Front. Agr. Sci. Eng. 2021, 8(2): 236–246 https://doi.org/10.15302/J-FASE-2020368

RESEARCH ARTICLE

OVEREXPRESSION OF *PTRLEA7*, A LATE EMBRYOGENESIS ABUNDANT FAMILY GENE FROM *PONCIRUS TRIFOLIATA*, CONFERS ENHANCED DROUGHT TOLERANCE BY ENHANCING ANTIOXIDANT CAPACITY

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KEYWORDS

abiotic stress, antioxidant, drought, late embryogenesis abundant, *Poncirus trifoliata*

HIGHLIGHTS

- A LEA family gene (*PtrLEA7*) was cloned from *Poncirus trifoliata*.
- *PtrLEA7* was strongly induced by stresses and ABA.
- *PtrLEA7* played a positive role in modulation of drought tolerance.
- Overexpression of *PtrLEA7* elevated antioxidant capacity.

Received September 3, 2020;
Accepted November 3, 2020.

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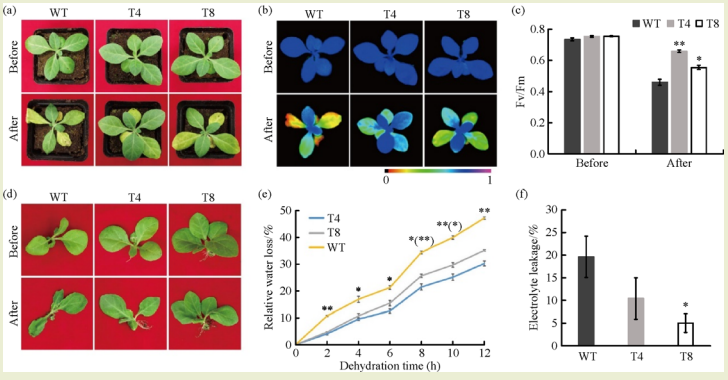
GRAPHICAL ABSTRACT



ABSTRACT

Late embryogenesis abundant (LEA) genes encode highly hydrophilic proteins that are essential in abiotic stress responses. However, most LEA genes in higher plants have not yet been investigated. This study identified an LEA family gene (*PtrLEA7*) from *Poncirus trifoliata* and studied its function in drought tolerance. The full-length coding sequence of *PtrLEA7* was 420 bp encoding a protein of 139 amino acids. Phylogenetic analysis shows that *PtrLEA7* protein belongs to the LEA_4 subfamily. Expression profiling by qPCR found that *PtrLEA7* was strongly induced by dehydration, cold and ABA treatments, and slightly induced by salt stress. Subcellular localization reveals that *PtrLEA7* protein was located in both cytoplasm and nucleus. To investigate its function, transgenic plants of both tobacco and *Poncirus trifoliata* overexpressing *PtrLEA7* were obtained. Stress tolerance assays show that overexpression lines had enhanced dehydration and drought tolerance compared with wild type plants, indicating that *PtrLEA7* positively regulates drought tolerance. In addition, transgenic plants had much

higher expression levels of three antioxidant enzyme genes (*CAT, SOD* and *POD*) and significantly increased catalase enzyme activity, accompanied by reduced reactive oxygen species accumulation in comparison with wild type plants. Collectively, this study demonstrates that *PtrLEA7* can confer enhanced drought tolerance partially via enhancing antioxidant capacity.

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1 INTRODUCTION

Plants are immobile and are challenged by multiple abiotic and biotic stresses throughout their lifespan. They have evolved precise mechanisms including launching stress-responsive genes to cope with these stresses and avoid serious injury. These identified stress-responsive genes can be classified into functional genes (like late embryogenesis abundant family gene, genes encoding enzyme or protease) and regulatory genes (like genes encoding transcription factor and protein kinase)[1]. Functional genes, encoding crucial proteins, have direct roles in plant stress resistance. At present there remain many functional genes of unknown function to be identified in higher plants.

Late embryogenesis abundant (LEA) family genes encode functional proteins that have been investigated for more than 35 years[2]. Since their discovery in cotton seeds during late stages of embryogenesis many more LEA proteins have been identified[3]. For example, in *Arabidopsis* there are 51 LEA encoding genes which can be divided into nine subfamilies including LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, dehydrin, PvLEA18, AtM and SMP[4]. Despite the great diversity among LEA subfamilies, all LEA proteins have some common properties such as hydrophilicity and structural complexity, and are often glycine-rich but lacking in cysteine and tryptophan residues[5,6].

More importantly, an increasing number of studies have demonstrated that LEA proteins are significantly induced by various abiotic stresses such as drought/dehydration, low temperature and high salinity[7–9], indicating the potential role of LEA proteins in stress response and resistance. In recent years, some LEA genes from different organisms have been identified, and their functions in abiotic stress response verified. For example, overexpressing *OsLEA3-1* in rice can confer enhanced drought tolerance without reducing yield[10]. The *LEA3* gene from *Brassica napus* was identified to be a positive regulator of drought tolerance by reducing reactive oxygen species (ROS) accumulation and enhancing photosynthetic efficiency[11]. *ZmLEA3* from maize can confer enhanced low temperature and osmotic stress tolerance by protecting lactate dehydrogenase activity and binding metal ions[12,13]. Consequently, the role of LEA proteins in plant stress response is well known, but the underlying functional mechanisms remain elusive. It is expected that LEA proteins might function as protectants of biomolecules and membranes under abiotic stresses[6,14,15]. In addition, some LEA proteins were found to work in stress tolerance by regulating metal ions and ROS levels[11,16]. Furthermore, LEA proteins have a ubiquitous subcellular distribution across *Arabidopsis* cells which may provide protection extensively for every cellular compartment, revealing that different LEA proteins might work synergistically under abiotic stresses[15].

Abiotic stresses can induce the accumulation of ROS, resulting in oxidative stress which will cause detrimental damage to plant cells. To cope with this, plants have evolved multiple enzymatic and non-enzymatic systems to prevent ROS damage. Antioxidant enzymes are crucial ROS scavengers and include superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT). Many studies have revealed that plants under abiotic stresses can enhance the activities of antioxidant enzymes by upregulating antioxidant enzyme-coding genes to improve tolerance to abiotic stresses[1,11].

Trifoliate orange (*Poncirus trifoliata* (L.) Raf) has higher cold and disease resistance than many other citrus species and is the most important and extensively used citrus rootstock[17]. However, its drought tolerance is relatively poor and its drought responsive genes are poorly identified. Given the crucial role of LEA genes in drought tolerance, it is meaningful to identify key LEA genes from trifoliate orange and study their functions in drought stress. In our previous study we compared the transcriptome of trifoliate orange before and after drought stress, and identified many drought responsive genes of which one LEA family gene named *PtrLEA7* was most significantly upregulated[17]. Here, therefore, we have investigated the function of *PtrLEA7* by overexpressing this gene in both tobacco and trifoliate orange. We found that *PtrLEA7* could confer enhanced drought tolerance and this might be partly due to an increase in antioxidant capacity.

2 MATERIALS AND METHODS

2.1 Plant materials

Seeds of trifoliate orange under natural photoperiod were collected from the nursery of the National Center of Citrus Breeding at Huazhong Agricultural University (Wuhan, China). Tobacco seeds used were from a sample stored in our laboratory. Three-month-old trifoliate orange seedlings were used to examine gene expression patterns under stress treatments. Six-week-old tobacco (*Nicotiana benthamiana*) plants were used for transient expression of subcellular localization. The plants were grown in pots of soil under a photoperiod of 16 h light/8 h dark at 25°C. The shoot segments of trifoliate orange and the leaf discs of tobacco (*Nicotiana tabacum*) in tissue culture were used for transformation to obtain transgenic plants.

2.2 Stress treatments

Three-month-old trifoliate orange was used for stress treatments with 20 seedlings in each treatment. In dehydration treatments, seedlings of trifoliate orange were placed on clean filter paper at room temperature and leaves were collected over a range of time intervals (0, 0.5, 1, 3, 5 and 7 d). In salt treatments the roots of the seedlings were immersed in a 300 mmol·L^{-1} NaCl solution and leaves were collected over a range of time intervals (0, 3, 6, 12, 24 and 48 h). In low temperature treatments, potted seedlings were placed in a growth chamber at 4°C and the leaves were collected over a range of time intervals (0, 0.25, 1, 3 and 5 d). In abscisic acid (ABA) treatments the seedling roots were immersed in a 100 μmol·L^{-1} ABA solution and the leaves were collected over a range of time intervals (0, 3, 6, 12, 24 and 48 h). The fully expanded healthy leaves in the middle position were collected. Every treatment used three plants as three biological replicates. The collected samples were immediately frozen in liquid nitrogen and stored at −80°C.

2.3 Cloning and *PtrLEA7* sequence analysis

The full-length ORF (open reading frame) of *PtrLEA7* was cloned from trifoliate orange based on the homologous gene sequence of sweet orange (ID: Cs1g13160.1, citrus.hzau.edu.cn/orange). Protein sequences of all LEA family genes from sweet orange[9] and *Arabidopsis*[4], and cloned *PtrLEA7* were analyzed collectively by constructing a phylogenetic tree with MEGA 5.

2.4 Quantitative real-time PCR (qPCR)

Total RNA was isolated using a RNAiso Plus Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions with 0.1 g of each leaf sample. First-strand cDNA was synthesized based on the extracted total RNA (0.1 μg total RNA per sample) using a Prime-Script First-Strand cDNA Synthesis Kit (TaKaRa). A qPCR assay was conducted with the synthesized cDNA (0.6 μL), gene-specific primers (0.2 μL), and a SYBR Green Master Mix (5 μL, TaKaRa), and the reaction was conducterd using a QuantStudio 7 Flex system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR reaction process included one cycle of 94°C for 60 s, and 45 cycles of 94°C for 5 s, 60°C for 30 s, followed by a dissociation stage. Each sample used four identical reaction systems as four biological replicates. *ACTIN* was used as the internal reference gene and the gene-specific primers (for *PtrLEA7* and the three antioxidant enzyme genes: *SOD*, *POD* and *CAT*, Table S1) were designed using the Primer-Blast tool in NCBI. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.5 Subcellular localization

First, the subcellular localization of PtrLEA7 was predicted with two online tools, Plant-mPLoc and YLoc^{+}. Then, the open reading frame (ORF) of *PtrLEA7* without the stop codon was cloned into the vector 101LYFP at *Xba*I and *Sal*I restriction sites, forming a fusion construct (35S: PtrLEA7-YFP) co-expressing PtrLEA7 and YFP (yellow fluorescent protein) proteins. After sequencing, the fusion construct (35S: PtrLEA7-YFP) and the control vector (35S: YFP) were separately transferred into *Agrobacterium tumefaciens* GV3101 and transient expression was performed in tobacco (*N. benthamiana*) leaves according to methods described previously[18]. The fully expanded leaves of six-week-old tobacco plants were injected with the bacterial suspension. Three leaves of each tobacco plant were injected and four tobacco plants in total were used for injection. Subsequently, all plants were incubated in a growth chamber (16 h light/8 h dark at 25°C) for two days. The fluorescence signals were observed with a laser scanning confocal microscope (Leica TCS SP8, Wetzlar, Germany).

2.6 Genetic transformation of tobacco and trifoliate orange

The ORF of *PtrLEA7* was cloned into the vector pBI121 at *Xba*I and *Sma*I restriction sites and the constructed vector was transferred into GV3101. For genetic transformation, six-week-old tobacco on MS (Murashige and Skoog[19]) medium (under 16 h light/8 h dark at 25°C) and three-week-old trifoliate orange on MT (Murashige and Tucker[20]) medium (in the dark at 25°C) were prepared in advance. Genetic transformation of tobacco was performed using leaf discs as explants[21] and shoot

segments of trifoliate orange were used as explants according to methods described previously[22]. The transformants were grown on MS (tobacco) or MT (trifoliate orange) medium containing 10 μg kanamycin mL^{-1}. For transgenic identification, DNA was extracted and semiquantitative RT-PCR was conducted with two pairs of primers (35S-F + *PtrLEA7*-R and *NPTII*-F/R). qPCR was performed as described previously using *Ubiquitin* and *ACTIN* as internal controls, respectively, to examine the expression levels of *PtrLEA7* in transgenic tobacco and trifoliate orange plants.

2.7 Drought/dehydration tolerance assays

T2 transgenic tobacco plants were generated by sowing seeds from T1 positive plants. Three-week-old T2 transgenic and wild type plants (grown in pots of soil under a photoperiod of 16 h light/8 h dark at 25°C) were used to simulate drought conditions by withholding water for 20 d. All plants were photographed and chlorophyll fluorescence recorded with an IMAGING-PAM chlorophyll fluorimeter (Heinz Walz GmbH, Effeltrich, Germany) before and after drought treatment. The maximum quantum efficiency of the photochemistry (Fv/Fm) was obtained according to the Imaging WinGegE software (Heinz Walz GmbH, Effeltrich, Germany)[23].

The three-week-old tobacco and four-month-old trifoliate orange plants grown in pots of soil under the natural photoperiod (16 h light/8 h dark at 25°C) were used to simulate dehydration conditions. Dehydration treatment assays were conducted by placing detached plants on clean filter paper at room temperature (25°C) for 48 h. Relative water loss was measured by weighing plants after each time interval[17]. Electrolyte leakage was measured after treatment using a conductivity meter (DSS-307, SPSIC, Shanghai, China) according to the instrument specification and a previously described method[23].

2.8 CAT activity measurement and histochemical staining

Leaves of the four-month-old wild type (WT) and transgenic trifoliate orange plants without treatment were used to examine CAT activity using kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. Total protein was measured according to the Coomassie Brilliant Blue G-250 staining method. Histochemical staining was carried out to observe the in situ accumulation of H_2O_2 and O_2^- by immersing leaves in 3,3'-diaminobenzidine and nitrotetrazolium blue chloride solutions, respectively, according to methods described previously[24].

2.9 Statistical analysis

Each experiment was conducted at least twice and every sample had at least three biological replicates. Statistical analysis was conducted in Microsoft Office Excel and IBM SPSS 22.0 (IBM SPSS STATISTICS, IBM Armonk, NY). The values are presented as mean±SE and statistically significant differences were determined using one-way analysis of variance in IBM SPSS, and shown as * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

3 RESULTS

3.1 Cloning and bioinformatic analysis of *PtrLEA7*

We previously sequenced the transcriptome of trifoliate orange before and after drought stress and identified some drought responsive genes[17]. From these genes we identified one LEA family gene (designated *PtrLEA7*) which was significantly drought-induced[17]. The full-length ORF of this gene was cloned from trifoliate orange and was 420 bp encoding a protein of 139 amino acids. A phylogenetic tree was constructed based on the protein sequences of all LEA family genes from *Arabidopsis*[4] and *Citrus sinensis*[9] and the protein sequence of *PtrLEA7*. The tree shows that PtrLEA7 was classified into the LEA_4 subfamily and was most closely related to orange1.1g045955m and AtLEA7 (Fig. 1), which also explains the designated name PtrLEA7.

3.2 *PtrLEA7* was induced by multiple stresses and abscisic acid treatment

The expression patterns of *PtrLEA7* were examined in trifoliate orange seedlings under different abiotic stresses and ABA treatment by qPCR. The results show that *PtrLEA7* was gradually upregulated during 7 d of dehydration, reaching a maximum at 5 d (Fig. 2(a)). Also, under low-temperature stress *PtrLEA7* was induced, peaking at 3 d, and then declining after recovery for 1 d (Fig. 2(b)). During 48 h of salinity (NaCl) treatment, *PtrLEA7* was slightly induced with a maximum expression level after 3 h (Fig. 2(c)). ABA signaling has a key role in abiotic stress response[25]. When examining expression patterns during ABA treatment we found that *PtrLEA7* was downregulated during the first 12 h and then upregulated after 24 h (Fig. 2(d)). These results show that *PtrLEA7* can be strongly induced by dehydration, low-temperature, and ABA treatments.

3.3 PtrLEA7 was localized in cytoplasm and nucleus

We first analyzed the subcellular localization of PtrLEA7 to

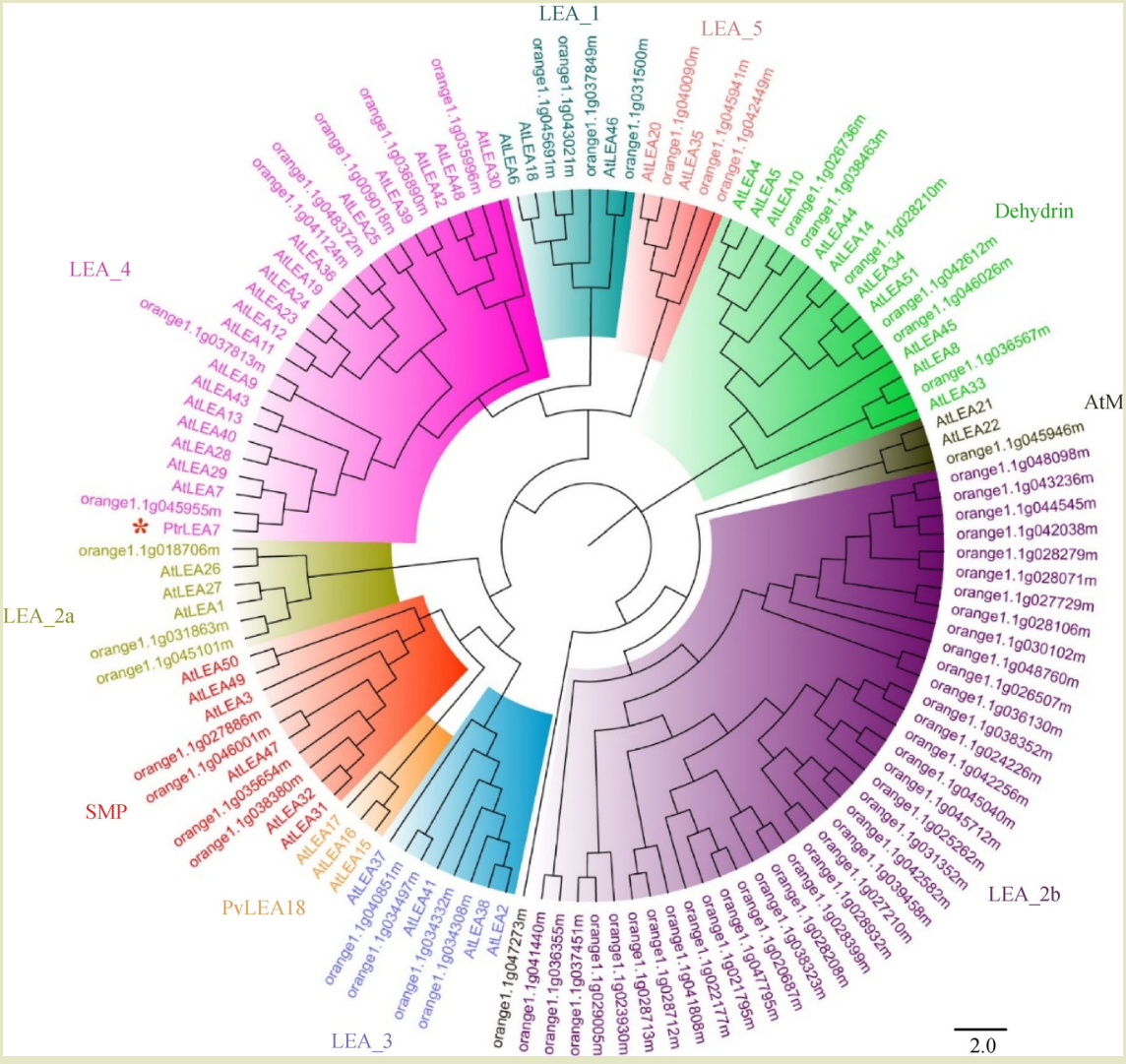

Fig. 1 Phylogenetic analysis of LEAs. Phylogenetic tree is constructed based on LEA protein sequences of *Arabidopsis thaliana*, *Citrus sinensis* and PtrLEA7. Different subfamilies are shown with different colors. The LEAs are divided into 10 subfamilies based on the taxonomy proposed in previous reports[4,8]. The scale bar indicates the relative amount of change along branches.

study its function. Two online tools (Plant-mPLoc and YLoc$^+$) predicted that PtrLEA7 protein could be localized in cytoplasm or nucleus (Table S2). To verify this, a constructed vector expressing both PtrLEA7 and YFP, along with control vector (expressing only YFP), were transiently expressed in tobacco leaves via mediation of *A. tumefaciens*. By microscopic observation the fluorescence of the control protein (YFP) was found to be distributed over the whole cell (Fig. 3(a–d)). The fusion protein (PtrLEA7-YFP) was found to be localized both in the nucleus and in other intracellular regions (Fig. 3(e–h)), which was considered to be a cytoplasm region based on the online prediction (Table S2). These results indicate that PtrLEA7 was localized in both nucleus and cytoplasm.

3.4 Overexpression of *PtrLEA7* conferred enhanced drought and dehydration tolerance

PtrLEA7 was dramatically induced by dehydration treatment (Fig. 2(a)). To further identify the function of *PtrLEA7*, transgenic tobacco plants were generated by overexpressing *PtrLEA7* via an *Agrobacterium* mediated transformation system (Fig. S1(a–d)). Three positive transgenic lines (T4, T6 and T8) were obtained in which the expression levels of *PtrLEA7* were significantly upregulated by more than 35 times that of the WT (Fig. S1(e–g)). The T2 transgenic tobacco seedlings in pots of soil were used to assess drought tolerance. Before treatment, no

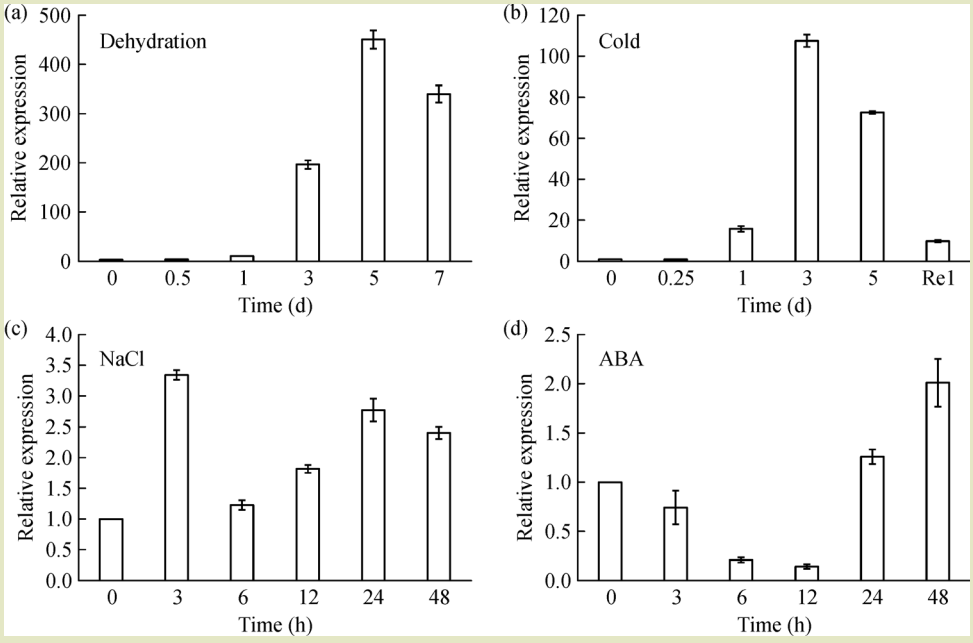

Fig. 2 Expression profiling of *PtrLEA7* under abiotic stresses and ABA treatment. Relative expression levels are measured by qPCR at each time point of different treatments comprising dehydration (a), low temperature (b), salt (c), and ABA (d).

morphological differences were observed between WT and two transgenic lines (T4 and T8). However, after drought treatment the WT plants showed a more sensitive phenotype with more severe yellowing in the leaves (Fig. 4(a)). The maximum quantum yield of PSII can evaluate the photoinhibition in plants under environmental stress, so the fluorescence image and the corresponding Fv/Fm values can reflect stress tolerance of plants. The fluorescence image shows that WT and transgenic lines had the same fluorescence before drought stress, whereas WT was impaired more severely than the two transgenic lines after drought stress (Fig. 4(b)). In line with the fluorescence image, Fv/Fm values of two transgenic lines were significantly higher than of WT after drought stress (Fig. 4(c)). Additionally, seedlings separated from soil were subjected to dehydration

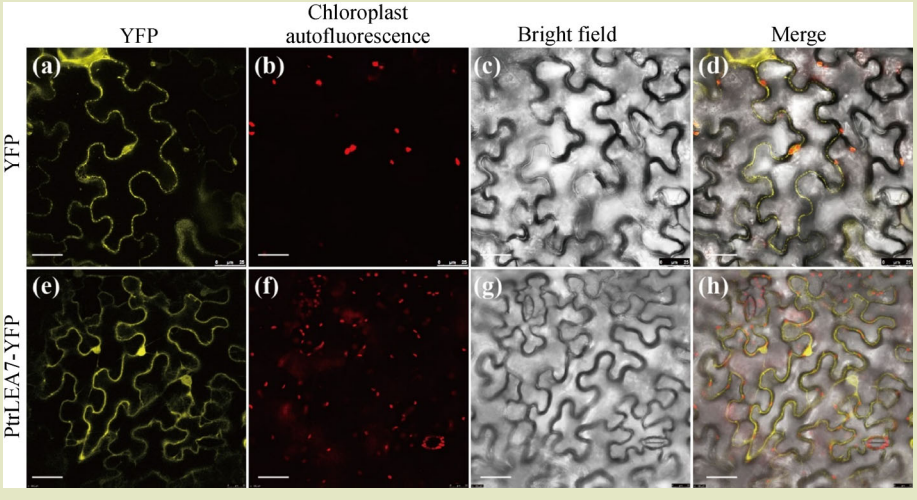

Fig. 3 Subcellular localization of PtrLEA7. The upper and lower images indicate the fluorescence of the control (YFP, a–d) and the constructed vector (PtrLEA7-YFP, e–h), respectively. The images show representative cells under UV field (a, e), autofluorescence (b, f), bright-field (c, g), and merged field (d, h). Bar = 25 μm.

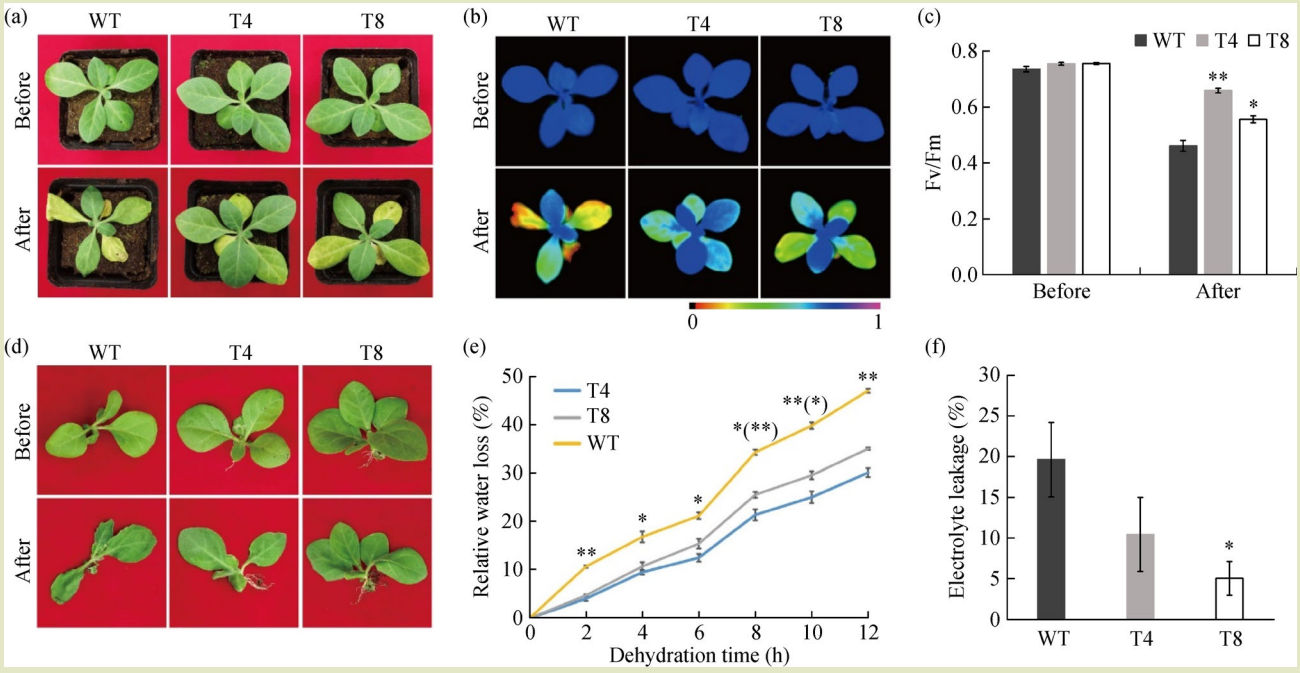

Fig. 4 Drought and dehydration tolerance assays of transgenic tobacco plants overexpressing *PtrLEA7*. (a–c) Phenotype (a), chlorophyll fluorescence (b), and corresponding Fv/Fm values (c) of transgenic and wild type (WT) plants before and after drought treatment. (d–f) Phenotype (d), relative water loss (e), and electrolyte leakage (f) of transgenic and wild plants during or after dehydration treatment. Lines T4 and T8 are transgenic.

treatment, and WT leaves had more pronounced shrinkage than the transgenic lines after treatment (Fig. 4(d)). During this treatment the relative water loss of the two transgenic lines was significantly lower than the WT (Fig. 4(e)). Also, after dehydration treatment the electrolyte leakage of the two transgenic lines was lower than of WT (Fig. 4(f)). Thus, the transgenic tobacco plants overexpressing *PtrLEA7* were more tolerant to drought and dehydration stresses than the WT.

To further identify the function of *PtrLEA7* in trifoliate orange we also overexpressed this gene in trifoliate orange and obtained ten positive transgenic plants (Fig. S2(a–e)). The qPCR analysis shows that gene expression levels of *PtrLEA7* in two transgenic lines (OE5 and OE8) were much higher than in the WT (Fig. S2(f)), suggesting that *PtrLEA7* was successfully overexpressed in trifoliate orange. Dehydration treatment was applied to shoots detached from the transgenic plants. Before treatment the transgenic plants and WT had the same phenotype, but after treatment WT shoots wilted more severely than the transgenic plants (Fig. 5(a)). In agreement with this phenotype, the two transgenic lines also displayed less impaired fluorescence and much higher Fv/Fm values than the WT plants (Fig. 5(b,c)). During the 48-h dehydration treatment the relative water loss of transgenic lines ase significantly lower than in the WT (Fig. 5(d)). These results demonstrate that overexpressing *PtrLEA7* in trifoliate orange can enhance dehydration tolerance. Collectively, these results in transgenic tobacco and trifoliate orange plants reveal that overexpression of *PtrLEA7* can confer enhanced drought and dehydration tolerance.

3.5 Transgenic lines show enhanced reactive oxygen species (ROS) scavenging capacity

To determine the mechanisms underlying the enhanced drought tolerance of *PtrLEA7*-overexpressed plants, expression of some drought-related genes was examined. Three antioxidant enzyme genes (*CAT*, *SOD* and *POD*) were screened as their expression levels were higher in the two transgenic lines than in the WT, especially *CAT* (Fig. 6(a)). The *CAT* gene encodes catalase, which showed significantly higher enzyme activity in the two transgenic lines than in the WT (Fig. 6(b)) in accordance with the gene expression levels. Catalase functions in the scavenging of ROS, which is adverse in plants under stresses [26]. Thus, the distribution of two major ROS (H_2O_2 and O_2^-) in leaves were observed by histochemical staining. The results show that WT leaves had much deeper and more extensive staining than leaves of the two transgenic lines (Fig. 6(c)), indicating less ROS

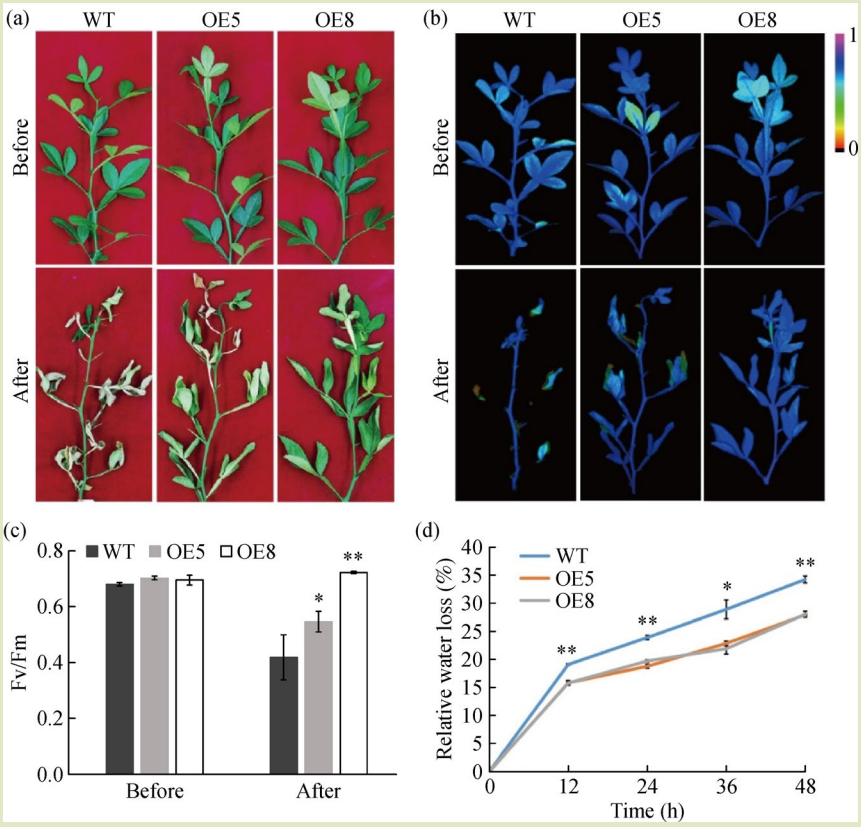

Fig. 5 Dehydration tolerance assays of transgenic trifoliate orange plants overexpressing *PtrLEA7*. (a–c) Phenotype (a), chlorophyll fluorescence (b), and corresponding Fv/Fm values (c) of transgenic and wild type (WT) plants before and after dehydration treatment. (d) Relative water loss over a 48-h dehydration treatment. OE5 and OE8 represent two transgenic lines.

accumulation in transgenic plants after drought stress. Taken together, these results demonstrate that transgenic plants overexpressing *PtrLEA7* had enhanced ROS scavenging capacity.

4 DISCUSSION

LEA proteins have been known for more than 30 years[27]. They

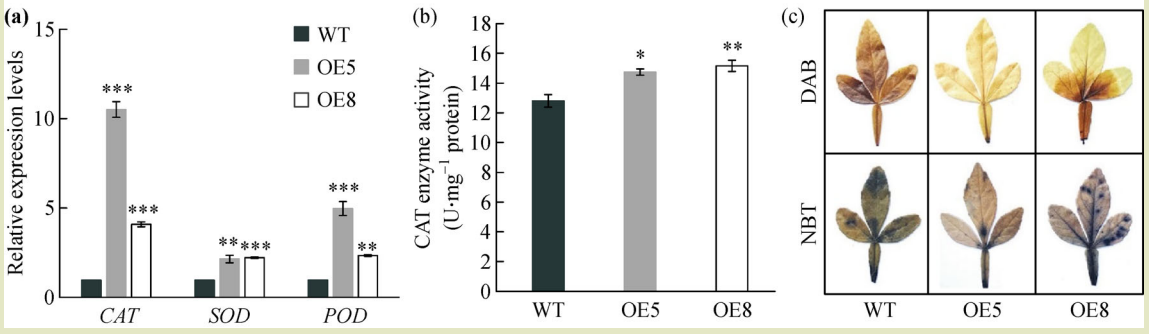

Fig. 6 Investigations of antioxidant capacity and ROS accumulation in *PtrLEA7*-overexpressed trifoliate orange plants. (a,b) Relative expression levels of three antioxidant enzyme genes (*CAT*, *SOD* and *POD*) (a) and catalase (CAT) enzyme activity (b) in WT and two transgenic lines. (c) *In situ* ROS accumulation in the leaves of WT and two transgenic lines after dehydration treatment by 3,3′-diaminobenzidine and nitrotetrazolium blue chloride staining.

were initially identified from cotton seeds during late embryogenesis, which is also the reason for the name LEA. Subsequently, some LEA proteins were identified from other vegetative tissues, especially those under abiotic stress[28]. LEA proteins are not plant specific as they have been found in some bacteria and insect larvae[27]. LEA proteins have some important properties being highly hydrophilic in structure and significantly heat stable[15]. Although increasing LEA proteins have been investigated in a range of plant species, their function in most higher plants remains elusive.

Here, one LEA family gene named *PtrLEA7* was cloned from trifoliate orange and phylogenetic analysis classified this gene into the LEA_4 subfamily (Fig. 1). LEA family proteins in *Arabidopsis* were classified into nine subfamilies comprising AtM, dehydrin, LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, PvLEA18, and SMP[4], and latter studies in other plant species mostly adopted this taxonomy. Of these subfamilies, LEA_4 has the most members in *Arabidopsis*[4]. The well-known genes *COR15A* and *COR15B* and the newly cloned gene *PtrLEA7* all belong to this subfamily[29]. The protein members in this subfamily have large differences in protein size and hydrophilicity, and therefore diverse functions in plants. To systematically analyze the classification of LEA proteins, here we collected all the LEA proteins from sweet orange and *Arabidopsis* and reclassified them based on previous studies[4,9]. The results show that all LEA proteins could be placed in 10 groups: AtM, dehydrin, LEA_1, LEA_2a, LEA_2b, LEA_3, LEA_4, LEA_5, PvLEA18 and SMP (Fig. 1). Here,LEA_2 was divided into LEA_2a and LEA_2b as these two subfamilies were found to belong to LEA_2 in a previous study[9]. Hence, a large number of LEA proteins have complicated classification, indicating that the functions of LEAs may be diverse.

This study shows that *PtrLEA7* may be induced by multiple abiotic stresses, especially dehydration and low temperatures (Fig. 2), and this is consistent with previous studies[30,31]. An increasing number of studies have revealed that most LEA proteins are dehydration/drought and low temperature induced, for example the dehydrin subfamily which are typically drought induced, and COR15A/B which are cold induced[29]. This stress-induced property indicates that LEA proteins might be involved in the abiotic stress response. While existing studies indicate that LEA proteins have no enzymatic activity, they likely function in stress response as protectants of macromolecules or membranes[15]. For example, some specific LEA proteins can protect enzymes from degradation[15]. Overexpression of *PtrLEA7* in this study also shows enhanced antioxidant enzyme activity (Fig. 6). This protective mechanism reveals that LEA proteins might serve as molecular chaperones by interacting with other proteins. Thus, searching for LEA-interactive proteins is important in future studies. Additionally, LEA proteins can segregate harmful metal ions and ROS to reduce their damaging effects under stress conditions[11,12,15,32]. As shown in this study, ROS accumulation in *PtrLEA7* overexpressed plants was significantly reduced (Fig. 6). Overall, LEAs are critical functional proteins and can directly function in plant stress tolerance, but the underlying mechanisms remain elusive and need further investigation.

By overexpressing *PtrLEA7* we found that transgenic plants exhibited enhanced drought and dehydration tolerance in both homologous trifoliate orange (Fig. 5) and heterogeneous tobacco (Fig. 5). Similar results were obtained in other studies in which ectopic expression of individual LEA proteins can significantly increase drought[10,12,33], cold[33] and salt tolerance[34]. Thus, LEA family genes have the potential to be used in breeding new germplasm with increased stress tolerance by genetic engineering. For example, it was confirmed that overexpression of LEA genes could confer enhanced drought tolerance without yield and quality reduction in rice[10,35]. With increasing numbers of LEA genes being identified from various plant species, their functions in abiotic stress response will be understood and utilized extensively in the future.

5 CONCLUSIONS

This study identified one LEA family gene (*PtrLEA7*) from trifoliate orange and investigated its function in drought tolerance. The results show that PtrLEA7 belongs to the LEA_4 subfamily and is located in both cytoplasm and nucleus. Its expression may be significantly induced by multiple abiotic stresses and ABA treatment especially dehydration and low temperatures. Overexpression of *PtrLEA7* conferred enhanced dehydration and drought tolerance in both trifoliate orange and tobacco plants. Further investigations revealed that *PtrLEA7* functioned in drought tolerance mainly by reducing ROS accumulation via increasing antioxidant capacity.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2020368 contains supplementary materials (Tables S1–S2; Figs. S1–S2).

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2018YFD1000300), the National Natural Science Foundation of China (31972377), Hubei Provincial Natural Science Foundation for Innovative Group (2017CFA018), and Leading Talents Fund in Science and Technology Innovation in Henan Province (194200510007).

Compliance with ethics guidelines

Tonglu Wei, Dalong Guo, and Jihong Liu declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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