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CHARACTERIZING AND DESIGNING PROMOTER OF GENE *ZmDREB2.7* INTO PLANT EXPRESSION VECTOR

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ABSTRACT

In the regulation of gene expression, particularly abiotic-stress tolerance characteristics in plants, inducible promoters play an essential role in ensuring the endurance and productivity of crops. In several previous studies, the *ZmDREB2.7* gene and some DNA polymorphisms in its promoter have been shown to enhance drought tolerance traits in maize (*Zea mays* L.). With the goal of creating a strong drought-tolerant promoter region derived from maize, in this study, the 1 kilobase 5'-upstream region of the *ZmDREB2.7* coding sequence from the drought resistant maize line Tevang1 was isolated and characterized. Essential putative *cis*-regulatory elements (as TATA box or CAAT box) and other elements that regulate drought tolerant properties (as RY/G box, ABRE element or MYB core) were investigated. After that, we designed the transgenic plant vector containing the isolated promoter and the *ZmDREB2.7* gene which were isolated in our earlier study. The promoter segment was removed 20 nucleotides ahead of the transcription start site and transferred into the recombinant pZY vector containing the *ZmDREB2.7* gene. In conclusion, we created a recombinant vector containing the modified ZmDREB2.7 promoter and the *ZmDREB2.7* gene segment derived from maize. This is a promising resource for the creation of a transgenic cultivar with effective resistance to dry stress in the future. Besides, we will keep on examining other potential genetic regions and promoters to diversify sources of genetic materials as well as understand deeper the stress-resistant transgenic plants.

Keywords: *Zea mays* L., drought tolerance, ZmDREB2.7 promoter, genetic modification, transgenic engineering.

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INTRODUCTION

Promoters, the most important factor in the regulation of gene expression, are divided into three groups: constitutive promoters, tissue-specific (or development-stage-specific) promoters and inducible promoters. The inducible promoters only express under a certain condition or in response to effects such as light, temperature, or chemicals. Early in the construction of genetically modified plants, the constitutive one is preferred and used for most studies in different species. The tissue-specific promoters are also preferred for protein generation that requires expression in certain tissues such as seeds or xylem (Yu et al., 2005; Huynh et al., 2016). However, the inducible promoters have the advantage of being able to control the timing and intensity of gene expression to prevent nutrient and energy wastage, demonstrating the potential in creating traits to help plants cope with non-biological disadvantages that do not affect plant growth and development under normal conditions (Xiao et al., 2000; Huang et al., 2005; Cominelli et al., 2008; Msanne et al., 2011).

Different *ZmDREB* genes vary in expression levels in various tissues and at separate developmental stages, as well as in response to dehydration. When studying the expression of *ZmDREB* genes in various tissues at different developmental stages, we were surprised to find numerous different cases of gene expression. Although the *ZmDREB2.7* gene had a very low expression level in various tissues and was induced only slightly in the root and leaf tissue under lack of water conditions, the ZmDREB2.7 protein was highly capable of transcriptional activation among ZmDREB proteins. At the same time, mutations in the *ZmDREB2.7* gene were considered to be significantly related to this tolerant phenotype in maize. Therefore, it has been suggested that *ZmDREB2.7* is a crucial gene in maize drought response independent from abscisic acid (Shengxue et al., 2013). Under mild stress and onset of stress, the expression level of the *ZmDREB2.7* gene was linearly proportional to survival. However, under adequate watering or extreme desiccation, there was no significant relationship between gene expression levels and viability. Early induction of the *ZmDREB2.7* gene in response to stress is crucial, which is extremely important in maize under water scarcity. The most important factor that plays a central role in regulating this trait lies in the untranslated region (UTR) is the promoter of the gene. For example, when studying *ZmDREB* genes polymorphism in maize based on SNPs, the *ZmDREB2.7* gene had a number of polymorphisms in which the 20-nucleotide indel preceded the starting point of transcription with important implications related to tolerance and the drought tolerant lines do not have these polymorphisms (Liu et al., 2013). In a previous study, we used the RD29A promoter to regulate the *ZmDREB2.7* gene (Huynh et al., 2019). It is also an inducing promoter, derived from *Arabidopsis thaliana*.

In this study, we studied the ZmDREB2.7 promoter segment of Q30 maize and modified it to lose 20 nucleotides preceding the transcription initiation point, in order to design plant transformation vectors containing the *ZmDREB2.7* gene and the ZmDREB2.7 promoter with similar characteristics as the water-scarcity adapt lines that have been studied.

MATERIALS AND METHODS

Plant materials

We used the Tevang1 maize cultivar (Q30) that was provided by the Maize Research Institute, Vietnam Agricultural Science Institute. This cultivar is well known for its good drought tolerance characteristics in some mountainous regions in northern Vietnam.

DNA extraction

Total DNA was extracted from leaf tissue according to the method of Roger et al. (1989) with some modifications to our samples. The extraction buffer consisted of 100 mM Tris

pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 0.2% (v/v) β-mercapto-ethanol, 1% PVP, 2% CTAB. Up to 3 g of fresh leaves was ground into a fine powder in liquid nitrogen, then 15 ml of extraction buffer were added and mixed well. The mixture was incubated at 65 ºC for 1 hour, then kept at room temperature for 15 minutes and centrifuged at 10,000 rpm at 4 ºC for 10 minutes. Protein and impurity were removed by the mixture of phenol/chloroform/isoamyl alcohol (25:24:1). The resulting composition was treated with RNase and centrifuged at 12,000 rpm at 4 ºC for 20 minutes before being purified with a chloroform/isoamyl alcohol (24:1) mixture. After centrifugation, the above solution was collected and precipitated at -20 ºC for at least 3 hours with 100% EtOH and 0.3 M CH_3COOH. Precipitation was collected after centrifuges at 12,000 rpm and 4 ºC for 20 minutes and was dried by SpeedVac and re-dissolved in DNase-free water. The DNA product was stored at -20 ºC.

ZmDREB2.7 promoter isolation

The upstream sequence region of the *ZmDREB2.7* gene was isolated by Polymerase Chain Reaction (PCR) using total DNA as a template and the specific primer pair ProZmDREB2.7F and ProZmDREB2.7R (Table 1). The primer pair was designed based on maize genome data published in the Maize genetics and genomics database (http://maizegdb.org). The PCR amplification steps were described according to Sambrook et al. (2001) with some adjustments to optimize the reaction conditions, as follows: 25 µL of total volume, containing 50 ng of total DNA, 2.5 µM each primer, 1 unit Taq DNA Polymerase, 1 mM dNTPs and corresponding buffer. A thermal cycle of the PCR reaction consisted of one denaturation cycle of 95 ºC/3 minutes, 35 cycles (95 ºC/30 seconds; 52 ºC/30 seconds; 72 ºC/1 minute) and the final synthesis step 72 ºC/5 minutes. The PCR product was purified by GeneJET^{TM}Gel Extraction Kit and checked by electrophoresis on 0.8% agarose gel to ensure the quality of the amplified product.

Sequencing and sequence analysis

After the purification quality was ensured, the ZmDREB2.7 promoter (ProZm2.7) was sequenced by the ABI 3500 Genetic Analyzer system with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), based on Sanger's principle. The composition of the reaction consisted of 200 ng DNA, 3.2 pM primers respectively, Big Dye and the corresponding buffers with a total volume of 15 µL. Thermal cycle for GeneAmp™ PCR System 9700 was as follows: 96 ºC - 1 minute, (96 ºC - 10 seconds, 50 ºC - 5 seconds, 60 ºC - 4 minutes) × 25 cycles.

Nucleotide sequence data were examined using SeqScape 2.5, Sequencing Analysis 5.2, BioEdit software and NCBI web BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequence was compared with the reference gene from B73 RefGen_v4 on the Maize database (http://maizegdb.org). Putative *cis*-acting elements in a sequence were analysed using the PLACE database (https://www.dna.affrc.go.jp/PLACE/).

Plasmid DNA extraction

Bacteria were grown overnight in a test tube containing 2 mL of liquid LB medium with suitable antibiotics at 37 ºC on a shaker 200 rounds per minute (rpm). Collect bacteria by centrifuge at 6,000 rpm for 6 minutes, then adding 150 µL Solution II and III. Centrifuge 12,000 rpm at 4 ºC, 15 minutes; collect suspension and add 1,000 µL EtOH 100 %. The next steps for precipitation and DNA collecting were similar to Section 1.

After extraction, the plasmids were checked on 0.8% agarose gel. Restriction enzyme digest reaction was performed to verify the plasmids capable of carrying the DNA segment of interest, which were higher than the control line. This reaction consisted of 20 ng of plasmids, buffer solution, distilled water, conducted at the right temperature and time conditions for the restriction enzymes. The product was checked again by electrophoresis.

Transformation of the recombinant vector into competent bacterial cell by electric pulse method and selecting the line containing the vector of interest

The competent cell was cooled for 30 minutes. About 200 ng of Ti-plasmid (already purified to remove salt) was added and cooled for 10 minutes, then transferred into an electrical impulse cuvette (sterilized by direct UV light). An electric pulse was generated at 25 Ω, 2.5 V, 400 W. 800 µL Super Optimal Broth medium was added and shaken for 2 hours at 28 ºC. Cells were spread-inoculated on a culture plate containing YEB medium (with the selected antibiotic of the corresponding vector) and incubated overnight at 28 ºC or 37 ºC. Plasmids were re-examined by PCR reaction and those capable of carrying the inserted vector were checked by restriction enzymes. Cell lines containing the recombinant vector were re-cultured in large numbers, and the plasmid was isolated and preserved at -80 ºC for further study.

RESULTS AND DISCUSSION

Isolation and sequence analysis of ZmDREB2.7 promoter

Total DNA was extracted from young leaves of Q30 maize which is known for its stable drought tolerance. The good DNA quality allowed us to use it as a template to further isolate the ZmDREB2.7 promoter by PCR, using ProZmDREB2.7F and ProZmDREB2.7R primers (Table 1). The PCR product obtained after purification is approximately 1.0 kb in length, equivalent to the theoretically calculated size (Fig. 1).

Table 1. List of primers

Primer name	Sequence (5'-3')	Length
ProZmDREB2.7 F	GGG TTC GCC CAA TTC CC	1,077 bp
ProZmDREB2.7 R	TGC ATC GAG CCA CCT GC	
ProZm2.7 *Hind*III F2	A GCT AAG CTT CCA TTG CCA TAT ACG	997 bp
ProZm2.7 *Sac*I R	ACT GAG CTC GCA GAT CGA TAT GG	
Zm2.7 *Sac*I F3	ATA GAG CTC ATG GAT CGG GTG CCG CCG CCG G	1,089 bp
Zm2.7 *Sac*I R2	ATA GAG CTC TCA AAG AGG GAC GAC GAG CTG C	
Restriction enzyme recognition sites are underlined		

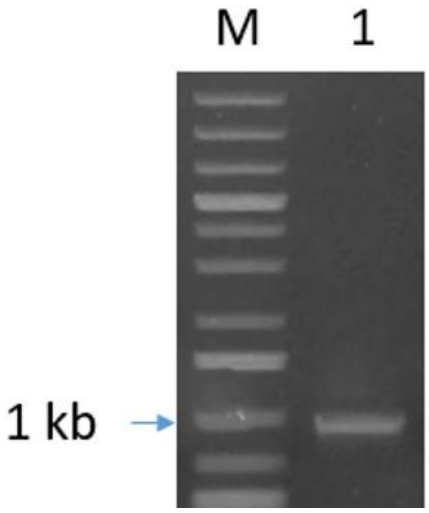


Figure 1. ZmDREB2.7 promoter amplified and purified result;
M: 1 kb DNA Ladder; 1: PCR product

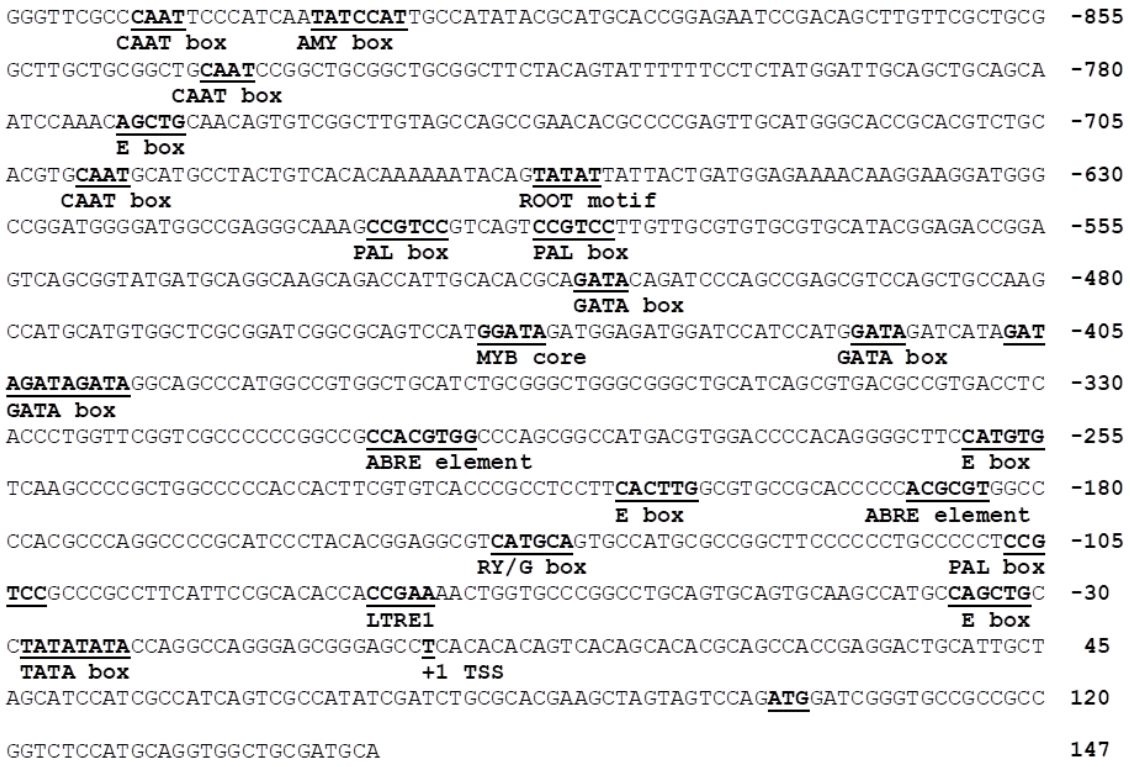


Figure 2. Basic *cis*-element factors in the ZmDREB2.7 promoter region

Table 2. Essential *cis*-regulatory elements in the ZmDREB2.7 promoter

Elements	Sequences	Position	Functions
TATA box	TATATATA	-29/-22	*cis*-regulatory element
CAAT box	CAAT	-921/-918, -841/- 838 and -700/-697	*cis*-regulatory element
E box	CANNTG	-768/-773, -261/-256, -211/-206 and -37/-32	*cis*-regulatory element
AMY box	TATCCAT	-908/-902	Related to amylase expression
PAL box	CCGTCC	-604/-599, -592/-587 and -108/-103	Related to phenylalanine ammonia-lyase expression
GATA box	GATA	-514/-511, -419/-416, and -408/-397	Related to light responsiveness and tissue specific expression
MYB core	GGATA	-446/-441	Involved in the water stress responsiveness
ABRE element	ACGCGT or CCACGT	-304/-299 and -190/-185	Involved in the abscisic acid responsiveness
RY/G box	CATGCA	-145/-140	Involved in the abscisic acid responsiveness
LTRE1	CCGAA	-79/-75	Related to low-temperature responsiveness

The ZmDREB2.7 promoter segment was then sequenced, resulting in a 1 kb read. After sequencing, the promoter segment was analyzed for the putative *cis*-acting regulatory elements using the analysis tool in the PLACE database. Figure 2 demonstrates

the isolated sequence contains promoter region (-930/+127), in which putative transcription start site (+1) and potential *cis*-acting elements are noted. The length from the transcription start site to the initiation codon (ATG-Met) of the *ZmDREB2.7* gene is 80 nucleotides. The main domains of a promoter, such as the TATA box and the CAAT box, were all identified in relative locations that were consistent with the properties of an eukaryotic promoter. In addition, a number of domains specific to an inductive promoter were found to be specified for many different characteristics; particularly those *cis*-regulatory elements with drought-resistant properties, abscisic acid responsiveness and water-stress resistant have been studied in previous research, such as RY/G box, ABRE element or MYB core. Several other *cis*-element factors were found on this promoter: LTRE1, GATA box, PAL box, etc. related to other properties have also been noted.

Details of the putative *cis*-regulatory elements are fully listed in Table 2. Although this promoter segment needs further verification for its ability to regulate genes under water-shortage conditions, we expect this will be a valuable resource for research into generating new drought tolerant crop varieties through genetic engineering.

pZY/ProZm2.7-ZmDREB2.7 vector design

After sequence analysis, *cis*-element recognition and referencing the results in Liu et al. study as mentioned; we decided to remove 20 nucleotides ahead of the transcriptional start site. Thus, the generation of a recombinant vector containing the *ZmDRE2.7* gene and the modified ProZm2.7 promoter and this modified promoter ProZm2.7 corresponding to the gene was the aim of the study. Besides, Liu et al. (2013) had reported higher endurance in the water-shortage condition of maize containing DNA polymorphisms, significantly 20-bp InDel upstream of the start codon, compared with other sensitive cultivars. Therefore, the ProZm2.7 promoter was synthesized based on the sequence we had isolated with 20 nucleotides elimination as designed and contained in the pUC cloning vector. Comparing the sequence of the modified promoter showed 95% similarity with the 5' UTR of the published gene from the B73 cultivar (Fig. 3). This synthetic promoter is expected to carry both the pre-existed drought resistance trait of the Q30 variety, as well as the strengthening tolerance in Liu's study.

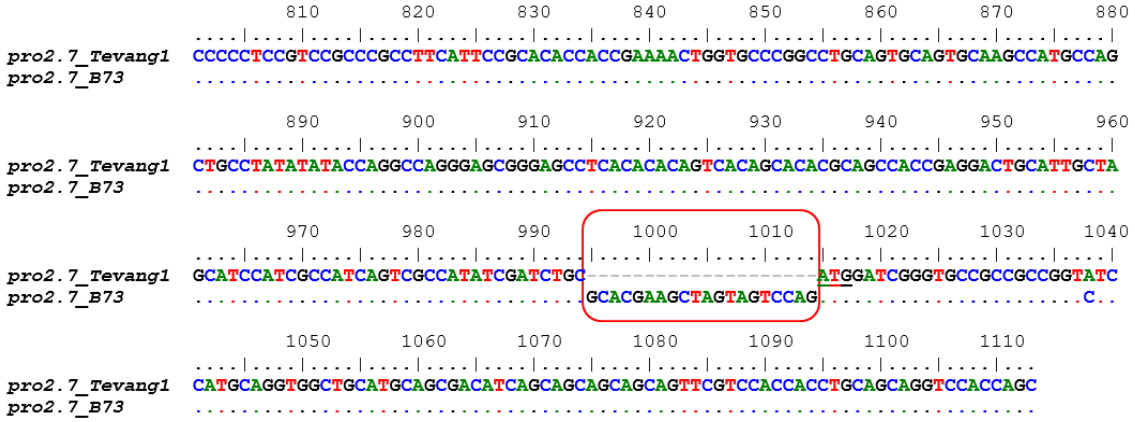


Figure 3. Sequence alignment between ZmDREB2.7 promoter isolated from Q30 and reference B73 maize line

This vector was used as the template for PCR reaction using ProZm2.7 *Hind*III F2/*Sac*I R primer pair to produce ProZm2.7 segment with *Hind*III and *Sac*I enzyme cut-off points

at both ends. Product about 1 kb in size, equivalent to a 997-bp-length theoretical ProZm2.7, replaced into the RD29A promoter position in the vector pZY/RD29A by two enzymes, *Hind*III and *Sac*I. Through screening by treatment with these two restriction enzymes, we have selected recombinant plasmid lines containing ProZm2.7 (Fig. 4). Thus, the pZY/ProZm2.7 vector was successfully selected as a material for the addition of the *ZmDREB2.7* gene. This designed diagram is shown in Figure 5.

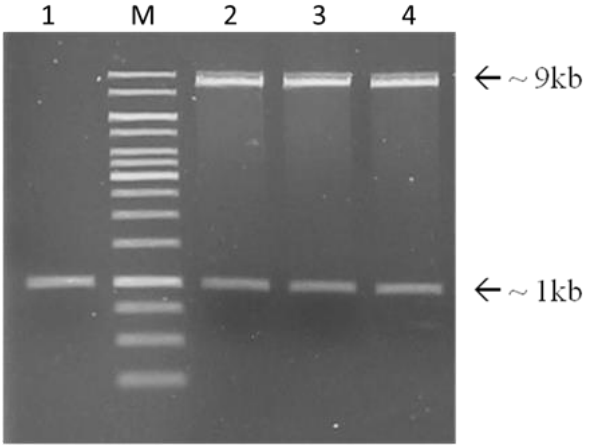


Figure 4. pZY/proZm2.7 vector screening result; M: 1 kb DNA ladder; 1: pUC-proZm2.7 PCR product with ProZm2.7 HindIII F2/SacI R primer pair; 2-4: Products treated with a restriction enzyme of plasmids from corresponding lines

With the ZmDREB2.7 gene segment had already cloned from our study (Huynh et al., 2019), we performed a PCR reaction using Zm2.7*Sac*I F3/R2 primer pair to make a recognizable position of the *Sac*I enzyme at both ends of the sequence. The PCR product was then treated with the *Sac*I enzyme. At the same time, vector pZY/proZm2.7 was also processed with the enzyme above. The product containing both the gene segment and the cyclic open vector was transformed into *Escherichia coli* bacteria. Colonies were selected randomly for plasmid isolated examination. We selected three plasmids to perform a PCR reaction test with two primer pairs: (1) Zm2.7 *Sac*I F3/R2 was used to check for the presence of genes and (2) proZm2.7 *Hind*III F2/*Sac*I R2 primers was used to check gene direction. Theoretically, if the plasmid containing the *ZmDREB2.7* gene segment is inserted correctly, the PCR product with the primer pair Zm2.7*Sac*I F3/R2 is approximately 1.1 kb, while the PCR product with the primer pair proZm2.7 *Hind*III F2/*Sac*I R2 will present a band of approximately 2 kb in size. The results in Figure 6A show that PCR products from plasmid numbers 1 and 3 have the correct size as expected. Thus, we have successfully designed a vector pZY/proZm2.7-*ZmDREB2.7* with a 2,400 bp insertion, including ZmDREB2.7 promoter, 35S terminator and coding sequence of *ZmDREB2.7* gene segment with 1,080 bp length. The total length from the left to the right border of the T-DNA repeat is 4,320 bp (Fig. 5).

This vector was further transformed into the *Agrobacterium tumefaciens* EHA105 competent cell. Screening results by PCR amplification experiment for *ZmDREB2.7* gene showed that except for colony number 3, the remaining colonies appeared a product band of approximately 1.1 kb in size, equal to theoretical size (Fig. 6B). The electrophoresis outcome proves that we have successfully obtained transformation *A. Tumefaciens* carrying vector pZY/proZm2.7- *ZmDREB2.7*. The plasmid, hypothetically, contains this vector is about 11 kb (Fig. 7).

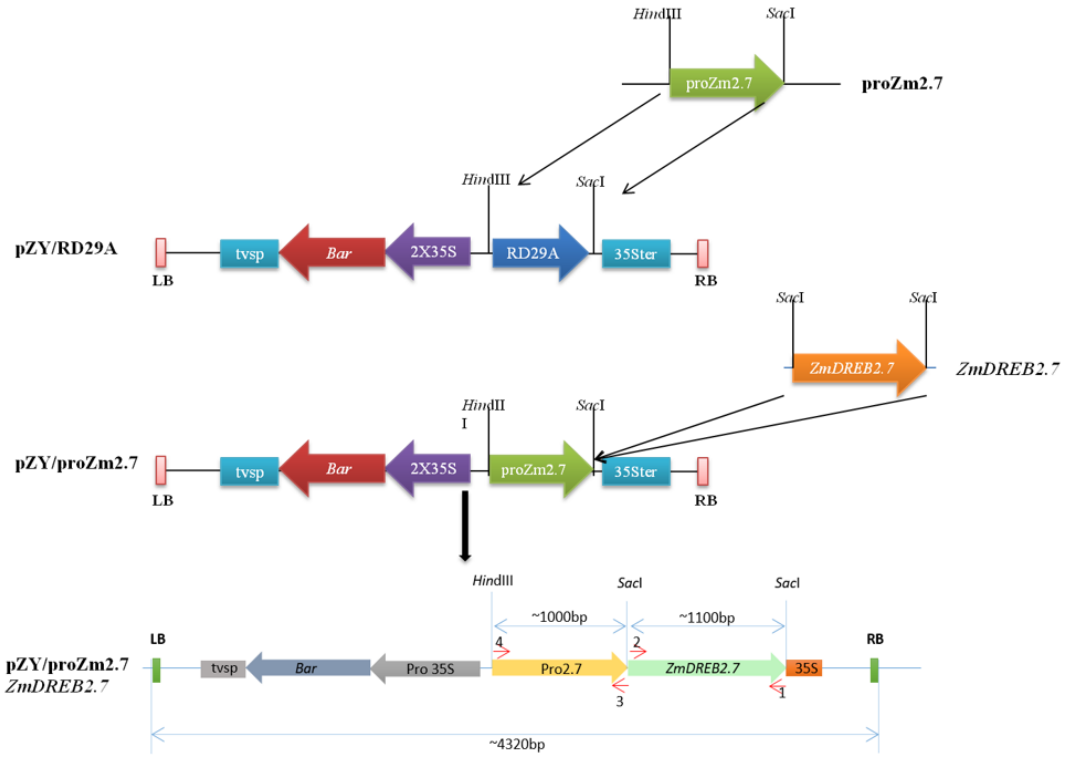
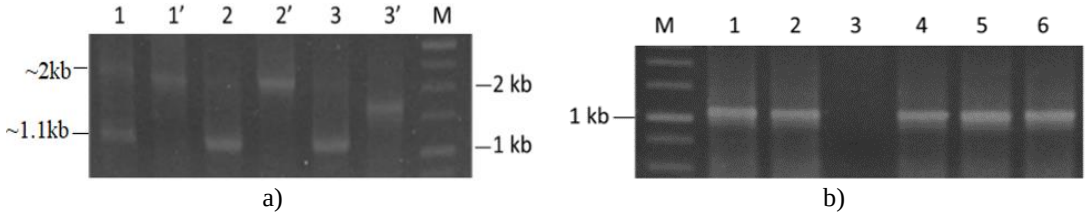


Figure 5. Schematic diagram of pZY::proZm2.7::*ZmDREB2.7* structure; List of primer: 1: Zm2.7SacI R2, 2: Zm2.7SacI F3, 3: proZm2.7SacI R, 4: proZm2.7HindIII F2

From this transgenic vector, we expect to create transgenic plant lines with favourable drought tolerance properties as the Q30 cultivar, while also retaining well-growth characteristics in the original line. This is a valuable genetic material with high exploitation value for further in-depth research purposes on genetic function, as well as the creation of Vietnamese-branded drought-tolerant plant lines. This available resource for the creation of genetically modified plants would help to reduce costs, workload and encourage the development of similar studies. Therefore, in the future, we will continue to follow up investigation to transfer this recombinant DNA into other plant varieties, in order to create a Vietnamese-brand drought tolerant line.



a) b)

Figure 6. The structure of proZm2.7::ZmDREB2.7::35Ster into the pZY vector; a) PCR checks for the presence of genes and the size of the recombinant plasmid lines. M: 1 kb DNA Ladder; 1, 2, 3: PCR products of Zm2.7SacIF3/R2 primer pair; 1', 2', 3': PCR products of proZm2.7 HindIII F2/SacI R2 primer pair; b) PCR screening for A. tumefaciens line containing pZY/proZm2.7-ZmDREB2.7 vector. M: 1 kb DNA Ladder, 1-6: PCR products of ZmDREB2.7 gene amplifying by corresponding colonies

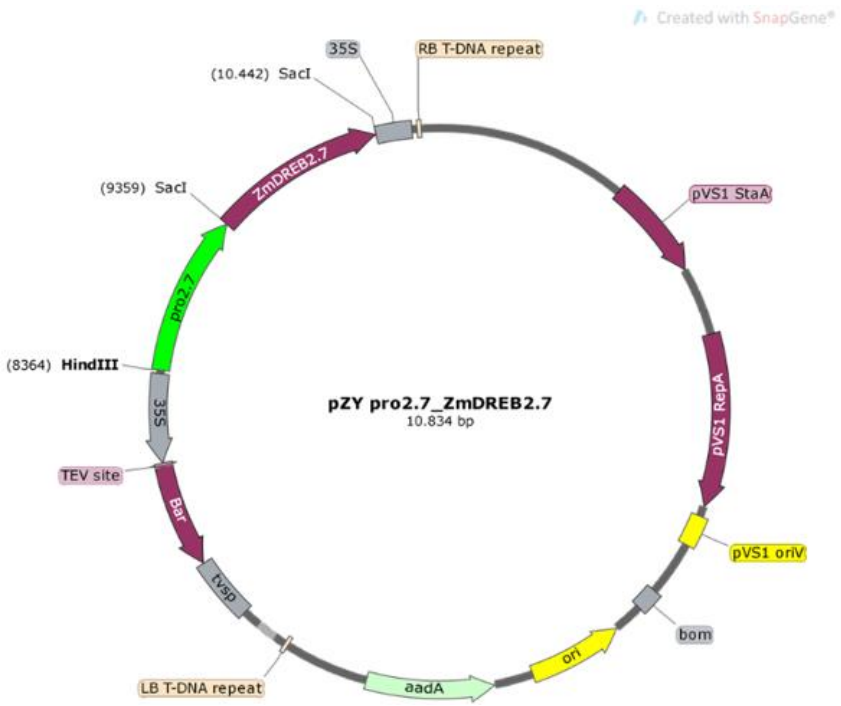


Figure 7. The full structure of the pZY::proZm2.7::*ZmDREB2.7* vector

In the context of the increased global water crisis and climate change, results such as in this study will be the available materials for selecting and improving plant varieties, enhanced vitality and yield under general adverse conditions, and water-stress survivability in particular. In the future, these highly adaptive crop varieties promise to contribute to the food security capabilities of Vietnam and the world.

CONCLUSION

In this study, we designed specific primers to successfully isolate the promoter segment of the *ZmDREB2.7* gene from the Q30 maize line with a stable drought tolerance trait. Hypothetical *cis* factors on this promoter were analyzed and showed great drought tolerance potential demonstrated in previous studies, as well as a number of potential *cis*-regulatory elements in tolerance ability against other abiotic stress. Next, we constructed the pZY vector carrying the ProZm2.7 promoter and the above gene sequence, and then successfully transformed it into the *A. tumefaciens* competent cells. This is a valuable source of genetic material for creating new plant varieties with good water-scarcity resistance and still retaining the advantages of the original cultivar.

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