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ISOLATION AND CHARACTERIZATION OF A *DREB* HOMOLOG GENE FROM A LOCAL DROUGHT-TOLERANT MAIZE CULTIVAR

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Clarifying the genetic background of the drought-tolerance trait is a crucial task that may help to improve plant performance under stress by a genetic engineering approach. Dehydration-responsive element-binding protein (DREB) is a transcription factor family which modulates many stress-responsive genes. In this study, we isolated a *DREB* homolog gene named *ZmDREBtv* from *Zea mays* var. *Tevang-1*. Using bioinformatic tools, a number of InDels and SNPs in *ZmDREBtv* sequence different from the reference accession were identified. In addition, based on deduced protein sequence similarity, ZmDREBtv was assigned to transcription factor DREB2 class as featured by a conserved DNA binding domain – AP2. The *ZmDREBtv* construct under thecontrol of the rd29A promoter was transformed into a drought-sensitive maize plant, K7 line. The transgenic plants were assessed with reference to molecular and phenotypic characteristics related to the drought-tolenrance trait. The results proved that the maize plants carrying *ZmDREBtv* gene showed enhanced tolerance and better performance to the water-deficit environment at different stages, compared to the wild-type plants.

Keywords: drought tolerance, maize, transformation, *DREB* gene, *Tevang-1* cultivar

INTRODUCTION

Nowadays, several approaches are used to improve crop ability to stand against water stress. There are two ways to achieve it: the genetic approach that creates stress-resistant crops and the agronomic approach reducing the chance that the plant will face stresses. The latter manages to increase water reservation and decrease water loss of farming systems by means of irrigation and planting practices. Unfortunately, because of limited water supplies and the increasing demand from non-agricultural sectors (Gleick, 2003), there is little opportunity to increase crop yield via this conventional way. Therefore, crops will need to be improved through the genetic approach. One of such approaches is traditional breeding which is labor-intensive and time-consuming. Meanwhile, genetic engineering, which transfers genes/alleles/traits from one plant to another, exhibits many advantages (Yang et al., 2010). In addition, the fundamental knowledge about pathways and specific genes involved in drought tolerance is expanding rapidly (Todaka et al., 2015). This holds the potential that genetic engineering can manifestly improve drought-tolerant phenotypes in crops in general, and in maize in particular. In fact, several promising maize transgenic events which are likely to enhance yield stability under water-deficit conditions were created (Shou et al., 2004; Castiglioni et al., 2008; Shi et al., 2017).

Drought resistance is a complex trait of many genes, including those with products

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such as functional proteins, e.g., osmotin, chaperones, late embryogenesis abundant proteins (LEA) and those encoding transcription factors (TFs) and proteins involved in signal transduction (Umezawa et al., 2006). The TFs are proteins that can specifically bind to *cis*-acting elements in the promoter region of downstream genes. The interaction might up/down-regulate the stress-related genes, thus contributing to stress tolerance. The stress-induced TFs are classified into many families, such as basic leucine zippers (bZIPs), WRKYs, MYBs, NACs, AP2/ERFs (APETALA 2/ethylene-responsive element binding factors). Among them, AP2/ERF is one of the largest families that modulate many abiotic stress-responsive genes. The DREB TFs belonging to the AP2/ERF consist of two classes, DREB1, and DREB2 (Umezawa et al., 2006).

Even though DREB1 and DREB2 proteins are similar in DNA binding domain and interact with the same promoter *cis*-element, each DREB group functions in response to different abiotic stimulations (Yamaguchi-Shinozaki and Shinozaki, 2006). The two TFs classes induce targeted genes under low temperature and dehydration conditions, respectively. Therefore, it is important to clarify the participation of DREB genes in resistance pathway of plants by cloning and studying their structures and functions. In maize, the two first isolated *DREB* genes were *ZmDREB1A* and *ZmDREB2A*. It was found that *ZmDREB1A* has a similar function to *DREB1s/CBFs* in *Arabidopsis* that plays a role in response to drought, high salinity, and low-temperature conditions. Meanwhile, ZmDREB2A can induce genes involved not only in dehydration but also in temperature changing (Qin et al., 2007). A microarray analysis on the ZmDREB2A-transgenic plants revealed 44 genes whose expressions increased by more than sevenfold. Those up-regulated genes included LEA proteins which protect plant cells from desiccation and salt stresses. Liu et al. (2013) identified *DREB* genes by genome sequencing, then cloned ten *ZmDREB1* genes (*ZmDREB1.1-1.10*) and eight *ZmDREB2* genes (*ZmDREB2.1-2.8*) from the B73 line. The phylogenetic relationship of DREBs was revealed, as well as their expression patterns under drought conditions. However, the information about homologs of genes in the valuable local cultivars that tolerate drought is limited. Therefore, our study aimed to characterize a *DREB* homolog gene from *Tevang-1* maize in Vietnam.

The *Tevang-1* maize cultivar was found in high-altitude rocky mountains located in the north of Vietnam, which are characterized by low temperature and annual precipitation. Because it grows in the water-limited environment, this variety adapts to drought and becomes a valuable genetic resource for crop improvement. Here, we isolated a *DREB* homolog gene from *Z. mays* var. *Tevang-1* and analyzed the sequence. Then, the *Tevang 1*-derived *DREB* homolog gene was employed to create maize transgenic events via *Agrobacterium*-mediated transformation. The gene of interest was inserted into the pCAMBIA1300 expression vector and driven by the drought-inducible rd29A promoter. Transgenic plants were assessed at the molecular level and with respect to agronomic characteristics related to the drought tolerance trait.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of *Z. mays* var. *Tevang-1* collected from Dien Bien Province, Northern Vietnam were germinated in a climate chamber at 25°C with day length of 12 hours to the three-leaf stage. Genomic DNA was extracted from the leaves of *Tevang-1* maize and used for gene cloning. Meanwhile, embryo donor and pollen donor plants, which were drought-sensitive K7 genetic background, were grown in a greenhouse with suitable conditions (temperature of 25°C, day length of 12 h). Fifty-five to 60 days after germination, embryo donor plants were sib-pollinated to produce embryo donor ears for transformation. White or light yellow pollen from sibling plants was used for pollination. After 9–13 days, ears could be harvested to supply immature embryos for the maize transformation experiment. Transgenic plants were grown in a greenhouse and monitored carefully. They were self-pollinated to produce progeny. Mature ears were harvested and seeds were preserved under the proper conditions. *Escherichia coli* DH10β, *Agrobacterium tumefaciens* EHA105 and expression vector pCAMBIA1300 were preserved in our laboratory for vector construction and transformation.

ISOLATION OF *DREB* HOMOLOG GENE AND BIOINFORMATIC ANALYSIS

Genomic DNA was extracted from leaves of *Tevang-1* maize cultivar using a GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Scientific, CA, USA). The sequence containing a *DREB* homolog gene was amplified using the specific primers (Forward: 5'-TCCGCCCGCCTTCATTCC-3'; Reverse: 5'-CTGGGAACGAAGCAGTAGC-3'). PCR product was cloned into pJET1.2 vector provided by a CloneJET PCR Cloning Kit (Thermo Fisher Scientific, CA, USA), according to the manufacturer's instructions. The recombinant plasmids were sequenced using a Big Dye

Terminator kit (ABI Foster City, USA) on an Applied Biosystems™ 3500 system. The sequencing results were assembled and edited by Bioedit software (http://www.mbio.ncsu.edu/bioedit/). The *DREB* homolog gene from *Tevang-1* cultivar – *ZmDREBtv* was annotated using a BLASTX tool (https://blast.ncbi.nlm.nih.gov/B-last.cgi). The obtained sequence was compared with the reference gene from B73 RefGen_v4 on http://maizegdb.org. The nucleotide sequence was translated into the putative protein sequence by ExPASy translate program (https://web.expasy.org/cgi-bin/translate/dna2aa.cgi). The deduced ZmDREBtv protein was subjected to programs PredictProtein 2013 (https://www.predictprotein.org/) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) to predict secondary structures and functional annotation. The phylogenetic relationship was established based on AP2 sequences of ZmDREB proteins downloaded from NCBI and ZmDREBtv. The protein alignment was proceeded by Bioedit software and then manually edited to final consistency. The phylogenetic tree was constructed using the maximum-likelihood method by MEGA7 software (https://megasoftware.net/).

VECTOR CONSTRUCTION AND MAIZE TRANSFORMATION

The coding sequence of *ZmDREBtv* was amplified and inserted into the expression vector pCAMBIA1300, in which the gene of interest was driven by a drought-inducible promoter rd29A. The resulting vector harboring a *HygR* gene serving as a selectable marker gene was then transformed into *A. tumefaciens* strain EHA105. The immature embryos derived from the K7 background were used for *Agrobacterium*-mediated transformation procedure adopted from Frame et al. (2015).

MOLECULAR ANALYSIS OF TRANSGENIC PLANTS

Genomic DNA was extracted from leaf tissue of transgenic plants. The presence of rd29A::*ZmDREBtv* cassette was identified using rd29A-specific primers (5'-TGTCCCTTTATCTCTCTCAGTCTC-3'; 5'-ATAGAGCTCTCAAAGAGGGACGACGA-GCTGC-3'). The transgene copy number was estimated using an absolute quantitative method. The standard curve was generated by a series of diluted solutions of plasmid and non-transgenic plant genomic DNA to final concentration of 10^5, 10^4, 10^3, and 10^2 copies/μL. The sequences of primers were as follows: rd29A-F: 5'-CTTAGTGAGACCCTCCTCTGTT-3'; DREBtv-R: 5'-CTCGTGCTGCG TACCTT-3'.

The total RNA from leaf tissue of transgenic plants was isolated and then used for the first-strand synthesis using a USB® First-Strand cDNA Synthesis Kit for Real-Time PCR (Affymetrix, USA). The relative expression level of internal and *Tevang-1*-derived *ZmDREB* genes was evaluated by relative quantitative real-time PCR, where *actin-1* served as the internal control gene. The primers used in gene expression analysis were the following: DREB-F: 5'-AGTTCGTCCACCACCTGC-3'; DREB-R: 5'-TGCCCTTCATGCACCCCTTG-3'; actin-F: 5'-AGGATACACACTTCCTCATGC-3'; actin-R: 5'-CTGTTCATAATCAAGGGCAACG-3'. A LightCycler® 96 System (Roche, Switzerland) was used for the real-time PCR using Luna® Universal qPCR Master Mix (New England Biolabs, USA).

DROUGHT TOLERANCE ASSESSMENT OF TRANSGENIC PLANTS

To assess the drought tolerance at the germination stage, thirty seeds of both non-transgenic and T2 transgenic plants were germinated on filter paper in a Petri dish wetted with 0% PEG (as control) or 10% PEG, 20% PEG (w/v) solution for 1 day at 25°C, respectively. The shoot length and root length (cm) were measured after 10 days of the treatment. For drought tolerance analysis at the early developmental stage, thirty-four-leaf-stage maize seedlings were subjected to shortage of water for 14 days, followed by rewatering. However, the control group was watered as usual during the period. The relative expression level of the *ZmDREB* was determined 4 days after withholding water (Camacho and Caraballo, 1994). Other drought assays were conducted when plants were at the stage of seven-day prior-tasseling and one-week post-pollination. Phenotypic characteristics relating to yield components were measured, including plant height, tassel length, number of tassel branch, ear length and diameter, number of kernel rows in each ear, number of kernels in each row, weight of 1000 seeds, ear-wet weight, and theoretical yield. Each experiment was performed in triplicate. The significance of the difference between groups was checked by Student's T-test.

RESULTS

ISOLATION OF *DREB* HOMOLOG GENE FROM *TEVANG-1* MAIZE CULTIVAR

The DNA fragment containing a gene homologous to *DREB* family was 1337 bp in length. The result of the BLASTX analysis confirmed that there was a homology between the *Tevang-1*-derived gene and genes encoding dehydration-responsive element-binding protein from rice, barley, *Arabidopsis thaliana*, and other plants. All of the above proteins contained a featured DNA binding

domain of AP2/ERF family. The isolated gene was named *ZmDREBtv*. The *ZmDREDtv* sequence was deposited in Genbank with the accession number of MH181238.

The 1337 bp *ZmDREBtv* sequence contained an open reading frame of 1077 bp. The 5'UTR and 3'UTR of 207 bp and 53 bp, respectively, were also isolated (Fig. 1). In the gene upstream region, we found some functional *cis*-elements of the promoter region, including TATA box, LTRE1 (low-temperature-responsive element 1), and E box (Fig. 1). A potential transcriptional start site was predicted at -100 position to the initial codon ATG. Nucleotide alignment of *ZmDREBtv* and B73 RefGen_v4 on maizegdb.org showed that the *ZmDREBtv* was about 98% homologous with the reference sequence (1:204458352-204459695). As shown in Fig. 1, several variations in the coding sequence were noticed. Among them, some led to amino acid changes. An insertion of GGC codon at +157 position and a deletion of GGTGGT at +532 position did not affect the reading frame but reduced Gly residues one by one. Other nucleotide changes, such as c.598A>G, c.664T>A, c.719T>C caused the transition of p.M200V, p.C222S, p.M240T (Fig. 1).

The deduced ZmDREBtv peptide consisted of 358 amino acids and had the molecular mass of 38.1 kDa with the theoretical isoelectric point of 6.42. Protein prediction by a Phyre2 tool revealed 22% of residues modeled with at least 90% confidence. Meanwhile, 63% of the sequence was predicted to be disordered. The conserved DNA binding domain AP2 was observed from 84 to 143 amino acid positions, including three β-sheets connected by loops and one α-helix which was almost parallel to β-sheets (Fig. 2a). The alignment of AP domains in DREB TFs from maize indicated conserved amino acids in DNA binding domain (Fig. 2a). The V14 and E19 residues critical for specific binding of DREB TFs to DRE core sequences (Liu et al., 1998) were observed in the AP2 domain of ZmDREBtv and most of ZmDREB proteins. Some ZmDREB1B and one ZmDREB1D had a replacement of p.E9V. Other amino acids, such as R6, R8, W10, E16, R18, R25, and W27, which were thought to directly contact with DNA, were also conserved (Allen et al., 1998).

A phylogenetic tree of ZmDREBtv and other DREB proteins from maize was constructed based on AP2 sequences. The result suggested that ZmDREBtv in this study belonged to DREB2 class and had the closest distance with ZmDREB2G protein followed by ZmDREB2C protein (Fig. 2b). As far as we know, ZmDREB2G was automatically annotated and deduced from the same locus as ZmDREBtv (https://ncbi.nlm.nih.gov). In addition, ZmDREBtv protein shared a high similarity in the AP2 domain with other DREB2C and DREB2G proteins from different plants. The phylogenetic analysis also indicated a close evolutionary relationship among these proteins (Fig. 2c).

VECTOR CONSTRUCTION AND TRANSFORMATION

Transgenic maize plants expressing *ZmDREBtv* gene were generated by *Agrobacterium*-mediated transformation with the binary vector pCAMBIA1300 rd29A::*ZmDREBtv*. The vector harbored *ZmDREBtv* and *HygR* genes driven by the drought-inducible promoter rd29A and the constitutive-expressing promoter CaMV 35S, respectively. The transformation experiments were processed in triplicate, each with approximately 1000 embryos. In total, the transformation experiments proceeded on 3037 immature embryos and 280 calli survived after the selection stage. After two weeks of cultivation under the light conditions, green spots on calli developed into hygromycin-resistant transgenic shoots with the regeneration frequency of 48.93% on average. Transgenic plantlets were placed into rooting medium; then 91 independent regenerative plants were moved to a greenhouse. Among them, 30 plants were positive to the presence of rd29A::*ZmDREBtv* fragment as the results of the PCR-based screening. Therefore, the transformation efficiency was 0.99% and transgenic events were denoted as D1 to D30.

The presence of the *ZmDREBtv* gene in the next generation of transgenic plants was examined by PCR (Fig. 3). In addition, the absolute quantitative real-time PCR was conducted to estimate the number of transgene integration sites and their zygosity. As the result, a number of homozygous *ZmDREBtv* transgenic T2 plants were obtained (Fig. 3). T3 generation of homozyous T2 plants was subjected to further analysis of transgene expression and drought tolerance.

DROUGHT TOLERANCE ASSESSMENT OF TRANSGENIC PLANTS

T3 generation of three events (D4, D10, and D11) that harbored homozygous *ZmDREBtv* gene at one locus was subjected to the drought tolerance analysis. To assess the protective ability of *ZmDREBtv* at the germination stage, the lengths of roots and shoots were recorded after 10 days post-germination (Fig. 4). When sprouted in water, the transgenic lines performed better than the non-transgenic line. Though there were no significant differences in the shoot length between groups in the normal conditions (Figs. 4a, 4b), the root lengths of three lines expressing ZmDREBtv were significantly higher

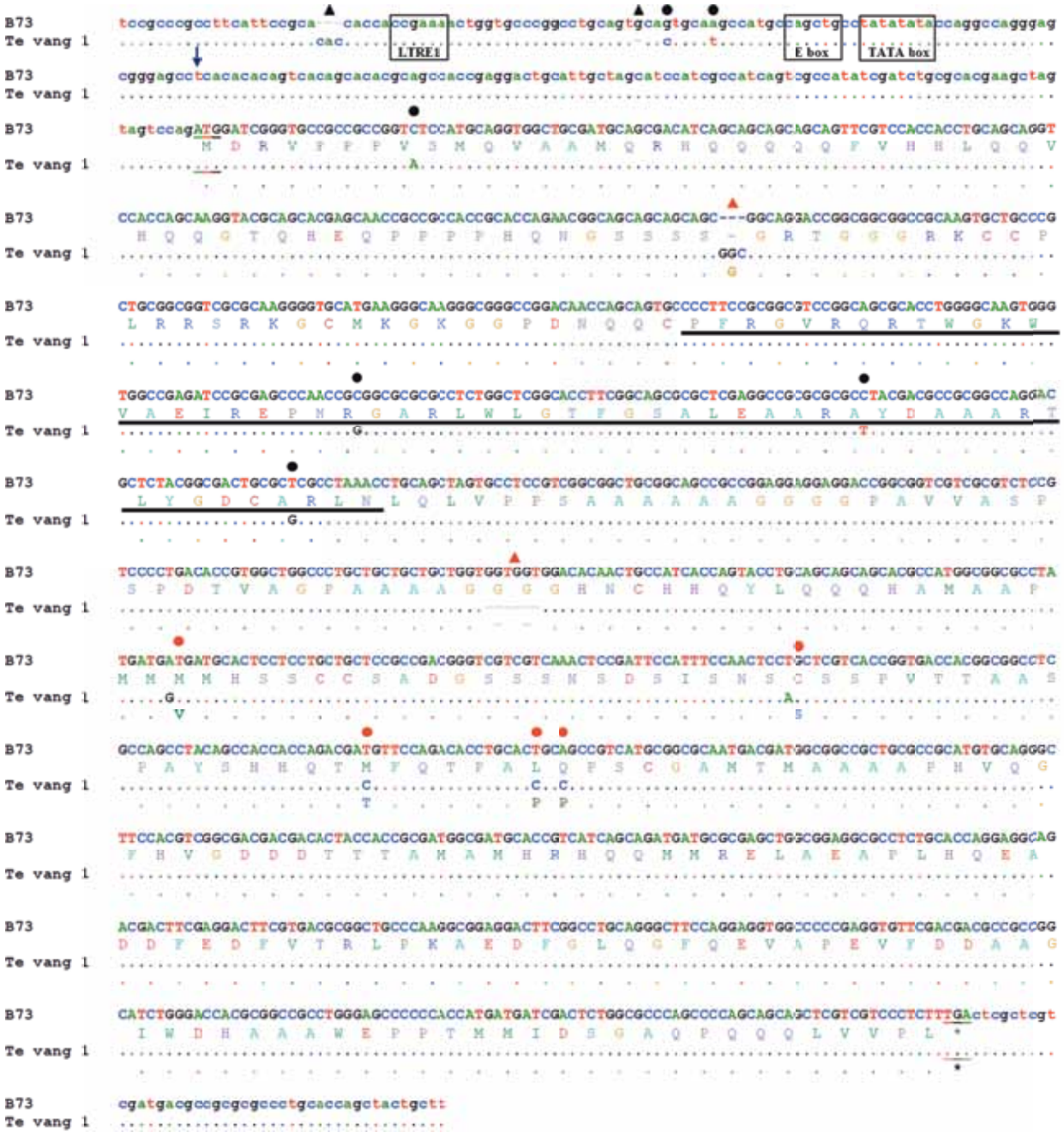

Fig. 1. The sequence of *ZmDREBtv* gene isolated from the maize cultivar *Tevang-1* (accession number on NCBI of MH181238.1) and its putative protein. The coding sequence is in upper cases and the 5'UTR and 3'UTR are in lower cases. The initial codon ATG is marked as "+1". The B73 reference sequence was downloaded from http://www.maizegdb.org/. The DNA binding domain AP2 is underlined. Promoter elements are denoted in the rectangular box. Dots represent SNPs, triangles represent insertion/deletion mutation.

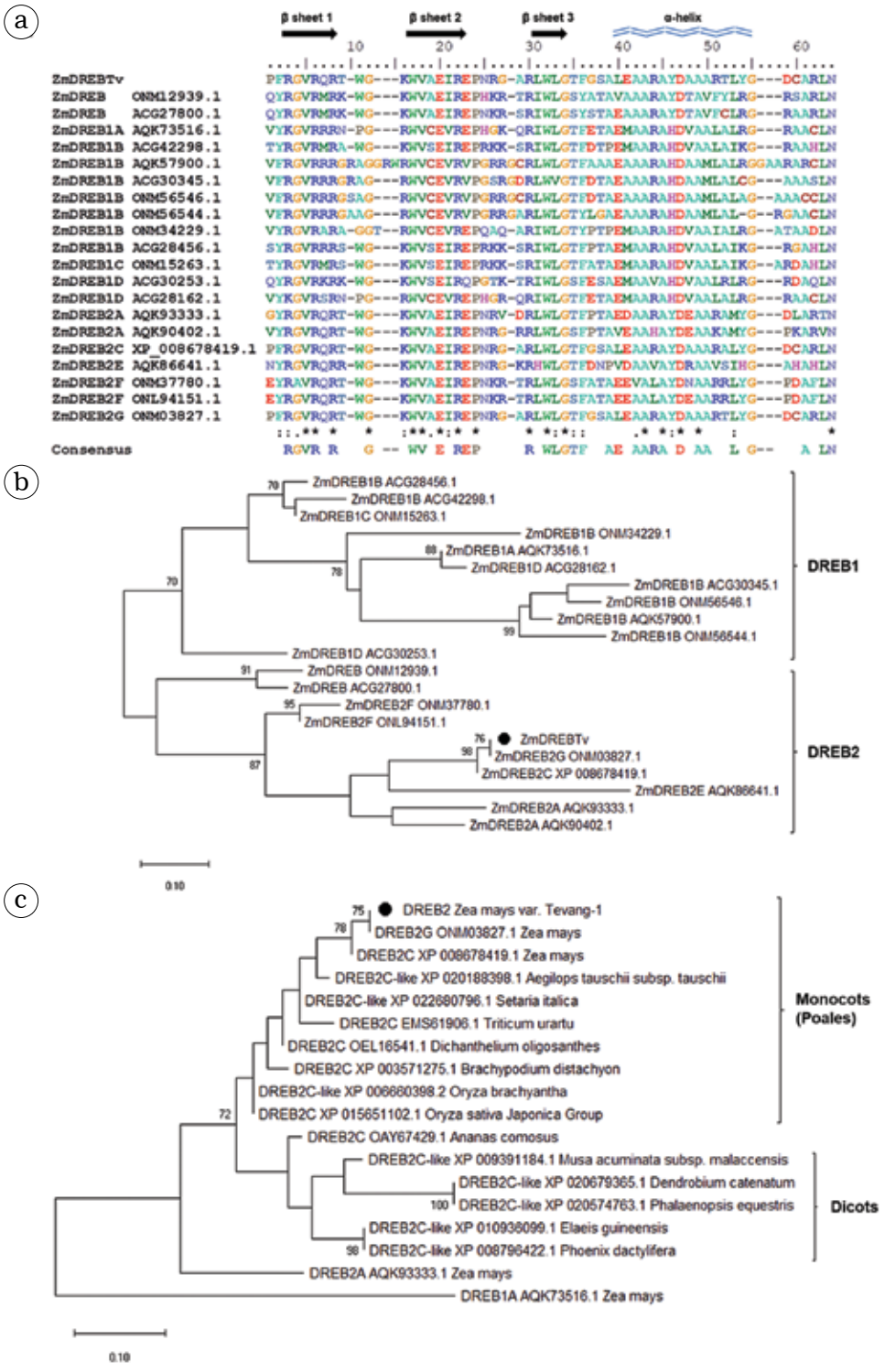

Fig. 2. Analysis of ZmDREB protein sequences. (**a**) Multiple ZmDREB transcription factors alignment of AP2 domains. ZmDREBtv sequence was translated using EMBOSS transeq program. Others DREB sequences were downloaded from NCBI with indicated accession numbers. The secondary structures are indicated above the corresponding sequences. The consensus sequence is at the bottom with the index of 70%. The asterisk, dot, and colon show single fully, highly, and weakly conserved residues, respectively. (**b**) Phylogenetic analysis of the AP2 domain of ZmDREB proteins is published in the NCBI and ZmDREBtv protein (indicated by a black dot). (**c**) Phylogenetic analysis of the AP2 domain of DREB2C, 2G proteins from different species and DREBtv from *Z. mays* var. *Tevang-1* (indicated by a black dot).

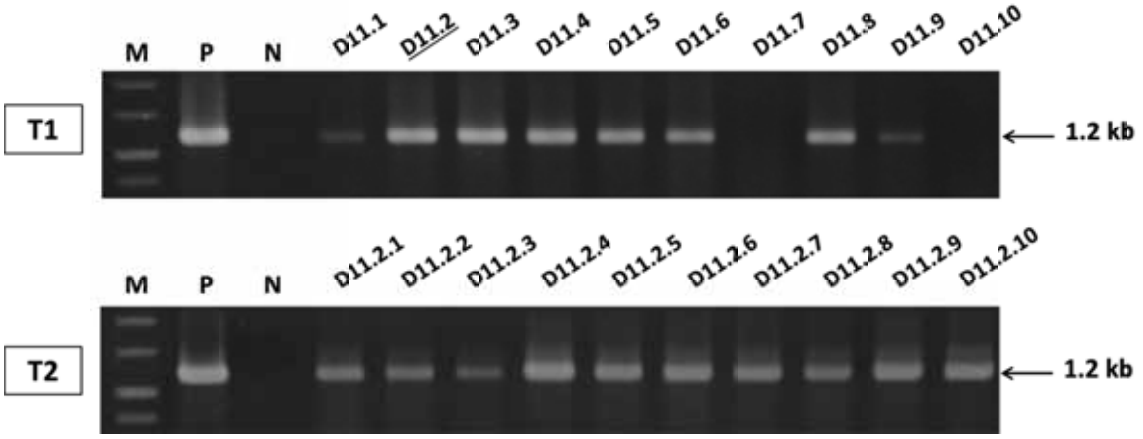

Fig. 3. The presence of the *ZmDREBtv* gene in T1 and T2 generation assessed by PCR. An example of D11 line in which, T1D11 and T2D11 were the progeny of T0D11 (one copy, heterozygous) and T1D11.2 (one copy, homozygous) which is underlined, respectively. P – plasmid control; N – no DNA added to the PCR mix.

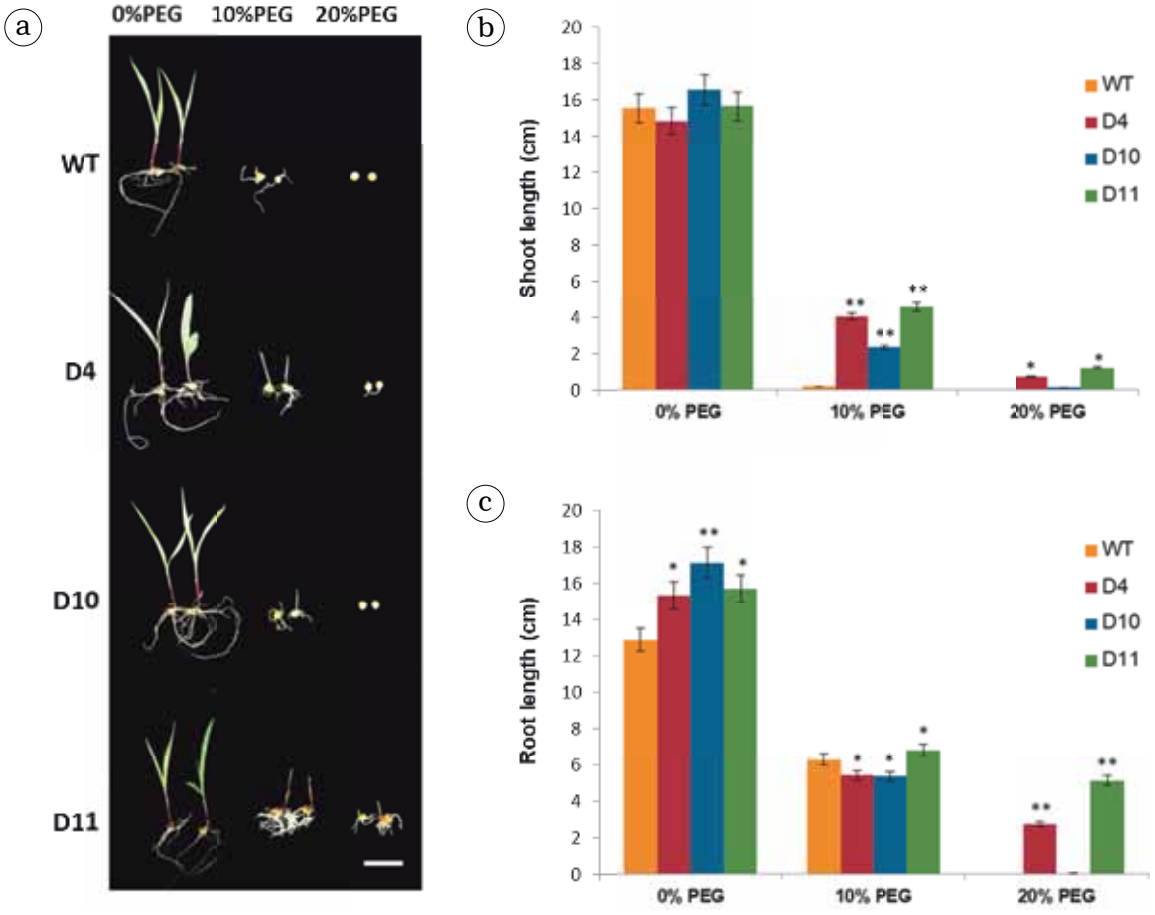

Fig. 4. Drought tolerance assessment of the wild-type (WT) and three transgenic lines (D4, D10, D11) at the germination stage using PEG6000 solution. (**a**) The phenotype of *ZmDREBtv* transgenic and wild-type seeds under various osmotic stresses. (**b**) and (**c**) The shoot and root lengths of transgenic and non-transgenic plantlets germinated under various osmotic stresses. Statistical significance was determined by Student's T-test. *P < 0.05; **P < 0.01.

than in the control line (Figs. 4a, 4c). Nevertheless, under drought conditions which was induced by 10% and 20% PEG6000, the germination of both the transgenic and the wild-type seeds was suppressed noticeably (Fig. 4). The D10 line exhibiting the best performance under the normal conditions germinated weakly under the water-stress induced by PEG6000 solutions. By contrast, an improved germination ability even under the severe stress (20% PEG6000) was observed in the transgenic lines, D4 and D11. Interestingly, the D11 seeds developed huge root systems when exposed to high osmotic conditions, despite the mean length of the shoot and the longest root difference between the two conditions (10% and 20% PEG6000) (Figs. 4a, 4c).

The D11 line was further studied to examinethe tolerance to dehydration at the early developmental stage. The transgenic and wild-type plants which had four leaves were subjected to drought by withholding water for 14 days (Fig. 5). It was observed that under the well-watering conditions, there were no significant differences between the two groups, although the mean figures of the recorded parameters in the transgenic plants were slightly higher than in the non-transgenic ones (Fig. 5). During the drought period, the wild-type line showed wilt symptoms earlier and they were more severe, compared to the transgenic one (Fig. 5a). In addition, only 20% of non-transgenic plants survived the no-watering period, whereas, the figure for the transgenic group was nearly two thirds (Fig. 5b). Furthermore, others morphological characteristics of the drought tolerance trait revealed that the transgenic plants had a better performance under the stress than the counterparts (data not shown). These results were positively correlated with the high expression level of *ZmDREB* genes in the transgenic plants (Fig. 5c). While the amount of internal *ZmDREB* transcript doubled after a 4-day period of no-watering in non-transgenic plants, the transcript level of internal and *Tevang-1 ZmDREB* genes in the D11 plants was about 3.5 times higher than that before the

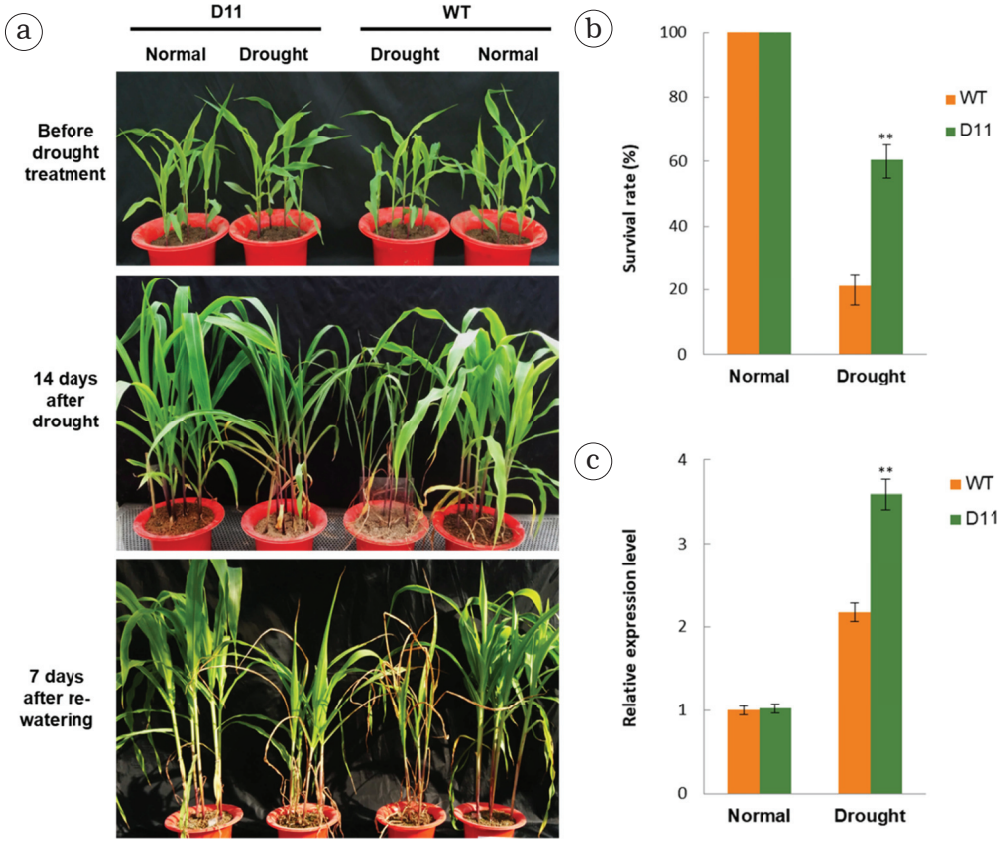

Fig. 5. Drought tolerance assessment of the wild-type (WT) and the transgenic plants (D11) at the early developmental stage. (**a**) The phenotype of *ZmDREBtv* transgenic and wild-type plants during the experiment. (**b**) The survival rate of transgenic and wild-type plants under the control and drought conditions. (**c**) The relative expression level of *ZmDREB* genes including the internal *ZmDREB* gene and *ZmDREBtv* gene in transgenic and wild-type plants 4 days after withholding water. Statistical significance was determined by Student's T-test. *P < 0.05; **P < 0.01.

drought treatment. As the result, the expression of *ZmDREBtv* induced by drought accounted for the disparity of the relative expression level of *ZmDREB* genes between the wild-type and transgenic plants.

The impact of *ZmDREBtv* gene on yield components was also assessed by drought-treatment at the tasseling and grain-filling stage of the D11 transgenic line. Apparently, the drought occurring during the former stage did not change the number of leaves in the foliage but significantly reduced the plant height in both experimental groups. The non-transgenic lines' height dropped from 134.2 cm to 67.52 cm while in the transgenic ones it only lowered from 135 cm to 72.8 cm (Table 1). Moreover, despite the fact that no significant difference was observed in the number of tassel branches between the normal and drought conditions in all plants, the size of the tassel went down noticeably when the plants were subjected to the water-limited conditions. Indeed, the tassel of the wild-type plants was 10.44 cm shorter, compared to that of the D11 line during the drought (Table 1). Additionally, when plants at the grain-filling stage were subjected to drought treatment, the non-transgenic line experienced more severe crop failures than the transgenic one (Table 2). Although the number of grain rows was the same in all the plants, some data about the ear slightly decreased during the drought. The ear length of the wild-type plants in the water-deficit environment was nearly 85% of that in the normal environment, while that ratio of the D11 plants was approximately 95% (Table 2). Similar results could be seen in such parameters as ear diameter and ear wet weight. Furthermore, the number of kernels per row was found to decrease in all plants under the dehydration conditions. Among them, the control line lost more seeds than the transgenic counterpart, and the figure of reduction was 3.4 kernels (from 18.4 to 15.0) and 1.8 kernels (from 18.0 to 16.2) per row, respectively. In addition, because drought adversely affected accumulation of starch in the endosperm, the weight of 1000 kernels of all drought-treated groups was lower, compared to the corresponding well-watered groups. As predicted, the figure for the transgenic plants was 14.6 gram higher, compared to that of the non-transgenic plants. It led to the theoretical yield of the *ZmDREBtv*-transgenic line, which is predicted to reach 2.91 ton/ha while 2.54 ton/ha is the value for wild-type line under drought conditions.

TABLE 1. Mean values for transgenic and non-transgenic lines under well-watered and drought conditions at tasseling stage.

Lines	Condition	Number of leaves	Plant height (cm)	Tassel length (cm)	Number of tassel branches
Transgenic lines	Drought	16.0 ± 0.0	72.8 ± 1.65	40.98 ± 1.32	7.6 ± 0.5
	Well-watered	16.4 ± 0.5	135 ± 2.1	57.34 ± 1.61	7.8 ± 0.5
Non-transgenic lines	Drought	16.0 ± 0.2	67.52 ± 1.39	30.3 ± 1.34	7.4 ± 0.5
	Well-watered	16.0 ± 0.2	134.2 ± 2.47	57.1 ± 1.97	7.6 ± 0.5

Table 2. Mean values for transgenic and non-transgenic lines under well-watered and drought conditions at grain-filling stage.

Lines	Condition	Ear length (cm)	Ear diameter (cm)	Number of kernel rows	Number of kernels per row	Weight (g) of 1000-kernels	Ear wet weight (g)	Theoretical yield (ton/ha)
Transgenic lines	Drought	7.22 ± 0.42	3.26 ± 0.08	10 ± 0.0	16.2 ± 1.4	142.8 ± 3.8	43.3 ± 1.3	2.91 ± 0.56
	Well-watered	7.58 ± 0.39	3.44 ± 0.12	10 ± 0.2	18.0 ± 1.5	152 ± 4.1	46.25 ± 1.7	3.13 ± 1.12
Non-transgenic lines	Drought	6.44 ± 0.46	2.7 ± 0.06	10 ± 0.0	15.0 ± 1.5	129.8 ± 3.2	39.12 ± 0.9	2.54 ± 0.97
	Well-watered	7.6 ± 0.41	3.46 ± 0.14	10 ± 0.0	18.4 ± 1.7	153.6 ± 3.9	46.27 ± 2.0	3.16 ± 1.32

DISCUSSION

In plants, *DREB* gene family encodes transcription factors which are significantly involved in drought and cold tolerance pathways by inducing the expression of many stress-driven genes. Sequencing of the maize genome contributes to identification of *DREB* genes (Liu et al., 2013). Nevertheless, a number of *DREB2* genes had not been analyzed functionally, especially in local drought-tolerant maize cultivars. In this study, we isolated a *DREB* gene from *Z. mays* var. *Tevang-1* and investigated the sequence characteristics and its deduced protein, using bioinformatic tools. The relationship between ZmDREBtv and other ZmDREB proteins suggested that *ZmDREBtv* belonged to the DREB2 transcription factor gene (Fig. 2b). It was also established that ZmDREBtv protein shares a high similarity with other monocots' DREB2C and 2G proteins (Fig. 2c). Meanwhile, many variations were identified between the *ZmDREBtv* gene from the local maize and B73 reference maize (Fig. 1). In facts, many studies reported that *DREB* genes show a high level of sequence variation. For instance, Wu et al. (2011) identified two, 15 and 16 SNPs, in three genes of *CBF/DREB1* family in barley (*Hordeum vulgare* L.) with the average figures of 0.3, 2, and 2.4 SNPs in each 100 bp of the coding region, respectively. In rice, 11 SNPs and 133 InDels were found in the *OsDREB1F* gene. Among them, one transversion mutation (C363G) led to replacement of aspartate by glutamate and enhanced the drought tolerance in the rice plant carrying that allele (Singh et al., 2015). Our study revealed that the *ZmDREBtv* coding region of *Tevang-1* cultivar had many variants, compared to that of the B73 reference maize including five transversion mutations and four transition mutations. Besides, we observed an addition of three nucleotides and a deletion of six nucleotides resulting in 358 amino acids instead of 359 amino acids in the ZmDREBtv protein sequence. Similarly, a deletion of the 18 bp fragment in the coding region was observed in *HvCBF3* gene from barley (Wu et al., 2011). Nevertheless, these mutations changed some amino acids of the ZmDREBtv without causing a frameshift mutation which might lead to the loss-of-function protein. Additionally, the missense and InDel mutations did not occur in the DNA binding domain coding region. The highly conversed DNA binding domain observed at DREB2 proteins showed that they have the same affinity and specificity as GCC-box. However, the difference in other polypeptide regions might change the way ZmDREBtv interacts with other components of the transcriptional complex.

In this study, we used the stress-inducible rd29A promoter to control the *ZmDREBtv*. Since this promoter allowed the low expression level of *ZmDREBtv* during the non-inducing conditions (Fig. 5c), the presence of the *ZmDREBtv* gene did not negatively affect the transgenic plants. Although the T0 plants exhibited an abnormal phenotype and poorly developed reproductive organs, T1 and T2 plants were normal in reproductive performance and phenotypic morphology. It was also observed that the development and seed production of T1 and T2 plants were similar to the wild-type plants. Hence, the sporadic fertility of T0 plants was postulated to have an adverse consequence for embryonic tissue culture which lasted for nearly three months. Indeed, the presence of the *ZmDREBtv* enabled plants to perform the same as and even better than the non-transgenic plants in the normal conditions (Figs. 4, 5). Additionally, the rd29A promoter permitted a high-level expression of the *ZmDREBtv* gene when plants were exposed to drought (Fig. 5c). This result was similar to *DREB1A* from *Arabidopsis* where its transcript showed a similar accumulation pattern following water-deficit and cold stresses. Kasuga et al. (2004) used RNA-gel blot analysis to confirm the expression of *DREB1A* driven by the rd29A promoter in tobacco plants under stress conditions. The study revealed that the *DREB1A* was expressed strongly 5 hours after dehydrating of the transgenic seedlings (Kasuga et al., 2004). In our study, the expression of *ZmDREBtv* gene was examined when water was withheld from four-leaf D11 plants for 4 days. It was also established that the *ZmDREBtv* from *Tevang-1* expressed at a higher level than the internal *DREB* gene. The possible reason for the difference in the expression level of the two homologous genes were the promoters driving those, an internal promoter and the inducible strong promoter – rd29A. Moreover, it was expected that once the transgenic plants were exposed to drought, the rd29A promoter would induce the ZmDREBtv production, which in turn would bind to the rd29A promoter. Therefore, a regulatory loop would magnify the expression level of the *DREB* gene from *Z. mays* var. *Tevang-1*.

Studies on different species showed that the mechanism of the protective ability against dehydration of DREB2 transcription factor induces the expression of other stress-inducible genes. Then, those gene products could directly help plants to survive and resume growth after the osmotic stress. Overexpression of *AtDREB2A CA*, a truncated version of *DREB2A* gene in *Arabidopsis thaliana*, increased the expression level of 14 genes under the drought stress (Sakuma et al., 2006). Products of the upregulated genes included LEA proteins which protected cell components from dehydration and improved the survival rate of the transgenic plants. Meanwhile, tobacco plants

overexpressing *CpDREB2* from papaya tree (*Carica papaya*) showed a high level of proline accumulation under the water-limited conditions (Arroyo-Herrera et al., 2016).

Here, the expression of *DREBtv* gene in the transgenic maize plants significantly improved the drought tolerance as the result of increase in the survival rate and the productivity of experimental plants. In general, it was observed that transgenic lines germinated better than the wild-type seeds in water. Noticeably, seedlings of the D11 line developed a huge root system under both mild and severe drought conditions (10% and 20% PEG6000 solution, respectively) (Fig. 4). As roots are organs that perceive the osmotic stress signal and uptake water and nutriens, their size and structure are critical for a plant. Under drought stimulation, the root system is prone to maximize the length and minimize the number of fibrous roots (Nejad et al., 2010). Besides, the expression of *ZmDREBtv* gene led to the observation that transgenic plants withstood longer when watering was halted. Sakuma et al. (2006) reported that *A. thaliana* plants harboring rd29A::*AtDREB2A CA* tolerated a 2-week period of no-watering better than the wild-type plants, with the survival rate of 83.3-88.3% and 21.3%, respectively. In this study, the percentage of the recovered transgenic maize plants was about three times higher than that of the control group. It was found out that the transgenic plants performed better than the non-transgenic plants at different examined stages due to the presence of *ZmDREBtv* gene.

Plants are negatively impacted by drought at every developmental and reproductive stage and at many levels. In consequence, plants adapt by co-ordination of many strategies at morphological, physiological, and biochemical levels (Tardieu et al., 2011). In the present study, the parameters related to agronomic traits and yield components were selected to assess the response of the transgenic plants expressing ZmDREBtv under water-deficit conditions in comparison with non-transgenic plants. The differences in their behavior indicated that the presence of ZmDREBtv empowered maize plants to maintain better performance during water stress.

CONCLUSION

In this research, we isolated a *DREB* homolog gene from *Z. mays* var. *Tevang-1* which encoded a transcription factor. The sequence of *ZmDREBtv* was 98% homologous with the reference gene and contained many variations in the coding region. The ZmDREBtv transcription factor belonged to DREB2 class that is actively involved in plant abiotic response pathway. The transgenic maize plants expressing *ZmDREBtv* under the control of the drought-inducible promoter rd29A showed improved performance compared to the non-transgenic plants under drought conditions at different stages.

AUTHORS' CONTRIBUTIONS

TTHH and VHN designed experiments and were involved in paper revision. NTL, LHL, NHH, HDL, TBTD, MMB, THP, HHH, TTHL conducted experiments and collected data. TTHH, TNL wrote the first draft of manuscript. TTHH, VHN completed the manuscript. The authors declare no conflict of interest.

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