Plant Science 176 (2009) 90–98

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

LEA 4 group genes from the resurrection plant *Boea hygrometrica* confer dehydration tolerance in transgenic tobacco

Xia Liu [a], Zhi Wang [a], Lili Wang [a], Renhua Wu [a], Jonathan Phillips [b], Xin Deng [a,*]

[a] *Research Center of Plant Molecular and Developmental Biology, Key Laboratory of Photosynthesis and Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Beijing 100093, PR China*

[b] *IMBIO (Molekulare Physiologie und Biotechnologie der Pflanzen), University of Bonn, Kirschallee 1, D-53115 Bonn, Germany*

ARTICLE INFO

Article history:
Received 11 June 2008
Received in revised form 22 September 2008
Accepted 24 September 2008
Available online 8 October 2008

Keywords:
Boea hygrometrica
Dehydration
LEA proteins
Resurrection plant
Transgenic tobacco

ABSTRACT

The resurrection plant *Boea hygrometrica* can survive extreme dehydration and is used as a model system to study desiccation tolerance. Screening of a cDNA library prepared from desiccated leaves via a macroarray technique has resulted in the identification of two dehydration responsive genes that encode group 4 late embryogenesis abundant (LEA) proteins, designated as *BhLEA1* and *BhLEA2*, respectively. *BhLEA1* and *BhLEA2* were induced by dehydration and signaling molecules, including abscisic acid (ABA). Transgenic tobacco that ectopically express *BhLEA1* and *BhLEA2* were generated and used to study the role of LEA proteins in dehydration tolerance. After a period of drought, the relative water content of leaves and photosystem II activity in transgenic tobacco were higher than wild-type plants. Furthermore the membrane permeability was lower in selected transgenic lines that expressed *BhLEA1* and *BhLEA2* than in wild-type plants. Superoxide dismutase and peroxidase activities were increased in transgenic plants relative to that observed in the wild-type control and proteins including ribulose-bisphosphate carboxylase (large subunit), light-harvesting complex II and photosystem II extrinsic protein were stabilized in transgenic plants compared to wild-type plants. Surprisingly, the steady state levels of BhLEA1 and BhLEA2 protein substantially increased in response drying, despite being under the transcriptional control of the CaMV 35S promoter. Data presented here suggests that *BhLEA* genes are likely to play a role in the general protection of the plant cell during dehydration and affect membrane and protein stability.

1. Introduction

Water deficit is a common environmental stress worldwide. Plants undergo many physiological and molecular responses to minimize the possible damages caused by water shortage. These reactions generally include the accumulation of osmolytes, scavengers of reactive oxygen, and putative osmoprotective proteins such as late embryogenesis abundant (LEA) proteins and small heat shock proteins [1,2]. LEA proteins were first characterized during the desiccation stage of seed development [3]. Plant vegetative tissues also accumulate LEA proteins in response to drought, freezing, salt stress, or by treatment with the phytohormone abscisic acid (ABA) [4,5]. A growing body of evidence has indicated a positive correlation between the accumulation of LEA proteins and drought tolerance [6–8]. Several LEA proteins from barley, *Tamarix androssowii* and *Brassica napus* have been shown to improve drought tolerance in transgenic plants [9–12]. However, the precise function of LEA proteins in drought tolerance has not yet been clarified.

Most LEA proteins are highly hydrophilic and remain soluble after boiling [13]. LEA proteins have been classified into at least five groups based on amino acid sequence homology and specific structural features [14]. Groups 1, 2 and 3 proteins share specific motifs within each respective group, while groups 4 and 5 lack significant signature motifs or consensus sequences [14,15]. Some LEA proteins may have roles in the protection of membranes and proteins and thus are thought to minimize damage as a result of stress conditions [1,4,5]. It has been speculated that LEA proteins may protect cellular and macromolecular structures or detoxifying molecules to alleviate the increase in ion concentration during stress [4,5,16–19]. LEA proteins may also be associated with the formation of a "glassy" state with sugars in desiccated cells [20].

Boea hygrometrica belongs to a group of so-called "resurrection plants", which have evolved the ability to recover from severe water loss in vegetative tissues [1,21]. Physiological and biochemical studies have demonstrated that specific mechanisms exist to

* Corresponding author. Tel.: +86 10 62836261; fax: +86 10 62590833.
E-mail address: deng@ibcas.ac.cn (X. Deng).

0168-9452/$ – see front matter © 2008 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.plantsci.2008.09.012

maintain the stability of macromolecules including membranes, chlorophylls, proteins, and pigment–protein complexes from degradation during dehydration so as to limit vegetative cell damages in a repairable level [22,23]. Differential expression screening of cDNA clones from a library prepared from dried leaves via a macroarray technique has resulted in the identification of two dehydration responsive genes that encode LEA proteins [24], designated as *BhLEA1* and *BhLEA2*. The aim of the present study was to characterize the *BhLEA* genes and determine functional information regarding the encoded gene products using a transgenic approach. Here we report that the ectopic expression of either *BhLEA1* or *BhLEA2* enhances drought tolerance of transgenic tobacco plants by slowing down water loss, and ameliorating the effects of stress, as observed by a decrease of photosystem II activity and the stabilization of proteins that are sensitive to water stress. On the basis of these observations, we propose that group 4 LEA proteins from *B. hygrometrica* are able to protect the plant cell and stabilize proteins during drought stress.

2. Materials and methods

2.1. Plant materials and growth condition

B. hygrometrica were collected in Beijing and grown in a greenhouse with regular irrigation. In dehydration experiments, excised leaves of *B. hygrometrica* were dehydrated for 0–8 h and rehydrated as described in [23]. For hormone and stress treatments, detached leaves were placed in solutions containing 100 μM ABA, 200 mM NaCl, 200 μM H_2O_2, 0.5 mM SA, 40% ethephon, 50 μM 2,4-D, 5 mM $CaCl_2$, 10 mM EGTA, respectively, in a climate chamber for 0.5, 8 and 24 h. For cold- and heat-stress, leaves were kept on pre-wetted filter papers in Petri dishes for indicated periods at 4 and 37 °C.

Tobacco cultivar SR1 seeds were sterilized for 2 min in 70% [v/v] ethanol and incubated for 30 min in 7% [w/v] sodium hypochlorite, 0.1% [w/v] SDS. Seeds were rinsed with sterile distilled water for three times, and sown on MS-agar for germination. Seedlings were grown in soil with vermiculite under controlled condition (16/8 h light period) at 22 °C.

2.2. Cloning of the BhLEA cDNAs and sequence analysis

Two LEA cDNA clones, designated *BhLEA1* and *BhLEA2*, were identified by differential screening of dehydration-inducible genes from a cDNA library prepared from desiccated leaves of *B. hygrometrica* using the macroarray hybridization method described in [24]. *BhLEA2* contained a complete open reading frame (ORF) (Genbank Accession No. EU669184), whereas the *BhLEA1* cDNA was truncated at the 5′ end. The complete *BhLEA1* open reading frame was subsequently obtained (Genbank Accession No. EU122334) by 5′ RACE using the System for Rapid Amplification of cDNA Ends kit (Invitrogen, USA). Two micrograms of total RNA from desiccated leaves was reverse transcribed using the gene specific primer 5-AATCTACATCCTCCTCCTAT-3′; the first and second rounds of PCR amplification used primer 5′-CGGATCCTTGCCATAGATAACT-3′ with anchor primers 5′-GGCCACGCGTCGACTAGTACG14-3′ and 5′-GGCCACGCGTCGACTAGTAC-3′, respectively. The conditions were 94 °C denaturation for 2 min, 35 cycles of 94 °C 30 s; 55 °C 30 s; 72 °C 1 min, and 72 °C elongation for 10 min for both the first and second PCR amplifications. cDNA and protein sequences were analysed using the BLAST algorithm [25]. Protein hydropathy was predicted by Kyte–Doolittle hydropathy plot analysis [26]. Motif analysis was performed using the Pfam program [27]. Amino acid comparison and multiple alignments were performed using the ClustalW program [28]. Phylogenetic tree was performed using the Neighbor Joining method using the MEGA4.0 program [29].

2.3. Construction of the plant expression vectors

BhLEA1 forward primer (5′-GGAATTCAAGATGCAAGCTGTGA-3′) and reverse primer (5′-GCTCGAGTCATTTCAGGCCATGG-3′), *BhLEA2* forward primer (5′-AGAATTCATGCAGACTGCGAAGC-3′) and reverse primer (5′-ACTCGAGCTACTGAGTCGGAGCT-3′) were designed to amplify the full-length open reading frame of the *BhLEA1* and *BhLEA2* genes, respectively. *Eco*RI and *Xho*I sites were incorporated into the forward and reverse primers, respectively. The amplified products were digested with *Eco*RI and *Xho*I and ligated downstream of the CaMV 35S promoter in a pBin19 series binary vector [30]. The recombinant plasmids were transformed into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation.

2.4. Plant transformation

Tobacco leaf discs were transformed using an *Agrobacterium* mediated method [31]. The infected explants were co-cultivated in MS-glucose medium in the dark for 2 days at 25 °C. After co-cultivation, explants were transferred to selective medium (MS + 0.1 mg/L NAA + 0.5 mg/L 6-BA + 100 mg/L kanamycin + 300 mg/L cefotaxime). After 20–30 days, the kanamycin resistant shoots were generated on selective medium and were transferred to rooting medium (MS + 0.1 mg/L NAA + 100 mg/L kanamycin + 300 mg/L cefotaxime) for rooting. Transgenic shoots were confirmed by PCR amplification using gene specific primers for *BhLEA1* and *BhLEA2* as described above. Homozygous lines were selected through two further rounds of selection on plates containing 100 mg/L kanamycin. T1 seeds giving a 3:1 ratio of survival on kanamycin were selected and grown to maturity, and T2 seeds were collected and were sown again on plates containing 100 mg/L kanamycin, and lines giving 100% survival were homozygous and T3 seeds were used for further experiments.

2.5. Isolation of RNA and DNA, PCR and semi-quantitative RT-PCR

Genomic DNA was extracted using a cetyltrimethyl ammonium bromide (CTAB)-based method [32]. Total RNA was extracted using an acidic guanidinuim thiocyanate–phenol–chloroform-based procedure [33]. Two micrograms of total RNA was heated to 70 °C for 5 min and then reverse transcribed with random primers using M-MLV Reverse Transcriptase (Promega, USA) for 60 min at 42 °C in a volume of 25 μL. PCR was performed on cDNA samples (1 μL of a 1:5 dilution) using rTaq polymerase (Takara, Japan). *BhLEA1* and *BhLEA2* were amplified with specific forward and reverse primers as described above. Semi-quantitative RT-PCR was repeated at least twice for each sample. 18s rRNA was amplified using the following primer pair: 5′-TTGTGTTGGCTTCGGGATCG-GAGTAAT-3′ and 5′-TGCACCACCACCCATAGAATCAAGAA-3′ as a control. The linear range of detection for each transcript was monitored and samples run for 32 cycles for *BhLEA1* and *BhLEA2* and 18 cycles for 18S rDNA were compared.

2.6. Protein extraction and Western blots

Total proteins were extracted from fresh leaves with protein extraction reagent (625 mM Tris–HCl, pH 6.8, 10% glycerol, 2% [w/v] SDS, 5% [v/v] β-ME). The slurry was boiled for 10 min, cooled to room temperature, and centrifuged at 12,000 × *g*, 4 °C for 10 min. Proteins concentration was measured using the Coomassie Brilliant Blue (CBB) G250 assay [34].