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Characterization of *AhLea-3* and its enhancement of salt tolerance in transgenic peanut plants

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ABSTRACT

Background: Late embryogenesis abundant (LEA) proteins were reported to be related to adversity stress and drought tolerance. *Lea-3* from *Arachis hypogaea* L. (*AhLea-3*) was previously found to be related to salt tolerance according to the result of transcriptome profiling and digital gene expression analysis. So, *AhLea-3* was cloned and the salt tolerance was validated by transgenic peanut plants.
Results: *AhLea-3* was isolated from M34, a salt-resistant mutant of peanut, with its cDNA as the template. *AhLea-3* contains one intron and two extrons, and the full-length cDNA sequence contains 303 bp. *AhLea-3* was ligated to pCAMBIA1301 to obtain the overexpression vector pCAMBIA1301-*AhLea-3*, which was then transferred into peanut variety Huayu23. The expression level of *AhLea-3*, as determined by qRT-PCR analysis, was >10 times higher in transgenic than in non-transgenic plants. Five days after they were irrigated with 250 mM NaCl, the transgenic plants showed less severe leaf wilting, higher activities of antioxidant enzymes (superoxide dismutase, peroxidase, and catalase), and lower malonic dialdehyde content than non-transgenic plants. Relative to non-transgenic plants, the transgenic plants had a higher photosynthetic net rate, stomatal conductance, and transpiration rate, and a lower intercellular CO_2 concentration after salt stress treatment (250 mM NaCl).
Conclusions: These results indicate that overexpression of *AhLea-3* increased the salt tolerance of transgenic peanut plants. *AhLea-3* might become a useful gene resource for the variety breeding of salinity tolerance in peanut.
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1. Introduction

Late embryogenesis abundant (LEA) proteins, which were first identified in cotton (*Gossypium hirsutum*), have low molecular weights, are usually hydrophilic, and accumulate, as their name implies, during late embryogenesis of plant seed development [1,2]. LEA proteins are generally assigned to seven groups based on their amino acid sequences and corresponding conserved motifs [3]. Except for LEAs in group 5 (D-95, D-73, D-34), which are hydrophobic, LEAs in groups 1 (D-19), 2 (D-11), 3 (D-7, D-29), 4 (D-113), 6 (PVLEA-18), and 7 (ASR-1) are hydrophilic (1). Lea 3 proteins belong to group 7 and are characterized by having a repeated 11-mer amino acid motif ΦΦE/QXΦKE/QKΦXE/D/Q (where Φ represents a hydrophobic residue) [4], and exhibit typical hydrophilic characteristics [3,5,6,7].

Many LEA proteins or their genes have been characterized from different species, and their accumulations have been found to be positively correlated with stress tolerance [8]. On the basis of the LEA gene expression pattern and protein analysis, researchers have proposed that LEA proteins might play an important role in the response of maturing seeds and plants to osmotic stress [9,10]. In maize, *ZmLEA3* is significantly upregulated in response to abiotic stresses [11,12]. Transgenic tobacco plants that overexpressed *CaLEA6* from pepper showed enhanced tolerance to dehydration and salt stress [13]. Overexpression of *OsLEA5* increased drought tolerance in rice [14], and overexpression of *LsEm1* increased

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tolerance to drought and high salinity in rice and *Escherichia coli* [15]. *SmLEA* overexpression enhanced salt and drought tolerance in *E. coli* and *Salvia miltiorrhiza* [8]. Transgenic sweet potato non-embryogenic calli that overexpressed *IbLEA14* showed enhanced tolerance to drought and salt stress, while RNAi calli exhibited increased stress sensitivity [16]. Overexpression of *ZmLEA5C* enhanced the tolerance of transgenic yeast to osmotic and low temperature stresses [17,18]. As indicated by these previous studies, increasing evidence indicates that the expression of *LEA* may be induced by drought and salinity stress and may increase tolerance to drought and salinity stress in many kinds of plants.

Peanut (*Arachis hypogaea* L.) is an important oil and food legume, and salt and drought stress are the major factors limiting peanut growth and yield. In this study, we identified a new *Lea-3* gene from peanut. Expression of the gene, which we named *AhLea-3*, significantly differed between the peanut salt-tolerant mutant M34 and the mutagenic parent control Huayu20 after salt stress treatment. *AhLea-3* gene was isolated from the salt-tolerant mutant M34, and an overexpression vector was constructed and transformed into peanuts. Transgenic plants that overexpress *AhLea-3* were generated and treated with high salinity stress, and physiological and biochemical characteristics were compared between the transgenic and the control plants. The results indicated that overexpression of *AhLea-3* increases salt stress tolerance in peanut.

2. Materials and methods

2.1. Plant materials

M34, a salt-resistant mutant, was identified from a population of pingyangmycin (PYM)-mutagenized Huayu20 peanut plants. Huayu23, a small pod-type peanut cultivar, is used for the transformation acceptor. All plants were grown at the Peanut Research Center, Qingdao Agricultural University (36°N, 120°E), China.

2.2. Validation of AhLea-3 expression in M34 and control plants

Specific qRT-PCR primers were designed according to the BLAST result in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and AhLea-3-F1 and AhLea-3-R1, together with control primers AhActin-F1 and AhActin-R1, were then synthesized by TSINGKE Co. (Beijing, China) (Table 1). Salt-resistant mutant M34 plants and mutagenic parent Huayu20 plants were irrigated with 250 mM NaCl. Total RNA was extracted from young leaves at 0, 6, 12, 24, and 48 h after the salt stress treatment, and was then reverse-transcribed to cDNA. The expression level of *AhLea-3* in M34 and Huayu20 was determined by qRT-PCR amplification with *Ah-Actin* as an internal control (Table 1).

2.3. Isolation and characteristics of AhLea-3 from M34

The specific PCR primers AhLea-3-F2 and AhLea-3-R2 were designed by primer5.0, and were then synthesized by TSINGKE Co. (Beijing, China) (Table 1). *AhLea-3* was isolated by PCR amplification with cDNA of M34 as template. The amplification program was as follows: 94°C denaturation for 4 min; followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. The amplification products were cloned into the pMD-18T vector for sequencing. The AhLea-3 protein sequence was submitted to NCBI for BLAST comparison, and corresponding phylogenetic trees construction. AhLea-3 protein structure, amino acid composition, and properties were predicted by Prot Param (https://web.expasy.org/protparam/).

2.4. Vector construction and genetic transformation

A 490-bp fragment from the mutant M34 cDNA was obtained by PCR amplification and was inserted into the vector pMD18-T to develop the construct pMD18-T-*AhLea*-3. Then, the corresponding product, 305 bp coding sequence was obtained by PCR amplification using primers AhLea-3-F3 and AhLea-3-R3 (containing the restriction site of *Bgl* II and *Bst*E II, Table 1) with pMD18-T-*AhLea*-3 as template. The vector pCAMBIA1301 was digested by *Bgl* II and *Bst*E II, and the 305-bp fragment of *AhLea*-3 was then ligated to the pCAMBIA1301 to obtain the recombined overexpression vector pCAMBIA1301-*AhLea*-3. Finally, the plasmid pCAMBIA1301-*AhLea*-3 was transferred into *Agrobacterium tumefaciens* strain EHA105. Primers used for cloning are listed in Table 1.

The recombined *A. tumefaciens* with pCAMBIA1301-*AhLea*-3 was cultured in YEB solution (with 100 mg/L kanamycin and 50 mg/L rifampicin). When OD_{600} = 0.6–0.8, the precipitate was collected by centrifugation and was then mixed with the same volume of MS medium with 100 pmol/mL acetosyringone (AS), 10 μmol/mL 2-(*N*-morpholino)ethanesulfonic acid (MES monohydrate), and 10 μmol/mL $MgCl_2{\cdot}6H_2O$. At 6–8 am every day, a disposable sterile injector was used to inject the carinas of receptor blossom with the resuspended recombined *Agrobacterium*; this was done until the full filling of the carina. The pods that developed from the injected flowers were marked by binding nylon cord and were then harvested when mature.

2.5. Molecular screening of positive transgenic peanut plants

Harvested seeds from transformed plants and non-transformed control plants were planted in sterile soil in a greenhouse. The genomic DNA was extracted from the harvested seeds, and the positive seeds were determined and screened by PCR amplification with 35S primer sequence 35S-F1: 5′-GCCGTAAAGACTGGCGAACA-3′ and 35S-R1: 5′-TCCCCCGTGTTCTC TCCAAA-3′. The PCR reaction conditions were as follows: 95°C for 5 min; followed by 35 cycles

Table 1
Primers used for qRT-PCR and PCR amplification of *AhLea-3*. Lower case showed the site of restriction endonuclease *Bgl* II and *Bst* EI, respectively.

Primer name	Primer sequence (5′ → 3′)	Product and its size
AhLea-3-F1	CGCTCTCTCTCACAAGCAAGCCGCC	qRT-PCR, 258 bp
AhLea-3-R1	CGGGGTGTGGTCTGATGTGAGTTTG	
AhActin-F1	TCTTCCAGCCATCCATGATCGGG	qRT-PCR, 190 bp
AhActin-R1	GCTACTCGGTGCCAATGCTGT	
AhLea-3-F2	AATCCAGAGGGAACAGAGCC	PCR, 490 bp
AhLea-3-R2	CCTATGATCGTATCCGAAAGCA	
AhLea-3-F3	actcttgaccatggtagatctATGGCTCGCTCTCTCTCACAAG	PCR, 305 bp
AhLea-3-R3	ggggaaattcgagctggtcaccTTATGAGTGTGGGCCCGGG	

at 95°C for 45 s, 55°C for 45 s, and 72°C for 40 s; and a final extension at 72°C for 10 min. The amplified products were detected by 2% agarose gel electrophoresis. Total RNA was extracted from the young leaves of transgenic plants and non-transgenic controls, and the expression level was determined by qRT-PCR amplification using the primers AhLea-3-F1 and AhLea-3-R1 indicated in Section 1.3 (Table 1).

2.6. Assessing the salt resistance of transgenic peanut plants

Four-week-old transgenic and non-transgenic control seedlings were irrigated with 250 mmol/L NaCl. The following properties were assessed for the transgenic and control plants using previously described methods [19,20]: the degree of leaf wilting; the enzymatic activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT); and malonic dialdehyde (MDA) content. The following photosynthesis-related parameters were also assessed for transgenic and control plants with a TARGAS-1 Portable Photosynthesis System (PP Systems International, MA, USA): photosynthetic net rate, stomatal conductance, transpiration rate, and intercellular CO_2 concentration.

3. Results

3.1. Expression of AhLea-3 in M34 and control plants

The expression level of *AhLea-3* in both M34 mutant and the control parent Huayu20 increased following salt stress treatment, and the difference was significant between before and after salt stress treatment (Fig. 1). The increase was greater in M34 than in Huayu20 in different time points after salt stress treatment (Fig. 1), which indicated that *AhLea-3* responded to salt stress.

3.2. Isolation and characteristics of AhLea-3 from M34

A 490-bp product was obtained by PCR amplification with M34 cDNA as template (Fig. 2a). The product was then ligated to the pMD18-T vector and transformed into *E. coli* DH5α. The 490-bp target fragment was also obtained both by PCR amplification with recombinant *E. coli* DH5α as template (Fig. 2b), and by *Bg*L II and *Bst*E II digestion (Fig. 2c). These results showed that *AhLea-3* had been cloned into the pMD18-T vector, which was then named pMD18-T-*AhLea-3*.

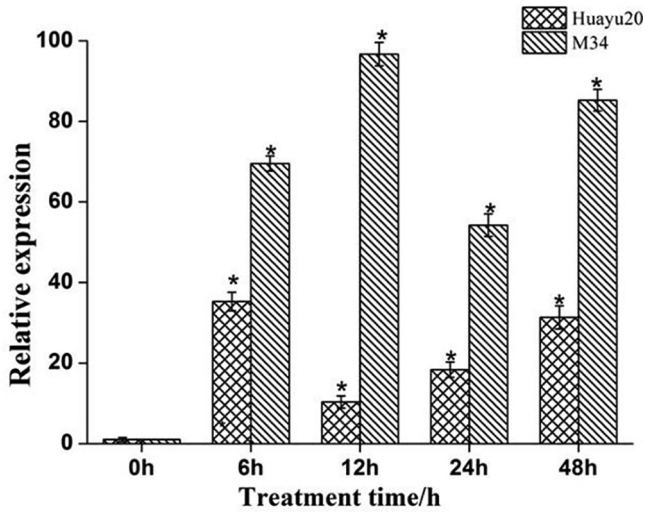

Fig. 1. The expression level of *AhLea-3* in M34 and control Huayu20 plants following salinity stress treatment. Values are means + SE of three replicate plants. Asterisks indicate a significant increase ($P < 0.05$) in expression relative to expression before treatment (0 h).

AhLea-3 was inferred to be an acidic protein containing 100 amino acids, with a molecular weight of 10.78 kDa and an isoelectric point of 9.84 as predicted by ExPASy (https://web.expasy.org/compute_pi/). The sequence alignment of Lea-3 proteins of various origin was made, and a phylogenetic tree was constructed (Fig. 3 and Fig. 4). The results showed that the AhLea-3 protein was highly homologous to Lea-3 from XP_015938428.1 (*Arachis duranensis*), XP_016197527.1 (*Arachis ipaensis*), and ABC41130.1 (*Arachis hypogaea*). The similarity coefficients between AhLea-3 and NP_001235600.1 (*Glycine* max), AAC06242 (*Nicotiana tabacum*), XP_004508204.1 (*Cicer arietinum*), and XP_023763079 (*Lactuca sativa*) were 54.29%, 51.79%, 47.12%, and 45.95%, respectively (Fig. 4).

3.3. Construction of the overexpression vector pCAMBIA1301

The overexpression vector pCAMBIA1301-*AhLea-3* was also constructed by digesting and ligating *AhLea-3* to pCAMBIA1301. The 305-bp target fragment was also obtained by PCR amplification with recombinant *E. coli* DH5α containing pCAMBIA1301-*AhLea-3* as template (Fig. 5a), and by *Bgl* II and *Bst*E II digestion (Fig. 5b). The result showed that the overexpression vector pCAMBIA1301-*AhLea-3* had been successfully obtained.

3.4. Screening of positive transgenic peanut plants

Fifty of the harvested seeds from injected flowers were subjected to PCR amplification with 35S primers 35S-F1: 5′-GCCGTA AAGACTGGCGAACA-3′; 35S-F2: 5′-TCCCCCGTGTTCTCTCCAAA-3′. The PCR reaction conditions were as follows: 95°C for 5 min; 32 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 40 s; and a final extension at 72°C for 10 min. A 495-bp amplification product was obtained from the seeds of the transgenic positive plants, and no target band was obtained from the seeds of non-transgenic, control plants (Fig. 6). Among the 50 seeds from transformed plants, 23 were positive for transformation, i.e., the transformation rate was 46%.

The expression level of *AhLea-3* was >10 times greater in transgenic plants than that in non-transgenic control plants (Fig. 7), which showed the transferred *AhLea-3* was overexpressed at the mRNA level.

3.5. Salt resistance of the transgenic peanut plants

Growth was clearly differed between transgenic plants and non-transgenic control plants that had been irrigated with 250 mM NaCl. The leaves of non-transgenic control plants were substantially wilted and were yellow-green, while the leaves of the transgenic plants were only slightly wilted and were normal green (Fig. 8). The result indicated that the overexpression of *AhLea-3* increased salt resistance.

Transgenic peanut plants overexpressing *AhLea-3* showed higher enzymatic activities of SOD, POD, and CAT, and lower MDA content than non-transgenic plants (Fig. 9). This suggested that overexpression of *AhLea-3* improved the plant's ability to eliminate reactive oxygen species (ROS), which might help explain the enhanced salt tolerance of the transgenic peanut plants.

Photosynthesis-related parameters were also measured after transgenic and control plants had been subjected to salt stress. The transgenic plants had a higher photosynthetic net rate, a higher stomatal conductance, a higher transpiration rate, and a lower intercellular CO_2 concentration than the non-transgenic plants (Fig. 10). These results indicated that overexpression of *AhLea-3* might help maintain photosynthesis during salt stress.

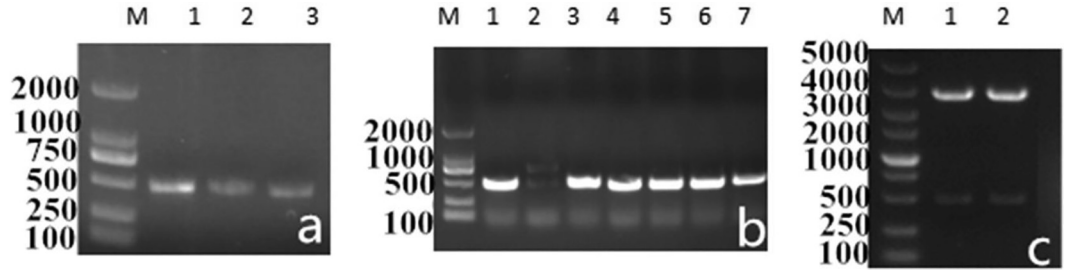

Fig. 2. The cloning and molecular identification of *AhLea-3* from M34. (a) Amplified bands with M34 cDNA as template; M: DL2000; 1–3: M34 cDNA. (b) Amplified bands with *E. coli* DH5α with pMD18-T-*AhLea-3* as template; M: DL2000; 1–7: *E. coli* DH5α with pMD18-T-*AhLea-3*. (c) Products of pMD18-T-*AhLea-3* by *Bg*L II and *Bst*E II digestion; M: DL5000; 1–2: recombinant plasmid pMD18-T-*AhLea-3*.

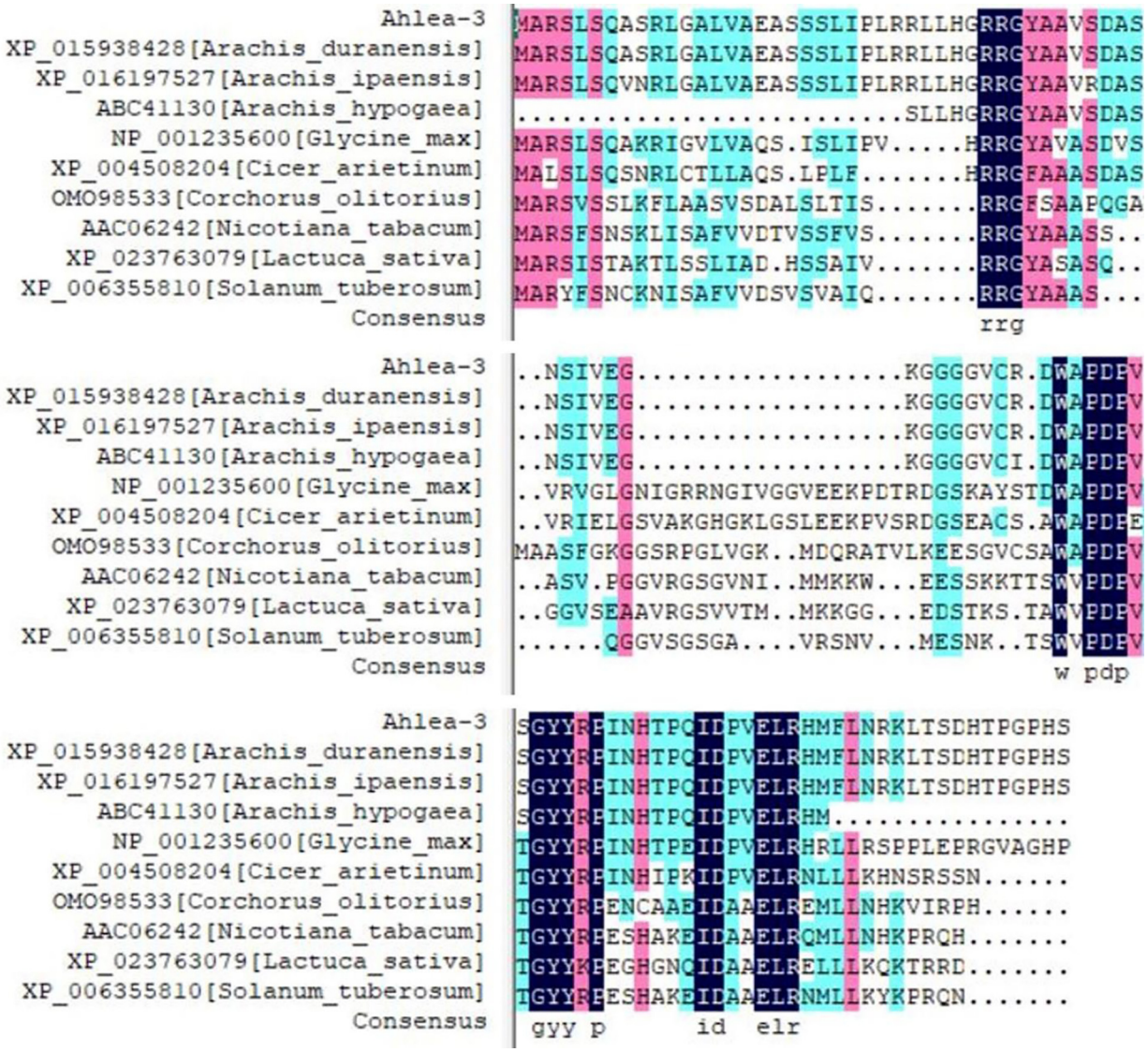

Fig. 3. Sequence alignment of Lea-3 proteins from different plant species. Dark blue indicates 100% homology, pink indicates >75% homology, and green indicates >50% homology.

4. Discussion

The large accumulation of LEA protein in the late stage of seed embryo development is thought to be related to stress resistance and osmotic regulation in plants. It has been frequently reported that *Lea-3* might participate in the hydration and drought tolerance of higher plants. To determine the role of *AhLea-3* from *A. hypogaea* in salinity stress tolerance, we cloned *AhLea-3* from a peanut mutant with salinity tolerance and predicted its corresponding functions. The AhLea-3 protein was found to contain a large number of hydrophilic amino acids and was predicted to have an average coefficient of hydrophilicity of −0.411. Its instability index

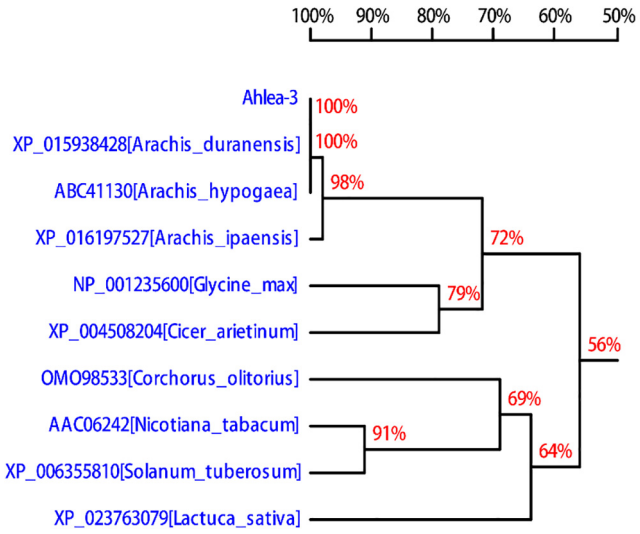

Fig. 4. A phylogenic tree of Lea-3 protein sequences in peanut and other plants. The percentages indicate the genetic variation between Lea-3 in peanut vs. other species. Vertical distance of different species represents the distance and proximity of genetic relationship.

exceeded 40, suggesting that it is unstable and hydrophilic. The high content of hydrophilic amino acids in AhLea-3 allows the protein to exist in random-coiled structures, which are beneficial for conserving water and resisting osmotic stress [21]. We then transformed *AhLea-3* into the peanut variety "Huayu23" with overexpression vectors under the control of a constitutive *CaMV35S* promoter. In response to salt stress, the overexpression transgenic lines grew better than the non-transgenic control plants.

Plants respond to abiotic stresses by increasing ROS, which reduce plant development by damaging proteins, lipids, DNA, and other cellular components. By balancing the rate of ROS production and ROS scavenging, plants can maintain normal development under abiotic stresses. Previous studies reported that SOD is a key enzyme for scavenging harmful reactive oxygen that accumulates during stress [22,23]. In the current study, we found that the activities of antioxidant enzymes (SOD, POD, and CAT) in response to salt stress were much higher in the transgenic plants than in the non-transgenic control plants. Similarly, MDA content, which is an indicator of oxidative stress, was much lower in the transgenic plants than in the non-transgenic control plants following salt stress treatment. These results suggested that overexpression of *AhLea-3* in peanut helped protect the activities of SOD, POD, and CAT, and thereby reduced the damage caused by salinity stress.

High salt levels can cause intracellular dehydration and can damage both proteins and cellular membranes [24]. Plants, however, have evolved some common mechanisms to reduce stress under adverse conditions [24]. For example, tobacco that overexpresses *CaLEA6* showed enhanced tolerance to dehydration and salt stress [8,13]; overexpression of *OsLEA5* confers tolerance to drought and high salinity in rice [14]. In the current study, peanut plants that overexpressed *AhLea-3* had a higher photosynthetic net rate and more efficient photosynthesis than control plants, which probably contributed to the increased salinity tolerance of the

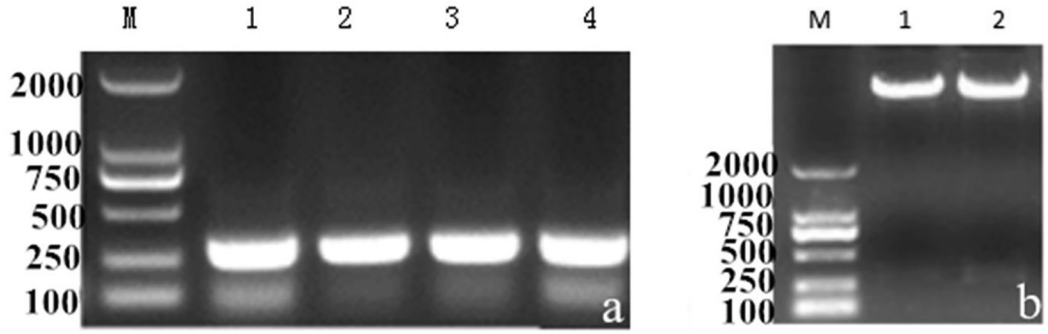

Fig. 5. Construction of the overexpression vector pCAMBIA1301-*AhLea-3*. (a) PCR amplification product with *E. coli* DH5α containing pCAMBIA1301-*AhLea-3* as template; M: DL5000; 1: control without DNA template; 2–5: *E. coli* DH5α with PCAMBIA 1301-*AhLea-3*. (b) Products of pCAMBIA1301-*AhLea-3* by *Bg*L II and *Bst*E II digestion; 1: pCAMBIA 1301-*AhLea-3*; M: DL15000.

Fig. 6. PCR amplification of harvested seeds from infected flowers. M: DL2000; 1–9: genomic DNA of peanut seeds from infected flowers; 10: Huayu23; 11: negative control; 12: *AhLea-3* recombinant plasmid.

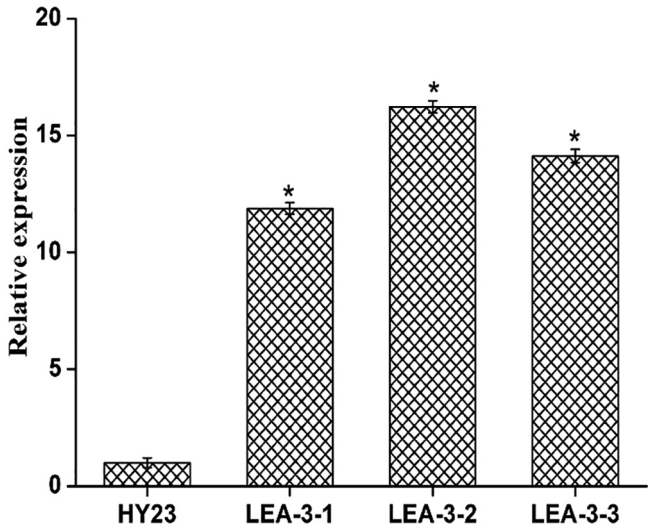

Fig. 7. Expression level of *AhLea-3* in three independent transgenic plants (LEA-3-1, LEA-3-2, and LEA-3-2) relative to the expression in the non-transgenic parent (Huayu23). Values are means + SE of three replicate plants. An asterisk indicates that the mean of the transgenic plants was significantly greater ($P < 0.05$) than the mean of the Huayu23 plants.

transgenic plants. Our results clearly suggest that salt tolerance was enhanced by overexpression of *AhLea-3* in the transgenic lines. This finding provides evidence that some protective mechanisms in plants are important for plant tolerance to adverse conditions.

There lie some cis-elements in the upper promoters of functional genes, which are reported to play key roles in the transcriptional regulation of related genes. The promoter of *AhLea-3* contains the following cis-elements: four MYB elements, eight MYC elements responsive to drought stress, one TCA element responsive to salicylic acid (SA), one seed-specific expression element, and four elements responsive to light. The increased expression level of *AhLea-3* after salinity treatment also indicates that these cis-elements might respond to external stress. Additional research is needed to identify the corresponding transcriptional regulatory factors.

In conclusion, we isolated and identified a novel *AhLea-3* gene that could be involved in the response of peanut plants to salinity stress. Overexpression of *AhLea-3* enhanced the tolerance of transgenic peanut plants to salinity stress by maintaining high activities of antioxidant enzymes (SOD, POD, and CAT), by decreasing MDA content, and by inhibiting chlorophyll damage in young leaves. *AhLea-3* could therefore be useful for engineering plant tolerance to salinity stress.

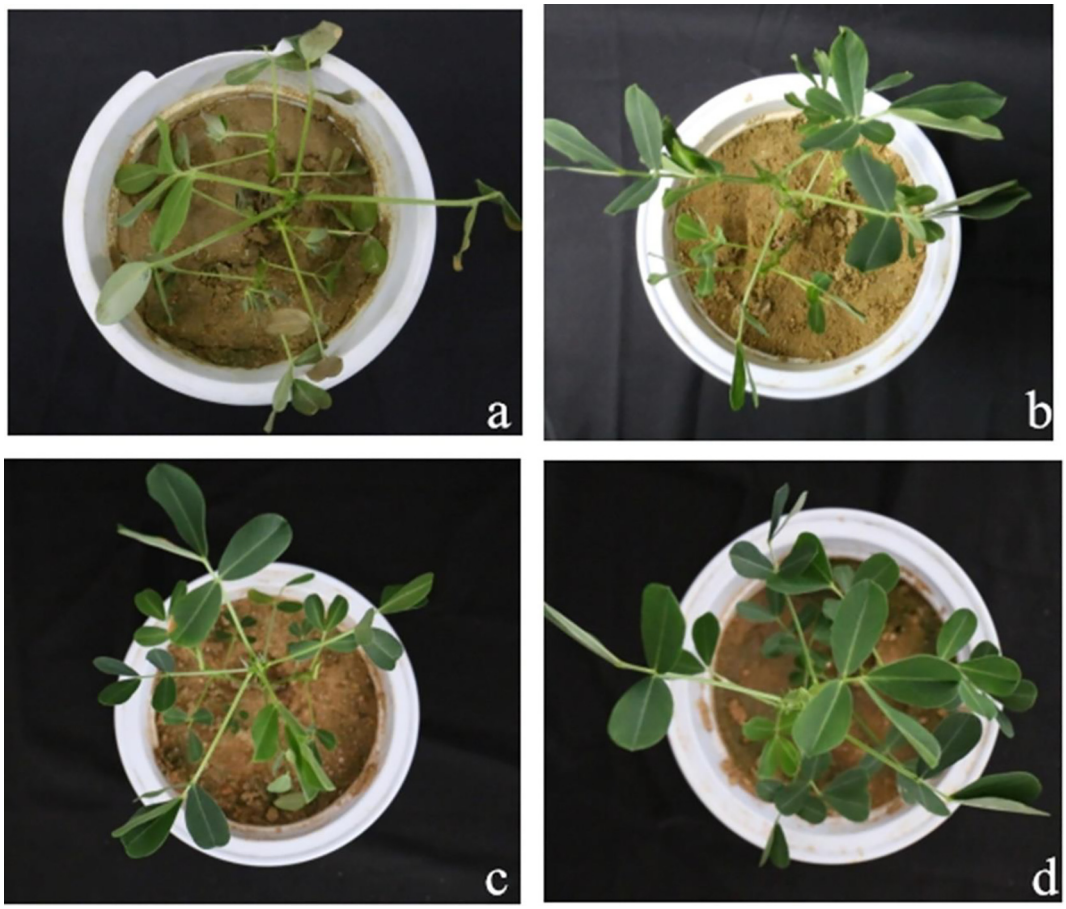

Fig. 8. Effects of salt stress treatment on control and transgenic plants that overexpress *AhLea-3*. (a) Huayu23 (the non-transgenic parent); (b–d) *AhLea-3* overexpression plants.

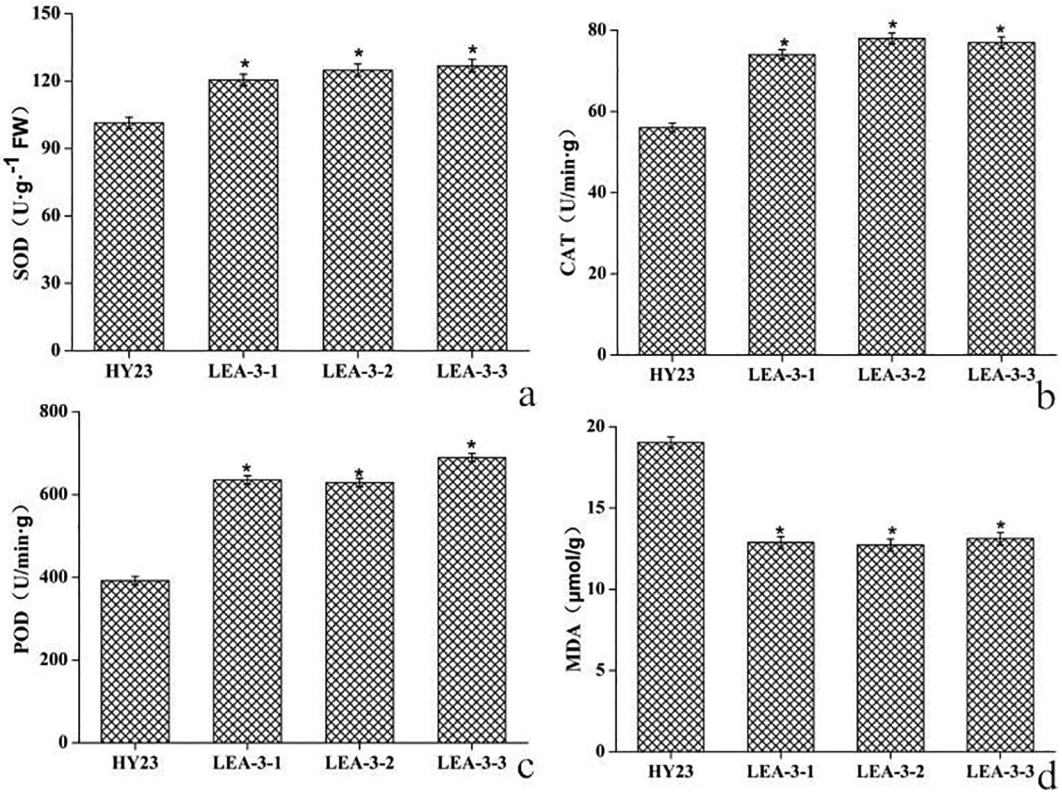

Fig. 9. Antioxidant enzyme activity and MDA content of *AhLea-3* transgenic plants and control plants in response to salt stress. (a) SOD activity; (b) CAT activity; (c) POD activity; (d) MDA content. Values are means + SE of three replicate plants. An asterisk indicates that the mean of the transgenic plants was significantly greater ($P < 0.05$) than the mean of the Huayu23 plants.

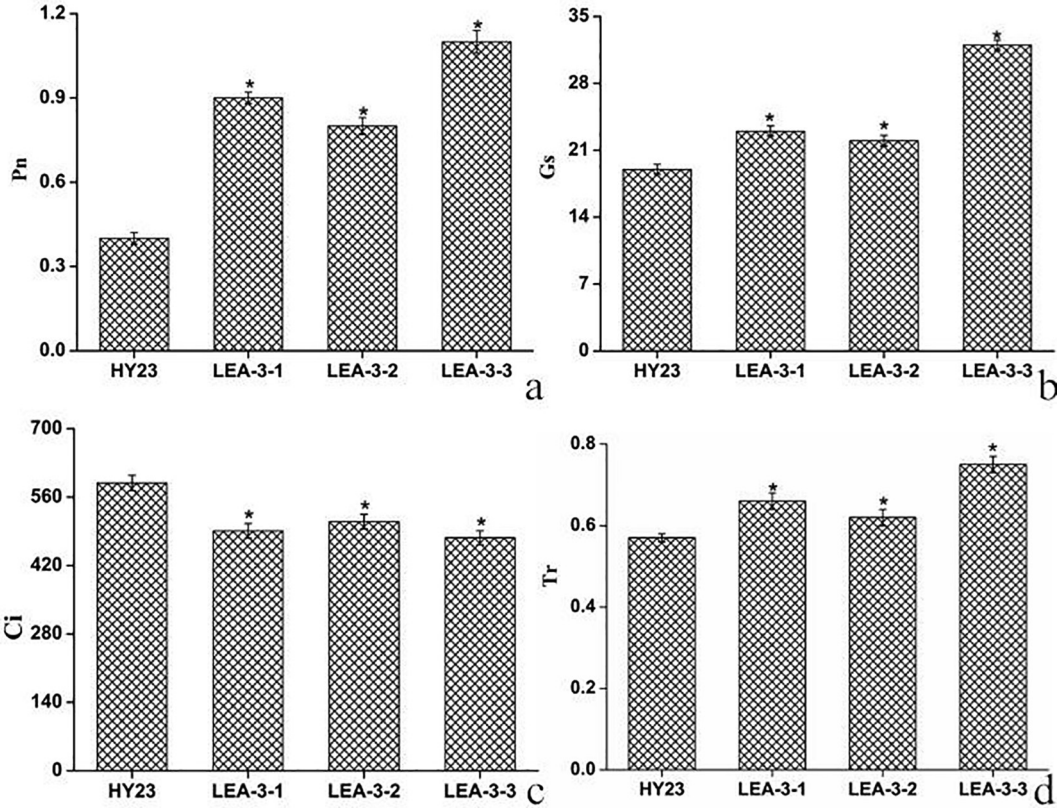

Fig. 10. Gas exchange parameter values of control plants (Huayu23, the non-transgenic parent) and AhLea-3 overexpression plants in response to salt stress. (a) Photosynthetic net rate (Pn); (b) stomatal conductance (Gs); (c) intercellular CO_2 concentration (Ci); (d) transpiration rate (Tr). Values are means + SE of three replicate plants. An asterisk indicates that the mean of the transgenic plants was significantly greater ($P < 0.05$) than the mean of the Huayu23 plants.

Conflict of interest

The authors declare that they have no conflict of interest.

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