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A *LEA* Gene from a Vietnamese Maize Landrace Can Enhance the Drought Tolerance of Transgenic Maize and Tobacco

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Abstract: Maize (*Zea mays*) is a major cereal crop worldwide, and there is increasing demand for maize cultivars with enhanced tolerance to desiccation. Late embryogenesis abundant (LEA) proteins group 5C is involved in plants' responses to various osmotic stresses such as drought and salt. A putative group 5C LEA gene from *Z. mays* cv. Tevang 1 was isolated, named *ZmLEA14tv*, and cloned into a T-DNA for expression in plants. The deduced amino acid of ZmLEA14tv showed a conserved Pfam LEA_2 domain and a high proportion of hydrophobic residues, characteristic of group 5C LEA proteins. Transgenic tobacco and maize plants expressing *ZmLEA14tv* were generated. During drought simulation conditions, the *ZmLEA14tv*-expressing plants of tobacco showed improved recovery ability, while those of maize enhanced the seed germination in comparison with the non-transgenic control plants. In addition, the survival rate of *ZmLEA14tv* transgenic maize seedlings was twice as high as the control. These results indicated that *ZmLEA14tv* might be involved in the drought tolerance of plants and could be a candidate gene for developing enhanced drought-tolerant crops.

Keywords: drought tolerance; *LEA*; Tevang 1 maize; tobacco

1. Introduction

Late embryogenesis abundant (LEA) proteins are mostly hydrophilic proteins, which can reduce the damage caused by severe environmental conditions. LEA proteins were reported to contribute to various developmental processes and to accumulate in response to drought, low temperature, salt stress, or treatment with the phytohormone ABA [1–4]. The first LEA was reported in cotton seeds [5,6]. LEA proteins accumulated during the late stages of embryogenesis and associated with the desiccation of seeds' embryos [7,8]. The members of the LEA protein family are also expressed during water deficit in bacteria (*Escherichia coli*) and yeast (*Saccharomyces cerevisiae*), suggesting a ubiquitous protective role of these proteins against osmotic stresses [9,10].

Following the Battaglia's classification, LEA proteins are categorized into seven different groups [11]. The LEA proteins of groups 1, 2, 3, 4, 6, and 7 are hydrophilic or typical LEA proteins, which have a low proportion of cysteine and tryptophan residues, and a high proportion of glycine, glutamic acid, lysine, and threonine residues. In contrast, the group 5 LEA protein has high content of hydrophobic residues. Based on amino acid sequences and conserved motifs, the group 5 LEA protein

was classified into three subgroups, namely 5A, 5B, and 5C [11]. Subgroup 5C LEA proteins were characterized by a low instability index, low proportion of polar (hydrophilic) and small residues, a higher proportion of non-polar residues, and heat-unstable conformation [11–13]. Moreover, the 5C LEA proteins are folded intrinsically and have more β-sheets than α-helices, which is also different from group 5A and 5B [8,13]. These differences in residue proportion and physical characteristics of group 5C from other LEA protein groups may refer to alternative functions involving stress tolerance.

Recently, due to the development of new sequencing technologies, the whole genome sequences of valuable plants such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), and cotton were published and made available to researchers [14–16]. Based on the conserved domains of LEA proteins, LEA protein families could be identified and characterized through whole-genome prediction approaches. In rice, 34 rice candidate *LEA* (*OsLEA*) genes were identified through a HMMER search (http://hmmer.janelia.org/) [17]. By using a similar method, 242, 136, and 142 candidate DNA regions that encode for LEA proteins were identified in three upland cotton namely *Gossypium hirsutum*, *G. arboreum*, and *G. raimondii*, respectively [18]. The LEA protein profile of maize was also reported with 32 LEA genes distributed non-randomly across chromosomes [19]. The accumulation of LEA profiles in various plants provided fundamental knowledge for functional analysis and *LEA* gene engineering in the future.

A small number of group 5C LEA proteins have been characterized, but their physical characteristics and biological functions are largely unknown. Some members of group 5C were identified in other plants such as cotton LEA14A, soybean D95-4, tomato ER5, hot pepper CaLEA6, *Arabidopsis* AtLEA14A, sweet potato IbLEA14A, rice OSLEA5, foxtail millet SiLEA14A, and wild peanut LEA [8,13,20–26]. The expression of LEA 5C proteins is upregulated by ABA and multiple abiotic stresses including salt and drought [13,23]. Functional studies of group 5C proteins showed that an overexpression of CaLEA6 protein, which originates from hot pepper (*Capsicum annuum*), could improve drought and salt tolerance significantly in tobacco [23]. In addition, the overexpression of other *LEA14A* genes such as *IbLEA14A* and *SiLEA14A* remarkably raised the level of lignification, free proline, and soluble sugar in transgenic sweet potato (*Ipomoea batatas*) calli, *Arabidopsis*, and foxtail millet (*Setaria italica*) [13,23,24]. Recombined OsLEA5 in *E. coli* could protect lactate dehydrogenase from misfolding under different abiotic stresses, resulting in stress tolerance [25].

Maize (*Zea mays* L.) is an important monocot crop worldwide; its production was more than 1.06 billion tons in 2016 [27]. Drought is the major factor that accounts for significant losses in maize productivity. A water reduction of 40% could decrease maize production by 39.3% [28]. Recently, a predicted profile of LEA family in maize has been reported through both bioinformatic and practical approaches [19]. A putative maize *LEA* gene located on chromosome 8 that contained a Pfam LEA_2 domain was described; however, any function of this gene in protecting plants against osmotic stress remains unknown [19]. In the current study, a putative *LEA* gene was isolated from the Tevang 1 landrace, designated as *ZmLEA14tv* and cloned into a T-DNA for expression in plants. The expression patterns of transgenic *ZmLEA14tv* in both tobacco and maize models were investigated to determine the importance of this gene in enhancing drought tolerance in selected plants.

2. Materials and Methods

2.1. Plant Materials and Drought-Stimulating Growth Conditions

Maize seeds (*Z. mays* cv. Tevang 1) were provided by the Maize Research Institute in Vietnam. Tevang 1 cultivar is a well-known landrace maize from the rocky mountain region in Northern Vietnam. The cultivar is well adapted to low annual rainfall and water-deficit cultivation. Maize seeds were germinated and grown under greenhouse conditions at 22/26 °C (night/day) and a photoperiod of 14/10 h (day/night) for two weeks. The genetic background line for transformation was K7, a selected maize that has a higher rate of regeneration and successful transformation through *A. tumefaciens*-mediated methods. The plant material was evaluated for several morphological and physiological traits. For analysis of drought tolerance at the germination stage, 30 seeds of both WT

and homozygous *ZmLEA14tv* maize (T2 generation) were germinated on filter paper in a Petri dish wetted with water (as control) or 10% PEG, 20% PEG (*w*/*v*) solution for one day at 30 °C. Shoot and root lengths were measured after eight days of the treatment, followed by taking photographs. For the drought tolerance assay, the five-leaf-stage maize seedlings were assigned to a withholding water period for 14 days followed by a three-day re-watering. At least five seedlings were grown in each plot. The survival rate, fresh stem weight, and fresh root weight of drought-treated and control seedlings were measured. The experiment was replicated three times.

Seeds of WT tobacco (*Nicotiana tabacum*) cultivar K326 and transgenic tobacco were sterilized with 70% ethanol and 5% bleach, which follows a method described previously [29]. The tobacco explant was germinated in Murashige and Skoog (MS) medium containing 200 mg/µL kanamycin under light/dark cycle conditions of 16/8 h at 25 °C. The 20-day-old T3 tobacco plants were put under drought conditions for 15 days and then re-watered to observe the morphological modification.

2.2. Isolation of the Putative LEA Coding Region in Tevang 1 Landrace Maize

Based on the putative *ZmLEA14A* sequence published in GenBank (accession number EU976614.1) and the flanking sequence of this gene located on maize chromosome 8 in the maizeGBD database (http://www.maizegdb.org/), PCR-specific primers for amplification of this region were designed. The DNA regions containing the putative *ZmLEA14A* open reading frame (ORF) and 5′ UTR of this gene were amplified from genomic DNA of Tevang 1 cultivar using the *ZmLEA14tv_F* forward primer sequence (5′TCACTTCTCTTCCAGCGAGTAC3′) and *ZmLEA14tv_R* reverse primer sequence (5′ TCTCGTACTACTCAAGCAGCAC3′). The PCR product was denoted as *ZmLEA14tv* and purified by Thermo Scientific PCR product purification kit (Cat. number #K0702, Waltham, MA, USA) then cloned into a pJET1.2 cloning vector following the manual of the producer. The cloning vector pJET1.2, which contains the PCR fragment, was transformed into *E. coli* DH10β competent cell by heat shock at 42 °C for 1 min. The colonies harboring the pJET1.2–ZmLEA14tv plasmid were checked by colony PCR with isolation primers and the restriction enzyme *Bgl*II.

2.3. ZmLEA14tv Expression Vector Construction and Transgenic Plant Generation

The coding region of *ZmLEA14tv* was amplified with the *ZmLEA14tv_CloneF* forward primer sequence (5′ ATTACCATGGCGCAGTTGGTG3′) and *ZmLEA14tv_CloneR* reverse primer sequence (5′ ATTAGCGGCCGCGAAGATGCTGG3′) that generates the recognition sites of *Nco*I and *Not*I at the 5′ and 3′ ends of the PCR products, respectively. The thermocycler program (Eppendorf, Hamburg, Germany) starts at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C for 1 min; 56 °C for 30 s; 72 °C for 1 min); the final extension step was 72 °C for 10 min. The 463-bp PCR product was then treated with *Nco*I and *Not*I, purified, and ligated into the pRTRA7/3 vector to generate a 35S promoter-ZmLEA14tv-35S terminator construct. The cassette was cut and combined into the T-DNA region of the pCAMBIA1300 (8958 bp) binary vector. The recombined binary vector pCAMBIA1300/ZmLEA14tv was transformed into *A. tumefaciens* strain EHA 105 after validation by sequencing. The transgenic tobacco and maize plants were regenerated by modified *Agrobacterium*-mediated transformation methods described in the studies of Topping (1998) and Frame et al. (2011), respectively [30,31].

2.4. Sequence Alignment and Gene Evolution Analysis

The sequence of PCR products was verified by 3500 Series Genetic Analyzers (Applied Biosystems, Foster City, CA, USA) followed the Sanger method. A deduced amino acid sequence of ZmLEA14tv protein was generated using the ExPaSy web tool (https://web.expasy.org). The isoelectric point molecular mass, the proportion of amino acid, and grand average of hydropathy (GRAVY) index of the putative ZmLEA14tv peptide were estimated using the ProtParam web tool (https://web.expasy.org/protparam/). Motif analysis was performed using the Pfam program (http://www.ebi.ac.uk/Tools/InterProScan/). The completed amino acid and deduced amino acid sequences of subgroup 5C

LEA proteins were used to construct a phylogenetic tree. Sequence alignment was carried out with ClustalW and adjusted manually. The phylogenetic tree was constructed with the neighbor-joining bootstrap method using the MEGA v6.0 program [32].

3. Results

3.1. Isolation and Vector Construction of ZmLEA14tv Gene

A putative LEA coding sequence from a native maize landrace Tevang 1 (*Z. mays* cv. Tevang 1) was isolated and named *ZmLEA14tv*. The isolated *ZmLEA14tv* had a size of 693 base pairs (bp) with an open reading frame of 459 bp in length encoding for a deduced 152 amino acid (aa) protein. Sequence analysis exhibited the highest similarity at 99% with a *Z. mays LEA14A* coding sequence (accession number NM_001159174), followed by *LEA14A* of *Shorgum bicolor* (XM_002454858.2) and *LEA14A*-like gene of *S. italica* (93% and 87%, respectively). The comparison with the reference sequence number NM_001159174 in GenBank showed two variations, G381A and C456T; however, the deduced amino acid sequence was not changed. The putative protein was predicted at 15.96 kDa in molecular weight with a pI of 6.08. The protein was rich in Val (12.6%), Leu (11.3%), and Gly (9.2%), but contained low quantities of Trp (0.7%), Asn (1.3%), Cys (0.7%), and Gln (1.3%). The GRAVY and instability index of the putative *ZmLEA14tv* were 0.047 and 16.01, respectively, suggesting stability and hydrophobic nature. A conserved "LEA_2" motif (PF03186), which was classified into subgroup 5C according to Battaglia's classification of LEA proteins, was found on the ZmLEA14tv through an InterProScan search [11]. Further analysis showed that ZmLEA14tv contained a low percentage of polar amino acids (23.02%) and a high level of hydrophobic residues (47.02%). The ZmLEA14tv protein displayed diverse homology with other group 5C LEA proteins and broadly matches similar segments in related LEA proteins, indicating a close evolutionary relationship among these proteins (Figure 1A,B). The phylogenetic analysis also showed that the ZmLEA14tv protein has the closest relationship with maize LEA14A protein (accession number NM_001159174), followed by an LEA-like protein from rice, namely Os01g0225600 (accession number NM_001048996), supported by high bootstrap values (99% and 95%, respectively).

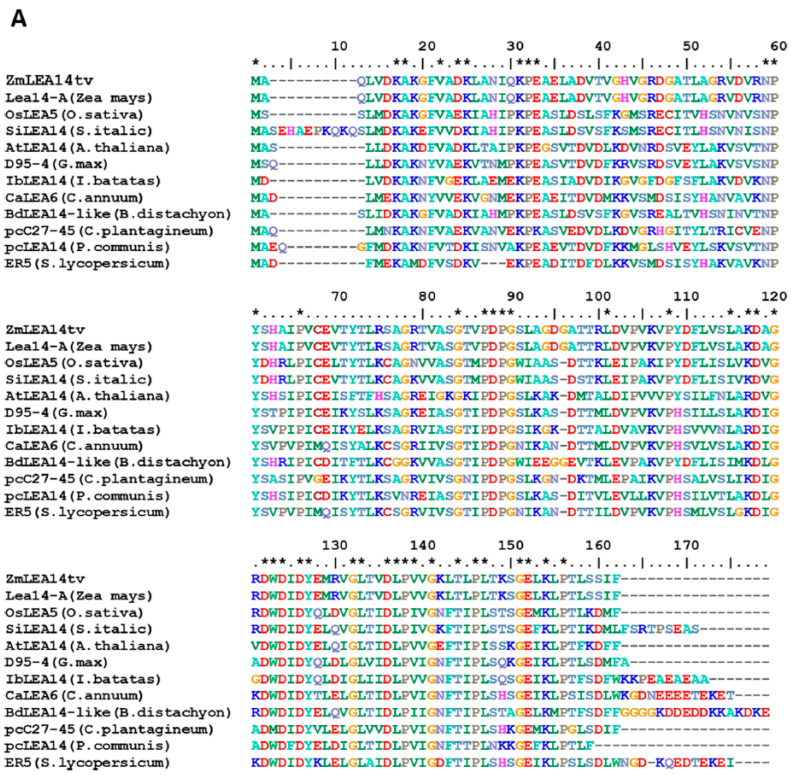

Figure 1. *Cont.*

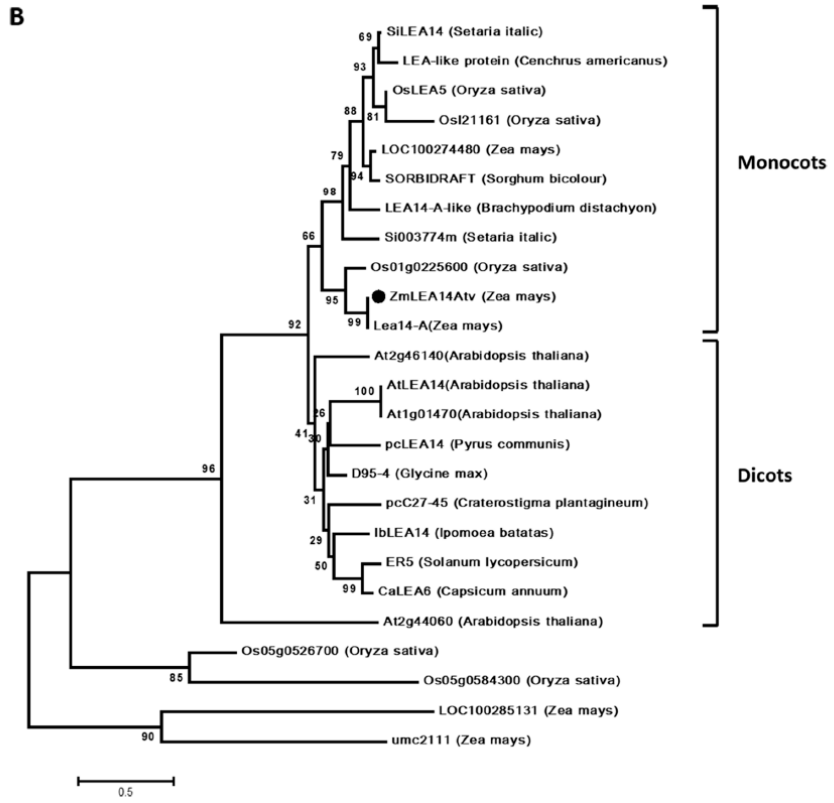

Figure 1. Sequence alignment and phylogenetic relationship for the putative ZmLEA14tv protein and its homologs. (**A**) Multiple sequence alignment of ZmLEA14tv with its homologs (LEA group 5 protein) from various plant species. The conserved positions were marked as stars. (**B**) Neighbor-Joining phylogenetic trees of ZmLEA14tv and its homologs. The clades of monocots and dicots are marked. ZmLEA14tv in *Z. mays* cv. Tevang 1 branch is highlighted by a solid dark circle. The GenBank accession numbers are as follows: SiLEA14 (*S. italic*, KJ767551), AtLEA14 (*Arabidopsis thaliana*, NM100029), Lea14-A (*Z. mays*, NM001159174), D95-4 (*Glycine max*, U08108), IbLEA14 (*Ipomoea batatas*, GU369820), ER5 (*Solanum lycopersicum*, U77719), Lemmi9 (*S. lycopersicum*, Z46654), CaLEA6 (*Capsicum annuum*, AF168168), OsLEA5 (*Oryza sativa*, JF776156), pcC27-45 (*Craterostigma plantagineum*, M62990), pcLEA14 (*Pyrus communis*, AF386513), At1g01470 (*A. thaliana*, BT015111), D95-4 (*G. max*, U08108), At2g46140 (*A. thaliana*, NM130176), Os01g0225600 (*O. sativa*, NM001048996), LEA14-A-like (*Brachypodium distachyon*, XM003567779), BdLEA14-like (*B. distachyon*, XM003567779), LOC100274480 (*Z. mays*, NM001148839), SORBIDRAFT (*Sorghum bicolour*, XM002441543), LEA-like protein (*Cenchrus americanus*, AY823547), OsI21161 (*O. sativa*, CM000130), Os05g0526700 (*O. sativa*, NM001062639), Os05g0584300 (*O. sativa*, NM001062985), At2g44060 (*A. thaliana*, BT024723), LOC100285131 (*Z. mays*, EU970969) and umc2111 (*Z. mays*, NM001155750).

3.2. *ZmLEA14tv* Gene Expression in Drought Resistance for Transgenic Tobacco

To evaluate the function of the *ZmLEA14tv* transgenic structure in plant osmotic tolerance, the transgenic tobacco plants that expressed ZmLEA14tv under the control of the CaMV 35S promoter were selected for further analysis (Figure 3A). Thirty transgenic plants were obtained, and three homozygous T3 transgenic lines (LEAtv-L1, LEAtv-L3, LEAtv-L7) with high expression levels of ZmLEA14tv (Figure 2B) were chosen for further investigation. To investigate the drought tolerance of the transgenic tobacco, the seedlings were treated with a shortage of water for 15 days. Subsequently, the plants were re-watered and grown for three days. Under normal and drought conditions, there were no significant differences in morphological features such as height, weight, and leaf surface between the transgenic and non-transgenic wild-type (WT) plants. Leaves of the transgenic and control plants became curled and wilted after 15 days of drought. However, 100% of the transgenic tobacco was

restored after a three-day re-watering, unlike the control plant (Figure 2C). The fastest recovery was observed in LEAtv-L1, which also expressed the highest level of the *ZmLEA14tv* transgene. This result indicated a correlation between the *ZmLEA14tv* expression and the recovery ability of the plant after the drought conditions.

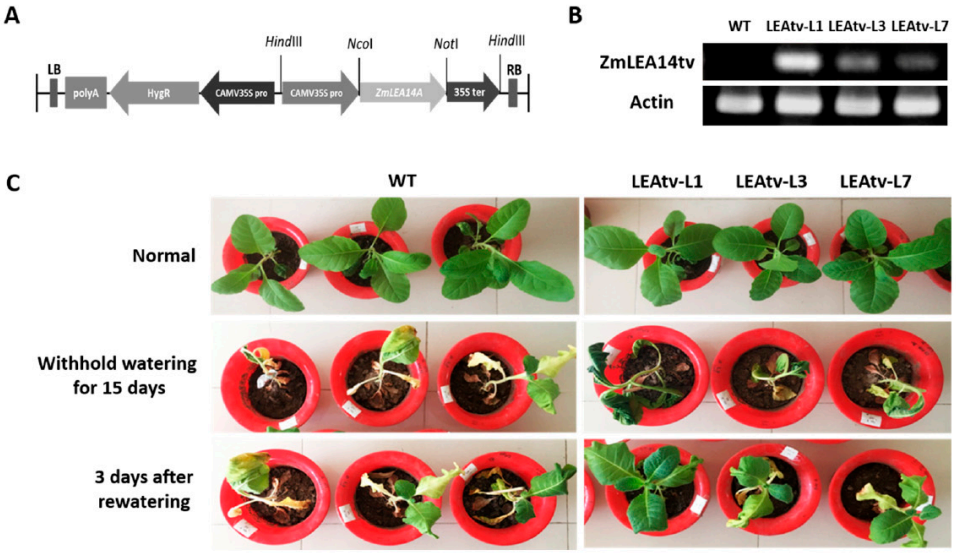

Figure 2. The expression of the *ZmLEA14tv* in tobacco (*Nicotiana tabacum*). (**A**) Schematic description of T-DNA involving in the pCAMBIA1300/ZmLEA14tv plasmid for the expression of *ZmLEA14tv* in plants. LB: left T-DNA border; RB: right T-DNA border; HygR: Hygromycin resistant gene; CaMV35S pro: Cauliflower mosaic virus 35S promoter; 35S ter: 35S terminator; *ZmLEA14A*: coding region of *ZmLEA14tv* gene; *Nco*I, *Not*I, and *Hin*dIII: restriction site of *Nco*I, *Not*I và *Hin*dIII, respectively. (**B**) RT-PCR analysis of ZmLEA14tv in transgenic tobacco lines (LEAtv-L1, LEAtv-L3, LEAtv-L7). WT: the wild type was used as a control. (**C**) The phenotype of transgenic and WT tobacco explants under normal and drought stress conditions.

3.3. Transgenic Maize with ZmLEA14tv Gene in Drought Tolerance

The ZmLEA14tv integration was confirmed by genomic PCR using pairs of primer sets specific to the Hpt and 35S promoter regions, respectively. RT-PCR and qRT-PCR of ZmLEA14tv were performed to validate the expression of the transgenic structure of T2 transgenic maize lines. Three T2 lines, namely L1453, L1482, and L1510, showed the highest expression level (2.7-, 9.2-, and 5.8-fold higher than the control, respectively) and the T2 seeds of these re-watered lines were used for further analysis (Figure 3A,B).

The drought tolerance of transgenic maize seeds during germination was examined. When germinated in water for eight days, the transgenic lines showed better growth than the WT line used as the regeneration material. Under normal conditions (H_2O), no significant differences in shoot height were observed between the transgenic lines and the WT ones. However, the root length of the L1510 and L1482 lines, which have higher ZmLEA14tv transcripts accumulation, was significantly higher than that of the wild type. In comparison with the control group in water, the germination of both the WT and transgenic lines was severely suppressed under 10% and 20% PEG stress (Figure 3C). None of the experimental seeds could germinate in 20% PEG. However, better germination and subsequent development were observed with the seeds of L1510 and L1482, which had higher expression of ZmLEA14tv transcripts in 10% PEG than the wild type (Figure 3D).

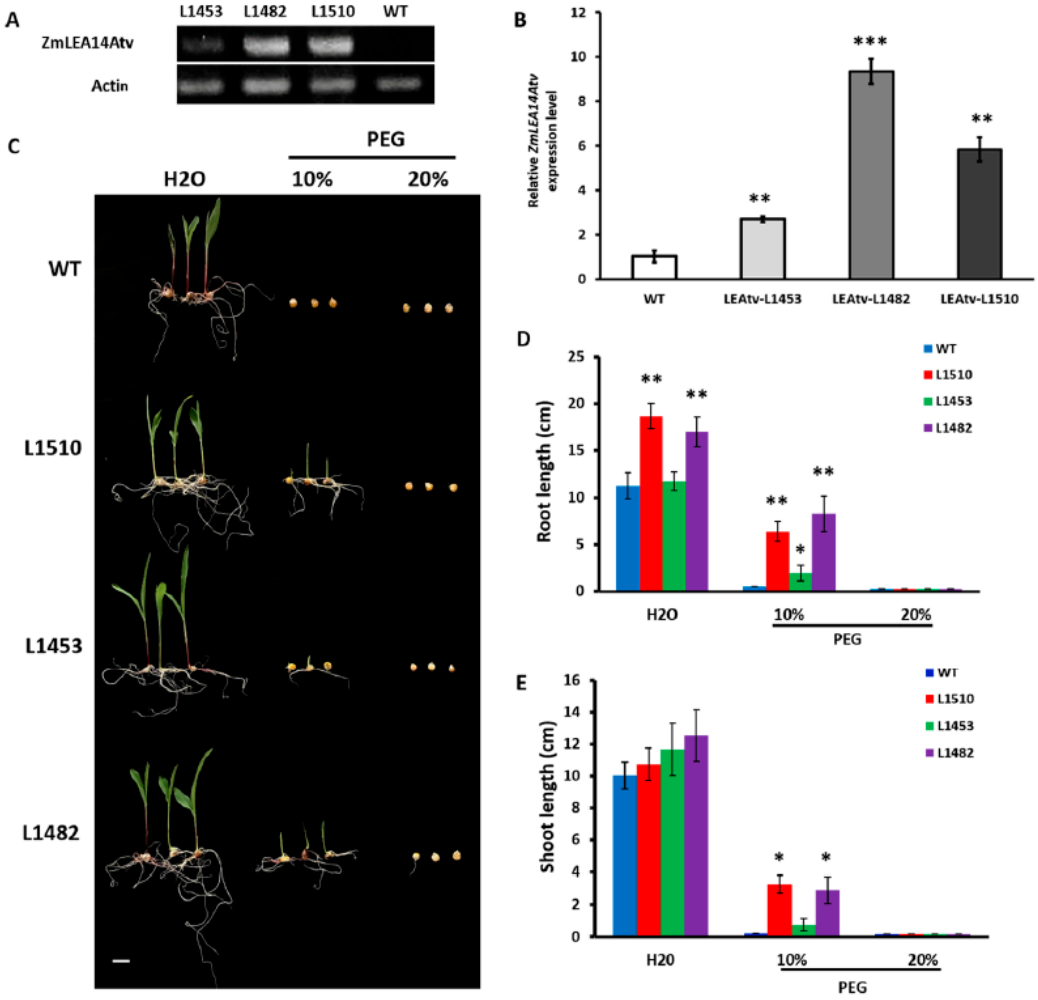

Figure 3. The expression of *ZmLEA14tv* in maize (*Z. mays*). (**A**,**B**) *ZmLEA14tv* expression in three lines of T2 transgenic maize (L1453, L1482, and L1510) determined by RT-PCR (**A**) and qRT-PCR (**B**). Data in (**B**) represent means and standard errors for three biological replicates. (**C**) The phenotype of transgenic and WT maize under various abiotic stress treatment during the germination stage. The T2 of transgenic seeds were soaked in water (as control) or in 10% PEG, 20% PEG solution for drought simulation for one day at 30 °C and then placed on filter paper in plastic boxes wetted with the same solutions mentioned above for eight days. Each experiment was replicated three times. (**D**,**E**) The root and shoot length of transgenic and wild-type maize germinated under control (H_2O) and drought-simulating conditions (10% PEG and 20% PEG). Statistical significance was determined by Student's *t*-test. * $p < 0.05$; ** $p < 0.01$.

Furthermore, the drought tolerance of transgenic maize seedlings in soil was examined (Figure 4B–E). No significant differences in survival rate and fresh weight were observed between the transgenic and WT plants under well-wateredconditions (H_2O). However, after drought stress for 14 days and re-watering, only 40% of WT seedlings could be restored, while this ratio in transgenic lines (LEAtv-L1 and LEAtv-L2) was almost doubled (87% and 80%, respectively) (Figure 4C). In addition, the fresh stem weight and fresh root weight of transgenic lines were significantly higher than the K7 wild type, suggesting a better growth rate of these lines in drought condition (Figure 4D,E). Taken together, these results indicate that the *ZmLEA14tv* gene showed improved drought resistance in transgenic maize.

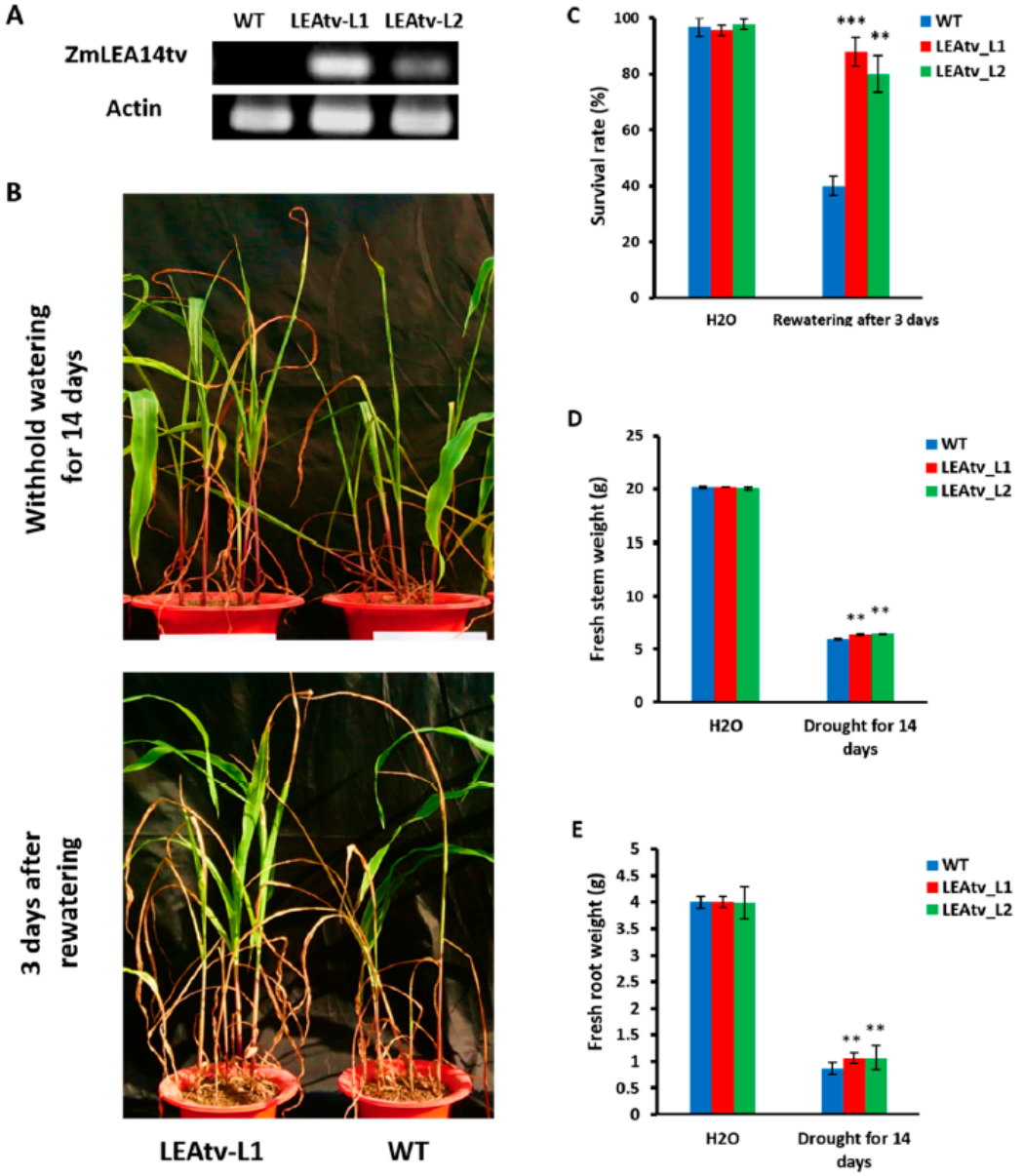

Figure 4. Drought tolerance of maize seedlings overexpressing *ZmLEA14tv*. (**A**) The RT-PCR analysis of *ZmLEA14tv* expression in transgenic maize lines (LEAtv-L1, LEAtv-L2). (**B**) The phenotype of transgenic and WT maize seedlings under drought stress treatment and re-watering conditions. The five-leaf-stage maize seedlings had watering withheld for 14 days, followed by a three-day re-watering. At least five seedlings were grown in each plot and the experiment was replicated three times. WT: wild type. (**C**–**E**) The survival rate, fresh stem weight, and fresh root weight of WT and transgenic maize seedling after the drought and re-watering treatments. Each experiment was replicated three times. Statistical significance was determined by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The identification and characterization of ZmLEA14tv, a putative atypical LEA group 5C member of the Tevang 1 maize cultivar, were reported in the present study. The deduced amino acid sequence of ZmLEA14tv possessed characteristics of a 5C LEA protein that contains a "LEA_2" domain (Pfam cluster PF03168). The LEA proteins are normally known as hydrophilins with a hydrophilicity index of more than 1 and a glycine (Gly) content more than 6% [11]. Typical LEA proteins can retain water

and protect other soluble protein from the aggregation due to their highly hydrophilic properties [33]. Group 5C LEA proteins had a higher proportion of hydrophobic residues than typical LEA proteins; however, they were also involved in various kind of stress tolerance. The estimated GRAVY index of ZmLEA14tv was 0.047, much lower than typical LEA proteins. The ZmLEA14tv protein sequence deduced from the isolated DNA showed a "LEA_2" domain that was characteristic of 5C LEA proteins and a high level of homology with other 5C members.

Functional analysis of 5C LEA proteins showed that the molecular mechanisms of the protective ability against desiccation stress were diverse. The overexpression of AdLEA, a 5C LEA protein from wild peanut, could help maintain the photosynthetic efficiency, reduce the ROS level, and induce the expression of some drought-responsive genes in transgenic tobacco [26]. Meanwhile, overexpressing *SiLEA14* from foxtail millet enhanced a higher level of proline and sugar accumulation in transgenic *Arabidopsis* [13]. The present study showed that the expression of *ZmLEA14tv* in tobacco significantly improved the recoverability of transgenic plants suffering from a short desiccation (Figure 2). This result suggested that *ZmLEA14tv* could function properly in tobacco and enhance its drought tolerance.

One of the methods to generate drought-tolerant maize is enhancing the expression of superior drought-tolerant genes in commercial lines. The overexpression of the *OsSta2* gene, encoding for a AP2/ERF protein, under a maize ubiquitin promoter improved the salt tolerance and grain yield of transgenic rice [34]. Furthermore, enhanced expression of rice dehydrin, namely *OsDhn1*, could increase the tolerance to oxidative stress under salt and drought conditions [35]. Group 5C LEA is well known as an atypical group of proteins that are involved in various abiotic stress response in plants [8,13,24]. In this study, a putative LEA gene namely *ZmLEA14tv* was isolated from the genomic DNA of *Z. mays* cv. Tevang 1, which is well adapted to drought stress in the northern mountains of Vietnam. Our data revealed the potential application of *ZmLEA14tv* in genetic engineering for improving crop performance in the context of climate change. Interestingly, transgenic maize seeds expressing *ZmLEA14tv* showed an improved germination ability in drought-simulated condition in comparison with the WT and did not cause any delay in shoot and root development in transgenic plants.

5. Conclusions

In summary, the coding region of *ZmLEA14tv* was isolated from *Z. mays* cv. Tevang 1 and then cloned into an overexpression cassette. This gene encoded a hydrophobic deduced protein that has a high similarity in structure and a close relationship with other 5C LEA proteins. In addition, the expression of *ZmLEA14tv* in model dicot plants such as tobacco significantly improved the recovery ability, while the enhanced *ZmLEA14tv* transgenic maize showed better germination and growth in drought simulation conditions. These results suggested that *ZmLEA14tv* could act as a potential candidate for genetic engineering to improve drought and other osmotic stress tolerance.

Author Contributions: N.V.H. and H.T.T.H. conceived and designed the experiments; B.M.M., N.T.L., H.H.H., and N.X.T. performed the experiments; B.M.M., L.T.T.H., and H.T.T.H. analyzed the data; B.M.M., N.V.H., and H.T.T.H. prepared the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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