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GmLEA2-1, A LATE EMBRYOGENESIS ABUNDANT PROTEIN GENE ISOLATED FROM SOYBEAN (*GLYCINE MAX* (L.) MERR.), CONFERS TOLERANCE TO ABIOTIC STRESS

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Late embryonic proteins (LEA) gene family was abundant mainly in higher plant embryos, which could protect the embryos from the damage caused by abiotic stress, especially drought and salt stresses. In the present study, *GmLEA2-1* was cloned from soybean leaf tissue treated by 10% polyethylene glycol 6000 (PEG6000). The results of quantitative real-time PCR (qRT-PCR) revealed a variety of expression patterns of *GmLEA2-1* in various tissues of soybean (root, stem, leaf, flower, pod, early embryo and late embryo). *GmLEA2-1* gene shared a lower sequence similarity with other typical LEA genes of same group from different species, but similar functions. Overexpression of *GmLEA2-1* in transgenic *Arabidopsis thaliana* conferred tolerance to drought and salt stresses. The fresh weight and dry weight of seedling, the primary root length and the lateral root density of transgenic *Arabidopsis* plants were higher than those of wild type *Arabidopsis* (WT) under drought and salt stresses. *Cis*-acting regulatory elements in the *GmLEA2-1* promoter were also predicted. These data demonstrate that *GmLEA2-1* protein play an important role in improving drought and salt tolerance in plants.

Keywords: *GmLEA2-1* – Stress tolerance – Soybean-promoter – Transgenic *Arabidopsis thaliana*

INTRODUCTION

Late embryogenesis abundant (LEA) proteins were first discovered in cotton 30 years ago [9]. They are expressed during the later stages of embryo development (post-abscission) in plant seeds and induced in vegetable tissues in the presence of abiotic stress such as drought, low temperature, salt and abscisic acid (ABA) [15]. With the improvement of deep sequencing technology, an increased number of LEA genes have been identified [3, 22, 23, 26, 29]. LEA proteins are categorized in seven distinct families, according to the results of Battaglia's group on the basis of their amino acid sequence similarities and conserved motifs [4].

The main physical characteristic of most LEA proteins are that they are generally hydrophilic and intrinsically unstructured or disordered. The structural feature of LEA proteins suggested that some of LEA play an important role in the regulatory pathway of defending adverse environment in the organism. The functions of some

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LEA groups have been studied in details [5, 6, 12, 17, 28, 30]. However, compared with other categories of LEA proteins, LEA_2 is rather more hydrophobic and possesses relatively stable three-dimensional structure [16]. The biological functional studies related to LEA_2 family are considerably fewer than in other groups. Only some of the atypical LEA_2 group genes from several different species have been investigated. The overexpression of atypical LEA_2 genes could improve the stress tolerance to drought and salt in transgenic plants [18, 29]. The difference both the hydrophobic content and the structure between LEA_2 group proteins and other LEA groups is not obvious. It was compelling for us to elucidate whether LEA_2 gene from soybean plays different or similar role in withstanding adverse environment.

In the present study, we aimed to isolate a novel member of LEA genes from soybean and characterize the role of *GmLEA2-1* in regulation tolerance to abiotic stresses. We investigated the expression of *GmLEA2-1* induced by abiotic stresses and the resistance to salt and drought stresses in transgenic *Arabidopsis* overexpressed *GmLEA2-1*. *Cis*-acting regulatory elements in the *GmLEA2-1* promoter were also predicted. These data could further define the role of *GmLEA2-1* in the responses to abiotic stresses and the potential involvement of *GmLEA2-1* in the genetic modification of plants in enhanced tolerance to drought and salt.

MATERIALS AND METHODS

Plant materials and growth conditions

Soybean seed (*Glycine max* (L.) Merr.) ‘Dongnong 50’ was obtained from Northeast Agricultural University (Harbin, China). To analyze the tissue-specific expression of *GmLEA2-1*, the root, stem, leaf, flower, pod, early embryo, and late embryo from soybean plants were harvested, frozen in liquid nitrogen and stored at -80°C . To analyze the expression profile of *GmLEA2-1* under abiotic stress conditions, soybean plants, with the first fully expanding trifoliolate leaves, were transplanted into 1/4 Murashige and Skoog (MS) liquid medium supplemented with 10% PEG6000 and 100 mM NaCl, respectively, 1/4 MS liquid medium without PEG6000 and NaCl as control. The leaves were sampled at 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 hours after transferring.

Seeds of *Arabidopsis thaliana* (Col-0), were cultured on 1/2 MS medium with 2% sucrose for three weeks at $21\text{--}22^{\circ}\text{C}$ with a 16 h/8 h (day/night) photoperiod and 60–70% relative humidity. Then, the young seedlings were transplanted on fertilized soil and grown in the same conditions.

Cloning and sequence analyses of GmLEA2-1

The total RNA was isolated from soybean seedlings treated by 10% PEG6000 using RNAiso Plus™ kit (TaKaRa, Japan). The first strand cDNA was synthesized accord-

ing to the protocol of the First Strand cDNA Synthesis Kit™ (TaKaRa, Japan). To obtain the open reading frame (ORF) of *GmLEA2-1*, PCR was performed with the primers 5'-TCGCTTGGATCACTGATCTCATCTC-3' and 5'-GCCAGTAATA-ACGAGCTCCAATTCTTCAAT-3'. The PCR products were purified and cloned into the pMD18-T vector (TaKaRa, Japan) for sequencing. The isoelectric point and molecular mass predictions were estimated using the compute Pi/Mw toll. Analysis of protein hydrophathy was done by constructing hydrophathy plots with the Kyte and Doolittle algorithm. Motif analysis was performed using the Pfam program. Sequence similarities were determined using the BLAST program and the GenBank database on the NCBI web server. Sequence alignment was performed with DNAMAN.

GmLEA2-1 expression analysis

Total RNA was isolated from soybean leaves using RNAiso Plus™ (TaKaRa, Japan). qRT-PCR was performed on a Chromo4 Real-Time PCR System (Bio-Rad, USA) using SYBR Green PCR Master Mix Reagent (TaKaRa, Japan). The primers used for *GmLEA2-1* amplification were 5'-TTAGAGGAAGGGGAACAGGTTACAC-3' and 5'-TGCCCATCAGAACTCAGAAAG-3'. Soybean *Actin4* gene was used as the internal control. The primer used for *Actin4* gene amplification was 5'-GTGTCAGCCATACTGTCCCCATTT-3', and the reverse primer was 5'-GTTTCAAGCTCTTGCTCGTAATCA-3'. Three replicates of each reaction were performed, the expression ratio was calculated as $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (C_{t_{target}} - C_{t_{actin}})_{PEG} - (C_{t_{target}} - C_{t_{actin}})_{CK}$.

Overexpression of GmLEA2-1 in Arabidopsis

GmLEA2-1 cDNA was sub-clone into the binary vector pCAMBIA3301. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* strain L4404 and was introduced into *Arabidopsis* using the floral dip method. Transgenic Arabidopsis plants were tested by PCR, using the primers 5'-CACTATCCTTCGCAAGACCC-3' and 5'-TGAATCGCAGTAACGTCAGC-3'.

For drought tolerance assay in MS medium, one-week-old seedlings were transformed to 1/2 MS medium with 0, 100, 200, 300 mM of mannitol. Photographs were taken after 10 days, and root lengths were measured. The up-part of seedlings was collected and used to measure proline content.

For salt tolerance experiment in MS medium, one-week-old seedlings were transformed into 1/2 MS medium containing 0, 50, 100, 150 mM of NaCl, respectively, for two weeks to evaluate salt tolerance in MS medium. The fresh weight and dry weight were measured, respectively. And, the average root length and the lateral root density were calculated afterward.

Biomass and proline accumulation

The proline content was tested according to Liu's method [21]. The content of proline was calculated as $\mu\text{g/g}$ using a proline standard (L-proline, Sigma-Aldrich Inc., MO, USA).

Statistical analysis

The survival rate, fresh weights, dry weights, root lengths, lateral root density and proline content were subjected to Student's *t*-test analyses using SPSS 12. All of the experiments were biologically repeated three times.

RESULTS

Isolation and sequence analysis of GmLEA2-1

A cDNA containing 1024 bp was isolated from soybean leaves treated with 10% PEG6000 for 6 h, named *GmLEA2-1*. GmLEA2-1 encoded a putative 320 amino acids with a calculated molecular weight of 35.65 kDa and a theoretical pI of 4.8. GmLEA2-1 was predicted to have highly hydrophobic structure with the exception of the N-terminal region [19]. The GmLEA2-1 protein contained a "Lea_2" motif (PF03186), which was classified into subgroup 5C (D-95) according to Battaglia's classification of LEA protein [7, 8]. Further analysis showed that GmLEA2-1 contained a lower percentage of polar amino acids (48.7%) and higher percentage of non-polar amino acids (25.8%) than other groups' LEA proteins [13, 29]. All these characteristics were consistent with those of other members of subgroup 5C [13, 29].

The deduced amino acid sequences shared very low identity with a typical group of LEA_2 (Fig. 1a). GmLEA2-1 was more closely related with At2g446060 (67.69%) than At1g01470 (13.53%) and At2g46140 (16.17%). The homologous rate between GmLEA2-1 protein and soybean endogenous LEA_2 protein D95-4 was only 8.2% [23].

Expression of GmLEA2-1 in different tissues of soybean and under drought and salt stress

GmLEA2-1 was strongly expressed in root and pod, and relatively lower in stem and leaf (Fig. 2a), which was consistent with the previous microarray and EST data [20]. Interestingly, *GmLEA2-1* expression in early and late embryos was strikingly low, in accordance with the results, of the expression of At2g44060 [17].

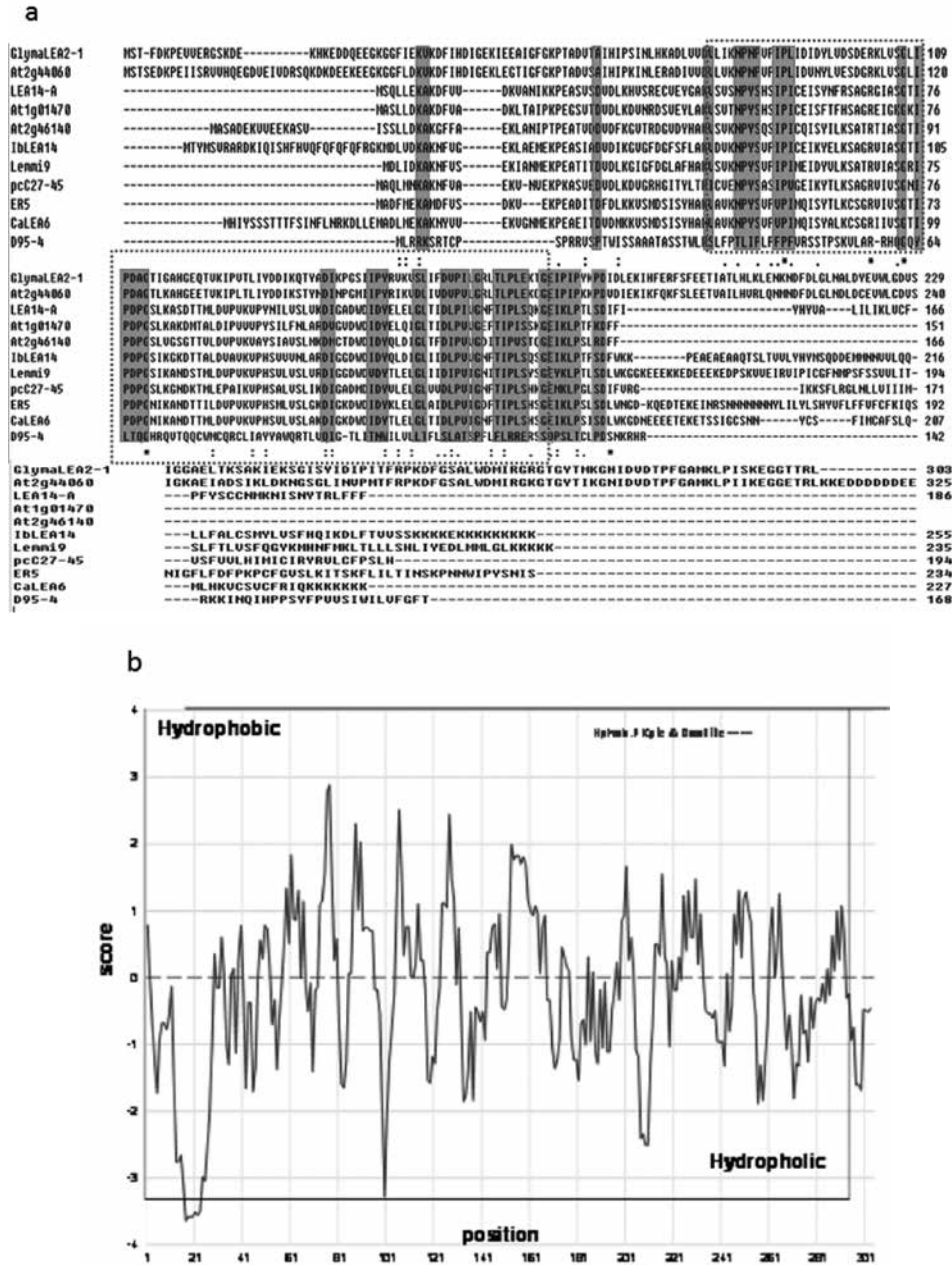


Fig. 1. Multiple sequence alignments, phylogenetic tree and hydropathicity plot for deduced amino acid sequences of soybean GmLEA2-1 and other closely related plant proteins. a – Multiple sequence alignments of GmLEA2-1 with other LEA14 proteins. The conserved “LEA₂” motif (PF03168) is boxed. b – Hydropathicity plot of the deduced GmLEA2-1 amino acid sequence analyzed using the KyteDoolittle algorithm

The mRNA level of *GmLEA2-1* in soybean leaf under 10% PEG6000 treatment did not exhibit obvious reduction or increase until soybean plants suffered from 9 hours dehydration (Fig. 2b). The peak of mRNA accumulation occurred at 12 hours from the initiation of stress. Along with the consistent stress of PEG6000, the level of *GmLEA2-1* mRNA began to drop down gradually. Additionally, 100 mM NaCl treating 8 hours resulted in increased accumulation of *GmLEA2-1* to a maximum level (14-fold) (Fig. 2c). The above results indicated that *GmLEA2-1* might play important roles in the responses to abiotic stress.

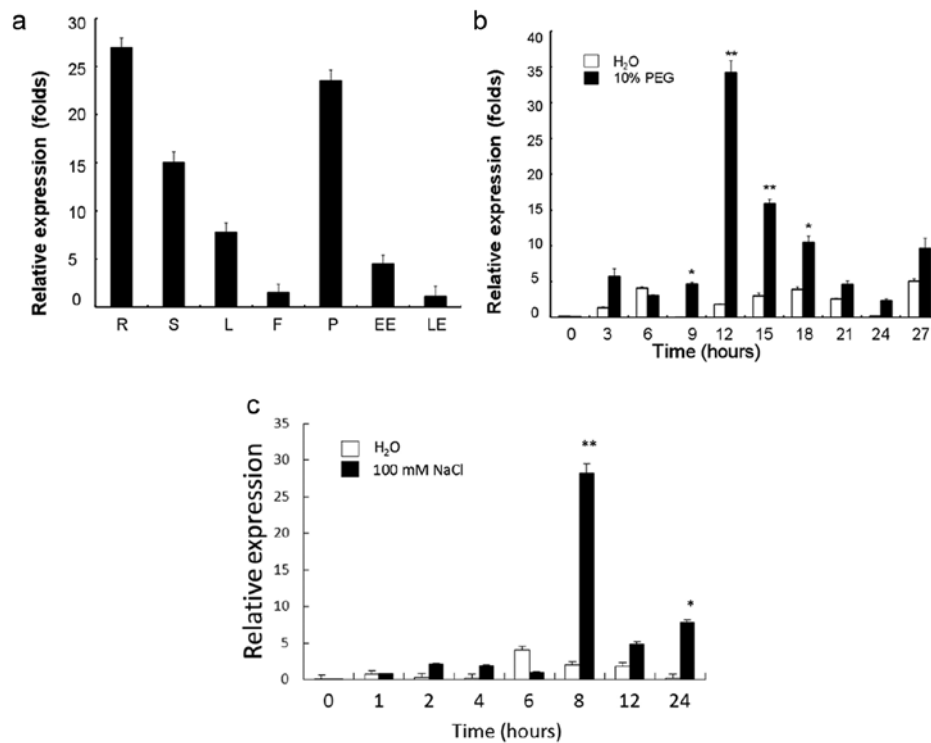


Fig. 2. Expression patterns of *GmLEA2-1* in various tissues and under different dehydration conditions. a – Tissue-specific expression of *GmLEA2-1*. Total RNA was extracted from roots (R), stems (S), leaves (L), flowers (F), pods (P), early embryos (EE), and late embryos (LE). b – *GmLEA2-1* transcripts accumulation levels in response to 10% (v/v) PEG 6000 treatment. c – *GmLEA2-1* transcripts accumulation levels in response to 100 mM NaCl (v/v). The ACTIN gene was used as a control gene. Each data point is average of three biological replicates and bars indicate SD. *Indicates significant differences in comparison to the wild type at $P < 0.05$, **the same at $P < 0.01$

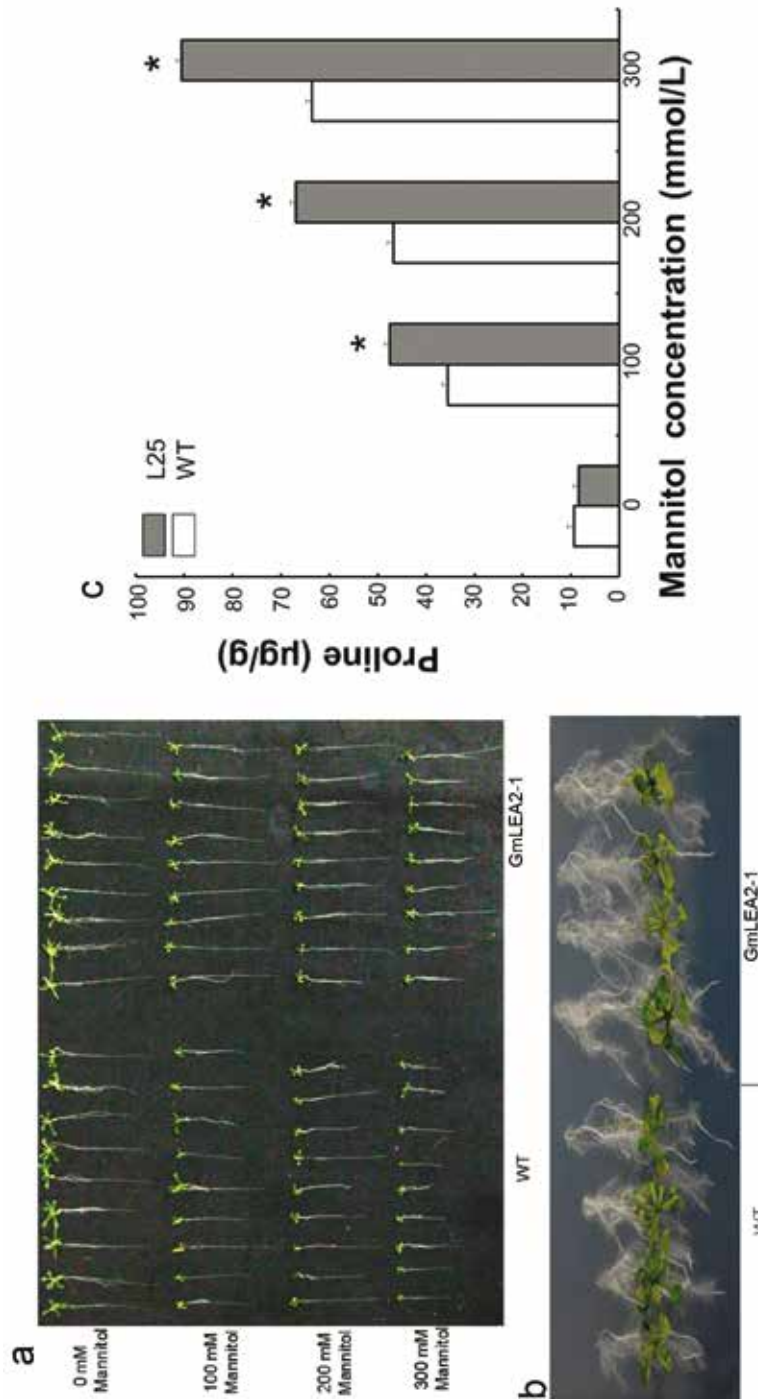


Fig. 3. Performance of the *GmLEA2-1* overexpressing seedlings under different concentration mannitol treatment. **a** – Photograph of representative wild type (WT) and transgenic plants (L25) treated by mannitol for 10 days. **b** – Root bending assay of WT and transgenic plants on MS medium supplemented with 300 mM mannitol. **c** – Proline contents of WT and transgenic Arabidopsis at different concentration of mannitol. Each data point is average of three experiments and bars indicate SD. *Indicates significant differences in comparison to the wild type at $P < 0.05$

GmLEA2-1 overexpression in *Arabidopsis* increased resistance to drought

Ten-day-old WT and transgenic seedlings were transferred into 1/2 MS medium with 0 mM, 100 mM, 200 mM, and 300 mM mannitol. The transgenic seedlings overexpressed *GmLEA2-1* showed a stronger growth performance than WT under high concentration mannitol stress (Fig. 3a). Transgenic lines showed a significantly stronger root growth than the WT with 200 mM and 300 mM mannitol stresses for 10 days (Fig. 3b).

The free proline content of WT and transgenic plants all increased after the treatment with mannitol for 10 days. However, a significantly higher increase of free proline content ($P < 0.05$) was observed in the transgenic lines L25 compared with the WT (Fig. 3c). Taken together, these results indicated that *GmLEA2-1* overexpression in *Arabidopsis* showed more drought resistance.

Table 1
Putative *cis*-acting regulatory elements associated with ABA and various stresses in *GmLEA2-1* promoter region

<i>Cis</i> -element name	Sequence	Numbers	Function
ABRE	ACGTG ACGT	1 4	ABA-mediated regulation of transcription. Involved in ABA response
DRE	RCCGAC ACCGAC	2 2	DRE/CRT regulatory element involved in dehydration, cold or senility responsiveness. DBF1 and DBF2 binding site in the maize <i>rab17</i> gene promoter involved in ABA induction
CRE	GTCGAC	4	DNA binding is regulated by temperature; "Core CRT/DRE motif"
CBF	RYCGAC GTCGAC	4 2	Binding site of barley CBF1 and barley CBF2; Preferred sequence for AP2 transcriptional activator HvCBF2 of barley; "Core CRT/DRE motif"; DNA binding is regulated by temperature
LTRE	CCGAC	2	ABA responsiveness; Involved in cold induction of BN115 gene from winter <i>Brassica napus</i> ; Light signaling mediated by phytochrome is necessary for cold- or drought-induced gene expression through the C/DRE in <i>Arabidopsis</i>
MYB	WAACCA TAACTG YAACKG CNGTTR	4 2 2 3	Binding site for ATMYB2, ATMYB2 is involved in regulation of genes that are responsive to water stress in <i>Arabidopsis</i> . Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2, both isolated from <i>Arabidopsis</i>
MYC	CANNTG	14	Binding site of ICE1 that regulates the transcription of CBF/DREB1 genes in the cold in <i>Arabidopsis</i>
WRKY	TGAC		Binding site of WRKY71, a transcriptional repressor of the gibberellin signaling pathway

M = C/A; Y = T/G; B = T/C/G; N = A/T/G/C; R = A/G; W = A/T; V = A/C/G; K = G/T.

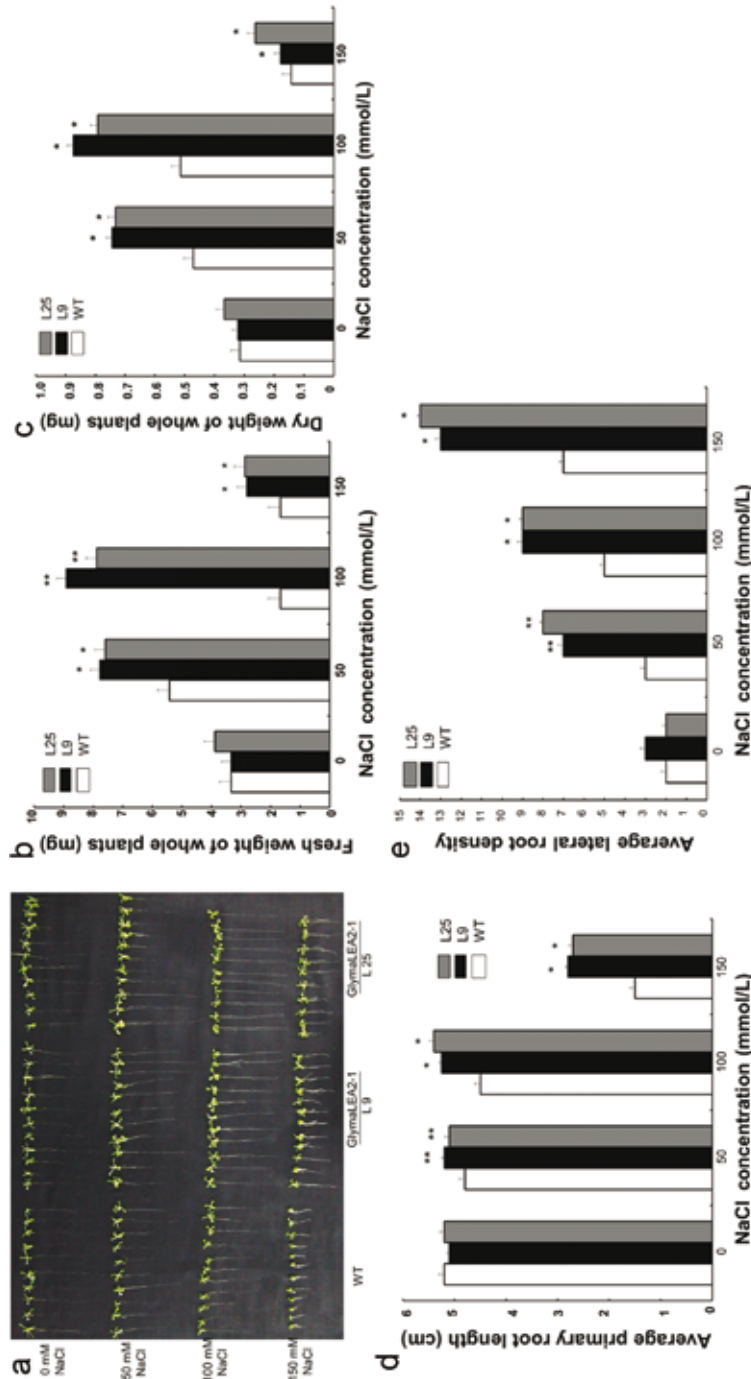


Fig. 4. Morphogenesis of *GmLEA2-1* transgenic seedlings under different concentrations of NaCl. a – Photograph of representative WT and transgenic Arabidopsis (L9, L25) treated by salt stress for two weeks. b – Comparison of fresh weight between WT and transgenic lines. c – Comparison of dry weight between WT and transgenic plants. d – Comparison of average primary root length between WT and transgenic plants. e – Comparison of average lateral root density between WT and transgenic plants. Each data point is average of three biological experiments and bars indicate SD. *, **Indicates significant differences in comparison to the wild type $P < 0.05$ and $P < 0.01$, respectively

GmLEA2-1 overexpression in *Arabidopsis* during increased resistance to salt

When L25 and L9 transgenic seedlings and WT were grown on 1/2 MS medium with 50 mM, 100 mM and 150 mM NaCl for two weeks, the transgenic seedlings possessed significantly larger leaves and longer roots than those of the WT (Fig. 4a). Consistently, the fresh and dry weights of transgenic seedlings were significantly higher than that of the WT (Fig. 4b and 4c). In addition, the transgenic lines L9 and L25 showed longer primary root length and more lateral root density than that of the WT ($P < 0.01$) (Fig. 4d, 4e). These results suggest that *GmLEA2-1* improves salt stress resistance of transgenic *Arabidopsis*.

GmLEA2-1 promoter contained stress associated cis-elements

A 2000 bp *GmLEA2-1* promoter fragment (including 5'UTR) was subjected to a putative *cis*-acting regulatory element search using the Plant *Cis*-acting Regulatory DNA Elements (PLACE) database [14]. In the *GmLEA2-1* promoter, one ABRE-like sequence, four ABA- and dehydration-responsive ACGT-box motifs, fourteen MYC, eleven MYB-like sequences, DRE-like sequences and CRT-like sequences were all identified (Table 1).

DISCUSSION

Typical LEA proteins can retain water molecules and protect other proteins from aggregation caused by desiccation because of their highly hydrophilic properties [11]. Conversely, atypical LEA proteins have higher content of hydrophobic residues than typical LEAs. The latter have been speculated to be involved in diverse stress tolerances, although few studies have been carried out to characterize their functions [13, 18, 28]. LEA_2 group gene, *GmLEA2-1*, was cloned from soybean in the present study and named as a novel atypical LEA member, like *SiLEA14* [28].

A previous study showed that drought, salt and root-knot nematode infection could induce *LEA* expression, among others in hot pepper [18], tomato [31], *Craterostigma plantagineum* and soybean [22], tomato [10] and *Arabidopsis* [17] etc. In the present study, although a large difference was found in amino acid distribution between *GmLEA2-1* and other atypical LEA_2 group members mentioned above, the expression of *GmLEA2-1* can also be induced by PEG6000 and NaCl. We speculate that these proteins represent the same important structure domains, even though their amino acid identity was not high, and they might be similar in the regulation of their expression or the pathway involved in environmental change.

In the present study, the overexpressed *GmLEA2-1 Arabidopsis* plants exhibited stronger stress-tolerance to dehydration and high salt. *GmLEA2-1* gene are assumed to play an important role in the pathway against abiotic stress, and this seems to be is

in agreement with previous data reporting that the overexpression of both *CaLEA6* and *IbLEA14* could improve the tolerance of transgenic plants to drought and salt stress through comparable phenotype difference and biological index change [18]. Therefore, most hydrophobic LEA_2 proteins might play very important role in the stress response.

The identification of *cis*-acting elements in the *GmLEA2-1* promoter may help obtain insight into the molecular mechanism of *GmLEA2-1* function. The ABA responsive element (ABRE), which has been identified in the promoters of many ABA-inducible genes of plants such as the cotton LEA gene D-113 [24], plays a key role in ABA signaling during seed development and under abiotic stresses [15]. The drought responsive/C-repeat/low temperature response (DRE/CRT/LTRE) element was identified as the second prominent *cis*-element in relation to the expression of stress regulated genes in general and LEA genes in particular, which binds the CBF/DREB1 transcription factors [15, 27]. MYC and MYB binding sites have been identified as regulatory elements associated with ABA, drought, or cold responsiveness in plants [1, 2, 26, 30]. These results suggest that *GmLEA2-1* might stand under the regulation of DREB-, DBF-, CBF-, MYC- or MYB-like transcription factors in soybean.

In summary, *GmLEA2-1* gene was isolated from soybean. It was induced by PEG6000 and NaCl. Overexpression of *GmLEA2-1* resulted in enhanced resistance to abiotic stresses in transgenic *Arabidopsis*. The results described above show that *GmLEA2-1* plays an important role in the pathway against abiotic stress. Our results also provide further important information for the development of crop tolerance to environmental stresses. Future studies are needed to deliver insight into the relationship between function and structural domains, and the molecular mechanisms of soybean LEA genes in plant defense responses and development.

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