection of recombinant Agrobacterium tumefaciens into soybean cotyledons. Multi-shoot regeneration, rooting and generation of transgenic soybean plants.

2) Determination of the presence and transcription of *GmDREB6* transgene in T0 transgenic soybean plants.

4. New contributions of the thesis

The thesis is the study of the characteristics of the *DREB* gene subfamily in the soybean genome and the analysis of *GmDREB6* gene expression in transgenic tobacco plants. The new contributions of the thesis are specifically shown as:

1) Identified 18 GmDREB genes belonging to the DREB subfamily of soybean (Glycine max) located on 17 chromosomes. The *GmDREB3* gene has 8 copies, the remaining genes have 1-4 copies. The common AP2 domain has 59-60 amino acids and the PTPEMAARAYDVAALALKGPSARLNFPEL motif is present in all

soybean DREB proteins. AP2 contains 11 promoter binding sites of the common functional genes RGRRWKERRWT. The phylogenetic tree of the *GmDREB* genes and the AP2 domain represents the evolution of this gene family.

2) The expression of the *GmDREB6* gene from soybean increases the transcriptional levels of the *NtP5CS* and *NtCLC* genes of transgenic tobacco plants under saline stress as experimentally demonstrated. Under saline stress conditions, the transgenic tobacco lines had an increase in *GmDREB6* gene transcription from 2,40 to 3,22 (times) compared with the non-salinated condition; The transcription level of *NtP5CS* gene increased from 1,24 to 3,60 (times) of *NtCLC* gene increased 3,65 – 4,54 (times) compared to WT plants ($P < 0,05$).

3) Successfully transformed the structure carrying the transgene *GmDREB6* into the soybean variety DT22 through *A. tumefaciens*, resulting in 8 PCR-positive *GmDREB6* transgenic soybean plants and 5 RT-PCR analysis results.

5. Scientific significance and practical meaning of the thesis

The results of studying the *DREB* gene subfamily in soybean have clarified the characteristics as well as the phylogeny of the *DREB* gene subfamily in soybean, creating a database for functional studies of *DREB* genes in the soybean genome.

The results of the analysis of overexpression of *GmDREB6* gene that increase the transcription level of *NtP5CS* and *NtCLC* genes of transgenic tobacco plants demonstrated the role of transcription factor DREB6 in the salt tolerance of soybean in salt stress conditions.

Transgenic vector *pBI121_GmDREB6* can be used to transfer into soybean or other crops to enhance the salt tolerance of transgenic plants. At the same time, the initial results of *GmDREB6* transgenic plants have opened a direction for research and application of transgenic techniques to create salt-tolerant soybean varieties, contributing to the response to climate change and the frequently occurring salinity droughts in Viet Nam.

6. Composition of the thesis

The thesis has 128 pages (including appendices), divided into chapters and sections: Introduction (5 pages); Chapter 1: Study overview (40 pages); Chapter 2: Research materials and study methods (14 pages); Chapter 3: Results and discussion (33 pages); Conclusion and recommendations (2 pages); Published works related to the thesis (1 page); References (23 pages); Appendix (3 pages). The thesis includes 12 tables, 26 pictures, 1 appendix and 156 references and 8 web pages.

Chapter 1. LITERATURE REVIEW

The thesis has consulted and summarized 156 documents on three basic issues, which are: (1) Drought and salt tolerance characteristics of plants and soybeans; (2) DREB transcription factor in plants and soybeans; (3) Genetic engineering in research to improve the tolerance characteristics of soybean plants.

Soybean [*Glycine max* (L.) Merrill (2n=40)] is a crop that has both economic and nutritional value and is also a crop for improving agricultural land. Soybean belongs to the group of plants with poor tolerance to abiotic stresses, such as drought and salinity. Abiotic stress is a major cause of crop failure worldwide, causing yield losses in many crops. Among abiotic stresses, drought and salinity are the main factors that reduce crop yield. Drought and salt stress disrupt the homeostasis and ion distribution in the cell. Plants respond to drought and salinity stress through signaling pathways and cellular responses such as synthesis of stress proteins, enhancement of antioxidants, and solute accumulation. The mechanism of salt tolerance of plants has been identified, which is the accumulation and maintenance of high concentrations of soluble substances in the cell to ensure the competitiveness of water with the saline environment and against physiological drought. Plants can regulate osmotic pressure even when their roots are exposed to saline environments. However, when salinized, plants often limit the water permeability of cell membranes.

Different physiological and biochemical mechanisms in cells have helped plants to survive, grow and develop in environments with high salt concentration, including (1) homeostasis and ion prevention, (2) ion transport and uptake (3) biosynthesis of osmotic protectors and compatible solutes, (4) activation of antioxidant enzymes and synthesis of antioxidant compounds, (5) synthesis of polyamine synthesis, (6) production of nitric oxide (NO), and (7) modulation of hormones. Chloride channel proteins (CLCs) are important anion transporters found in bacteria, yeast, plants, and animals. Cl^- ions are important for several biological processes in cells, such as membrane depolarization, regulation of cell volume, resistance to salt stress, tolerance to metals. It can be speculated that the CLC protein may be involved in the movement of Cl^- across cellular organelles. The expression of CLC protein increased the transport of Cl^- from the cytoplasm to the vacuole and conferred resistance to NaCl in the cell.

Soluble sugars, proline, glycine betaine, organic acids and trehalose are some of the major osmotic agents. Multiple studies have found that proline content was increased in NaCl-tolerant cell lines. Proline accumulation was increased when plants lived under salt stress conditions. The proline biosynthetic pathway in plants involves two major enzymes: Δ'-pyrroline-5-carboxylate reductase (P5CR) and Δ'-pyrroline-5-carboxylate synthetase (P5CS). Proline accumulation is one of the main causes of increased osmotic pressure, thereby increasing the water holding capacity of plants.

Studies on DREB all confirm that DREB protein is a transcription factor that actively participates in the process of tolerance to abiotic adverse factors by activating the activity of genes directly involved in resistance. factors such as drought and salinity. Several members of the *DREB* gene subfamily have been identified in the soybean genome. Each gene in the DREB family has a different sequence and length but is strongly expressed when soybean plants are exposed to abiotic stresses. Among the *GmDREB* genes in the soybean genome, some have been functionally elucidated, such as *GmDREB1, GmDREB2, GmDREB3, GmDREB5*. In which, *GmDREB1* has the function of heat, drought, and

cold tolerance. *GmDREB2* was confirmed to increase the drought and salt tolerance of transgenic plants. *GmDREB3* enhances tolerance to cold, drought and salinity in transgenic *Arabidopsis*. *GmDREB5* is associated with salt tolerance. However, in this gene subfamily, information on the function of some *GmDREB* genes is incomplete, such as *GmDREB6, GmDREB7*…; Simultaneously, interventions to enhance *GmDREB* gene expression (overexpression) to increase transcriptional activation to change the expression level of functional genes, to improve drought tolerance of soybean are necessary issues. be solved. From the above studies, it is shown that DREB proteins are important transcription factors in the regulation of genes involved in abiotic stresses. Thus, *DREB* genes can be used to improve the tolerance of agriculturally important crops to adverse conditions such as drought and high salinity by transgenic engineering.

Gene transfer is a technique applied in the study of gene function. Two groups of methods are applied in gene transfer research in plants: direct gene transfer and indirect gene transfer. Indirect gene transfer in soybean through Agrobacterium is a technique that includes the following steps: (i) Research and collect information on genes related to the trait, trait of interest and gene isolation; (ii) Design of transgenic vectors; (iii) Creating bacteria with transgenic structure; (iv) Infection into plant tissue; (v) Selection of transformations and regeneration of transformed plants; (vi) Analysis of transgenic plants. Soybean DREBs of the AP2 family are an active transcription factor that binds to the cis sequence of the promoter to activate the expression of target genes in soybean in the presence of abiotic stress signals from the environment. The AP2 domain has about 58 or 59 amino acids, including some amino acids bound to the dehydration response element (DRE) or GCC box. Recently, research has shown that overexpression of the DREB protein increases tolerance to abiotic stress under abiotic stress conditions. Evaluation of drought tolerance of *DREB1A* and *DREB2A* transgenic soybean plants concluded that *DREB* transgenic plants had a higher survival rate than non-transgenic plants under severe water shortage in both greenhouse and field conditions. However, so far,

few reports on the analysis of strong expression of the *GmDREB6* gene that increases proline content and increases tolerance to drought and salt stress in transgenic plants have not been published.

Chapter 2. MATERIALS AND METHODS

2.1. Materials

Plant Materials: Tobacco variety K326 is provided by Tobacco Research Institute. Soybean variety DT22 was provided by the Bean Research and Development Center.

Bacterial strains and vectors: E. coli strains DH5α and *A. tumefaciens* C58/pGV2206. The vectors are kept at the Department of Plant Cell Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

2.2. Methods

2.2.1. Group of methods to analyze *DREB* gene subfamily in soybean

DREB gene subfamily analysis in soybean was performed using the Bioinformatics tool. Sequence data of the *GmDREB* gene and the AP2 region of the DREB protein were exploited from the NCBI Data Bank, in which there are a few *DREB* gene sequences isolated from Vietnamese soybean varieties.

2.2.2. Group of methods for designing plant transgenic vectors and analyzing the activity of *GmDREB6* gene expression vectors in tobacco

The transgenic vector carrying the *GmDREB6* gene is designed in two basic steps: (1) Design an independent structure including the GmDREB6 gene, the cmyc fragment and the cutting site of the Xbal/Sacl enzyme pair (GmDREB6_cmyc); (2) Insert the structure into the plant transgenic vector pBI121 to form the recombinant vector pBI121_GmDREB6.

The recombinant vector was transferred into A. tumefaciens by electrical pulse (2.5 kV, 25 μF, 200 Ω) to produce recombinant A. tumefaciens.

Structural transformation of the GmDREB6 gene through recombinant *A. tumefaciens* into tobacco variety K326 was performed according to the method of Topping (1998). After inoculation, the transformed samples were transferred to a co-culture medium and regenerated in vitro to produce transgenic tobacco plants.

Total DNA was extracted from the leaves of transgenic tobacco and WT plants according to the method of Saghai-Maroof et al (1984). The presence and transcription of the *GmDREB6* transgene in transgenic tobacco were determined by PCR with the primer pair *XbaI-DREB6-F/DREB6-SacI-R.*

2.2.3. Group of methods to analyze the expression levels of *GmDREB6, NtP5CS, NtCLC* genes in transgenic tobacco plants

Salt treatment method

The experiment was carried out in a culture room with the temperature maintained at 25°C and humidity at 80% with a light and dark cycle of 16h/20h. Wild-type tobacco (WT) and transgenic *GmDREB6*. The experimental plants were irrigated with 50 ml of 200 mM NaCl daily for 3 weeks. The control plants were irrigated with equal amounts of H_2O. Leaf samples were collected on the last day of the experiment for gene expression analysis.

Method of gene expression analysis using Real-time quantitative reverse transcription PCR (qRT-PCR)

The primer pairs of the actin reference gene and the target gene GmDREB6 were designed and synthesized to be used for qRT-PCR to analyze the transcription level and activity of the transgenic vector constructs in transgenic plants. The thermal cycle of the qRT-PCR reaction is as follows: 95°C for 3 min, amplification for 40 cycles (95°C for 10 s, primer conjugation at 60°C for 20 s, and extension at 72 °C for 20 seconds). Results were synthesized and analyzed using Q-Rex software version 1.0.0 (QIAGEN, Germany), and Livak's -ΔΔ Ct method was used to analyze gene expression data (Livak and Schmittgen, 2001).

2.2.4. Group of methods to transfer *GmDREB6* gene soybean

The method of transforming the vector structure carrying the transgene *GmDREB6* the soybean variety DT22 through *A. tumefaciens* was performed according to the method of Olhoft et al (2006) and Nguyen Thu Hien et al (2014). Determination of the presence and transcription of the *GmDREB6* transgene in transgenic soybean by PCR.

2.2.5. Data analysis and processing: Data were processed using SPSS software.

2.3. LOCATION OF RESEARCH AND COMPLETE THESIS

Gene transfer experiments were carried out at the Plant Cell Technology Laboratory, Department of Biology, University of Education - Thai Nguyen University. The gene transfer experiment was carried out from April 2018 to August 2021.

Experiments on designing transgenic vectors and analyzing gene expression in transgenic plants were conducted at the Department of Plant Cell Technology and the Key Laboratory of Gene Technology under the Institute of Biotechnology – Han Lam of Sciences. and Viet Nam Technology.

The thesis was completed at the Department of Genetics & Biotechnology, Department of Biology, College of Education - Thai Nguyen University.

Chapter 3. RESEARCH RESULTS AND DISCUSSION

3.1. CHARACTERISTICS AND PHARMACOLOGY OF THE DREB GENE FACTORY IN SOYBEAN

3.1.1. Results of identifying genes in the *DREB* gene subfamily in soybean

Mining from the NCBI database has identified 69 different GmDREB gene sequences on GenBank. Of the 69 DREB gene sequences in soybean extracted from NCBI data belonging to 18 GmDREB genes. Phang et al (2008) reported that there are more than 10 DREB genes in the soybean genome. However, in this study, we identified 18 GmDREB genes present

in the soybean genome. In this result, the GmDREB genes have from 1 to 8 copies (Table 3.1) distributed on 17 chromosomes.

3.1.2. Phylogenetics of members of the *GmDREB* gene subfamily in soybean.

The results of phylogenetic analysis of the *DREB* subfamily showed that 69 *GmDREB* gene sequences were divided into two branches A and B with 6 groups designated as I, II, III, IV, V, VI.

Table 3.1. Copy number and position of each *GmDREB* gene in the soybean genome

TT	Gene name	Number of copies	Location of genes on chromosome number
1	*GmDREB*	1	13
2	*GmDREB1*	2	9, 10
3	*GmDREB1B*	1	10
4	*GmDREB1D*	1	13
5	*GmDREB1E*	3	1,10, 17
6	*GmDREB1F*	4	5, 11, 12, 13
7	*GmDREB2*	2	4,6
8	*GmDREB2A2*	1	14
9	*GmDREB2C-like*	2	2, 6
10	*GmDREB2D*	2	4,6
11	*GmDREB2F*	4	2, 3, 10, 19
12	*GmDREB3*	8	1, 3, 4, 7, 9, 11, 13, 17
13	*GmDREB5*	2	12, 13
14	*GmDREB6*	1	5
15	*GmDREB7*	1	20
16	*GmDREBa*	1	12
17	*GmAP2-2*	1	4
18	*GmAP2-Ile-DBDP*	1	16

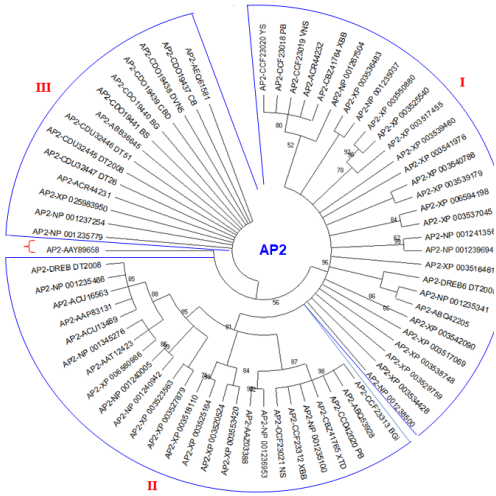
Group I includes *GmDREB1* genes (B, D, E, F), group II includes *GmDREB1* and *GmDREB7* genes, group III includes *GmDREB3* and *GmDREB6* genes, group IV has *GmDREB2* genes and one *GmDREB1* gene, group V includes genes GmDREB5, group VI includes the genes *GmDREB* and *GmDREB2*. Thus, there are 4 groups containing the gene pairs *GmDREB1-GmDREB7* (group II), *GmDREB3-GmDREB6* (group III), GmDREB2-GmDREB1 (group IV) and *GmDREB-GmDREB2* (group VI). It is interesting to note that some *GmDREB3* sequences are distributed in separate branches in the tree diagram. Sequence *GmDREB3* bearing code XM_003534380 is a sub-branch of clade A, *GmDREB3* (NM_001251571) is a separate clade from group IV, two *GmDREB3* genes (DQ055133 and NM_001250024) are distributed in a clade, separate from group IV. The GmDREB3 gene has 8 copies on different chromosomes, of which *GmDREB3* (XM_003534380) is located on chromosome 9, *GmDREB3* (NM_001251571) on chromosome 17 and *GmDREB3* (DQ055133 and NM_001250024) on chromosome number 4.

Characterization of the AP2 domain in the DREB subfamily in soybean

The amino acid sequence of the AP2 domain in the DREB protein is derived from 18 common soybean GmDREB genes with 59-60 amino acids. The PTPEMAARAYDVAALALKGPSARLNFPEL motif of the AP2 domain is present in all soybean DREB proteins. The results of comparing the AP2 domains of 18 DREB proteins in soybean all have 11 amino acids associated with DNA strands in the promoter region (DNA binding site) with 4 forms and the most common is RGRRWKERRWT, found in 13/18 DREB proteins. In addition, there are two forms KGRRNKERRWT and KGRRWKERRWT (present in 2/18 DREB proteins) and RGRRTKERRWT forms present in only one DREB protein.

3.1.3. Phylogenetic tree AP2 domain in soybean

Evolutionary analysis of the DREB protein subfamily based on the amino acid sequence of the AP2 domain in 69 DREB proteins of soybean using the Maximum Likelihood method and the JTT matrix-based model in MEGAX.



Hình 3.3. The AP2 domain phylogenetic tree of the DREB protein subfamily in soybean was established based on 69 amino acid sequences of the AP2 domain by the Maximum Likelihood method in MEGAX [87] and the JTT matrix-based model [78] with 1000 repeated bootstrap.

The results of analysis of AP2 domain evolution of DREB protein in soybean show that the phylogenetic tree of the AP2 domain is divided into 4 branches, in which there is only one branch with only one AP2 sequence of DREB2 protein (AAY89658) and the other three branches, each branch has a group of symbols I, II, III (Figure 3.3). Group, I include 29 AP2 sequences of the proteins DREB1, DREB1B, DREB1D, DREB1E, DREB1F, DREB3, DREB6, AP2-2, AP2-Ile-DBDP; group II includes 25 AP2 sequences of DREB, DREBA, DREB2A2, DREB2C-like, DREB3, DREB5 proteins; group III includes 14 AP2 sequences of DREB2 (13 sequences) and DREB1 (1 sequence) proteins.

3.2. DESING OF GmDREB6 GENE TRANSFORMED VECTOR AND ANALYSIS OF VECTOR ACTIVITIES ON TOBACCO

3.2.1. Construction of transgenic vector carrying *GmDREB6* gene

Synthesized artificial GmDRE

Based on the information of the *GmDREB6* gene with code EF551166 on GenBank, the artificial *GmDREB6* gene was designed with a size of 741 bp. In which, the coding sequence of the artificial *GmDREB6* gene has 693 nucleotides, adding 15 nucleotides as the cutting site of the

XbaI/SacI enzyme pair and 33 nucleotides encoding the cmyc tail, for a total of 741 nucleotides. The *GmDREB6* gene was inserted into the pUC18 vector (*pUC18_GmDREB6*).

Generating a transgenic vector carrying the GmDREB6 gene

The pBI121 transgenic vector contains the GUS gene (*pBI121_GUS*). The *Xba*l/*Sac*l enzyme pair was used to cut and remove the *GUS* gene from the *pBI121_GUS* vector, then insert the *GmDREB6* transgene into the pBI121 transgene, creating a *pBI121_GmDREB6* transgene carrier structure. The test results of the experiments on creating transgenic vectors are shown in Figure 3.6. Plasmid *pBI121_GmDREB6* extracted from E. coli DH5α was transformed into *A. tumefaciens*. Then recombinant *A. tumefaciens* was cloned, selected, and examined. Randomly selected 4 colony lines and analyzed by colony-PCR cloning the *GmDREB6* gene, resulting in a DNA band corresponding to the size of the *GmDREB6* transgene (Figure 3.7). Positive colonies of *A. tumefacines* were stored and used to infect tobacco and soybean to generate transgenic plants.

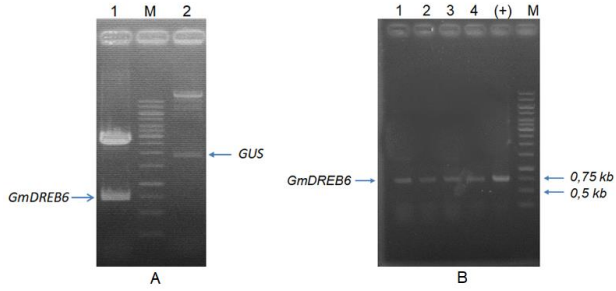


Fig. 3.6. A- Electrophoresis gel of cutting products from the pPU18_GmDREB6 and pBI121_GUS vector with *Sac*I/ *Xba*I enzyme pair. 1: pPU18_GmDREB6; M: DNA marker; 2: pBI121_GUS. B- Electrophoresis gel of colony-PCR product from *E. coli* DH5α colonies to test *GmDREB6* gene in *pBI121_GmDREB6* construct. M: DNA marker; (+): *GmDREB6* gene amplified from *pPU18_GmDREB6*; 1, 2, 3, 4: *GmDREB6* gene amplified from *E. coli* DH5α colonies.

Thus, the *35S-GmDREB6-cmyc* structure in the transgenic vector pBI121 was successfully designed and created recombinant *A. tumefaciens* AGL1 strains carrying the transgene structure *pBI121_GmDREB6*. The characteristics of the transgenic vector *pBI21_GmDREB6* are shown in Figure 3.8.

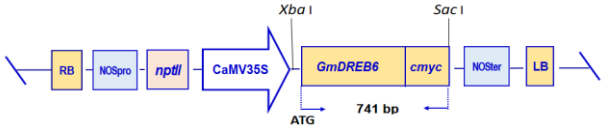


Fig. 3.8. Schematic diagram of the vector *pBI121_GmDREB6* used for gene transfer through Agrobacterium tumefaciens [126].
LB and RB: left and right border of T-DNA; nptII: neomycin-phospho-transferase II; CaMV35S: 35S promoter of cauliflower mosaic virus; GmDREB6_cmyc: The soybean DREB6 gene is associated with a nucleotide sequence encoding the cmyc peptide; XbaI and SacI: Recognition and cleavage sites of restriction enzymes XbaI and SacI

3.2.2. Structural transformation of pBI121-GmDREB6 into tobacco

Pieces of tobacco leaf that were about 1cm2 in size were cultured on MS media for 48 hours; after that, the leaf pieces were immersed in the *A. tumefaciens* suspension in 20 minutes. The bacterially infected leaf pieces were transferred to the co-cultivation medium, the multi-shoot regeneration medium, the rooting medium respectively, to create transgenic tobacco plants.

From 180 samples in three independent experiments, 539 shoots were induced on multi-shoot regeneration and shoot elongation media supplemented with kanamycin, in which 309 elongated shoots were rooted. And 101 rooted plantlets were planted on the pots, among which 48 plantlets were transferred to the greenhouse.

PCR analysis results of 13 transformed plants

PCR analysis results of 13 transformed plants with *XbaI-DREB6-F/ DREB6-SacIR* primer pair showed that nine positive plants with the emergence of a DNA band in the same size as *GmDREB6* (741 bp). The PCR- positive transgenic plants in the T0 generation were labeled as T0-4, T0-5, T0-6, T0-7, T0-8, T0-9, T0-11, T0-12, T0-13.

Conducting Southern blot analysis of 9 PCR-positive plants to confirm the fusion of the transgene *GmDREB6* into the transformed tobacco genome, the results showed that there were 8 T0 plants (T0-4, T0-5, T0- 6, T0-7, T0-9, T0-11, T0-12, T0-13) showed positive results with Southern hybridization, T0-8 plants did not appear DNA panel (Figure 3.11). Continuing to analyze the expression of the *GmDREB6* gene on 8 transgenic tobacco plants at Southern blot-positive transcription by RT-PCR, the results showed that 5 T0 plants were transcribed to produce mRNA, which is the trees T0-5, T0-7, T0-9, T0-12, T0-13.

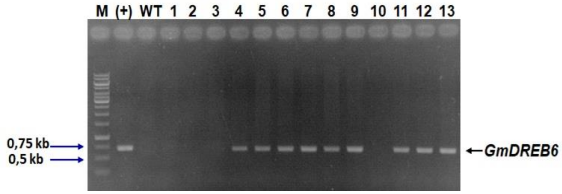


Fig. 3.10. *Image of electrophoresis results of PCR product cloning transgene GmDREB6 from transgenic tobacco plants in T0 generation. M: DNA scale 1 kb; (+): plasmid pBI121-GmDREB6; WT: non-transformed tree; 1-13: T0 generation transgenic plants (T0-1, T0-2, T03, T0-4, T0-5, T0-6, T0-7, T0-8, T0-9, T0-11, T0-12, T0-13)*

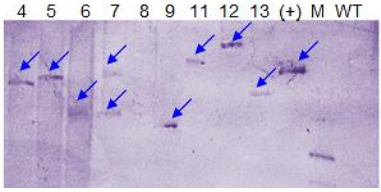


Fig. 3.11. Image of Southern blot analysis results examining the fusion of the transgene GmDREB6 into the genome of transgenic tobacco plants in the T0 generation. *M: DNA scale 1 kb; (+): plasmid pBI121-GmDREB6; WT: non-transformed tree; Lanes 4, 5, 6, 7, 8, 9, 11, 12, 13: Transgenic plants of the T0 generation (T0-4, T0-5, T0-6, T0-7, T0-8, T0-) 9, T0-11, T0-12, T0-13)*

The *35S-GmDREB6-cmyc* structure in the recombinant vector pBI121 was successfully transformed into tobacco plants mediated by *A.*

tumefaciens. The *GmDREB6* transgene was shown to be incorporated into the genome and expressed in transgenic tobacco at the transcriptional level.

3.3. ANALYSIS OF EXPRESSION OF *GmDREB6, NtP5CS, NtCLC* GENE IN TRANSGENIC TOBACCO BY REAL TIME qRT-PCR

3.3.1. Salt treatment of *GmDREB6* transgenic tobacco lines and WT plant.

In 5 lines of T0 plants (T0-5, T0-7, T0-9, T0-12, T0-13) that produced mRNA synthesis results, three good growing plant lines were selected, T0-5, T0. -9 and T0-13 to further analyze gene expression under salt stress by Real-time RT-PCR. The transgenic lines T0-5, T0-9 and T0-13 are denoted L1, L3 and L9, respectively. WT lines and three transgenic lines L1, L3, L9 with normal growth, development and uniform morphology were selected to handle salt stress in the grow room. Tobacco pots were irrigated with 50 ml of 200 mM NaCl daily for 3 weeks, then gene expression levels were analyzed by qRT-PCR. Controls were the GmDREB6 transgenic lines, and the non-transgenic plants were irrigated with 50 ml H_2O daily (Figure 3.13).

Fig. 3.13. Morphology of *GmDREB6* transgenic tobacco plants and nontransgenic plants under H_2O and NaCl irrigation in the plant growth cabinet after 3 weeks. *A: water 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: non-transgenic tobacco plants.*

Observation of experimental and control morphologies after 3 weeks showed that, in the watered plots (Figure 3.13A) the L1, L3, L9 transgenic plants had similar morphology, leaf color, and plant height; but in the

experimental plot (Figure 3.13B) irrigated with 50 ml of 200 mM NaCl, L1 plants showed stronger growth than L3, L9 and WT plants. The WT plants grew worse and had small, yellow leaves near the base, while the L1, L3, L9 transgenic plants had normal growth, with green, large leaves like those in the watered control plot. This expression also showed normal development of the transgenic tobacco lines under salt stress, while the WT plants showed yellow leaves, poor growth and stopped growth.

3.3.2. 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 3.14).

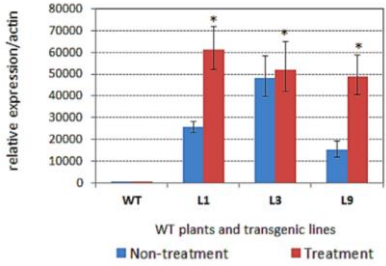


Fig. 3.14. xpression level of *GmDREB6* gene in transgenic tobacco lines under salt stress by qRT-PCR reaction using actin as reference gene *(P <0,05).*

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. Phân tích biểu hiện gen *NtP5CS* của cây thuốc lá chuyển gen (gen nội sinh), kết quả trên hình 3.15 cho thấy, trong điều kiện tưới nước bình thường (không xử lý NaCl), không có sự khác biệt về mức độ phiên mã giữa các dòng thuốc lá chuyển gen so với cây WT.

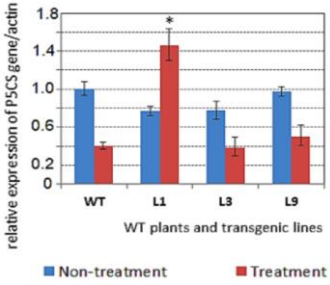


Fig. 3.15. Expression levels of NtP5CS gene in GmDREB6 transgenic tobacco lines under salt stress by qRT-PCR using actin as reference gene. *WT: non-transformed tobacco; L1, L3 and L9: transgenic tobacco lines. An asterisk (*) on each column represents a statistically significant difference (P <0,05).*

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. 3.15).

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. 3.16).

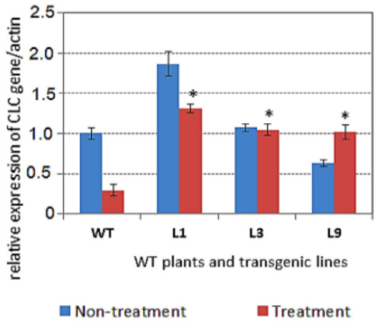


Fig. 3.16. Expression levels of NtCLC genes in *GmDREB6* transgenic tobacco lines under salt stress by qRT-PCR using actin as reference gene. *WT: non-transformed tobacco; L1, L3 and L9: transgenic tobacco lines. An asterisk (*) on each column represents a statistically significant difference (P <0,05).*

3.3.3. Discuss the results of *GmDREB6, NtP5CS, NtCLC* expression in transgenic tobacco lines

Previous studies have demonstrated that the DREB subfamily plays an important role in the plant salinity response through an ABA-independent pathway. Enhanced expression of the *DREB* gene triggered the expression of tolerance response genes under abiotic stress conditions. In

the results of our transgenic tobacco study at 50 ml 200 mM NaCl irrigation daily for 3 weeks, the transgenic tobacco lines expressed an increased GmDREB6 transcript level from 108.03 % to 321.95% compared with transgenic plants under normal watering conditions ($P < 0.05$). This result confirms the strong response of the GmDREB6 gene when transgenic tobacco plants receive salinity stress signals 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.) [97]. 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.

The chloride channel (CLC) protein is an important anionic carrier that exists in bacteria, yeasts, plants, and animals. 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. Expression of the CLC protein increased the transfer of Cl^- from the cytoplasm to the vacuole and created a tolerance to NaCl in the cells.

The results of expression analysis of *GmDREB6, NtP5CS*, and *NtCLC* genes on tobacco plants show that the *GmDREB6* gene is a potential

candidate that can be used to improve salt tolerance of plants, opening a new research direction. develop salt-tolerant crops in the context of climate change with increasing sea levels.

3.4. STRUCTURAL TRANSFER OF *GmDREB6* THROUGH *AGROBACTERIUM TUMEFACIENS* IN SOYBEAN CULTIVAR ĐT22

3.4.1. Transformation and generation of *GmDREB6* transgenic soybean plants from soybean cultivar ĐT22

The soybean cultivar DT22 is widely grown in the North of Viet Nam has a short growth period and is resistant to powdery mildew, however, the soybean cultivar DT22 together with cultivar DT12, DT94, W82, DT2003, DT2001, DT51, D2101, DT22, DT96, DT95, D8, DT90, DT83, DT84, DT30 were assessed to have low salt tolerance. In this study, the Viet Nam soybean cultivar DT22 was selected as a genetic recipient for implementation. Experimental transgenic *GmDREB6* to improve salt tolerance of this soybean cultivar.

The results of structural transformation carrying the *GmDREB6* transgene through infection with *A. tumefaciens* through the axils of the cotyledons are shown in Figure 3.17. In 450 transformed samples, repeated 3 times, 185 samples budged, and the total number of shoots was 583. Select 109 shoots to transfer to the rooting medium. As a result, 53 lines of T0 plants were transferred to the substrate and 12 lines of T0 plants survived in net house conditions.

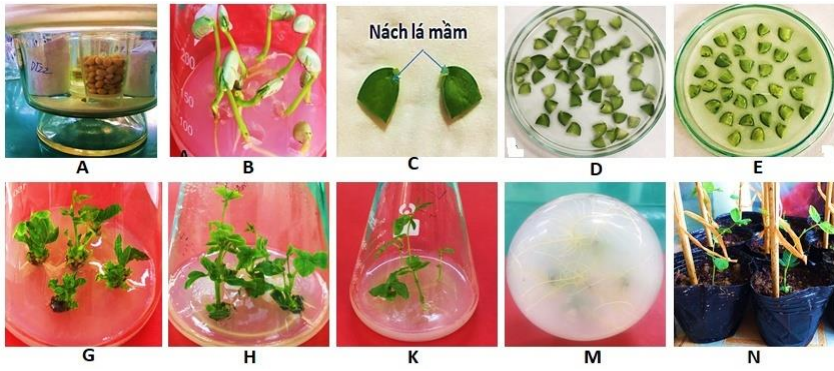


Fig. 3.17. Image of transformation and regeneration of transgenic soybean plants.

3.4.2. Analysis of the presence and transcription of *GmDREB6* transgene in transgenic soybean

The transgenic soybean plants in the T0 generation in the greenhouse were used to analyze the presence of the *GmDREB6* transgene in the transgenic soybean genome by PCR with the primer pair *GmDREB6_XbaI-F/GmDREB6_c-myc-SacI-R*. The results of electrophoresis analysis showed that the DNA band approximately 750 bp in size appeared in 8 transgenic soybean lines No. 1, 3, 4, 6, 8, 9, 11, 12, while lines No. 2, 5, 7, 10 did not appear DNA band. Continuing to analyze the transcriptional expression of 8 PCR-positive plants by RT-PCR reaction with primer pair *GmDREB6_XbaI-F/GmDREB6_c-myc-SacI-R*, the results are shown in Figure 3.19. In 8 T0 plants carrying RT-PCR analysis, 7 plants were positive, that is, T0 = 1, T0-3, T0-6, T0-8, T0-9, T0-11, T0-12 plants. Thus, the *GmDREB6* transgene expressed mRNA synthesis.

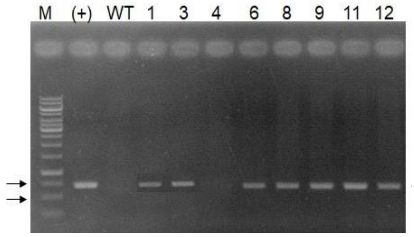


Fig. 3.19. Electrophoresis image of RT-PCR product amplified structure containing GmDREB6 gene in transgenic soybean genome. M: DNA scale 1 kb; WT; non-transformed soybean; (+) plassmid pBI121_GmDREB6 as a positive control; 1, 3, 4, 6, 8, 9, 11, 12: *GmDREB6* transgenic soybean lines.

3.4.3. Discussing the results of *GmDREB6* gene transfer in soybean

Research on gene transfer in soybean by *A. tumefaciens* containing the transgene transgenic structure through the damaged cotyledon has been published by many authors. In Viet Nam, Tran Thi Cuc Hoa (2007), Nguyen Thi Thuy Huong (2011), Nguyen Thu Hien (2011), Lo Thi Mai Thu (2014), Lo Thanh Son (2015), Nguyen HQ, et al (2019), Pham et al. (2020), also succeeded in gene transfer in soybean by infecting *A. tumefaciens* carrying the transgene structure through the damaged cotyledon. By this method, we also successfully transformed the vector *pBI121_GmDREB6* into the Vietnamese soybean cultivar DT22 with an average number of regenerated shoots of 3.15 shoots/sample. The results of

3 times of selection have selected 53 soybean plants grown on the substrate. Using PCR with primers *GmDREB6_XbaI-F/GmDREB6_c-myc-SacI-R* confirmed that the transgene *GmDREB6* was present in the genomes of eight T0 transgenic plants.

In the transgenic soybean genome, there are intrinsic *GmDREB6* gene and transgenic *GmDREB6* gene. The coding region of the intrinsic gene and the transgene *GmDREB6* is 693 bp in size, encoding 230 amino acids. If using PCR with primers designed to amplify the coding region, the only result is a DNA fragment of about 0.7 kb, and it is not possible to determine whether the *GmDREB6* transgene has been incorporated into the soybean genome. transgenic or not. Therefore, to analyze transgenic soybean plants, we designed PCR primer pairs to clone the DNA fragment including the *GmDREB6* gene, the sequences containing the XbaI and SacI restriction enzymes. In the transgenic vector *pBI121_GmDREB6*, the *GmDREB6-c-myc* construct consists of a *GmDREB6* gene fragment, containing a 693 bp coding region, an 8 bp (GCTCTAGA) fragment at the 5' end containing the cleavage site for XbaI, a sequence of 33 bp in size. encodes for c-myc antigen, and the 7 bp fragment (GAGCTCG) in the 3' end contains the cleavage site for SacI. Thus, the *pBI121_GmDREB6_cmyc* structure is 741 bp in size. The PCR primer pair *GmDREB6_XbaI-F/GmDREB6_c-myc-SacI-R* was designed to amplify the structure *GmDREB6-c-myc* and the results of agarose gel electrophoresis showed a single DNA band with a size of about 0,75 bp (Figure 3.15). At the same time, the results of RT-PCR analysis identified the *GmDREB6* transgene expressed in 7 T0 soybean plants. The efficiency of *GmDREB6* gene transfer at the PCR analysis stage was 1.78% and the RT-PCR analysis stage was 1,56%.

CONCLUSIONS AND RECOMMENDATIONS

1. Conclusions

1.1. Eighteen *GmDREB* genes belonging to the *DREB* subfamily in the soybean genome were identified from the NCBI database. Copies of GmDREB genes are distributed on 17 chromosomes, of which *GmDREB3* has 8 copies, the rest of other *GmDREB* genes have 1-4 copies. The AP2 domain of the ubiquitous DREB protein has 59-60 amino acids and the

PTPEMAARAYDVAALALKGPSARLNFPEL motif is present in all soybean DREB proteins. AP2 contains 11 promoter binding sites of functional genes, with 4 forms and the most common is RGRRWKERRWT (present in 13/18 DREB proteins).

1.2. The plant transgenic vector *pBI121_GmDREB6* containing the *35S-GmDREB6-cmyc* construct was designed and transformed into tobacco. The incorporation and expression of the *GmDREB6* transgene in the transgenic tobacco genome were determined by Southern blot analysis and RT-PCR.

1.3. By qRT-PCR analysis, it was demonstrated that the expression of the *GmDREB6* gene from soybean increased the transcript levels of *NtP5CS* and *NtCLC* genes of transgenic tobacco under saline treatment. The L1 and L9 transgenic tobacco lines had an increased *GmDREB6* gene transcription level from 2.40 to 3.22 (fold) compared with the untreated condition. Compared with WT plants, the transcription level of the *NtP5CS* gene in transgenic lines increased from 1.24 to 3.60 times and of *NtCLC* gene increased by 3.65 - 4.54 times ($P < 0.05$). Simultaneous enhancement of expression in both the *GmDREB6* transgene and two endogenous genes, *NtP5CS* and *NtCLC*, was confirmed in L1 transgenic tobacco lines.

1.4. The structure carrying the *GmDREB6* gene was successfully transformed into the soybean cultivar DT22 through the axils of the cotyledons thanks to *A. tumefaciens*, which regenerated 12 transformed plants with normal growth under net house conditions. Molecular analysis of the *GmDREB6* transgenic lines in the T0 generation identified 8/12 PCR-positive lines and the *GmDREB6* transgene in 7 lines showed transcriptional expression. The efficiency of *GmDREB6* gene transfer at the PCR analysis stage was 1.78% and the RT-PCR analysis stage was 1,56%.

2. Recommendations

Further research is needed to clarify the function of some *DREB* genes in the soybean genome experimentally. Continue to analyze the recombinant protein expression of *GmDREB6* transgenic soybean lines to select and create soybean lines with high salinity capacity.