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ZmLEA3, a Multifunctional Group 3 LEA Protein from Maize (*Zea mays* L.), is Involved in Biotic and Abiotic Stresses

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Regular Paper

Late embryogenesis abundant (LEA) proteins accumulate to high levels during the late stage of seed maturation and in response to water deficit, and are involved in protecting higher plants from damage caused by environmental stresses, especially drought. In the present study, a novel maize (*Zea mays* L.) group 3 LEA gene, *ZmLEA3*, was identified and later characterized using transgenic tobacco plants to investigate its functions in abiotic and biotic stresses. Transcript accumulation demonstrated that *ZmLEA3* was induced in leaves by high salinity, low temperature, osmotic and oxidative stress as well as by signaling molecules such as ABA, salicylic acid (SA) and methyl jasmonate (MeJA). The transcript of *ZmLEA3* could also be induced by pathogens [*Pseudomonas syringae* pv. tomato DC3000 (*pst dc3000*)]. ZmLEA3 is located in the cytosol and the nucles. Further study indicated that the ZmLEA3 protein could bind Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. Overexpression of *ZmLEA3* in transgenic tobacco (*Nicotiana tabacum*) and yeast (GS115) conferred tolerance to osmotic and oxidative stresses. Interestingly, we also found that overexpression of *ZmLEA3* in transgenic tobacco increased the hypersensitive cell death triggered by *pst dc3000* and enhanced the expression of *PR1a*, *PR2* and *PR4* when compared with the wild type. Thus, we proposed that the ZmLEA3 protein plays a role in protecting plants from damage by protecting protein structure and binding metals under osmotic and oxidative stresses. In addition, ZmLEA3 may also enhance transgenic plant tolerance to biotic stress.

Keywords: Hypersensitive response • Metal binding • Osmotic stress • Oxidative stress • Plant pathogens • ZmLEA3.

Abbreviations: APX, ascorbate peroxidase; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; CaMV, *Caulifower mosaic virus*; CAT, catalase; CS4, citrate synthase 4; GFP, green fluorescent protein; HR, hypersensitive response; IMAC, immobilized metal ion affinity chromatography; JA, jasmonate; LEA, late embryogenesis abundant; LDH, lactate dehydrogenase; MDA, malondialdehyde; MeJA, methyl jasmonate; MS, Murashige and Skoog; PEG, polyethylene glycol; POD, peroxidase; PR, pathogenesis related; *pst dc3000*, *Pseudomonas syringae* pv. tomato DC3000; qRT–PCR, quantitative real-time reverse transcription–PCR; ROS, reactive oxygen species; SA, salicylic acid; TBA, thiobarbituric acid; TCA, trichloroacetic acid; SOD, superoxide dismutase; WT, wild type; YNB, yeast nitrogen base.

Introduction

Environmental stresses such as drought, high salinity and disease induce changes in enzyme activities and gene expression in crop plants, leading to considerable reduction in their growth and productivity. In response to various stresses, plants produce a series of proteins to protect cell metabolism. The synthesis of hydrophilic proteins is a major part of the plant response to stress conditions. Late embryogenesis abundant (LEA) proteins are major hydrophilic proteins, which can reduce the damage caused by adverse conditions.

LEA proteins were first identified in cotton seeds 31 years ago (Dure and Galau 1981); they are accumulated during the late stages of seed development and are associated with the acquisition of desiccation tolerance in maturing seeds. LEA proteins have high hydrophilicity, a lack or low proportion of cysteine and tryptophan residues, and a preponderance of certain amino acid residues such as glycine, glutamate, lysine and threonine. According to amino acid sequences and conserved motifs, LEA proteins are categorized into seven distinctive groups (Battaglia et al. 2008). Three major groups (numbered 1, 2 and 3) of LEA proteins have been described in a range of different plants and plant tissues. Group 3 LEA protein functions have been extensively studied in transgenic plants, and are characterized by a repeating motif of 11 amino acids TAQAAKEKAGE (Dure 1993). Circular dichroism (CD) analysis

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of various group 3 LEA proteins indicated that they are in a randomly coiled structure in solution. However, they adopt an α-helical conformation in the presence of sucrose and glycerol, or after drying, and are thought to be involved in protein–protein and protein–lipid interactions under stress conditions (Tolleter et al. 2007, Battaglia et al. 2008). Group 3 LEA proteins might also contribute to the formation of a tight hydrogen-bonding network in the dehydrating cytoplasm, together with sugars, to promote the long-term stability of sugar glasses during anhydrobiosis (Wolkers et al. 2001, Battaglia et al. 2008).

LEA proteins have been reported to contribute to various developmental processes and to accumulate in response to drought, low temperature, salt stress or treatment with the phytohormone ABA (Shao et al. 2005, Tunnacliffe and Wise 2007). Overexpression of wheat *TaLEA2* and *TaLEA3* in yeast resulted in improved salt and freezing stress tolerance (Yu et al. 2005). Co-expressed plant and animal LEA proteins in mammalian cells, which could self-aggregating polyglutamine (polyQ) proteins, showing more protection at early time points (Liu et al. 2011). Overexpression of soybean PM2 in bacteria conferred tolerance to salt stress (Liu and Zheng 2005). A group 3 LEA protein from the desiccation-tolerant nematode *Aphelenchus avenae* was able to prevent the aggregation of many other proteins both in vitro and in vivo (Chakrabortee et al. 2007). The maize LEA3 protein Mlg3 can be induced by ABA and hyperosmolarity (Thomann et al. 1992), and it can reduce cell shrinkage effects during dehydration (Amara et al. 2012). Some LEA proteins have been reported to bind DNA and metal. Citrus dehydrin, CuCOR15, was reported to bind Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (Hara et al. 2005), which was thought to prevent the production of reactive oxygen species (ROS). *Vigna radiata* dehydrin, VrDhn1, can bind metal and DNA, and this might stabilize DNA during seed dehydration (Lin et al. 2012). The high correlation found between the accumulation of group 3 LEA proteins and their transcripts under stress conditions indicates that they are important factors for plants to adapt to environmental stress.

Maize (*Zea mays* L.) is an important monocotyledonous crop worldwide. Drought and plant disease are the major factors limiting the suitability of geographical locations for growing crops and horticultural plants, and they periodically account for significant losses in plant productivity. In this report, we present the molecular and functional characterization of a novel group 3 LEA gene, *ZmLEA3*, from maize, which was found to be significantly up-regulated in response to biotic stress [the pathogen *Pseudomonas syringae* pv. tomato DC3000 (*pst dc3000*)] and various abiotic stresses, such as hyperosmolarity, cold, high salt and ABA. Moreover, the ZmLEA3 protein bound Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, which may reduce oxidative damage. Overexpression of *ZmLEA3* in tobacco resulted in improved osmotic stress tolerance and enhanced tolerance for oxidation stress. Finally, overexpression of *ZmLEA3* also resulted in enhanced tolerance to the pathogen *pst dc3000*.

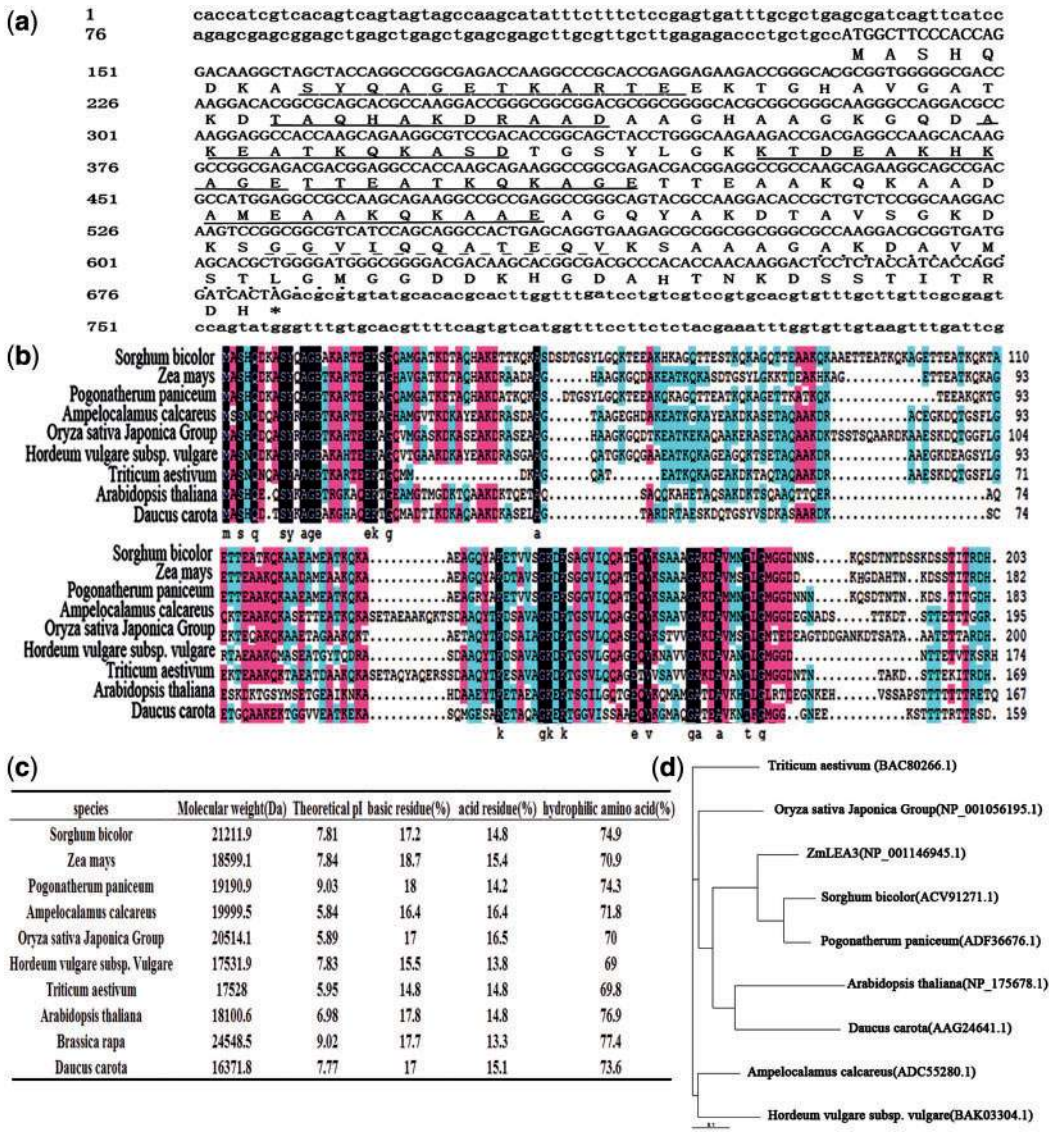
Results

Isolation and bioinformatic analysis of *ZmLEA3*

To elucidate the mechanism of group 3 LEA proteins that are involved in plant stress responses, we isolated the gene *ZmLEA3* (GenBank accession No. NM_001153473) from maize. The *ZmLEA3* cDNA has an open reading frame of 546 bp that encodes a protein of 182 amino acids with a predicted molecular mass of 18.59 kDa and a pI of 7.84 (http://www.expasy.org/tools/pi_tool.html). Analysis of *ZmLEA3* using the maizeGDB databases (http://www.maizegdb.org/) showed that ZmLEA3 was located on the long arm of chromosome 6 and consists of three exons and two introns. The variability in the 11-mer motif leads to a subclassification of the group 3 LEA proteins into two subgroups: 3A, represented by the cotton D-7 LEA protein; and 3B, represented by the cotton D-29 LEA protein, The 3A subgroup is highly conserved; motifs 5 (TAQ [A/S] AK [D/E] KT[S/Q] E) correspond to almost the same 11-mer described originally for this subgroup. Motif 4 (SYKAGETKGRKT) is present in the N-terminal portion of 3A proteins, and motifs 1 (GGVLQQTGEQV) and 2 (AADAVKHTLGM) are present in the C-terminal portion. The other subgroup (3B) is more heterogeneous; the motif (ESW [T/A] [E/G] WAK [E/D] KI) is highly conserved and is unique to this subgroup (Dure 2001, Battaglia et al. 2008). According to the analysis of the conserved domain (**Fig. 1a**), the ZmLEA3 protein is classified in the group LEA3A. The protein is rich in alanine (22%), glycine (10.4%), lysine (14.3%) and threonine (10.4%), while it lacks cysteine, tryptophan, phenylalanine and proline. As shown by hydropathy plots (Kyte and Doolittle 1982), the ZmLEA3 protein is highly hydrophilic, and contains 70.9% hydrophilic amino acid residues (**Supplementary Fig. S1a**). Using PONDR (Romero et al. 2004), the protein is predicted to be highly disordered (**Supplementary Fig. S1b**). The ZmLEA3 protein displays a diverse homology with other group 3 LEA proteins and broadly matches similar segments in related LEA proteins, indicating a close evolutionary relationship among these proteins (**Fig. 1b, c**). Selecting several representative proteins of group 3 LEA proteins, we compared the molecular weight, theoretical pI and amino acid content of the group. All the proteins are highly hydrophilic (from 69% to 77.4%), and the theoretical pI ranges from 5.84 to 9.03 (**Fig. 1c**).

Transcript accumulation of *ZmLEA3* under different stress treatments

Because LEA proteins can be induced by different abiotic stresses, the transcript accumulation of *ZmLEA3* was studied after the plant was exposed to various stress conditions for different periods of time. Quantitative real-time reverse transcription–PCR (qRT–PCR) was performed using cDNA derived from stressed and non-stressed leaves of *Z. mays*. The results demonstrated the inducibility of *ZmLEA3* transcript accumulation in response to salt, dehydration and low temperature, as well as treatment with ABA, salicylic acid (SA), methyl jasmonate



species	Molecular weight(Da)	Theoretical pI	basic residue(%)	acid residue(%)	hydrophilic amino acid(%)
Sorghum bicolor	21211.9	7.81	17.2	14.8	74.9
Zea mays	18599.1	7.84	18.7	15.4	70.9
Pogonatherum paniceum	19190.9	9.03	18	14.2	74.3
Ampelocalamus calcareus	19999.5	5.84	16.4	16.4	71.8
Oryza sativa Japonica Group	20514.1	5.89	17	16.5	70
Hordeum vulgare subsp. Vulgare	17531.9	7.83	15.5	13.8	69
Triticum aestivum	17528	5.95	14.8	14.8	69.8
Arabidopsis thaliana	18100.6	6.98	17.8	14.8	76.9
Brassica rapa	24548.5	9.02	17.7	13.3	77.4
Daucus carota	16371.8	7.77	17	15.1	73.6

Fig. 1 Sequence analysis of *ZmLEA3* derived from maize, and multiple sequence alignment and analysis of group 3 LEA proteins from several plant species. (a) The nucleotide sequence of *ZmLEA3* cDNA together with its predicted amino acid sequence. The putative different conserved segments are indicated as motif 1 (short-dashed line), motif 2 (dotted line), motif 4 (long-dashed line) and motif 5 (solid line). (b) Multiple sequence alignment of ZmLEA3 with other group 3 LEA proteins. (c) Comparison of the molecular weight (Da), pI and amino acid content (%) of different group 3 LEA proteins. (d) Phylogenetic relationship of ZmLEA3 with other closely related LEA proteins. The unrooted dendrogram was constructed with the Treeview tool using the maximum likelihood method based on a complete protein sequence alignment of LEA proteins from other species.

(MeJA) and *pst dc3000*. Organ-specific expression studies showed that the transcription of *ZmLEA3* was higher in seeds than that in roots, stems and leaves (**Fig. 2a**). Under ABA treatment, the transcript level of *ZmLEA3* was induced slowly within 24 h, reached its highest level suddenly at 36 h, maintained this level up to 48 h, and then reverted back to near its control level immediately when the signal was removed (**Fig. 2b**). The transcript accumulation of ZmLEA3 in response to H_2O_2 reached its highest level at 12 h and then was reduced to its usual normal level (**Fig. 2c**). The transcript level of *ZmLEA3* in response to NaCl treatment reached a high peak within 24 h and then was reduced at 36 h, but its transcript accumulation reached its highest level at 48 h and decreased to close to its uninduced level when the treatment was removed (**Fig. 2d**). Under 20% polyethylene glycol (PEG) treatment, there were two peaks; the transcript accumulation was induced at 6 h and reached a high level at 12 h, but the transcript accumulation was alleviated at 24 h, and reached its highest level at 48 h, suggesting the existence of a feedback adjustment (**Fig. 2e**). Cold treatment caused *ZmLEA3* expression to reach its highest level at 24 h and then reduce slowly to the pre-induction level (**Fig. 2g**). SA and jasmonates (JAs) are known to play major roles in regulating plant

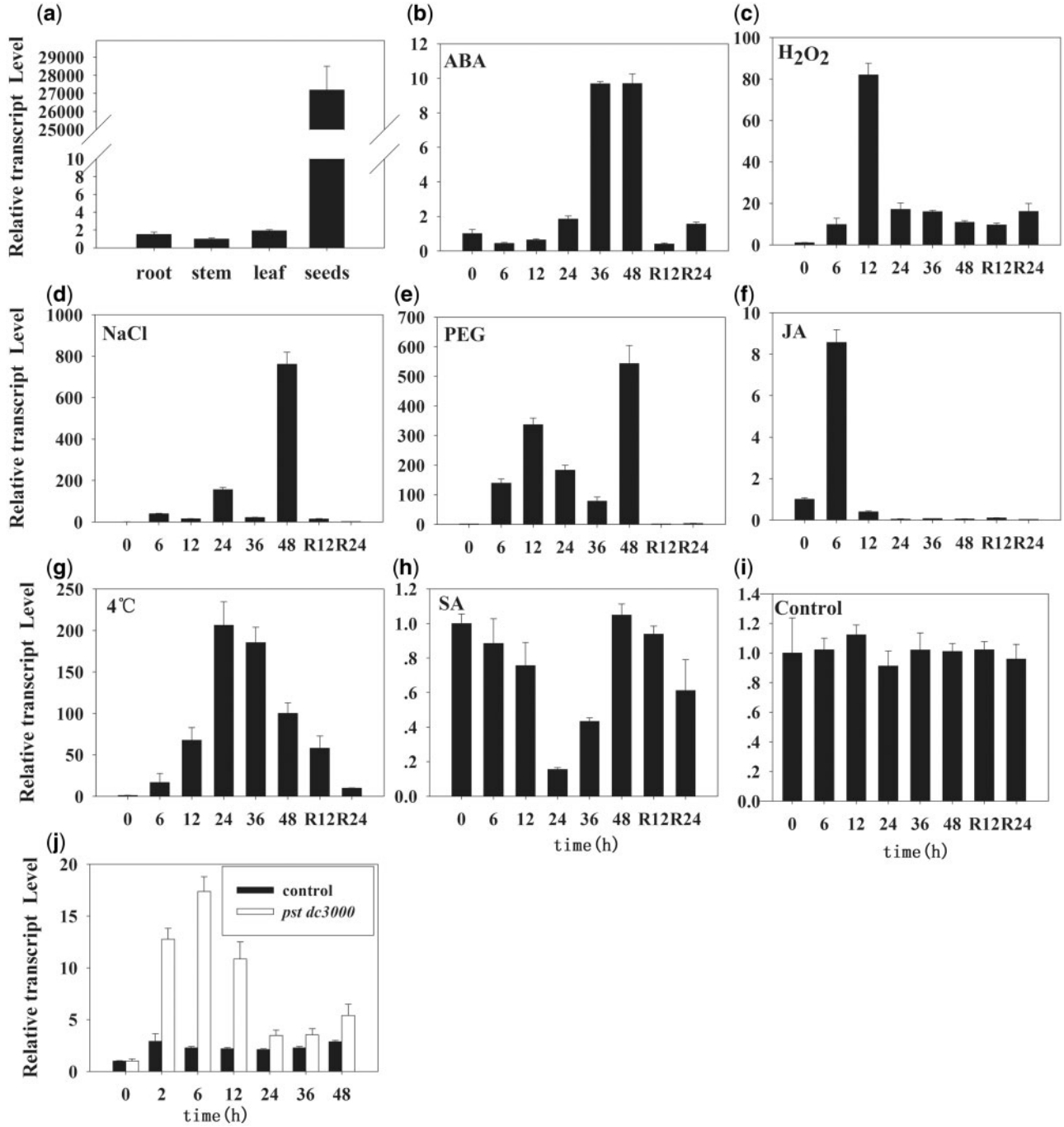


Fig. 2 Transcript accumulation of *ZmLEA3* in maize leaves in response to different abiotic and biotic stresses as determined by qRT–PCR. The maize seedlings were incubated with Hoagland's solution for 2 weeks; uniformly sized plants at similar growth stages were chosen for further study. (a) Tissue-specific expression of *ZmLEA3* under non-stress conditions; total RNA was isolated from leaves, stems, roots and seeds. Maize seedlings were treated with 100 μM ABA (b), 20 mM H_2O_2 (c), 150 mM NaCl (d), 20% PEG6000 (w/v) (e), 100 μM JA (f), 100 μM SA (j), 4°C (g) or water (control, h). (i) Maize seedlings were treated with *pst dc3000* or 10 mM $MgCl_2$ (control). Total RNA was isolated from leaves at the indicated times after the treatments. R represents recovery.

defense responses to various pathogens (Bari et al. 2009). In this study, *ZmLEA3* transcript was quickly induced within 6 h by MeJA treatment and then was reduced to the pre-induction level (**Fig. 2f**). However, *ZmLEA3* showed a negative response to SA treatment (**Fig. 2h**). Under control conditions, there were no significant differences at various time points (**Fig. 2i**). To gain more detailed insights into *ZmLEA3* expression upon pathogen infection, we determined the *ZmLEA3* mRNA levels in *pst dc3000*-inoculated plants at different time points. As shown in **Fig. 2i**, pathogen infection markedly increased

ZmLEA3 mRNA levels in comparison with the control (10 mM $MgCl_2$). The transcript accumulation of *ZmLEA3* reached its highest level suddenly at 6 h and then decreased. It should be emphasized that the transcript levels reached much higher levels (from 100- to 800-fold) under hyperosmolarity and salt treatment when compared with peroxide treatment, which produced an 80-fold increment. Under ABA, JA and pathogen treatments, lower increases were detected (from 10- to 20-fold).

The *ZmLEA3* proximal promoter sequence was subjected to a search for motifs using the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases. Consistent with the results of qRT–PCR, the promoter of *ZmLEA3* also contained various *cis*-acting elements involved in SA, MeJA, ABA, defense and stress responses (**Supplementary Fig. S3**).

Metal binding property of ZmLEA3 proteins

To confirm the metal binding property of the recombinant ZmLEA3 protein, immobilized metal ion affinity chromatography (IMAC) was used to test the binding affinity between proteins and metal ions. The ZmLEA3 protein was applied to IMAC columns that chelated Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} under high ionic strength (1 M NaCl). The ZmLEA3 protein was retained in the columns that immobilized Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, but not in the columns that immobilized Ca^{2+} or Mg^{2+} (**Fig. 3a**). The retained ZmLEA3 protein was eluted with the EDTA solution, indicating that the ZmLEA3 protein binds Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. To examine its metal binding activity further, we used imidazole elution instead of EDTA. The result was the same as that with EDTA (**Fig. 3b**).

Subcellular localization of ZmLEA3

LEA proteins are located in different subcellular compartments, including the nucleus, cytoplasm, chloroplasts, mitochondria and the endoplasmic reticulum (ER) (Roberts et al. 1993, Nylander et al. 2001, Ukaji et al. 2001, Hara et al. 2003, Tunnacliffe and Wise 2007). The subcellular localization of ZmLEA3 was investigated by generating stable transgenic tobacco carrying a *ZmLEA3*–green fluorescent protein (GFP) fusion protein driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. Leaf epidermal cells of the transgenic tobacco plants expressing the ZmLEA3::GFP fusion protein were examined with a Leica confocal laser scanning microscope. As shown in **Fig. 4a**, the ZmLEA3–GFP fusion protein specifically accumulated in the cytosol and nucleus. To examine the results further, nuclear proteins and cytoplasmic proteins were isolated for Western blotting. Western blotting produced the same results (**Fig. 4b**).

Overexpression of ZmLEA3 enhanced transgenic tobacco tolerance to osmotic stress

To evaluate the function of the ZmLEA3 protein in plant osmotic tolerance, the transgenic tobacco plants that overexpressed *ZmLEA3* under the control of the CaMV 35S

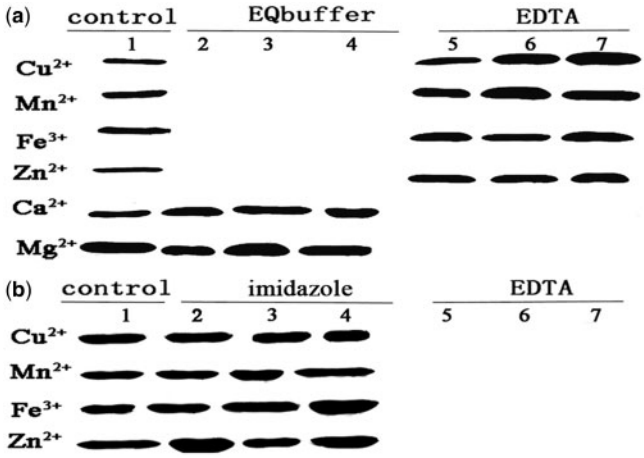


Fig. 3 Investigation of metal–ZmLEA3 binding using immobilized metal ion affinity chromatography (IMAC). The columns were charged with Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. Protein was loaded onto a column equilibrated with EQ buffer (50 mM Tris–HCl pH 7.4, 1 M NaCl). The unbound protein on the column was washed out with the EQ buffer. Then, bound protein was eluted with 100 mM EDTA (a) or 600 mM imidazole (b). The column not charged with metal was used as a positive control. Samples were collected and subjected to SDS–PAGE analysis and stained with Coomassie Brilliant Blue.

promoter were selected for further analysis. Independent transgenic lines were obtained by kanamycin resistance selection and confirmed by genomic PCR (data not shown). Five independent lines were selected, and their expression levels of *ZmLEA3* were detected by qRT–PCR, whereas no expression was detected in the wild-type (WT) plants (**Fig. 5**). Three of the *ZmLEA3* transgenic lines (LEA3-4, LEA3-5 and LEA3-13) were chosen for further analysis. When the mannitol concentration was 200 mM, a drastic decrease in the germination rate was observed for the seeds of WT plants after 10 d, in that only approximately 30% of the seeds germinated, whereas the transgenic lines retained between 40% and 65% of their germination capacity. As the mannitol concentration increased to 250 mM, approximately 30–45% seeds of the transgenic lines maintained their capacity to germinate, but only 20% of the WT seeds germinated (**Fig. 6a**).

A quantification of the differences in main root length of the WT and transgenic lines confirmed these results. The lengths of the primary root of the transgenic lines were >2 cm, while those of the WT were restricted to <1.4 cm under 200 mM mannitol. As the mannitol concentration increased to 250 mM, the primary root lengths of the transgenic lines were >1.5 cm, while those of the WT were restricted to <1 cm. It is important to mention that there were no phenotypic differences between the transgenic lines and WT plants under control conditions (**Fig. 6b–e**). Abiotic stresses result in the accumulation of ROS in plants. For this reason, we evaluated the accumulation of H_2O_2 in the transgenic and WT seedlings under osmotic stress. After treatment, the

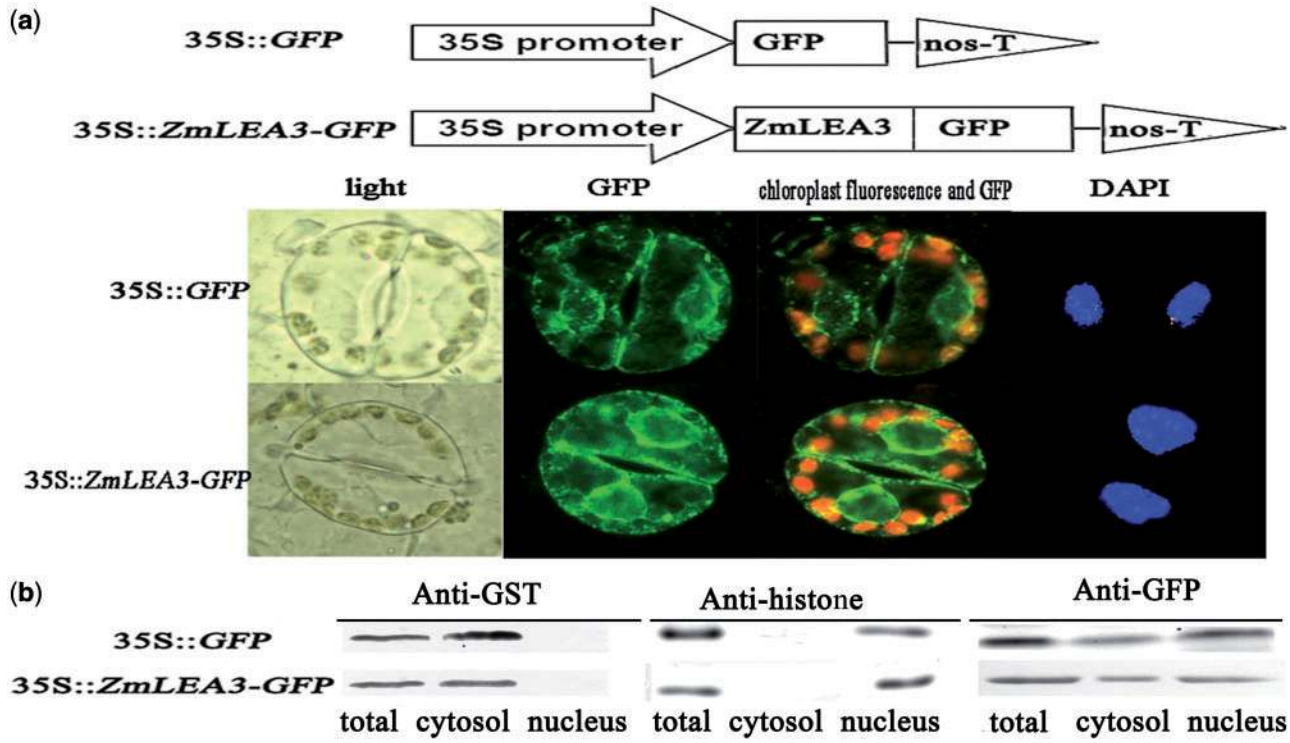


Fig. 4 Subcellular localization of the ZmLEA3–GFP fusion protein in transgenic tobacco. Subcellular distribution of the ZmLEA3–GFP fusions and control GFP in the epidermis guard cells of transgenic tobacco using confocal laser scanning microscope. The red is chloroplast fluorescence, and the green is GFP. The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI) (a). Western blotting analysis of the cytosol and nuclear fractions from transgenic tobacco probed with histone H1 antibodies, GST antibodies or GFP antibodies (b). Nuclear protein (histone H1) and cytoplasmic protein (glutathione *S*-transferase, GST) were used as controls.

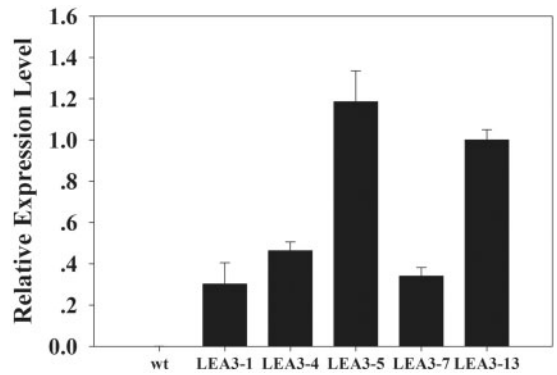


Fig. 5 *ZmLEA3* transcript accumulation in transgenic tobacco plants. Analysis of expression of *ZmLEA3* in five transgenic tobacco plants by qRT–PCR. The expression is relative to that of the WT. Total RNA was isolated from leaf samples collected from T_2 tobacco plants under normal conditions.

ZmLEA3-overexpressing lines showed much lower accumulation of superoxide radical (O_2^-) relative to the WT lines (**Fig. 6f**). These results indicated that the overexpression *ZmLEA3* could enhance the tolerance of the transgenic plants to osmotic stress.

Lipid peroxidation was measured by analyzing the malondialdehyde (MDA) levels in both the WT and transgenic tobacco under unstressed and osmotic-stressed conditions. No significant difference was observed between the WT and the transgenic lines under optimal growing conditions. The MDA content increased in all the plants upon application of osmotic stress; however, this increase was significantly more pronounced in the WT plants than in the transgenic lines under osmotic stress (**Fig. 6g**). Electrolyte leakage always occurs following membrane damage. The amount of electrolyte leakage in the WT plants was higher than in the transgenic lines under osmotic stress (**Fig. 6h**).

Overexpression of *ZmLEA3* enhanced transgenic tobacco tolerance to oxidative stress

As ZmLEA3 can bind metal, and therefore may reduce oxidative damage caused by free metals, we addressed the question of whether the oxidative tolerance was also enhanced in transgenic lines. To confirm this hypothesis, transgenic and WT seeds were sown on Murashige and Skoog (MS) medium containing 3.75 mM H_2O_2 for 4 weeks. There was a clear difference in root length; the growth inhibition in the transgenic lines was much less severe than in the WT plants, and the primary root length of the transgenic lines was longer than that of the WT plants (**Fig. 7a, b**). The amounts of relative electrolyte leakage (**Fig. 7c**) and MDA (**Fig. 7d**) in the WT plants were higher than in the transgenic lines after oxidation treatment. The results

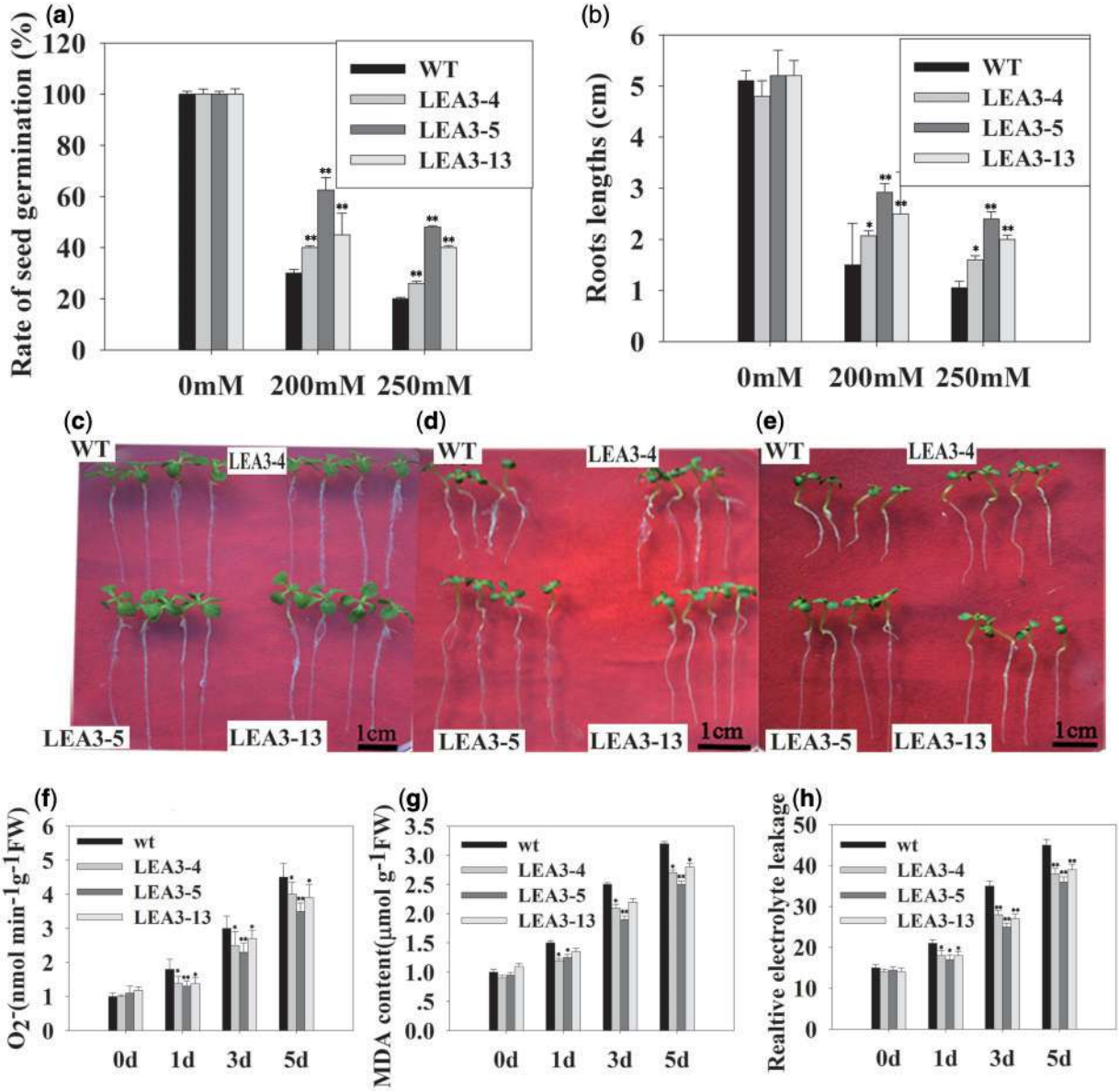


Fig. 6 Assay for water deficit tolerance in *ZmLEA3*-overexpressing transgenic tobacco plants. (a) The germination rate was counted after 30 seeds were sown for 10 d on MS medium containing different concentrations of mannitol (0 M, 200 mM or 250 mM). (b) The root length was measured 4 weeks after sowing on MS medium containing different concentrations of mannitol (0 M, 200 mM or 250 mM). The phenotype of transgenic and WT tobacco germinated on MS medium containing different concentrations of mannitol [0 M (c), 200 mM (d) or 250 mM (e)]. The seeds were allowed to grow for 4 weeks before the photographs were taken; 30 seedlings were measured. O_2^- (f), MDA (g) and electrolyte leakage (h) in 6-week-old *ZmLEA3*-overexpressing lines and the WT were measured at the indicated times after treatment with 20% PEG. The statistical significance of the difference was confirmed by Student's *t*-test, $^*P < 0.05$; $^{**}P < 0.01$.

indicated that the overexpression *ZmLEA3* in tobacco could also reduce the damage caused by oxidative stress.

Overexpression of *ZmLEA3* enhanced yeast cell tolerance to osmotic and oxidative stress

To investigate whether the ZmLEA3 protein plays the same role in yeast as in plants, the coding sequence of the *ZmLEA3* gene was cloned into vector pPI3.5K, and then transferred into yeast (GS115) cells. Expression of the *ZmLEA3* gene in yeast cells was induced by methanol. The growth curves of the yeast cell lines transformed with the pPI3.5k-*ZmLEA3* vector and the control lines containing the empty vector (pPI3.5k) were calculated under 800 mM mannitol or 20 mM H_2O_2. Under optimal conditions, there was no significant difference between the transformed yeast and the control; but, under stress conditions, the transformed yeast displayed improved growth compared with the control, and the lag phase of the transformed yeasts was

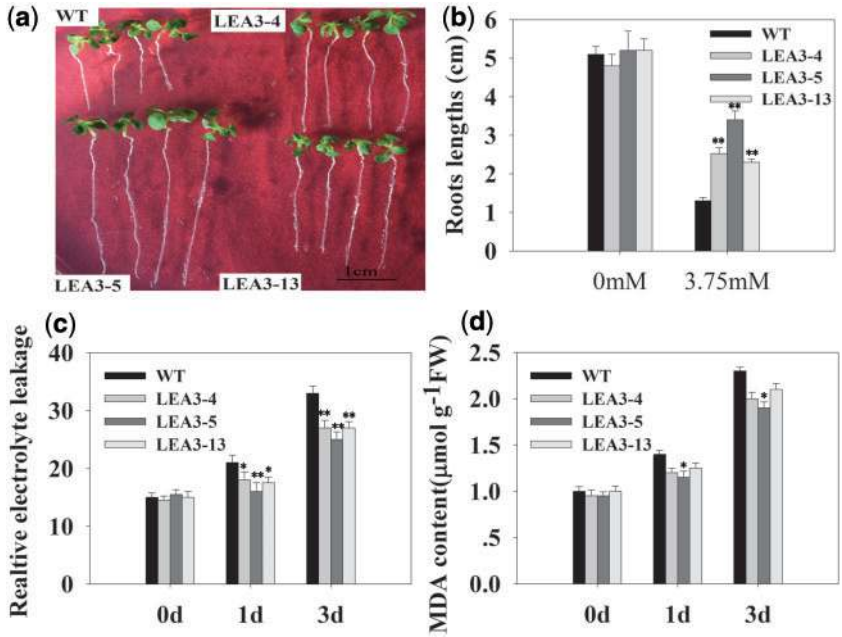


Fig. 7 Assay for oxidation stress tolerance in *ZmLEA3*-overexpressing transgenic tobacco plants. (a) Phenotype of transgenic and WT tobacco seeds germinated on MS medium containing 3.75 mM H_2O_2 for 4 weeks; 30 seedlings were measured. (b) The root length was measured on MS medium containing 3.75 mM H_2O_2 or on control MS medium. Electrolyte leakage (c) and MDA (d) in 6-week-old *ZmLEA3*-overexpressing lines and the WT were measured at the indicated times after treatment with 20 mM H_2O_2. The statistical significance of the difference was confirmed by the Student's *t*-test, $^*P < 0.05$; $^{**}P < 0.01$.

shorter than that of the control (**Fig. 8**). These results demonstrated that overexpression of the *ZmLEA3* gene in yeast could also significantly enhance tolerance to osmotic and oxidative stress.

ZmLEA3 protein protected LDH activity from damage caused by oxidation and water deficit stresses

Previous studies have shown that LEA proteins (AtD113) are able to protect enzymatic activities [lactate dehydrogenase (LDH)] during in vitro partial water limitation and freezing (Reyes et al. 2005, Reyes et al. 2008). We performed yeast two-hybrid screening to identify whether the ZmLEA3 protein could interact with certain enzymes [peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), LDH and citrate synthase 4 (CS4)]. However, there was no specific interaction with enzymes under control conditions (data not shown). To obtain more detailed information, the rate of inactivation of LDH activities during water loss and oxidative stress was determined with or without the ZmLEA3 protein. After two cycles of desiccation, LDH (alone) lost approximately 60% of its initial activity, whereas LDH retained 70% of its initial activity when additional ZmLEA3 protein was added (**Fig. 9a**). For oxidative stress, LDH (alone) lost approximately 30% of its activity when 100 or 200 μM H_2O_2 was added, but LDH with additional ZmLEA3 protein retained 90% of its activity during the process (**Fig. 9b**). These results demonstrated that the ZmLEA3 protein could preserve LDH activity under oxidative and water deficit stresses.

Overexpression of ZmLEA3 enhanced the plant defense response to *pst dc3000*

The up-regulation of *ZmLEA3* by JA and *pst dc3000* infection prompted us to evaluate the role of ZmLEA3 in the plant defense response. To determine the role of ZmLEA3 in plant resistance to pathogens, we analyzed the hypersensitive response (HR) caused by the infection with *pst dc3000* of the transgenic plants and the WT plants. After inoculation with *pst dc3000*, the leaves of transgenic plants exhibited more HR symptoms than the WT, plants as shown by trypan blue staining after 12 h (**Fig. 10**). As it is well known that HR at the site of infection can also activate systemic acquired resistance (SAR), which provides protection against a broad spectrum of pathogens throughout the plant and induces the accumulation of pathogenesis-related (PR) protein, the expression levels of four marker genes from different pathways (*PR1a*, *PR2*, *PR4* and *PR5*) were determined. As shown in **Fig. 11**, the expression levels of *PR1a*, *PR2* and *PR4* were significantly higher in the transgenic lines than in the WT plants, but there was no difference in *PR5* expression between the WT and the transgenic lines.

Thus, overexpression of *ZmLEA3* may enhance the plant defense response to bacterial pathogen, and the expression of many PR genes was also elevated; however, this fascinating possibility awaits further investigation.

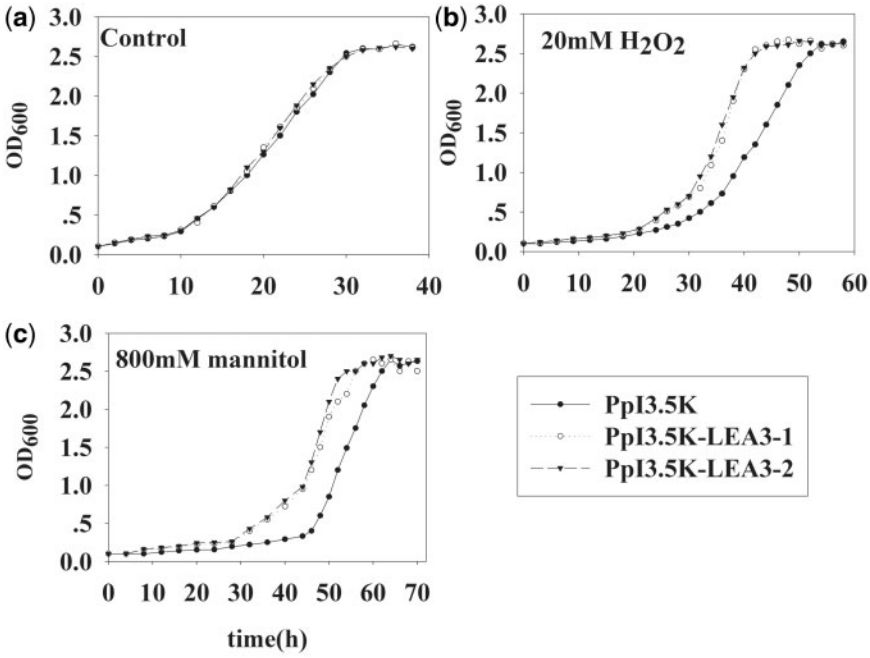


Fig. 8 Assay for oxidation stress and water deficit tolerance in *ZmLEA3* transformant yeast. Transformant yeasts (GS115) with empty vector or PpI3.5 K-*ZmLEA3* were grown in the non-stress BMGY medium (a) or in medium containing 20 mM H_2O_2 (b) or 800 mM mannitol (c) after induction. Two transformant yeasts (PpI3.5 K-*ZmLEA3*-1 and PpI3.5 K-*ZmLEA3*-2) were chosen for further study. The yeast were grown to OD_{600} = 0.8 in BMMY medium, then 1 ml of the culture was inoculated into BMGY (150 ml) supplemented with 20 mM H_2O_2 or 800 mM mannitol. At each time point, 3 ml of culture was used to measure the OD_{600} with a spectrophotometer. Growth was measured three times.

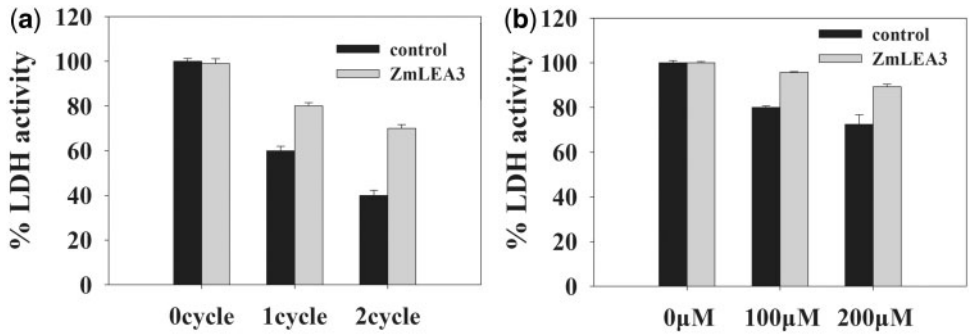


Fig. 9 ZmLEA3 could protect LDH activity during water deficit and oxidation stresses. (a) Effect of desiccation on LDH activity. One drying cycle corresponds to vacuum drying for 1 h in a modified tray freeze-dryer followed by immediate rehydration in water to the original volume. (b) Effect of oxidation on LDH activity after 100 or 200 μM H_2O_2 treatment. LDH activity was measured in the presence of 0.24 mg of group 3 LEA protein (ZmLEA3) or 0.24 mg of BSA protein (control).

Discussion

LEA proteins are highly hydrophilic; they are speculated to retain water molecules and protect other proteins from aggregation or desiccation (Tunnacliffe and Wise 2007). Here, we reported the identification and characterization of ZmLEA3 in maize. Database searches and sequence analysis suggest that ZmLEA3 belongs to the LEA3A group. It is located on the long arm of chromosome 6 and consists of three exons and two introns. It shares high amino acid sequence homology with other hydrophilic group 3 LEA proteins.

ZmLEA3 can be induced by various abiotic stresses including high osmotic, salinity, low temperature, oxidative stress and the application of ABA. ABA, as a key phytohormone, plays a vital role in the plant stress response. Abiotic stresses such as drought and high salinity result in strong increases in the ABA levels, accompanied by a major change in gene expression. However, under osmotic and NaCl treatment, the accumulation peak was consistent with the transcript peak due to H_2O_2 treatment rather than due to ABA treatment. From these data, we infer that the regulation of ZmLEA3 expression may operate via at least two pathways, one requiring ABA directly and a

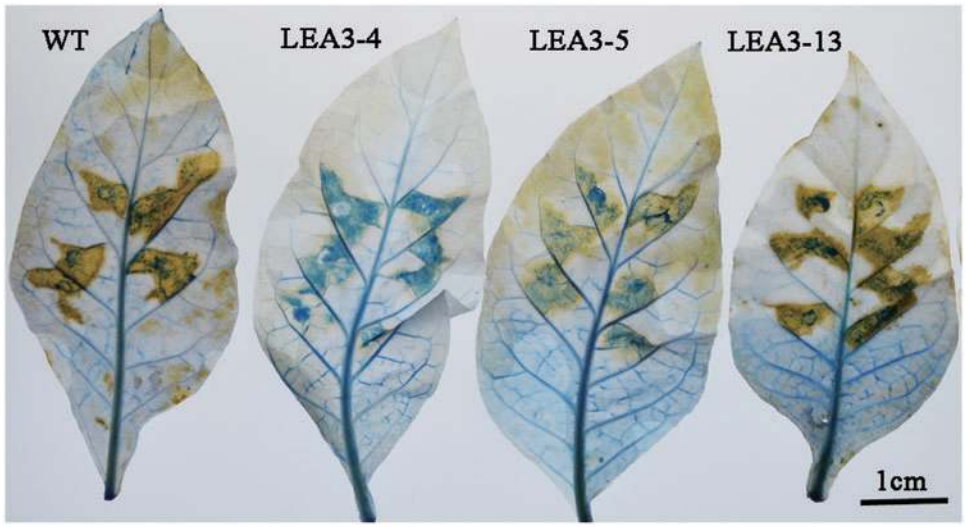


Fig. 10 The HR-induced cell death test by trypan blue staining. The WT and transgenic tobacco plant leaves were infiltrated with *pst dc3000* strains (1×10^7 c.f.u. ml^{-1}). These experiments were performed three times with similar results.

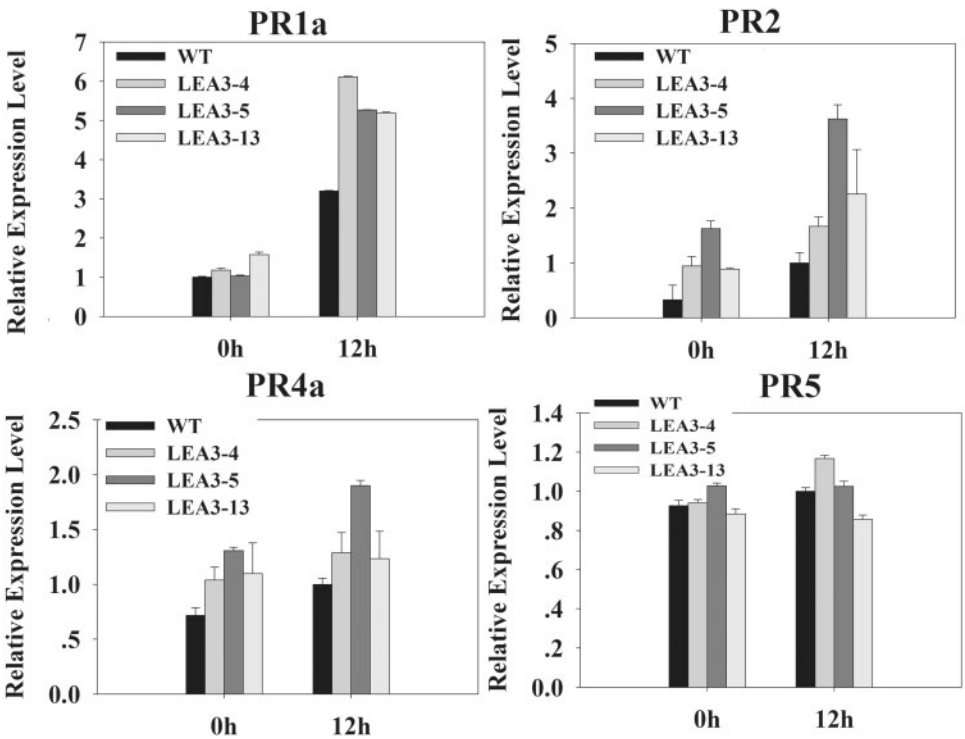


Fig. 11 Expression of PR genes by qRT–PCR after inoculation with *pst dc3000*. Total RNA was extracted from the leaves at the indicated times. All qRT–PCRs were performed using the same batch of cDNA and were repeated three times with identical results.

second that can be induced by osmotic or oxidative stresses independently of de novo ABA synthesis. As important signaling molecules, SA and JAs play important roles in defense responses against pathogens. In this study, *ZmLEA3* could be induced by the application of MeJA. However, SA had a strong negative effect on *ZmLEA3* expression. These results are consistent with a model in which the SA-dependent pathway and the JA-dependent pathway act antagonistically (Felton et al. 1999a, Felton et al. 1999b, Pieterse and van Loon 1999, Thomma et al. 2001). To confirm these roles, the expression of *ZmLEA3* was tested after *pst dc3000* injection. The result showed that *ZmLEA3* could also be induced by *pst dc3000*. These results indicated that *ZmLEA3* could respond to various abiotic and biotic stresses.

Catalytic metals, such as Cu^{2+} and Zn^{2+}, primarily exist as metal–protein complexes in plants under control conditions. However, they can be released as free ions when plants are exposed to environmental stresses. These free ions are required to produce ROS via the Haber–Weiss or the Fenton reaction (Jiang 1999, Mittler 2002). It has been reported that the group 4 LEA proteins GmPM1 and GmPM9 bind metal such as Fe^{3+}, Ni^{2+}, Cu^{2+} and Zn^{2+} (Liu et al. 2011). The group 2 LEA proteins CuCOR15 and VrDhn1 can bind metal, which promotes the interaction of DNA with LEA proteins (Hara et al. 2005, Lin et al. 2012). In the present study, we demonstrated that ZmLEA3 protein could bind Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} in vitro, and this role could be inhibited by the chelator EDTA or imidazole. Because ZmLEA3 protein contains a higher level of histidine (4%) than average (2%) and imidazole can compete with histidine to bind metal, we speculated that the histidine residues play an important role in binding metal. Therefore, it was reasonable to speculate that binding of ions by the ZmLEA3 protein in vivo plays an important role in reducing oxidative damage and ion toxicity when plants are exposed to abiotic stresses.

Many studies have successfully demonstrated that overexpression of LEA proteins results in the enhancement of abiotic stress tolerance in plants. Overexpression of barley HVA1 in rice enhanced tolerance to water deficit and salt (Rohila et al. 2002, Babu et al. 2004). The constitutive expression of the wheat group 3 LEA-L2 protein in *Arabidopsis thaliana* resulted in a significant increase in the freezing tolerance of cold-acclimated plants (NDong et al. 2002). In this study, the transgenic tobacco plants exhibited a higher germination rate and a lower increase in endogenous superoxide radical (O_2^-), MDA and relative electrolyte leakage levels than those in the WT tobacco under osmotic stress. These results indicated that ZmLEA3 could enhance transgenic tobacco tolerance to osmotic stress.

The ZmLEA3 protein can bind metals such as Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, which may reduce oxidative damage caused by free ions. To characterize this role in the stress response further, transgenic tobacco was treated with H_2O_2. A significantly lower increase in endogenous MDA and relative electrolyte leakage levels was also found in transgenic tobacco under oxidative stress (**Fig. 8**). These results implied that the ZmLEA3 protein could have antioxidant activity by binding free metal.

The group 3 LEA proteins are widely distributed in the plant kingdom and non-plant organisms such as the prokaryotes *Deinococcus radiodurans* (Battista et al. 2001), *Steinernema feltiae* (Solomon et al. 2000) and *A. avenae* (Goyal et al. 2003). For further characterization of ZmLEA3, we investigated whether ZmLEA3 could protect yeast from damage caused by osmotic and oxidative stress. The *Pichia* yeast GS115 was chosen for the study. The results showed that the overexpression of *ZmLEA3* in yeast significantly enhanced the transformed yeast's tolerance to osmotic and oxidative stress. Thus, the ZmLEA3 protein may play the same roles in both the plant and non-plant kingdom.

As is the case for molecular chaperones, determination of the function of LEA proteins has been approached via in vitro assays. The proteins LEA76 and COR15a of *A. thaliana* can protect the activity of malate dehydrogenase and LDH by avoiding the malate dehydrogenase and LDH conformational modifications during partial water deficit (Reyes et al. 2005, Nakayama et al. 2007). In this study, ZmLEA3 did not interact with SOD, APX, CAT, POD, LDH or CS4 under control conditions, but ZmLEA3 could protect LDH activity from the damage caused by oxidative and osmotic stresses. According to the results, we could surmise that ZmLEA3 could protect protein activities under environmental stresses.

Although the functions of LEA proteins in abiotic stresses have been studied in depth, their function in biotic stress is unclear. The LEA protein SAG21 was found to enhance the tolerance to bacterial pathogens (*pst dc3000*) and oxidative stress in *A. thaliana* (Salleh et al. 2012). Wheat LEA protein DHN-5 may possibly be involved in the pathogen response in *A. thaliana* by down-regulating three jasmonate-ZIM domain (JAZ) proteins that act as negative regulators of the JA signal (Brini et al. 2011). When plants are invaded by pathogens, the area in the vicinity of the infection site can form a zone of dead cells, termed the HR, which can limit the growth of the pathogen by killing the host cells (Goodman et al. 1994, Shirano et al. 2002, Pike et al. 2005, Nemchinov et al. 2008). In this study, the potential link between *ZmLEA3* and resistance to pathogens was investigated by using a bacterial pathogen *pst dc3000*. The enhanced HR and the increased expression of the *PR1a*, *PR2* and *PR4* genes after inoculation in transgenic lines compared with the WT indicated that the resistance of transgenic plants to the pathogen was increased. However, the results are preliminary, and to determine the possible role of ZmLEA3 in the pathogen response further, a wide range of pathogens should be used and the response of transgenic lines should be examined.

In conclusion, the study presented here identified a novel group 3 LEA gene *ZmLEA3*, which was inducible in response to a variety of abiotic and biotic stresses. The protein is localized in the cytosol and nucleus. This study identifies the group 3 LEA protein from *Z. mays* that possessed the ability to bind Mn^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+}. Furthermore, overexpression of *ZmLEA3* could enhance the tolerance of transgenic tobacco and yeast to osmotic and oxidative stresses. Further study indicated that ZmLEA3 could enhance the plant HR cell death triggered by pathogen infection and increase the expression of *PR1a*, *PR2* and *PR4*.

Materials and Methods

Plant materials and growth conditions

Maize (*Z. mays* L. cv Zhengdan958) was used in this study. It which was grown in Hoagland's solution (pH 6.0) under greenhouse conditions at 22/26°C (night/day), with a photosynthetic active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (day/night) for 2 weeks.

Seeds of WT tobacco (*Nicotiana tabacum* cv NC 89) and transgenic plants were treated with 70% ethanol for 30 s and

with 2.6% bleach for 10 min, washed six times with sterile water, and plated on MS medium (Murashige and Skoog 1962) under light/dark cycle conditions of 16/8 h at 25°C. The transgenic tobacco was germinated in MS medium containing 200 mg l^{-1} kanamycin under the same conditions.

Amplification and sequence analysis of *ZmLEA3*

Total RNA was extracted by using the RNeasy Plant Mini Kit (TIANGEN). The first-strand cDNA synthesis was performed using 5 μg of total RNA, oligo $(dT)_{12-18}$ primer and ThermoScript Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. The full-length coding sequence of *ZmLEA3* was amplified from the cDNA using gene-specific primers based on the sequence from NCBI (NM_001153473): forward GGATCCGCCATGGCTTCCCACCA (*Bam*HI site underlined) and reverse GAGCTCGACAGGATCGGACCAAGT (*Sac*I site underlined). The products were cloned into pMD18-T vectors (TAKARA) and sequenced. Multiple sequence alignment was performed using the CLUSTALWX program. Treeview software was used to construct a tree showing phylogenetic relationships for the ZmLEA3 proteins.

Transcript accumulation analysis of *ZmLEA3* using real-time PCR under different stresses

The maize seedlings were incubated with Hoagland's solution for 2 weeks, and then uniformly sized plants at similar growth stages were subjected to cold treatment, 100 μM ABA, 20% PEG6000 (w/v), 250 mM NaCl, 100 μM MeJA, 100 μM SA, 20 mM H_2O_2 and *pst dc3000*. Cold treatment was exerted by exposing the plants to 4°C in a growth chamber with a 16 h light/8 h dark regime. Leaf, stem and root tissues were collected at specific time points, and the materials were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the RNeasy Plant Mini Kit (TIANGEN) according to the manufacturer's instructions. The first-strand cDNA synthesis was performed as described above. All cDNAs were used in qRT–PCRs performed using primers (forward GTCCGTGACCCTGTTTGC and reverse CCGCCCGACTCGTTTA) with SYBR Green qRT-PCR SuperMix (TransGene). The maize actin gene (NM_001156990.1) was amplified using primers (forward CCACGAGACCACCTACAACT and reverse CCTTTCTGGAGGAGCAAC) along with the *ZmLEA3* gene to allow gene expression normalization and subsequent quantification. All qRT–PCRs were carried out using the Bio-Rad CFX96 Real-time system. Cycling parameters for all qRT–PCRs were 94°C for 10 min initially, followed by 40 cycles, each comprising 94°C for 30 s, 60°C for 30 s and 72°C for 30 s.

Protein purification

The expression of recombinant proteins with a 6×His tag at the N-terminus was performed following the manufacturer's protocol (pET system). The ZmLEA3 fusion protein was purified by an Ni-column (GE Healthcare), and the 6×His tag was then removed by on-column thrombin (GE Healthcare) digestion according to the handbook. The protein was then exchanged into a low-medium salt buffer (20 mM Tris–HCl, 100 mM NaCl at pH 8) using a HiPrep Desalting column (GE Healthcare).

Metal chelating affinity chromatography

Interactions between metal ions and polypeptides were measured by means of IMAC using HiTrap Chelating HP (Amersham Pharmacia Biotech) according to Ueda et al. (2003) and Hara et al. (2009). The columns were charged by applying 3 ml of 100 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_3$, $CuCl_2$ and $ZnCl_2$, respectively. Recombinant protein ZmLEA3 was loaded to bind the column. The unbound recombinant protein was washed out with EQ (50 mM Tris–HCl pH 7.4, 1 M NaCl) buffer, and the bound protein was eluted by the addition of 100 mM EDTA or 600 mM imidazole. A total of 20 μl of each protein was subjected to SDS–PAGE analysis.

Yeast two-hybrid interaction assays

For the two-hybrid assays, *ZmLEA3* was amplified by PCR and cloned into the pGBT7 vector (Clontech) by using specific primers (forward CATATGATGGCTTCCCACCAGGACAAGGC, *Nde*I site underlined, and reverse GAATTCCTAGTGATCCCTGGTGATGGTA, *Eco*RI site underlined). The sequences of CAT, SOD, APX, CS4, POD and LDH were cloned into the pGADT7 vector (Clontech). The genes were amplified and their respective primers are listed below: *Z. mays* APX (NM_001112030), forward CATATGATGGTGAAGGCCTACCCCACGGT (*Nde*I site underlined) and reverse GAATTCCACCACACTCTTCTGGTTCGTTA (*Eco*RI site underlined); *Z. mays* CAT (NM_001111945.1), forward CATATGATGGATCCATACAAGCACCGCCC (*Nde*I site underlined) and reverse CCCGGGTTACATGCTCGGCTTCAGGTTCA (*Sma*I site underlined); *Z. mays* CS4 (NM_001155598.1), forward CATATGATGGCGCAGGAGGCCACCACGC (*Nde*I site underlined) and reverse GAATTCTCAAGCAGCCTTGTTCTTGCAG (*Eco*RI site underlined); *Z. mays* LDH (NM_001155412), forward CATATGATGAAGAAGGCCACTTCGCTCTC (*Nde*I site underlined) and reverse GAATTCTCAGAGGCCGAGGAGCTGGCAG (*Eco*RI site underlined); *Z. mays* POD (NM_001254790.1), forward CATATGATGGCTACCTCCTCTGGTTCTTG (*Nde*I site underlined) and reverse GGATCCTTAATTAGTACTGGACACCCAAG (*Bam*HI site underlined); and *Z. mays* SOD (NM_001112272.1), forward CATATGATGGCTCTCCGCACCCTGGCATC (*Nde*I site underlined) and reverse GAATTCTCAAGCAAGAACATTTTCGTACA (*Eco*RI site underlined)

For interaction studies, plasmids containing fusion proteins were co-transformed into *Saccharomyces cerevisiae* Y2HGold and selected on medium lacking leucine, tryptophan, histidine and adenine. Galactosidase activity filter assays were performed according to the manufacturer (Clontech).

In vitro stress assays and LDH activity measurements

LDH (Roche) from rabbit muscle was diluted in 25 mM Tris–HCl, pH 7.5. Partial water loss was assayed according to the previously published method (Goyal et al. 2005, Reyes et al.

2005). For oxidation treatment, LDH was diluted in Tris–HCl (25 mM, pH 7.5) containing different concentrations of H_2O_2. The final enzyme concentration was 200 nM, corresponding to 6.7 μg ml^{-1} LDH. The proteins tested in the LDH assays were added at a monomer concentration of 200 nM, corresponding to a protein : enzyme molar ratio of 1 : 1. For LDH activity, the assay buffer was 25 mM Tris–HCl pH 7.5 containing 2 mM pyruvate (Sigma) and 0.15 mM NADH (Roche). LDH activities were monitored as the rate of decrease in absorbance at 340 nm for 1 min due to the conversion of NADH into NAD at 25°C. The rate determined for the untreated samples was considered to be 100% in all graphs.

Overexpressed *ZmLEA3* in tobacco

The coding regions of *ZmLEA3* were ligated into the binary pBI121 expression vector under the control of the CaMV 35S promoter. Constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404, and the transformation of tobacco plants was performed by the leaf disc transformation method (Li et al. 2008).

Subcellular localization of ZmLEA3–GFP fusion proteins

The entire coding sequence of *ZmLEA3* was amplified with specific primers (forward TCTAGAGCCATGGCTTCCCACCA, *Xba*I site underlined, and reverse GGTACCGTGATCCCTGGT GATGGT, *Kpn*I site underlined). The products were cloned into pMD18-T vectors (TAKARA) and sequenced. The gene was inserted into the reconstructed binary vector pBI121-GFP, which generated a C-terminal fusion protein with the GFP gene controlled by the CaMV 35S promoter. Constructs were transformed into *A. tumefaciens* strain LBA4404 and then transformed into tobacco plants as described above (Li et al. 2008).

To verify the localization result, the nuclear protein of transgenic plants was isolated for Western blotting. A nuclear protein (histone H1) and a cytoplasmic protein (glutathione *S*-transferase, GST) were used as controls. The method was performed as previously described (Folta and Kaufman 2000, Bae et al. 2003). A 30 μg aliquot of protein was separated by electrophoresis on a 12% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. The membranes were probed with specific antibodies.

Osmotic stress treatments and assays in plants

Seeds of WT and transgenic tobacco were treated with 70% ethanol for 30 s and with 2.6% bleach for 10 min, washed six times with sterile water, and plated on MS agar medium (Murashige and Skoog 1962) at 25°C under a 16 h light/8 h dark cycle. The seed germination assay was performed by plating 30 seeds of transgenic and WT plants on MS medium containing different concentrations of mannitol. After 10 d, the number of germinated seeds was expressed as a percentage of the total number of seeds plated. The experiment was repeated at least three times.

The comparative rates of lipid peroxidation were assayed from the leaves of control and osmotically treated tobacco seedlings by determining the levels of MDA. Six-week-old transgenic lines and the WT line were treated with 20% PEG6000 for the indicated time, and then 0.5 g of leaves were collected for MDA measurements. The MDA content was determined using the thiobarbituric acid (TBA) reaction. Leaf samples (0.5 g) of tobacco plants were homogenized in 5 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 r.p.m. for 10 min at 4°C. Subsequently, 2 ml of 0.6% (w/v) TBA in 10% TCA (w/v) was added to 2 ml of the supernatant. The mixture was heated in boiling water for 15 min and then quickly cooled in an ice bath. Following centrifugation at 12,000 r.p.m. for 10 min at 4°C, the absorbance of the supernatant at 450, 532 and 600 nm was determined spectrophotometrically. The concentration of MDA was calculated by the following equation: MDA content (μmol l^{-1}) = 6.45 ($A_{532} - A_{600}$) − 0.56 A_{450}.

Relative electrolytic leakage was determineed as previously described (Xing et al. 2011). Superoxide radical (O_2^-) concentration was measured as described by Jiang and Zhang (2001).

Oxidative stress treatment and assay in plants

For the plant seedling antioxidative active test, sterilized seeds were sown on MS agar plates containing 3.75 mM H_2O_2. All plates were placed in a controlled-environment growth chamber at 25°C with a photoperiod of 16/8 h (day/night) for 4 weeks, and then the lengths of the primary roots of 30 seedlings were measured. The experiment was repeated at least three times.

Six-week-old transgenic lines and the WT line were treated with 20 mM H_2O_2 for the indicated time, and then 0.5 g of leaves was collected for MDA measurements. The MDA content and the electrolyte leakage were measured as described above.

Expression of ZmLEA3 in *Pichia* yeast GS115

The coding sequence of *ZmLEA3* was amplified from the plasmid using gene-specific primers (forward GGATCCGCCATGGC TTCCCACCA, *Bam*HI site underlined, and reverse GAATTCGA CAGGATCGGACCAAGT, *Eco*RI site underlined). The products were cloned into pMD18-T vectors and sequenced. The right sequence was digested and ligated into the plasmid Ppic3.5K (Invitrogen) with the AOXI promoter. The *Pichia* GS115 strain was transformed with 5 μg of linearized plasmid by the LiCl method according to the EasySelect Pichia Expression Kit (Invitrogen). As a negative control, the same strain was transformed with the Ppic3.5K empty vector. All the competent cells were removed to minimal dextrose medium plates [1.34% yeast nitrogen base (YNB), 4×10^{-5} biotin and 2% glucose] after transformation.

Osmotic and oxidative tolerance assays of yeast transformants

The recombinant colonies were inoculated in 25 ml buffered glycerol-complex medium (BMGY: 1% yeast extract, 2%

peptone, 10 mM K_3PO_4, 1.34% YNB, 4×10^{-5} biotin and 1% glycerin). After 18 h of incubation at 28°C, the cells were collected by centrifugation and resuspended in 200 ml of induction medium [buffered methanol-complex medium (BMMY); 1% yeast extract, 2% peptone, 10 mM K_3PO_4, 1.34% YNB, 4×10^{-5} biotin and 0.5% methanol] incubated at 28°C for 4 d. Methanol was added every 24 h to a final concentration of 0.5%.

The yeast transformants Ppic3.5K-*ZmLEA3* and Ppic3.5K were grown to OD_{600} = 0.8, then 1 ml of the culture was inoculated into BMGY (150 ml) supplemented with 20 mM H_2O_2 or 800 mM mannitol. At each time point, 3 ml of culture was used to measure the OD_{600} with a spectrophotometer. Growth was measured at least three times.

Pathogen inoculation and disease development

Pseudomonas syringae pv. tomato DC3000 was cultured on King's B (KB) medium (Liu et al. 2010) containing 50 μg ml^{-1} rifampicin. Overnight log phase cultures were grown to an OD_{600} of 0.6–0.8 (OD 0.1 = 10^8 c.f.u. ml^{-1}) and diluted with 10 mM $MgCl_2$ to a concentration of 10^7 c.f.u. ml^{-1} before inoculation. Control inoculations were performed with 10 mM $MgCl_2$. The bacterial suspensions were infiltrated into the abaxial surface of a leaf using a 1 ml syringe without a needle, and the HR was detected by trypan blue staining as previously described (Koch and Slusarenko 1990, Katagiri et al. 2002).

For the *PR* gene analysis by qRT–PCR, the transgenic and WT lines were infiltrated with bacterial suspensions and the expression of the *PR* genes was examined after 12 h. The gene-specific primer pairs used are as follows: tobacco PR1a (X12737), forward TCTCTACACTTCTCTTATTC and reverse GTTCTACACCTACATCTG; tobacco PR2 (M60460), forward CATAACCTTCCACTCTTA and reverse GATACAATAATCTC CACATT; tobacco PR4a (X58546), forward AACAGTGAGAAT AGTAGAT and reverse CATAGTTGACAGTAAGGT; tobacco PR5 (AF154636), forward GCTAAGTCAATCCACCAT and reverse AGTCCAGTCTCACCATAA; and tobacco actin (U60495.1), forward GGCTATGTATGTTGCTATTC and reverse CAGATTCAGAAGATATTGTCAT.

Statistic analyses

Statistical analyses and plotting were performed using SigmaPlot and SPSS.

Supplementary data

Supplementary data are available at PCP online.

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