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ORIGINAL ARTICLE

Sweetpotato late embryogenesis abundant 14 (*IbLEA14*) gene influences lignification and increases osmotic- and salt stress-tolerance of transgenic calli

Sung-Chul Park · Yun-Hee Kim · Jae Cheol Jeong · Cha Young Kim · Haeng-Soon Lee · Jae-Wook Bang · Sang-Soo Kwak

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Abstract Late embryogenesis abundant 14 (*LEA14*) cDNA was isolated from an EST library prepared from dehydration-treated fibrous roots of sweetpotato (*Ipomoea batatas*). Quantitative RT-PCR revealed a variety of different *IbLEA14* expression patterns under various abiotic stress conditions. *IbLEA14* expression was strongly induced by dehydration, NaCl and abscisic acid treatments in sweetpotato plants. Transgenic sweetpotato non-embryogenic calli harboring *IbLEA14* overexpression or RNAi vectors under the control of *CaMV 35S* promoter were generated. Transgenic calli overexpressing *IbLEA14* showed enhanced tolerance to drought and salt stress, whereas RNAi calli exhibited increased stress sensitivity. Under normal culture conditions, lignin contents increased in *IbLEA14*-overexpressing calli because of the increased expression of a variety of monolignol biosynthesis-related genes. Stress treatments elicited higher expression levels of the gene encoding cinnamyl alcohol dehydrogenase in *IbLEA14*-overexpressing lines than in control or RNAi lines. These results suggest that *IbLEA14* might positively regulate the response to various stresses by enhancing lignification.

Keywords Abiotic stress · Late embryogenesis abundant protein · *Ipomoea* · Lignin · Transgenic callus

Abbreviations

ABA	Abscisic acid
CAD	Cinnamyl alcohol dehydrogenase
CCAOMT	Caffeoyl coenzyme A *O*-methyl-transferase
COMT	Caffeic acid-*O*-methyl-transferase
C4H	Cinnamate 4-hydroxylase
DW	Dry weight
4CL	4-Coumarate:coenzyme A ligase
LEA	Late embryogenesis abundant
PAL	Phenylalanine ammonia-lyase
WC	Water content
SAMS	s-Adenosyl-L-methionine synthetase

Introduction

Abiotic stresses such as dehydration, high salinity and extreme temperature conditions can reduce crop yields by as much as 60% and, thus, they represent critical factors that limit crop success worldwide (Bray 1997). Environmental stresses trigger physiological and molecular responses that enable plants to prevent or minimize exposure to stressful conditions, or to acclimate and minimize the effects these circumstances. The most common abiotic stress is drought, which adversely affects plant growth and development via changes in metabolism and gene expression (Ingram and

S.-C. Park and Y.-H. Kim contributed equally to this work.

S.-C. Park · Y.-H. Kim · J. C. Jeong · H.-S. Lee · S.-S. Kwak (✉)
Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Oun-dong 52, Yusong-gu, Daejeon 305-806, Republic of Korea
e-mail: sskwak@kribb.re.kr

C. Y. Kim
Bioindustrial Process Center Jeonbuk Branch Institute of Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeonbuk 580-185, Republic of Korea

S.-C. Park · J.-W. Bang
Department of Biology, Chungnam National University, Daejeon 305-764, Republic of Korea

Bartels 1996; Bray 1997). Cellular dehydration triggered by osmotic stress due to high salinity or low temperature conditions also leads to increased cellular abscisic acid (ABA) levels, which elicit stomatal closure and induce expression of stress-related genes. Therefore, a large number of dehydration-responsive genes are likewise induced by high salinity, low temperature, or ABA treatments. One of the most widely used strategies for improving tolerance to environmental stresses such as dehydration has been the overexpression of genes induced by stress treatments (Bartels and Sunkar 2005), including dehydration-induced genes from a wide range of plant species.

Dehydration conditions typically induce the expression of stress-responsive genes involved in the synthesis of various osmolytes and low-molecular-weight proteins, including late embryo genesis-abundant (LEA) proteins (Ingram and Bartels 1996). LEA proteins belong to a large group of plant proteins that are synthesized abundantly and stored during seed maturation (Ingram and Bartels 1996; Bray 1997). These proteins play a protective role under osmotic stress conditions (Baker et al. 1988; Ingram and Bartels 1996). LEA proteins can also play a role in the homeostasis of proteins and nucleic acids, stabilization of cell membranes and maintenance of redox balance (Tunnacliffe et al. 2010). LEA proteins were first identified during the desiccation phases of seed development, where they could protect specific cellular structures or ameliorate the effects of drought stress by their possible function of sequestering ions and maintaining minimum cellular water requirements (Baker et al. 1988; Wise and Tunnacliffe 2004). LEA proteins are highly expressed during the last stage of seed development in most plants, which is triggered by a natural reduction of seed water content. LEA gene expression is usually associated with the increase of embryonic abscisic acid and the acquisition of desiccation tolerance (Hughes and Galau 1989). Transcript analysis during all stages of cotton embryogenesis indicated that expression of LEA appeared during late embryogenesis and was maintained at a high level in the dehydrated mature embryos (Dure et al. 1981). After germination, expression of LEA genes decreased quickly (Dure et al. 1981). Interestingly, although LEA proteins were first identified from developing seeds, many LEA proteins are expressed in vegetative tissues in response to ABA or environmental stress conditions (Bartels and Sunkar 2005; Hundertmark and Hincha 2008). LEA proteins have been classified into nine groups, based on the amino acid sequence homology and specific motives in Arabidopsis (Battaglia et al. 2008; Hundertmark and Hincha 2008). These groups are expected to gather proteins performing different speculative functions during periods of water deficit (Bray 1997). According to the recent classification of LEA proteins using PFAM nomenclature based on the sequence motives, the Arabidopsis genome contain nine LEA groups: dehydrin, LEA_1, LEA_2, LEA_3, LEA_4, LEA_5, PvLEA18, SMP, AtM (Hundertmark and Hincha 2008). Dehydrin proteins were identified originally from developing cotton embryos (Close 1996). The *Arabidopsis* dehydrins ERD10 and ERD14 exhibit chaperone activity and ion-binding properties, which are modulated by phosphorylation (Kovacs et al. 2008). Overexpression of citrus or potato dehydrin proteins confers chilling tolerance in transgenic tobacco and cucumber, respectively (Hara et al. 2004; Yin et al. 2006). Tomato *LE25* of LEA_1 group showed increased tolerance to salt or chilling stress in yeast (Imai et al. 1996). Gene expression analysis of the *PAP260* and *PAP051* in the LEA_1 group during seed germination also has suggested that may be related to histone deacetylation (Tai et al. 2005). Legume *LjIDP1* of LEA_3 group prevented protein inactivation under dehydration and freezing conditions (Haaning et al. 2008). Barley HVA1 of LEA_4 group protected cell membranes from injury and transgenic expression of this protein also provides enhanced drought- and salt stress-tolerance in rice (Chandra Babu et al. 2004). PsLEAM of pea in the group not only stabilizes mitochondrial matrix proteins but also protects liposomes and interacts with membrane during dry state (Grelet et al. 2005). Other LEA_4 proteins, such as AavLEA1 protein from the anhydrobiotic nematode and ArLEA1A from a bdelloid rotifer prevented desiccation-induced protein aggregation in vitro and in vivo, respectively (Goyal et al. 2005; Chakrabortee et al. 2007). LEA_5 includes cotton D-19 and wheat Em proteins, which showed high levels of sequence homology and similar protein characteristics (Baker et al. 1988; Swire Clark and Marcotte 1999). In vitro studies indicate that wheat Em protein functions as a molecular chaperone under desiccation or freezing conditions (Gilles et al. 2007) and that it can confer dehydration-tolerance in yeast (Swire Clark and Marcotte 1999). In the case of a PvLEA-18 group protein, transcripts accumulated not only upon water-stress and ABA treatment but also during seedling development and growth under optimal irrigation conditions (Colmenero-Flores et al. 1999). *Medicago truncatula* MTPM25 protein, belonging to the SMP group, was found to prevent protein aggregation and dissolve cold-induced aggregates, suggesting a repair function (Boucher et al. 2009). Most proteins of LEA groups are cytosolic and highly hydrophilic, comprising α-helices or random coil (Tunnacliffe and Wise 2007; Battaglia et al. 2008). In contrast, LEA_2 group, such as soybean D95-4 (Maitra and Cushman 1994), cotton LEA14-A (Galau et al. 1993), tomato ER5 (Zegzouti et al. 1997), tomato Lemmi9 (Eycken et al. 1996), hot pepper CaLEA6 (Kim et al. 2005) and *Arabidopsis* At1g01470, At2g46140 and At2g44060 (Hundertmark and Hincha 2008) contain significantly higher numbers of hydrophobic

residues and three-dimensional structure. In fact, the solution structure of LEA14 from Arabidopsis has been resolved at high resolution by NMR (Singh et al. 2005). Expression of these proteins occurs under the conditions of drought, high salinity, low temperature, ABA, ethylene, nematode and mildew infection or high light stresses (Galau et al. 1993; Maitra and Cushman 1994; Eycken et al. 1996; Zegzouti et al. 1997; Dunaeva and Adamska 2001; Kimura et al. 2003; Kim et al. 2005; Hundertmark and Hincha 2008). Recently, increased leaf fresh weights and chlorophyll contents have been observed in *CaLEA6*-overexpressing transgenic tobacco plants under drought or salt stress (Kim et al. 2005). Since LEA14 proteins differ from the other LEA groups in both hydrophobic content and structure, they are also likely to function differently, despite involvement in the dehydration response. However, the biological and physiological functions of hydrophobic LEA14 proteins remain unknown.

Sweetpotato [*Ipomoea batatas* (L.) Lam.] is known as a relatively drought-resistant crop and globally it represents one of the most important root crops grown on marginal land. Although sweetpotato is recognized as a comparatively drought-tolerant plant, the molecular mechanisms underlying this tolerance are not well defined. In a previous study, we isolated and characterized expressed sequence tags (ESTs) from a full-length enriched cDNA library prepared from fibrous roots of sweetpotato subjected to dehydration stress (Kim et al. 2009). Expression analysis showed that some of these sweetpotato dehydration-responsive genes were also induced in response to other abiotic stresses such as NaCl and extreme temperature. Thus, investigation of dehydration-treated EST pools can provide valuable genetic information about the regulatory networks involved in stress-responsive processes. In this study, we isolated and functionally characterized *IbLEA14* from an EST library of dehydration-treated fibrous roots of sweetpotato. *IbLEA14* encodes a hydrophobic LEA14 protein. Its physiological functions under stress conditions were examined using overexpression or RNAi suppression of *IbLEA14* in transgenic sweetpotato calli.

Materials and methods

Plant materials

Sweetpotato (*Ipomoea batatas* L. Lam. cv. White Star, obtained from Bioenergy Center, National Crop Research Institute, RDA, Muan, Jeonnam, Korea) plants were cultivated in a growth chamber in soil at 25°C under a photocycle of 16 h light/8 h dark for 50 days. Non-embryogenic calli were induced from shoot meristems of sweetpotato (cv. White Star) cultured on MS (Murashige and Skoog 1962) medium supplemented with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose and 0.4% Gelrite. Calli were maintained at 26°C in the dark and proliferated by subculture into freshly prepared medium at 3-week intervals.

Stress treatment

Sweetpotato plants grown at 25°C for 50 days were used for stress treatments. For analysis of dehydration treatments, sweetpotato leaves and fibrous roots were collected at 0 (untreated control), 1, 2, 4, 8, 16, and 24 h after treatment. For treatments with NaCl and abscisic acid, the third leaves from the top were detached from each plant and placed into conical tubes containing 30 ml of sterile water (control), 100 mM NaCl or 0.1 mM ABA, and then incubated at 25°C for 24 h. For low temperature stress, plants were exposed to 15, 10, 4 and 25°C (control) for 24 h. All treated plant materials were frozen immediately in liquid nitrogen and stored at −70°C until further use. For salt and osmotic stress treatments of transgenic sweetpotato callus, 2-week-old calli were used. To mimic salt stress conditions, callus was incubated on half-strength MS medium including 3% sucrose and 7% Phyto agar with 300 mM NaCl for 72 h. To reproduce osmotic stress conditions, PEG 6000 (polyethylene glycol, Sigma, St. Louis, MO, USA)-infused plates were used, according to a modified version of the method reported in Verslues et al. (2006). PEG-infused plates were prepared by dissolving solid PEG in a sterilized solution of half-strength MS medium with 2 mM Mes buffer (pH 5.7), followed by overlaying of the PEG solution onto agar-solidified half-strength MS medium plates containing 7% Phyto agar. The agar medium and PEG solution were equilibrated for at least 12 h before the excess PEG solution was removed. Osmotic stress strength was expressed as the concentration of the overlayed 30% PEG solution. The addition of high concentration of sucrose can itself induce an osmotic response; we thus used half-strength MS medium without sucrose.

Analysis of DNA and protein sequences

Sequence identities were determined using BLAST on the NCBI web-server, and multiple sequence alignments were performed using Clustal X and the GeneDoc program. The isoelectric point (pI) and molecular weight, as well as the signal peptide and grand average of hydropathy (GRAVY) of deduced proteins, were predicted using the ExPasy (http://www.expasy.org/tools), PSORT (http://psort.ims.u-tokyo.ac.jp), and SoftBerry (http://www.softberry.com) programs.

Gene expression analysis

Total RNA was isolated from sweetpotato using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated extensively with RNase-free DNase I to remove any contaminating genomic DNA. For quantitative expression analysis of sweetpotato genes including *IbLEA14*, first-strand cDNA was generated from total RNA (2 μg) using MMLV reverse transcriptase (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. Quantitative RT-PCR was performed in a fluorometric thermal cycler (DNA Engine Opticon 2, MJ Research, Waltham, MA, USA) using EverGreen fluorescent dye, according to the manufacturer's instructions. The inter-experimental quality control comparisons of repeated samples were assessed using CT values between the three replications. Above 1.5 of differential values was removed from data set. In addition to, quality control of the reaction by gel electrophoresis confirmed the presence of a single product of the correct size, and multiple peaks had samples in the dissociation graph were also dismissed for exclusion of unspecific PCR reaction. Linear data were normalized to the mean CT of *α-tubulin* as reference gene, and the relative expression ratio was calculated using $2^{\Delta-\Delta Ct}$ method. The expression levels of *IbLEA14* and various other sweetpotato genes were analyzed by quantitative RT-PCR using the gene-specific primers listed in Table 1.

Vector construction and transformation

To construct the *IbLEA14* expression vectors, *IbLEA14*-specific primers containing attB1 and attB2 sequences (5′-ACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCTGGTGGACAAG-3′, 5′-ACCACTTTGTACAAGAAAGCTGGGTCTTAGGCAGCTTCTGCCTC-3′) were designed. PCR products were inserted into pDONR 207 using in vitro BP clonase recombination reactions, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). LR clonase reactions were then used to transfer DNA fragments from entry clones to destination vectors, i.e., an N-terminal FLAG-tag overexpression (pGWB12) vector (Nakagawa et al. 2007) or an RNAi (pH7GWIW2) vector (Karimi et al. 2002) those are under control of CAMV 35S promoters, according to the manufacturer's instructions (Invitrogen). Recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105, which was then used for *Agrobacterium*-mediated transformation of sweetpotato non-embryogenic calli, as described by Lim et al. (2004).

Immunoblot analysis

For analysis of protein expression in N-terminally FLAG-tagged IbLEA14 transgenic sweetpotato callus lines, callus tissues (0.1 g) were homogenized in extraction buffer containing 20 mM Hepes (pH 7.4), 0.5 mM EDTA, 1 mM $MgCl_2$ and protease inhibitor cocktail (Roche, Manheim, Germany). For immunoblot analyses, total proteins (10 μg) were separated on 12% SDS-polyacrylamide gels and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) via semi-dry electroblotting. Immunoblots were performed using monoclonal anti-FLAG antibody (Sigma) followed by anti-mouse IgG conjugated to horseradish peroxidase (Sigma). Blots were visualized via enhanced chemiluminescence (ELPIS, Seoul, Korea).

Analysis of water content

The degree of dehydration in tissues was assessed by the water content (WC) of transgenic calli after 30% PEG or 300 mM NaCl treatments. WC was measured using the following formula: WC (%) = (fresh weight − dry weight)/fresh weight (Zhao et al. 2004).

Table 1 Primer sequences used for expression analysis of *IbLEA14* and other sweetpotato genes

cDNA	Forward primer	Reverse primer
IbLEA14	GCCCTGGATGTGGCAGTGAA	GGCAGCTTCTGCCTCTGCTTC
swDREB1	TGACGTGGAGCTTGATGCTGAC	GGAATGGACGCTTTTCGCCT
PAL	CCTTGCACGGTGGCAACTTC	TTGGCAAAGCGCAACGAGAT
C4H	ATCGAGTGGGGCATTGCAGA	TTGGCGTCGTGGAGGTTCAT
4CL	TATTTGAATGATCCGGCGGC	ACAGCAGCATCTGCAATCATCG
SAMS	AATTGTGCGGGACACTTGCC	AGGGGTCTCATCGGTGGCAT
COMT	CGACAGCCCGATGACAAACC	GAATCCGGTCCAGCATGACG
CCAOMT	AAAGAAGGCCCAGCATTGCC	TTCCTCATTGGAGCATCGGG
CAD	TGGTTGGGGAACCAATGCAC	GCTTTTCGCCTTTTCCTGGG
α-tubulin	CAACTACCAGCCACCAACTGT	CAAGATCCTCACGAGCTTCAC

Analysis of lipid peroxidation

Lipid peroxidation was measured using a modified thiobarbituric acid method (Peever and Higgins 1989), whereby the specific absorbance of extracts was recorded at A_{532}. Non-specific absorbance was measured at A_{600} and then subtracted from the A_{532} readings. The concentration of malondialdehyde (MDA) was calculated as a measure of lipid peroxidation.

Quantitative and qualitative analysis of lignin content

For lignin staining, sweetpotato calli were incubated overnight in 70% ethanol containing 1% phloroglucinol (Mlícková et al. 2004). After rinsing in distilled water, the calli were bathed in 50% HCl. Lignin contents were determined using thioglycolic acid assays (Hatfield and Fukushima 2005). Dry tissues (20 mg) were powdered and treated with 0.5 ml of a 1:10 mixture of thioglycolic acid and 2 N HCl for 4 h at 100°C. After washing twice with water, lignothioglycolic acid was extracted from the pellet in 1 ml of 0.5 N NaOH for 18 h. Lignin contents were determined at A_{280} using a lignin standard (alkali, 2-hydroxypropyl ether, Aldrich).

Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA). The subsequent multiple comparisons were examined based on the least significant difference (LSD) test. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 12) and statistical significance was set at $P < 0.05$ and $P < 0.01$.

Results

Isolation and molecular characterization of *IbLEA14*

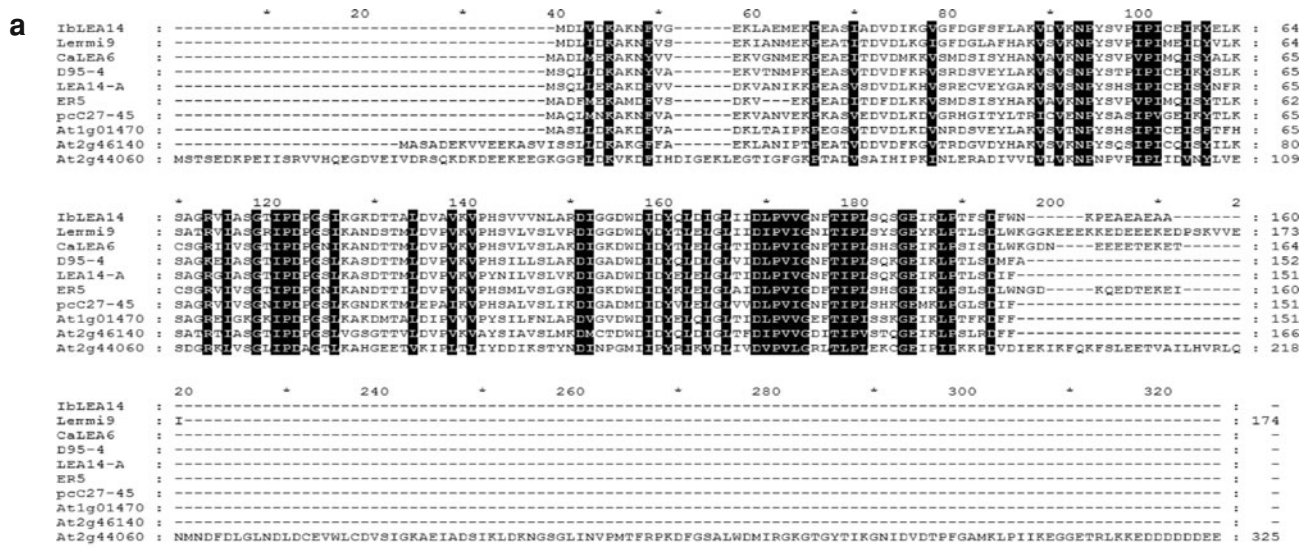
The *LEA* cDNA clone (GU369820) was obtained from an EST library of dehydration-treated fibrous roots of sweetpotato (Kim et al. 2009). According to the recent classification of LEA proteins (Battaglia et al. 2008; Hundertmark and Hincha 2008), this cDNA clone was named *IbLEA14* (*Ipomoea batatas* Late Embryo Abundant protein 14) of LEA_2 group since it encodes a novel LEA protein in sweetpotato. The cDNA contains 793 bp, of which 480 bp form a single ORF that encodes a putative cytoplasmic polypeptide of 160 amino acids with a calculated molecular weight of 17.2 kDa and a theoretical *pI* of 4.5. The deduced amino acid sequence (Fig. 1a) shares significant identity with an atypical group of LEA_2, i.e., 71% identity with Lemmi9 (Eycken et al. 1996), 70% with D95-4 (Maitra and Cushman 1994), 65% with CaLEA6 (Kim et al. 2005), 63% with Lea14-A (Galau et al. 1993), and 61% with desiccation-tolerant *Craterostigma plantagineum* pcC27-45 (Piatkowski et al. 1990) and tomato ER5 (Zegzouti et al. 1997) (Fig. 1b). Among *Arabidopsis* LEA14 s, IbLEA14 is more closely related with At1g01470 (62%), At2g46140 (58%) than At2g44060 (29%) (Hundertmark and Hincha 2008). According to the Kyte and Doolittle (1982) hydropathy plot, IbLEA14 is predicted to have a highly hydrophobic structure, with the exception of the C-terminal region (Fig. 1c). Among the LEAs shown in Fig. 1a, the hydrophobic LEA14 proteins show similar features (data not shown). The Grand average of hydropathy (GRAVY) index of IbLEA14 was 0.031, a value comparable to Arabidopsis LEA14 proteins (0.056 and 0.123 for At1g01470 and At2g46140), whereas At2g44060 was −0.314 (Hundertmark and Hincha 2008).

Southern blot analyses of genomic DNA digested with *Eco*RI, *Hin*dIII and *Bam*HI identified two or three hybridizing bands that did not include internal restriction sites for *IbLEA14* (data not shown), which suggests that *IbLEA14* may be a part of a small gene family in sweetpotato.

Expression of *IbLEA14* in different tissues and under various abiotic stresses

Quantitative RT-PCR analyses were used to investigate *IbLEA14* expression patterns in various whole plant tissues (leaf, stem, fibrous root, thick pigmented root and tuberous root) and different types of callus (non-embryogenic and embryogenic; Fig. 2a). *IbLEA14* expression levels varied considerably in different sweetpotato tissues, being expressed strongly in fibrous roots, thick pigmented root tissues and both types of calli, which showed similar levels of expression. To investigate *IbLEA14* expression under various abiotic stresses, quantitative RT-PCR analysis was conducted after dehydration, salt, low temperature and ABA treatments. Following dehydration, differential regulation of *IbLEA14* was observed in the leaves and fibrous roots (Fig. 2b). In fibrous roots, dehydration induced a weak increase in *IbLEA14* transcription, which occurred regardless of dehydration time. Expression levels in the fibrous roots were higher than in leaves under normal conditions. Interestingly, dehydration treatment elicited a strong and rapid increase in *IbLEA14* expression in leaves. The dehydration marker gene *swDREB1* was used as a positive control (Kim et al. 2008). Elevated *swDREB1* expression was observed 1 h after dehydration in fibrous roots and expression continued to be up-regulated for 24 h in leaves.

In sweetpotato leaves, *IbLEA14* expression was induced rapidly, i.e., within 4 h, by treatment by 100 mM NaCl or


a
IbLEA14
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ER5
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At2g46140
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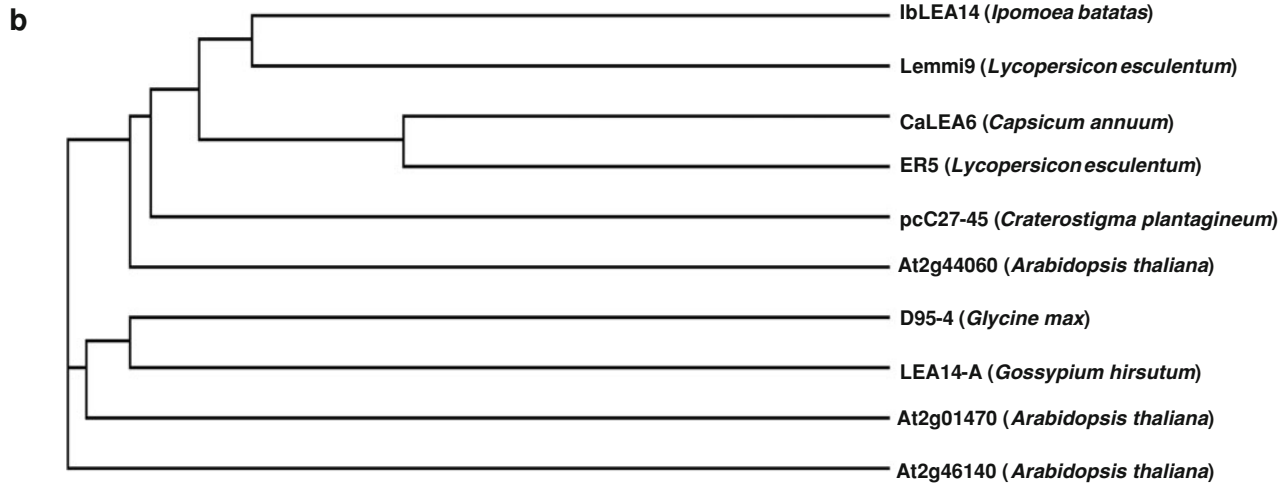
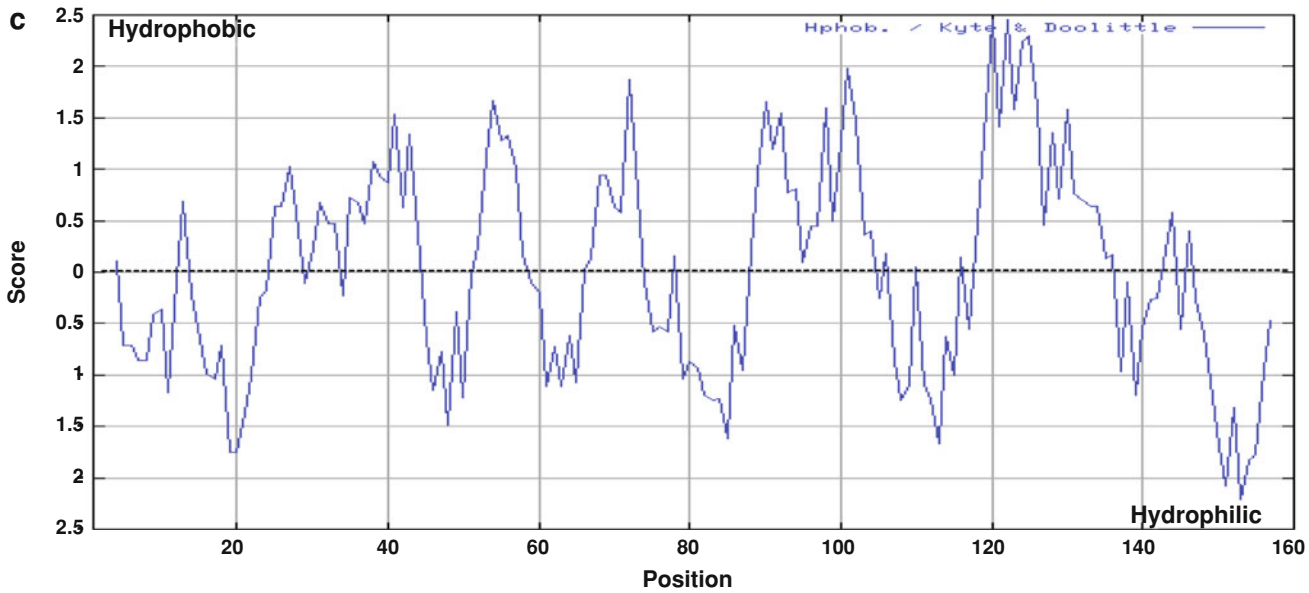

b
IbLEA14 (Ipomoea batatas)
Lemmi9 (Lycopersicon esculentum)
CaLEA6 (Capsicum annuum)
ER5 (Lycopersicon esculentum)
pcC27-45 (Craterostigma plantagineum)
At2g44060 (Arabidopsis thaliana)
D95-4 (Glycine max)
LEA14-A (Gossypium hirsutum)
At2g01470 (Arabidopsis thaliana)
At2g46140 (Arabidopsis thaliana)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120
140
160

◀ **Fig. 1** Multiple sequence alignment, phylogenetic tree and hydropathicity plot for deduced amino acid sequences of sweetpotato IbLEA14 and other closely related plant proteins. **a** Multiple sequence alignment of IbLEA14 with other LEA14 proteins. **b** Phylogenetic trees for IbLEA14 and other LEA14 proteins. **c** Hydropathicity plot of the deduced IbLEA14 amino acid sequence analyzed using the Kyte-Doolittle algorithm (Kyte and Doolittle 1982). The GenBank accession numbers are as follows: Lemmi9 (*Lycopersicon esculentum*, Z46654), D95-4 (*Glycine max*, U08108), CaLEA6 (*Capsicum annuum*, AF168168), LEA14-A (*Gossypium hirsutum*, M88322), ER5 (*Lycopersicon esculentum*, U77719), pcC27-45 (*Craterostigma plantagineum*, M62990), *Arabidopsis* [At1g01470 (BT015111), At2g46140 (NM_130176) and At2g44060 (BT024723)], and IbLEA14 (*Ipomoea batatas*, GU369820)

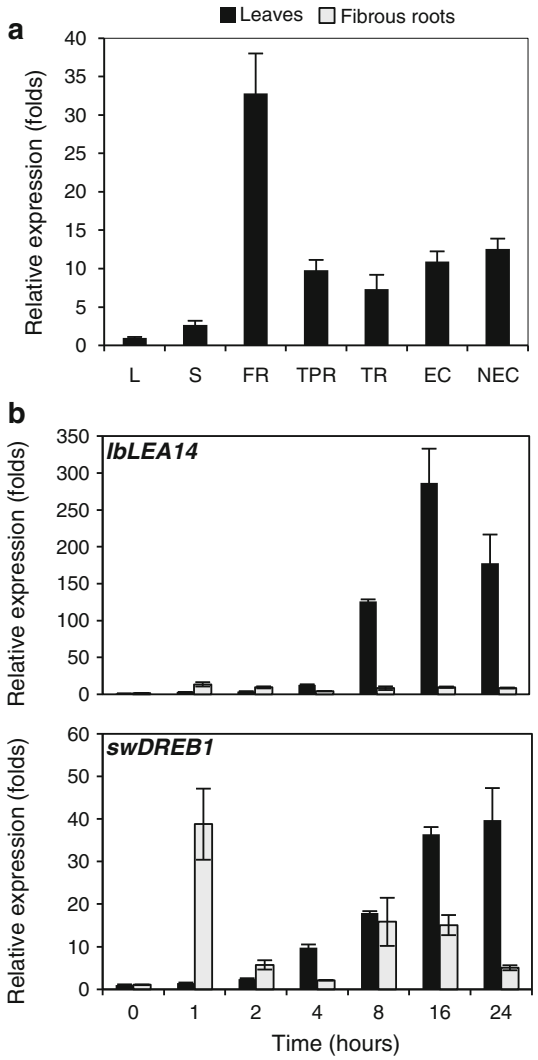

Fig. 2 Expression patterns of sweetpotato *IbLEA14* in various tissues and under different dehydration conditions. **a** *IbLEA14* expression in various tissues and different types of callus. Total RNAs were extracted from leaf (*L*), stem (*S*), fibrous root (*FR*), thick pigmented root (*TPR*), tuberous root (*TR*), embryogenic callus (*EC*), and non-embryogenic callus (*NEC*). **b** *IbLEA14* expression patterns in leaves and fibrous roots of sweetpotato plants under dehydration conditions for 24 h. The *swDREB1* gene was used as a dehydration marker

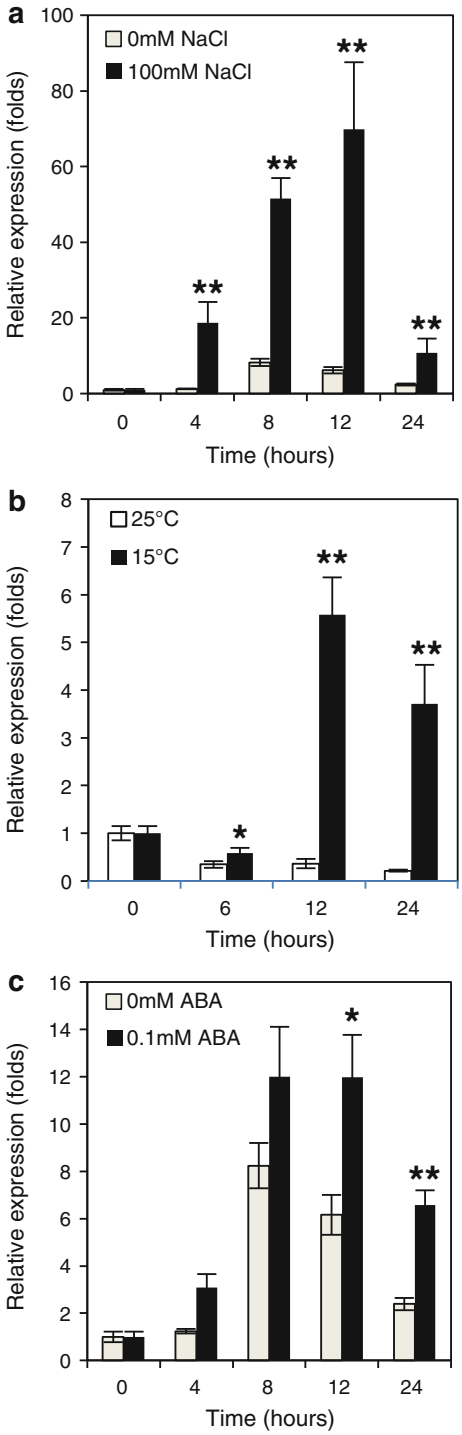

Fig. 3 *IbLEA14* expression patterns under a variety of abiotic stress conditions in sweetpotato leaves. **a** *IbLEA14* expression patterns in leaves treated with 100 mM NaCl for 24 h. **b** *IbLEA14* expression patterns in leaves under 15°C low temperature conditions. **c** *IbLEA14* expression patterns in leaves treated with 0.1 mM ABA for 24 h. Data presented represent the average of three replicates. Statistical significance of differences between the control and treatment groups were determined by one way ANOVA with LSD post hoc test (*$P < 0.05$; **$P < 0.01$)

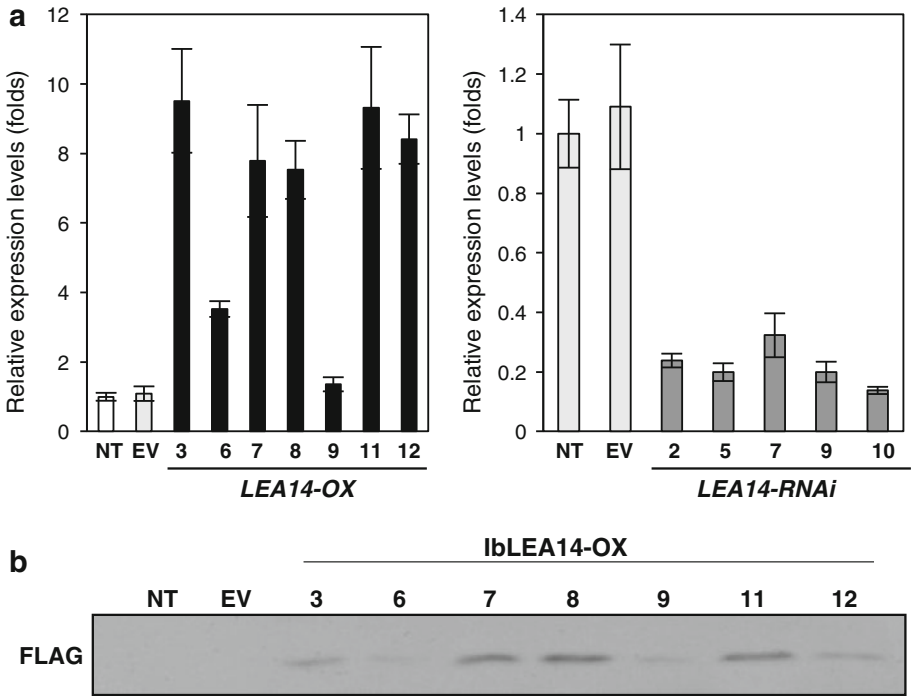

Fig. 4 Molecular and biochemical characterization of *IbLEA14* transgenic sweetpotato calli. **a** Quantitative RT-PCR analysis of *IbLEA14* in the independent overexpression (OX) and RNAi transgenic lines. **b** Immunoblot analysis of FLAG-tagged IbLEA14 protein in OX transgenic lines

0.1 mM ABA (Fig. 3a, c), and after 12 h, treatment at 15°C elicited stronger induction of *IbLEA14* expression than at 25°C (Fig. 3b). Expression of the *LEA14* also slightly increased under untreated control conditions (0 mM NaCl or ABA treatments). Expression of *IbLEA14* also increased moderately under control conditions, an effect that could may result from wounding. In tomato, expression of the *ER5* was also induced by drought, ABA and wounding treatment (Zegzouti et al. 1997). Taken together, these data suggest that an ABA-dependent pathway might be involved in the regulation of *IbLEA14* expression in response to dehydration, salt and low temperature treatments.

Generation of transgenic *IbLEA14* calli

Since dehydration and NaCl treatments strongly induced *IbLEA14* expression, the function of IbLEA14 protein was examined further under these stress conditions. Transgenic sweetpotato calli that overexpressed or RNAi-suppressed *IbLEA14* were generated. The expression vectors were introduced into sweetpotato via *Agrobacterium*-mediated transformation and transgenic calli were identified by genomic PCR analysis (data not shown), followed by confirmation with quantitative RT-PCR and immunoblot analysis. *IbLEA14* expression levels varied according to the transgenic line and correlated relatively closely to the amount of IbLEA14 protein that accumulated. The overexpression (OX) lines 3, 7, 8, 11, and 12 showed enhanced *IbLEA14* transcript levels (i.e., >sevenfold higher) compared to control lines, whereas RNAi lines 2, 5, 9, and 10 exhibited lower levels of expression (i.e., approximately 80% lower) (Fig. 4a). The OX lines 7, 8 and 11 showed strong accumulation of FLAG-tagged IbLEA14 protein, whereas expression was weak in lines 6 and 9, and intermediate in lines 3 and 12 (Fig. 4b). Interestingly, most transgenic calli were morphologically different from control lines, whereby OX lines showed stiffening of the callus and RNAi lines exhibited a softer callus (data not shown). To determine whether *IbLEA14* expression plays a role in the changes in cell growth, transgenic calli were analyzed with dry mass measurements for 3 weeks (Table 2). Over the first 2 weeks, the OX lines showed retarded cell growth rate (%), whereas the dry mass of the RNAi lines indicated faster growth rate (%) than the control lines such as NT and EV.

Response of transgenic *IbLEA14* calli to abiotic stresses

To assess the effects of altered *IbLEA14* expression on drought and salt stress tolerance, 2-week-old calli were treated with 30% PEG or 300 mM NaCl for 3 days (Fig. 5a). RNAi lines exhibited the most osmotic damage and greatest loss of water content (WC), whereas the OX lines showed higher WC than control lines (Fig. 5b). In addition, the OX lines showed the greatest tolerance to the presence of PEG or NaCl, as determined by the quantitative analyses of lipid peroxidation (Fig. 5c). Moreover, RNAi lines demonstrated increased sensitivity to drought and salt stress. Based on these results, it appears that the

Table 2 Cell growth of sweetpotato callus on the basis of cell dry weight (DW) and relative growth increase (%) during 21 days

Callus	Lines	Cell growth (mg DW/plate)				Relative growth (% increase in DW)			
		1 day	7 days	14 days	21 days	1 day	7 days	14 days	21 days
Control	NT	6.09 ± 1.33	22.03 ± 1.72	55.94 ± 6.09	118.18 ± 15.51	100.00 ± 5.48	361.86 ± 28.27	918.64 ± 100.08	1,940.99 ± 254.72
	EV	5.01 ± 0.23*	16.07 ± 1.22**	49.52 ± 3.28	104.85 ± 4.56	100.00 ± 4.53	320.62 ± 24.39	988.20 ± 65.54	2,092.27 ± 91.10
IbLEA14-OX	OX7	5.72 ± 0.53	14.77 ± 3.16**	31.45 ± 9.06**	77.68 ± 14.34**	100.00 ± 9.29	258.06 ± 55.16**	549.55 ± 158.28**	1,357.45 ± 250.53**
	OX8	4.89 ± 0.28*	13.07 ± 1.33**	39.92 ± 1.27*	82.95 ± 4.67**	100.00 ± 5.68	267.27 ± 27.24**	816.45 ± 25.93	1,696.77 ± 95.60
	OX11	5.13 ± 0.56*	12.20 ± 2.44**	35.39 ± 8.58**	90.66 ± 10.88**	100.00 ± 10.92	237.66 ± 47.60**	689.39 ± 167.12**	1,766.03 ± 211.90
IbLEA14-RNAi	RNAi-2	4.93 ± 0.58*	25.90 ± 1.68*	66.24 ± 11.49	126.55 ± 2.99	100.00 ± 11.70	525.00 ± 34.10**	1342.75 ± 232.98**	2,565.21 ± 60.58**
	RNAi-5	5.44 ± 0.17	22.57 ± 1.07	66.61 ± 3.11	126.39 ± 2.35	100.00 ± 3.14	414.49 ± 19.64	1223.51 ± 57.14*	2,321.36 ± 43.25*
	RNAi-7	6.34 ± 0.21	24.70 ± 1.08	67.46 ± 5.08	114.31 ± 18.04	100.00 ± 3.38	389.32 ± 17.05	1063.22 ± 80.07	1,801.79 ± 284.40

Data presented represent the average of three replicates. Statistical significance of differences between the control and transgenic lines were determined by one way ANOVA with LSD post hoc test (*$P < 0.05$; **$P < 0.01$)

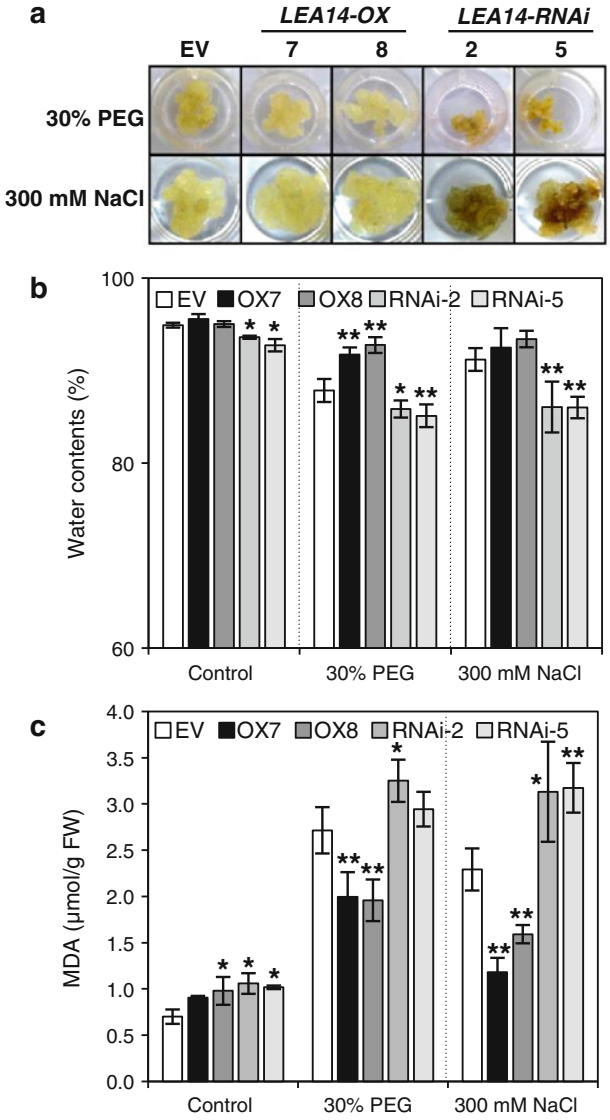

Fig. 5 Effect of drought (30% PEG) and salt (300 mM NaCl) stress on transgenic *IbLEA14* calli. **a** Visible damages of sweetpotato calli after treatments with PEG or NaCl for 72 h. **b** Water contents (WC) in sweetpotato calli after treatment with PEG or NaCl for 72 h. **c** Quantitative analysis of lipid peroxidation in sweetpotato calli after PEG or NaCl treatment for 72 h. Data presented represent the average of three replicates. Statistical significance of differences between the control and transgenic lines were determined by one way ANOVA with LSD post hoc test (*$P < 0.05$; **$P < 0.01$)

expression of *IbLEA14* is involved in enhanced tolerance to dehydration and salt stress conditions.

Expression of *IbLEA14* elicits increased lignin content

Cell rigidification results from the cross-linking of several compounds including lignin (Boudet 2000) and lignin accumulation have been associated with reduced cell elasticity and growth (Zhong and Ye 2007). The lignin accumulation of *IbLEA14*-overexpressing calli was

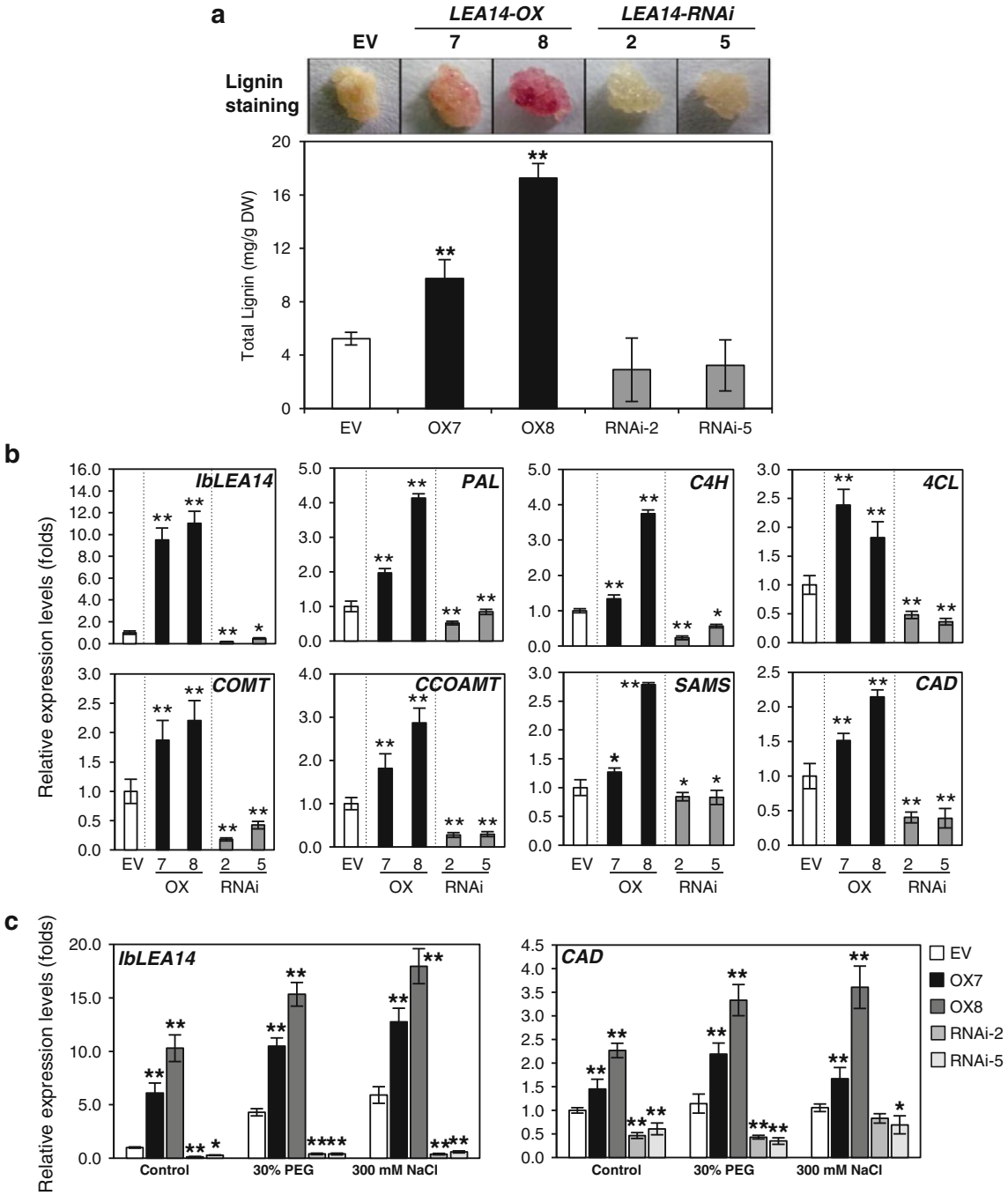

Fig. 6 Analysis of lignin content and lignification-related gene expression in *IbLEA14* transgenic calli. **a** Histochemical analysis of lignified callus tissues using phloroglucinol-HCl staining and quantitative analysis of lignin levels in sweetpotato calli. Data presented represent the average of three replicates. **b** Expression patterns of various lignin-related genes in sweetpotato calli. Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (*C4H*), 4-coumarate:coenzyme A ligase (*4CL*), caffeic acid-*O*-methyl-transferase (*COMT*), caffeoyl coenzyme A *O*-methyl-transferase (*CCAOMT*), S-adenosyl-L-methionine synthetase (*SAMS*), and cinnamyl alcohol dehydrogenase (*CAD*). **c** Expression patterns of *IbLEA14* and *CAD* in *IbLEA14* transgenic calli after treatment with 30% PEG or 300 mM NaCl for 24 h. Data presented represent the average of three replicates. Statistical significance of differences between the control and transgenic lines were determined by one way ANOVA with LSD post hoc test (*$P < 0.05$; **$P < 0.01$)

examined to determine whether lignin production was responsible for retarded growth in these lines (Fig. 6a). As expected, lignin contents correlated with *IbLEA14* expression levels, and higher levels of lignin accumulation were detected in *IbLEA14* OX lines, especially line 8, whereas RNAi lines showed similar levels of lignin accumulation to those of the controls. In comparison to the control calli, lignin accumulation was 1.9- and 3.3-fold

higher in the OX lines 7 and 8, respectively. As altered lignin contents were found in *IbLEA14* transgenic calli, quantitative RT-PCR analyses were conducted to characterize the expression patterns of a variety of lignification-related genes in these transgenic tissues (Fig. 6b). Substantially increased expression of cytosolic monolignol biosynthesis-related genes, such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:coenzyme A ligase (4CL), caffeic acid-*O*-methyl-transferase (COMT), caffeoyl coenzyme A *O*-methyl-transferase (CCAOMT), S-adenosyl-L-methionine synthetase (SAMS) and cinnamyl alcohol dehydrogenase (CAD), was observed in *IbLEA14*-OX transgenic lines. During stress treatments, expression levels of *IbLEA14* and *CAD* were higher in OX lines than in control or RNAi lines (Fig. 6c). These results suggest that, during stress conditions, *IbLEA14* overexpression results in elevated expression of monolignol biosynthesis-related genes in the transgenic callus, leading to an increase in lignin content. Thus, it may be concluded that *IbLEA14* expression induces increased lignin production as a protective response to various abiotic stress conditions.

Discussion

Agriculture is susceptible to damage from various abiotic stress conditions, and developing tolerance to different environmental factors represents a major challenge for crop development world-wide. To generate multiple stress-tolerant crops, superior stress-tolerance genes must be cloned from various plant species. Sweetpotato is a major root crop that can resist many adverse environmental conditions, including drought stress. In this study, the abiotic stress-responsive gene *IbLEA14* was isolated from an EST library generated from the fibrous roots of sweetpotato subjected to drought stress. Overexpression and RNAi suppression of *IbLEA14* in a transgenic sweetpotato non-embryogenic callus system provided a mechanism for examining the functional role played by IbLEA14 in abiotic stress tolerance.

The deduced amino acid sequence of IbLEA14 exhibited similarity to an atypical group of hydrophobic LEA_2 group proteins including Lemmi9 (Eycken et al. 1996), D95-4 (Maitra and Cushman 1994), CaLEA6 (Kim et al. 2005), LEA14-A (Galau et al. 1993), ER5 (Zegzouti et al. 1997), pcC27-45 (Piatkowski et al. 1990), *Arabidopsis* three LEA14 homologues (Hundertmark and Hincha 2008) (Fig. 1). These proteins are predicted to be relatively hydrophobic, except in the C-terminal region. Various environmental conditions modulate the expression of LEA_2 group proteins in different plant species. Kim et al. (2005) reported that, in hot pepper, *CaLEA6* expression is induced by PEG, NaCl, chilling and ABA treatments. In tomato, *ER5* expression is induced by ethylene, drought, ABA and wounding treatment (Zegzouti et al. 1997), and the genes *pcC27-45* and *D95-4* are expressed during dehydration or ABA exposure (Piatkowski et al. 1990; Maitra and Cushman 1994). Root-knot nematode infection induces expression of *Lemini9* in tomato (Eycken et al. 1996). Hundertmark and Hincha (2008) also reported that expressions of three *LEA14 Arabidopsis* homologue genes such as At1g01470, At2g46140 and At2g44060 induced under cold, heat, drought, salt and high light and ABA treatment conditions. These genes contain ABRE (ABA responsive element) and/or DRE/CRT/LTRE (drought responsive/C-repeat/low temperature response) element, which binds to the DREB/CBF or bZIP transcription factors, thus expression of the *LEA14* genes induced by various abiotic stress conditions (Hundertmark and Hincha 2008). In this study, comparison between stressed and unstressed sweetpotato plants revealed that *IbLEA14* expression increases under dehydration, NaCl and low temperature conditions (Figs. 2, 3). Since *IbLEA14* expression is induced by ABA, it is possible that *IbLEA14* responds to various abiotic stresses via an ABA-mediated pathway in sweetpotato.

Here, sweetpotato non-embryogenic calli were used to study IbLEA14 function, since this system is homologous to sweetpotato plants and transgenic materials can be generated quickly by transformation. To validate the role played by IbLEA14 in abiotic stress tolerance, non-embryogenic calli were transformed with vectors that performed *IbLEA14* overexpression or RNAi suppression under the control of a constitutive *CaMV35S* promoter (Table 2). The *IbLEA14* transgenic calli displayed unique phenotypes with respect to growth and stress tolerance. After 3 weeks of culture, it was clear that *IbLEA14* OX transgenic calli grew more slowly than control lines, whereas RNAi lines grew more rapidly (Fig. 4d). The growth retardation phenotype is considered to be a natural adaptive process that allows plants to prepare defense mechanisms against stress conditions. There are large numbers of genes that affect plant growth when overexpressed or repressed. Among them, overexpression of various dehydration-induced genes often results in reduced growth under normal growth conditions (Kasuga et al. 2004; Sakuma et al. 2006; Mlynárová et al. 2007), similar to the results of this study. It has been suggested that the hydrophobic LEA14 proteins perform important roles in plant responses to a variety of stress conditions. However, only a few studies have reported analysis of transgenic *LEA14* gene expression. In transgenic tobacco and Chinese cabbage plants, overexpression of hot pepper *CaLEA6* elicited enhanced tolerance to dehydration and salt stress (Park et al. 2003; Kim et al. 2005). In this study, *IbLEA14* OX lines exhibited enhanced tolerance to drought and salt stress relative to control lines, whereas RNAi lines showed

increased stress sensitivity, as evidence by WC and lipid peroxidation analyses (Fig. 5). Since high levels of *IbLEA14* expression provide transgenic calli with enhanced tolerance to drought and salt stress conditions, it is possible to suggest a working hypothesis whereby IbLEA14 participates in a subset of development involved in the stress response of sweetpotato.

It has been reported that abiotic and biotic stresses are responsible for increasing cell wall lignification (Lee et al. 2007), which is associated with decreased plant growth, as well as the increased structural rigidity and durability of plant tissues (Guenni et al. 2002). In the present study, *IbLEA14*-OX transgenic callus lines showed increased lignin contents (Fig. 6a). In addition, the transgenic calli exhibited morphological alterations in which OX lines showed stiffening of tissues, whereas RNAi lines demonstrated tissue softening (data not shown). *IbLEA14* expression was shown to result in the induction of a variety of monolignol biosynthesis-related genes, including *PAL, C4H, 4CL, COMT, CCAOMT, SAMS* and *CAD* (Fig. 6b). Lignin is generally derived from dehydrogenative polymerization of monolignols such as *p*-coumaryl alcohol, conifryl alcohol, and sinapyl alcohol (Amthor 2003). These monolignols are formed in the cytosol, and different enzyme-coding genes are involved at three different stages of monolignol biosynthesis. The first step involves enzymes for the common phenylpropanoid pathway such as PAL, C4H and 4CL, followed by methylation of monolignols, including COMT, CCAOMT, SAMS and ferulate-5-hydroxylase (F5H), after which the last steps of monolignol biosynthesis are performed by hydroxycinnamoyl-CoA:NADPH oxidoreductase (CCR) and CAD. This finding suggests that the increased *IbLEA14* expression correlates with cytosolic monolignol biosynthesis, which involves the induction of monolignol biosynthesis-related genes such as *PAL, C4H, 4CL, COMT, CCAOMT, CAD* and *SAMS*, and results in the accumulation of lignin contents.

It has been reported that lignin not only provides terrestrial plants with rigidity against compressive forces but also forms a mechanical barrier against pathogen infections and abiotic stresses such as drought, low temperature, high light and ultraviolet-B radiation (Moura et al. 2010). In particular, the effects of drought on lignin biosynthesis are well documented. The aromatic properties of lignin make the cell wall impermeable to water, which reduces transpiration and assists with maintaining normal turgor pressures under drought stress (Monties and Fukushima 2001). Therefore, it may be speculated that lignification represents an initial form of protection against drought stress. Several studies have shown that the process of lignification and high expression levels of lignin biosynthesis-related genes are important for drought tolerance. Hu et al. (2009) reported that drought-tolerant inbred maize lines showed a more active lignification response to drought stress than drought-sensitive inbred lines and that expression levels of lignin biosynthesis-related genes, such as *CAD*, *COMT* and *SAMS*, were also higher in drought-tolerant inbred lines than in drought-sensitive lines. Rice plants treated with drought stress for 72 h have shown increased expression of genes involved in lignin biosynthesis including PAL, 4CL, CCAOMT and CAD (Yang et al. 2006), and similar results have been obtained with *Citrullus lanatus* sp., which displays extraordinary tolerance to drought conditions (Yoshimura et al. 2008). CAD is involved in the last steps of monolignol biosynthesis, and in this study, *IbLEA14* OX lines treated with PEG or NaCl showed higher levels of *CAD* expression than control lines (Fig. 6c). Increased *IbLEA14* expression correlates with the upregulation of *CAD* and increased lignin accumulation, which provides a defense mechanism against drought and salt stress.

Although *IbLEA14* expression levels were reduced by approximately 80% in the RNAi lines, the lignification phenotypes of these lines were either similar or only slightly decreased with respect to control lines (Fig. 6a). Moreover, the expression levels of cytosolic monolignol biosynthetic genes were only slightly lower or similar to control lines (Fig. 6b). When considering the differences in *IbLEA14* expression levels, these results became even less remarkable compared with control and OX lines (Fig. 4a). It is possible that the lack of a significant lignification phenotype in RNAi lines may result from a threshold effect with respect to *IbLEA14* expression levels. In this study, *IbLEA14* overexpressing transgenic callus also showed reduced growth rate compared with control lines under optimal conditions (Table 2). Use of the stress-inducible promoter instead of the constitutive *35S CaMV* promoter for the overexpression of *IbLEA14* can minimize the negative effects on plant growth in transgenic plants (Kasuga et al. 2004). Therefore, we expect that the overexpression of *IbLEA14* under the control of stress-inducible *SWPA2* promoter in transgenic plants not only significantly enhances the tolerances of the plant to dehydration and salt stress but also minimizes the negative effects on plant growth (Kim et al. 2003).

According to the results in this study, transgenic sweetpotato calli exhibited a variety of phenotypes in response to the different levels of *IbLEA14* expression and these variations impacted cell growth, morphology, lignification and stress resistance. IbLEA14 may be involved in these functions as a consequence of regulating increased lignin production. Lignification performs crucial roles in many aspects of plant physiology, including plant rigidification, growth inhibition and the drought stress response. Therefore, we suggest that IbLEA14-mediated lignin

accumulation may be responsible for the physiological changes observed with LEA14 expression. Further investigation will be required to elucidate the exact role IbLEA14 performs in the regulation of lignin production in sweetpotato under stress conditions. For analyses of stress tolerance and lignification in woody plants, transgenic poplar expressing *IbLEA14* is being generated. We expect that our studies involving the overexpression or suppression of *IbLEA14* in transgenic plants will provide valuable information for the development of crops with enhanced tolerance to a variety of stresses.

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