Brief communication

Isolation and expression analysis of LEA genes in peanut (*Arachis hypogaea* L.)

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Late embryogenesis abundant (LEA) protein family is a large protein family that includes proteins accumulated at late stages of seed development or in vegetative tissues in response to drought, salinity, cold stress and exogenous application of abscisic acid. In order to isolate peanut genes, an expressed sequence tag (EST) sequencing project was carried out using a peanut seed cDNA library. From 6258 ESTs, 19 LEA-encoding genes were identified and could be classified into eight distinct groups. Expression of these genes in seeds at different developmental stages and in various peanut tissues was analysed by semi-quantitative RT-PCR. The results showed that expression levels of LEA genes were generally high in seeds. Some LEA protein genes were expressed at a high level in non-seed tissues such as root, stem, leaf, flower and gynophore. These results provided valuable information for the functional and regulatory studies on peanut LEA genes.

[Su L, Zhao C-Z, Bi Y-P, Wan S-B, Xia H and Wang X-J 2011 Isolation and expression analysis of LEA genes in peanut (*Arachis hypogaea* L.). *J. Biosci.* **36** 223–228] **DOI** 10.1007/s12038-011-9058-5

1. Introduction

Late embryogenesis abundant (LEA) proteins were first found in upland cotton (*Gossypium hirsutum* L.) seeds, accumulating at late stage of embryogenesis (Dure and Galau 1981). Most LEA proteins comprise highly hydrophilic amino acids, which lack or have a low proportion of Cys and Trp residues. In general, they are randomly coiled proteins in solution and therefore are considered intrinsically unstructured proteins (Battaglia *et al.* 2008). There are a few atypical LEA proteins that contain a significantly higher proportion of hydrophobic residues and are predicted to adopt more globular conformations. For example, cotton protein D34 (Baker *et al.* 1988), D73, D95 (Galau *et al.* 1993) and a D95-like protein from *Arabidopsis* are all atypical LEA proteins (Singh *et al.* 2005).

LEA proteins are found in both angiosperms and gymnosperms, as well as in moss (Machuka *et al.* 1999) and pteridophytes (Reynolds and Bewley 1993). LEA proteins are also found in a wide range of other organisms including bacteria (Stacy *et al.* 1999), yeast (Garay-Arroyo *et al.* 2000), cyanobacteria (Tanaka *et al.* 2004), nematode (Solomon *et al.* 2000), the brine shrimp (Sharon *et al.* 2009) and collembola (Bahrndorff *et al.* 2009). Several nomenclature systems have been reported to classify LEA proteins into different groups. In early studies, LEA proteins were classified according to their molecular weights and therefore were named as D7,

Keywords. *Arachis hypogaea* L.; cDNA library; ESTs; late embryogenesis abundant protein

Supplementary materials pertaining to this article are available on the *Journal of Biosciences* Website at *http://www.ias.ac.in/jbiosci/Jun2011/pp223–228/suppl.pdf*

D11, D19, D29, D34, D73, D95, D113, etc. (Hughes and Galau 1991). As more LEA proteins were identified, new classification systems based on protein amino acid composition or different sequence motifs was applied (Dure 1993; Machuka *et al.* 1999; Battaglia *et al.* 2008; Bies-Ethève *et al.* 2008). LEA proteins could be classified into five to nine groups by different nomenclature systems.

In vitro and *in vivo* experiments showed that LEA proteins play important roles in normal seed development and plant response to environmental stress condition, such as dehydration, salinity, osmosis and low temperature (Robertson *et al.* 1994; Ingram and Bartels 1996; Colmenero-Flores *et al.* 1997; Wang *et al.* 2002, 2003; Battaglia *et al.* 2008; Bies-Ethève *et al.* 2008; Hundertmark and Hincha 2008; Shimizu *et al.* 2010). However, the precise function of LEA proteins in plant development and stress response remains to be clarified.

Peanut (*Arachis hypogaea* L.) is widely grown in China, India, Nigeria and the United States. It ranks fifth among the world's oil crops (Moretzsohn *et al.* 2005). However, molecular biological studies, genomics and genetic modification of peanut are very limited compared with other crops like rice, cotton and soybean, because of its large unknown genome (2800 Mb) as well as the recalcitrant of transformation. EST sequencing is a flexible and effective method for gene cloning. It is widely used in plants such as soybean, maize, cotton, wheat and many other plant species. We constructed a cDNA library using immature seeds of a Chinese peanut cultivar, Luhua-14, and carried out an EST sequencing project using this library (Bi *et al.* 2010). In this study, we report the identification and analysis of 19 peanut AhLEA protein genes using direct EST sequencing. The sequence similarity with LEA proteins from other species and the expression patterns of peanut LEA protein genes were investigated. We provide important information for a global understanding of peanut LEA proteins, including their sequence conservation and variation as well as expression patterns in seeds at different developmental stages and in various peanut tissues. The possible roles of LEA proteins in peanut embryo development and stress tolerance are discussed.

2. Materials and methods

2.1 *Plant material*

Peanuts were grown in farmlands. Gynophores were labeled before their penetration in soil. Seeds at different developmental stages 10 to 90 days after pegging (DAP) were collected and immediately frozen in liquid nitrogen before being stored in −70°C. The root, stem, leaf, flower and gynophore were collected from the peanuts growing in the same field. All samples were immediately frozen in liquid nitrogen and stored at −70°C before RNA extraction.

cDNA library construction and ESTs sequencing: Immature peanut seeds (20–60 DAP) were collected for cDNA library construction. Total RNA was extracted from 200–500 mg of seed mixture by using TRIZOL reagent following the manufacturer's protocol (TaKaRa). Messenger RNA was isolated and purified from total RNA (Promega). Directional cDNA synthesis (using EcoRI, XhoI restriction sites adapters) and library construction were according to the protocol of the cDNA library construction kit (Stratagene). Colonies were randomly picked for plasmid preparation. Purified plasmid DNA was used as a template for EST sequencing by using T3 or T7 primers with BigDye^{R} Terminator v3.1 Cycle Sequencing Kit (ABI) on ABI 3730XL DNA Analyzer.

2.2 *Analysis of ESTs*

The vector and low-quality sequences were removed manually. Sequences were clustered and assembled into contigs using CAP3 (Huang and Madan 1999) and annotated using BlastX (e-value <1e−10).

2.3 *Cloning and classification of AhLEAs genes*

The sequences related to *AhLEA* genes were identified and their open reading frame (ORF) was determined by ORF Finder (*www.ncbi.nlm.nih.gov/gorf/gorf.html*) and subjected to multiple sequence alignments using ClustulW1.83 software (*http://www.ch.embnet.org/software/ClustalW.html*). Bies-Ethève's nomenclature system was used for AhLEAs classification (Bies-Ethève *et al.* 2008). The clones that had or were predicted to have full-length ORFs were sequenced again to get the accurate sequences or the full length of the ORF.

2.4 *Expression analysis of AhLEAs*

Total RNA was isolated from seeds at nine different developmental stages (10, 20, 30, 40, 50, 60, 70, 80 and 90 DAP) and various non-seed tissues including root, stem, leaf, flower and gynophore. Total RNA was extracted using RNAiso Reagent (TaKaRa) as described by the manufacturer. Five micrograms of RNA was reverse-transcribed using PrimerScript^{TM} 1st Strand cDNA Synthesis Kit (TaKaRa) with oligo (dT) primer according to the protocol provided by the supplier. The gene-specific primers were designed for each individual gene (supplementary table 2). The resulting cDNA was used as a template for semi-quantitative RT-PCR. PCR reactions were performed following the programme: 3 min at 94°C; 28 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C; 5 min at 72°C as final extension. Ten microliters of the PCR product was used for electrophoresis and visualized by ethidium bromide staining. Peanut actin was used as the control.

3. Results

3.1 *Cloning and classification of peanut LEA protein genes*

Immature seeds of peanut cultivar Luhua-14 were used to make a cDNA library, and subsequently, large-scale EST sequencing was carried out using this library. Random colonies were picked for sequencing and 6258 EST sequences were produced using T3 primer. In addition, 1198 clones were re-sequenced using T7 primer in order to obtain the full length of the insert sequence. All these ESTs were submitted to NCBI Genbank (EE123340-EE127745, EG372473-EG374270, EG529454-EG530705). In this work, we focused on the identification and characterization of LEA genes. Totally, 271 ESTs were predicted to represent LEA genes. They could be assembled into 18 contigs and 1 singleton using CAP3 software analysis.

From these EST sequences we obtained the full-length ORFs of 16 LEA genes. We could identify only the 5′ fragments of *AhLEA5-1* and *AhLEA5-2*. The corresponding clones for these ESTs were recovered for plasmid preparation and subsequently subjected to sequencing using the T7 primer from the 3′ end of the insert. After joining the two ESTs from one clone, we obtained the full-length ORFs of *AhLEA5-1* and *AhLEA5-2* genes. Collectively, full-length ORFs of 18 LEA protein genes except *AhLEA3-3* were cloned. Detailed sequence information of these 19 LEA protein genes is listed in supplementary table 1. According to the Bies-Ethève nomenclature system and phylogenetic analysis results, peanut LEA genes could be classified into eight different groups (supplementary figure 1). Only one member was found for the LEA2, LEA7 and LEA8 groups. Two members were found for the LEA4, LEA 5 and LEA6 groups. Three and seven members were identified for the peanut LEA1 and LEA3 groups, respectively (supplementary table 1). *AhLEA3-1* represented the most abundant peanut LEA gene (65 clones), followed by *AhLEA4-2* (38 clones) and *AhLEA3-7* (33 clones). The detailed sequence information including the molecular weight, isoeletric points and the conserved motifs, as well as the sub-cellular localization of AhLEA proteins, are listed in the supplementary material.

3.2 *Expression pattern analysis of AhLEA genes*

Expression patterns could provide insight into the function of LEA genes. In this work, semi-quantitative RT-PCR was used for expression analysis of peanut LEA genes. The results showed that most AhLEA genes were highly expressed in seeds from 30 to 90 DAP, and were not detected in non-seed tissue, which was similar to the results observed from *Arabidopsis* (Bies-Ethève *et al.* 2008). The expression of *AhLEA1*, *AhLEA4*, *AhLEA5* and *AhLEA8* genes was only detected in seeds. The expression of seven LEA protein genes (*AhDHN1*, *AhLEA3-1*, *AhLEA3-2*, *AhLEA3-4*, *AhLEA3-6*, *AhLEA6-1* and *AhLEA7-1*) could be detected both in seed and non-seed tissues including root, stem, leaf, flower and gynophore. The expression of *AhLEA3-2* and *AhLEA6-1* was observed in all the five non-seed tissues tested. *AhDHN1* was expressed weakly in leaf, flower and stem. *AhLEA3-1* and *AhLEA7-1* were expressed in root, stem, leaf and flower. *AhLEA3-6* was expressed in root, leaf and flower. Interestingly, *AhLEA3-4* was highly expressed in flower but not in other non-seed tissues (figure 1).

In 10 DAP seeds, the expression of most LEA genes was undetectable during seed development. However, the expression of *AhLEA6-1*, *AhLEA6-2* and *AhLEA7-1* was clearly detected in 10 DAP seeds (figure 1). The expression of 17 LEA genes could be detected in 30 DAP seeds. The results showed that different groups of LEA genes exhibited variable expression patterns during seed development, for example, the genes of LEA1, LEA5, LEA6, LEA7 and LEA8 groups. Even different members from one group of LEA showed distinct expression patterns, for instance, *LEA6-1* and *LEA6-2* (figure 1). *AhLEA1-3* and *AhLEA3-5* were weakly expressed in seeds, and more reaction cycles (32 cycles) of PCR amplification were used in order to obtain clear bands. For other genes, 28 reaction cycles were used for PCR amplification.

4. Discussion

Most of LEA proteins are highly hydrophilic, heat stable in solution and relatively small in molecular weight. The molecular weight of LEA proteins in *Arabidopsis thaliana* ranged from 10 to 30 kDa (Hundertmark and Hincha 2008). From our EST sequencing data, we obtained 19 LEA protein genes and classified them based on Bies-Ethève's nomenclature system. Like many LEA proteins from other species, most of these peanut LEA proteins are small peptides with molecular weights ranging from 6.98 to 36.76 kDa. Most of them, except LEA5, LEA6 and LEA7, are hydrophilic. We did not obtain sequences related to *Arabidopsis thaliana* LEA9, which is only found in Brassicaceae species (Bies-Ethève *et al.* 2008; Hundertmark and Hincha 2008). The abscisic acid stress ripening (ASR) proteins formed an independent LEA protein group. ASR genes have been discovered from various plant species, such as potato (Silhavy *et al.* 1995), tomato (Rossi *et al.* 1996), rice (Vaidyanathan *et al.* 1999; Yang *et al.* 2004), maize (Riccardi *et al.* 2004) and *Ginkgo biloba* (Shen *et al.* 2005). However, no ASR-like genes were found from *Arabidopsis* (Bies-Ethève *et al.* 2008; Hundertmark and Hincha 2008) and peanut. Fifty LEA proteins were identified in *Arabidopsis*, 35 in rice, 36 in grapevine, 33 in poplar and only 10 in *Chlamydomonas* (Hundertmark and Hincha 2008).

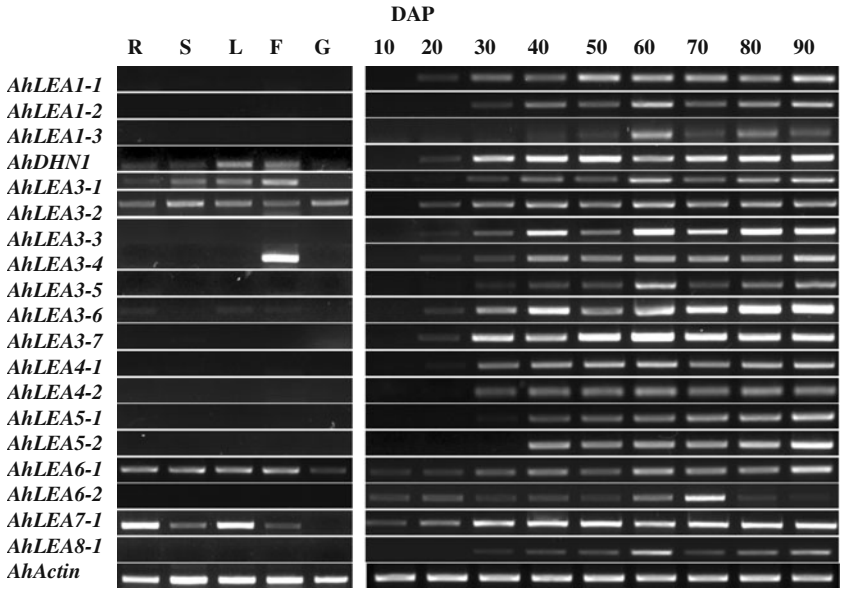

Figure 1. AhLEA gene expression analysis using semi-quantitative RT-PCR (R, root; S, stem; L, leaf; F, flower; G, gynophore). DAP 10 to 90 represent nine developmental stages of seed maturation; DAP, days after pegging. *AhActin* gene was used as amplification control.

With the growth of peanut EST sequences in the database, more peanut LEA protein genes could be identified.

The exact biological functions of most conserved motifs in LEA proteins are unknown. The function of only a small number of motifs could be proposed in plants. For example, the N-terminal motif of LEA1 proteins and N-terminal motif of LEA3 protein, and the motif1 of LEA4 (Kyte and Doolittle 1982; Shih *et al.* 2004), have the ability to form an α-helix, which may play a protection role on functional proteins and maintain normal physiological processes in plant cells under water deficiency. The motif studied most in-depth was the K-segment of dehydrin. It can form an amphipathic hydrophilic α-helix structure, which is probably involved in protein–protein and protein–lipid interactions. Experimental evidence showed that the K-segment was important to stabilize other cellular components under stress conditions (Koag *et al.* 2009). S-segment and RRKK sequence were considered to be the nucleus localization signal (Jensen *et al.* 1998). Y-fragment showed similarity to nucleic acid binding sites of chaperone proteins from bacteria and plants (Close 1996; Martin *et al.* 1993). Further investigations are required to clarify the function of peanut conserved motifs in LEAs.

Accumulation of LEA proteins is found to occur mainly during the late stages of seed development. Some LEA genes were expressed in non-seed tissues under normal or stress conditions. Most peanut LEA genes showed high expression levels during seed development, which is consistent with LEA genes from other plant species. The expression of most peanut LEA genes reached a peak level in 60 DAP seeds. The expression of only seven AhLEA genes was detected in vegetative tissues. It is reasonable that all 19 peanut LEA genes expressed in seed, because they were cloned from the peanut seed cDNA library. However, some of the *Arabidopsis* LEA genes were not expressed in seed, such as *AtLEA3-7*, *AtLEA5-3* and *AtLEA8-3* (Bies-Ethève *et al.* 2008). Some peanut LEA genes of LEA2, LEA6 and LEA7 groups, showed similar expression patterns to their *Arabidopsis* counterpart. Expression of *AhLEA1*, *AhLEA4*, *AhLEA5* and *AhLEA8* genes was only detected in seed, but most of these genes in *Arabidopsis* expressed both in seed and non-seed tissues. Some of these *Arabidopsis* LEA genes expressed only in non-seed tissue; for example, *AtLEA5-3* and *AtLEA8-3* expressed only in flower. The expression of all peanut LEA3 genes was detected in seed, while four of them were also detected in non-seed tissues. The majority of *Arabidopsis* LEA3 genes expressed in seed, while some *AtLEA3* expressed only in non-seed tissues. Interestingly, *AhLEA3-4* is found to be expressed not only in seed but also in flower in high amounts but not in other non-seed tissues. In contrast, *AtLEA3-7* expressed only in *Arabidopsis* flower but not in seed (Bies-Ethève *et al.* 2008). Different expression patterns of LEA in peanut suggested that these genes may play different roles in plant normal development and adaptation to stress conditions.

Accumulation of LEA proteins is thought to be one mechanism for plants stress tolerance as well as normal seed development. The precise functions of LEA protein are still rather enigmatic. In this study we presented sequences of 19 LEA genes and their expression profiles under normal growth conditions. Analysis of the expression patterns of these genes under stress conditions such as drought, salinity and low temperature is underway to understand the possible roles of these genes in peanut stress tolerance.

Acknowledgements

This work was supported by Foundation for Excellent Scientist in Shandong Province (2006BS06008), National Natural Science Foundation of China (30871324, 31000720), Shandong Province Natural Science Foundation (ZR2010CZ002, Y2008D44), Shandong Province 'Taishan Scholar' Foundation and Shandong Academy of Agricultural Sciences foundation (2006YBS001).

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MS received 18 August 2010; accepted 25 February 2011

*e*Publication: 16 May 2011

Corresponding editor: IMRAN SIDDIQI