論　文（Original article）

Characterization of late embryogenesis abundant (LEA) proteins involved in environmental stress response of black poplar

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Abstract

Late embryogenesis abundant (LEA) proteins are known to accumulate in the late stage of seed development and in vegetative tissues following environmental stress. They exist not only in plants but also some animals, fungi, and bacteria. LEA proteins have diverse structures and gene expression patterns and are considered to safeguard living cells against disadvantageous changes in environmental conditions. In this study, cDNAs encoding two LEA proteins, PnLEA1 and PnLEA2, were isolated and analyzed to characterize LEA proteins in black poplar (*Populus nigra* L.). The predicted PnLEA1 and PnLEA2 proteins were structurally different; namely, PnLEA1 was identified as an LEA_1 family protein with one LEA_1 domain, and PnLEA2 was identified as an LEA_2 family protein with two LEA_2 domains. The *PnLEA1* and *PnLEA2* genes were expressed in the roots and leaves. The expression of *PnLEA1* was up-regulated by drought, salinity, and cold stress. The expression of *PnLEA2* was also increased by drought and salinity stress, though the increment was smaller than *PnLEA1*. Both PnLEA1 and PnLEA2 conferred early tolerance to high salinity on the *Escherichia coli* that overexpressed them. These results suggest that PnLEA1 and PnLEA2 are involved in the mechanisms of tolerance to environmental stress in *P. nigra*.

Key words: environmental stress, *Escherichia coli*, gene expression, LEA proteins, mRNA, *Populus nigra* L.

1. Introduction

Woody plants in their natural habitats are exposed to various environmental stressors such as light, temperature, gravity, water, oxygen, carbon dioxide, soil nutrients, chemicals, disease, and herbivory. For example, water deficits inhibit shoot, cambial, and root growth in trees (Kozlowski and Pallardy 1997). The number and size of leaves are decreased by drought stress; it also affects reproductive growth, including the number of flower buds, fruit-bearing capacity, and the size and shape of fruits. At the cellular and molecular levels of higher plants, drought stress induces rapid accumulation of abscisic acid (ABA), which closes stomata and reduces CO_2 absorption (Osakabe et al. 2014). Lower concentrations of CO_2 cause defective photosynthesis and result in the generation of reactive oxygen species that damage intracellular molecules such as DNA, proteins, and membrane lipids. Salinity stress influences the growth of woody plants. Sodium chloride (NaCl) treatments reduce the growth of leaves, tree height, and leaf water potential in four genotypes of *Populus* (Fung et al. 1998). Salinity stress also decreases leaf water potential in *Eucalyptus grandis* and *Pinus radiata* (Myers et al. 1998). High concentrations of NaCl reduce seed germination and survival rates, shoot length, and fresh weight of seedlings of three conifers (Croser et al. 2001). Daily and seasonal changes in temperature can induce heat and cold stress. Photosynthesis and growth of trees decrease, but stomatal conductance and the consumption of carbohydrates increase due to heat stress (Teskey et al. 2015). Photosynthetic assimilation and the activation state of Rubisco in leaves of two *Populus* species were reduced at 40℃ compared to 27℃ (Hozain et al. 2010). Additionally, cold stress severely decreased the growth of hybrid aspen at 4℃ (Welling et al. 2002, Renaut et al. 2004). Drought, salinity, and temperature stresses are partially similar because they often induce osmotic or oxidative stress (Wang et al. 2003). Woody plants have been inferred to possess defense systems against environmental stress because they are long-lived, sessile organisms that cannot escape exposure to the environmental stressors that influence their growth, morphogenesis, survival, and reproduction. The molecular mechanisms of response to environmental stress in woody plants have been studied; however, many remained unknown.

The relationship of late embryogenesis abundant (LEA) proteins to environmental stress response has been previously reported (Wang et al. 2003, Battaglia et al. 2008, Shih et al. 2008, Hincha and Thalhammer 2012). LEA proteins were first found in wheat seed embryos (Cuming and Lane 1979), and cotton (Dure III et al. 1981). After cDNAs of LEA

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proteins were cloned from cotton (Dure III et al. 1989), their homologous genes have been isolated from other plant species. LEA proteins were initially divided into six groups based on their amino acid sequences (Dure III 1993, Bray 1993). At present, the classification of LEA proteins is complicated because many LEA protein genes have been identified in various plants. For example, 50 LEA protein genes discovered in the *Arabidopsis thaliana* genome were categorized into nine groups according to their sequence similarity and conserved repeated motifs (Bies-Ethève et al. 2008). On the other hand, Hundertmark and Hincha (2008) grouped 51 LEA protein genes of *Arabidopsis* into seven families and nine groups according to the Pfam protein families database and investigated their gene expressions. Taking the sequence information and previous studies into consideration, Battaglia et al. (2008) classified LEA proteins from different plant species into seven groups. As a result of whole genome sequencing, plant LEA protein genes have been comprehensively identified not only in model plants but also in the following crops and trees: legumes (Battaglia and Covarrubias 2013), tomato (Cao and Li 2015), potato (Charfeddine et al. 2015, Chen et al. 2019), orchid (Ling et al. 2016), rape (Liang et al. 2016), watermelon and melon (Altunoglu et al. 2017), cassava (Wu et al. 2018), cotton (Magwanga et al. 2018), xerophyte (Muvunyi et al. 2018), sorghum (Nagaraju et al. 2019), wheat (Liu et al. 2019, Zan et al. 2020), Moso bamboo (Huang et al. 2016), Chinese plum (Du et al. 2013), sweet orange (Pedrosa et al. 2015), Chinese red pine (Gao and Lan 2016), and tea (Jin et al. 2019, Wang et al. 2019).

Some plant LEA proteins and their mRNAs increase during seed maturation and in vegetative tissue under drought, osmotic or cold stress conditions; therefore, they are likely involved in stress responses to environmental stress (Battaglia et al. 2008). There are also some LEA proteins whose expression is regulated by ABA that participate in drought stress responses (Wang et al. 2003). Most LEA proteins are hydrophilic, including biased hydrophilic amino acids (Battaglia et al. 2008), and that might make it possible to inhibit aggregation and inactivation of other proteins under desiccation or freezing conditions (Hincha and Thalhammer 2012). Not only plants but also bacteria, fungi, and animals possess LEA proteins (Hand et al. 2011). Larvae of the sleeping chironomid (*Polypedilum vanderplanki*) can survive without water in a state of anhydrobiosis. Three cDNAs of LEA proteins have been isolated from this chironomid, and their mRNAs were increased by desiccation and salinity stress (Kikawada et al. 2006). The chironomid LEA protein is suggested to be involved in vitrification with trehalose (Shimizu et al. 2010). Consequently, LEA proteins are likely to protect cellular molecules from negative influences induced by environmental stress; however, the physiological functions of many LEA proteins in higher plants and other living organisms have not been sufficiently clarified. Hence, further investigation is needed to elucidate the various biological properties of LEA proteins.

In the present study, we aimed to characterize LEA proteins from black poplar (*Populus nigra* L.). *Populus* species have been used as model woody plants because of the ease of clonal propagation, tissue culture and transformation techniques, and genetic information acquisition. Responses to environmental stress in poplar trees have been studied to increase our understanding of tolerance mechanisms in woody plants (Nishiguchi et al. 2002, Nanjo et al. 2004, Nanjo et al. 2007, Nishiguchi et al. 2012, Miyazawa et al. 2017). In woody plants, cDNAs of dehydrin, a member of the LEA protein family, have been isolated from *Populus* species (Caruso et al. 2002, Bae et al. 2009). The whole genome sequence of *Populus trichocarpa* was read for the first time in woody plants (Tuskan et al. 2006). Based on that reading, 53 LEA protein genes were expected to be in the genome (Lan et al. 2013). However, the physiological functions of poplar LEA proteins under stress conditions remain unknown. Here we report the structure of two LEA proteins from *P. nigra*, their gene expressions under stress conditions, and the acquisition of salinity tolerance in *Escherichia coli* via the LEA proteins.

2. Materials and Methods

2.1 Plant materials

Approximately 10 cm were cut from the ends of young branches of *P. nigra* and rooted in moist vermiculite. For stress treatments, the rooted branches were transferred to plastic pots (75 mm upper diameter, 60 mm high) and grown in a phytotron (Koito Electric Industries, Nagaizumi-cho, Japan) at 25 ℃, 70% relative humidity under metal halide lamps (500 μmol m^{-2} s^{-1} of photosynthetically active radiation, 16:8 h light:dark photoperiod).

2.2 Cloning and sequencing analysis

The cDNA clones encoding LEA proteins, PnFL2-006_H01 and PnFL2-020_A03, were screened from the full-length enriched cDNA libraries of *P. nigra* (Nanjo et al. 2004, Nanjo et al. 2007) using the BLAST+ software (Camacho et al. 2009). The HMMER 3.3 software (http://hmmer.org/) and the Pfam protein families database 32.0 (El-Gebali et al. 2019) were used to identify the protein functional domains. The UniProtKB/Swiss-Prot Release 2020_01 database (The UniProt Consortium 2019) and the *A. thaliana* Araport11 protein database (Cheng et al. 2017) were searched for orthologs to the poplar LEA proteins using BLAST+. Global alignment of two protein sequences was executed using the EMBOSS Needle program of the EMBL-EBI web site (Needleman and Wunsch 1970,

Madeira et al. 2019), and their sequence identity was estimated. Multiple protein sequences were aligned by the ClustalW program, and phylogenetic analysis was carried out by the maximum parsimony method with MEGA X software (Kumar et al. 2018). The grand average of hydropathy (GRAVY) scores were calculated by the ProtParam tool on the ExPASy server (Kyte and Doolittle 1982, Gasteiger et al. 2005).

2.3 Stress treatments and gene expression analysis

Drought stress was induced in poplar plantlets (~30 cm high) by watering cessation. Leaves were collected during the treatment period and frozen in liquid nitrogen. After collecting the leaves, the plantlets were discarded to avoid the effects of leaf detachment. Vermiculites in the pot were weighed (wet weight), dried overnight at 120℃, and re-weighed (dry weight). The water content was calculated as (wet weight − dry weight)/(wet weight). For salinity stress, a hydroponic solution containing 200 mM NaCl was added to the pots once daily, while a hydroponic solution without NaCl was used for the control. Heat and cold stress were induced using an incubator at 40℃ and 4℃, respectively, under continuous dark conditions. Total RNA was prepared from the frozen leaves, and reverse transcription quantitative real-time PCR (RT-qPCR) was performed as described previously (Nishiguchi et al. 2012). The PCR primers are shown in Table S1. The relative mRNA level was normalized to the mRNA level of the poplar ubiquitin 5 homolog (*PnUBQ5*, cDNA clone PnFL2-047_P22, DDBJ accession numbers DB883027 and DB901131). For statistical analysis, Welch's *t*-test and Dunnett's test were performed using Excel 2016 and R software (Ver. 3.6.0), respectively.

2.4 Measurement of growth of *E. coli* overexpressing LEA proteins

The DNA fragments encoding LEA proteins on PnFL2-006_H01 and PnFL2-020_A03 were amplified using PCR primers (Table S1) to construct the expression plasmid vectors of LEA proteins for *E. coli*. The amplified DNA fragments were cut by NheI and XhoI and inserted into the NheI and XhoI sites in pET-24a (+) (Merck, Darmstadt, Germany). The constructed plasmids were verified by DNA sequencing and introduced into *E. coli* BL21 (DE3) (Merck). For preculture, *E. coli* BL21(DE3) harboring each plasmid was cultivated overnight in 4.5 ml of a medium containing 1% Bacto tryptone (Life Technologies, Carlsbad, CA), 0.5% yeast extract, 30 μg/ml kanamycin, 1 mM isopropyl-β-D-thiogalactopyranoside and 100 mM NaCl at 37℃, with shaking. Forty-five microliters of the precultured bacterial cells were inoculated into 4.5 ml of fresh media including 100 mM NaCl or 1 M NaCl and cultivated for 48 h to be used as the main culture. The growth of *E. coli* was measured as absorbance units (AU) at 660 nm of the culturing media using Miniphoto 518R (Taitec, Koshigaya, Japan). Dunnett's and Tukey-Kramer tests were utilized to compare *E. coli* growth statistically, using R software.

3. Results

3.1 Isolation and structural analysis of *P. nigra* LEA proteins

To identify the cDNAs of LEA proteins in *P. nigra*, we first investigated the predicted LEA protein genes in the genome of *P. trichocarpa* (Tuskan et al. 2006). Thirty-seven genes with the annotation of "late embryogenesis abundant" were picked up from the *P. trichocarpa* genome annotation (V2.0). Using these 37 amino acid sequences as the query for the tblastn program, we searched the full-length enriched cDNA libraries of *P. nigra* exposed to a variety of stressors such as dehydration, high salinity, chilling, and heat (Nanjo et al. 2004, Nanjo et al. 2007). After removing redundant clones, two cDNA clones, PnFL2-006_H01 (DDBJ accession numbers DB875794 and DB894098), and PnFL2-020_A03 (DB896353 and DB878123) were identified as the candidates.

PnFL2-006_H01 encoded a predicted protein of 177 amino acid residues (aa). Using the hmmscan program from HMMER, the N-terminal 1 to 70 amino acid sequences of the predicted PnFL2-006_H01 protein were annotated to the LEA_1 family only (Pfam ID: PF03760) in the Pfam database at an independent E-value (i-Evalue) of 6.8e-23. Using the PnFL2-006_H01 protein sequence as a query, we searched the UniProtKB/Swiss-Prot database and found five orthologs: *Glycine max* 18 kDa seed maturation protein (UniProt accession number: Q01417); *A. thaliana* LEA protein 46 (Q9FG31, AGI locus code: AT5G06760); *Helianthus annuus* 11 kDa LEA protein (P46515); *Gossypium hirsutum* LEA protein D-113 (P09441); and *Solanum lycopersicum* protein LE25 (Q00747). These five proteins are all classified within the LEA type 1 family in UniProtKB. They were homologous to the N-terminal LEA_1 domain of the predicted PnFL2-006_H01 protein (Fig. 1a). The AT5G06760 protein (158 aa) had been named LEA46 by Hundertmark and Hincha (2008) and was most similar to the PnFL2-006_H01 protein in the *A. thaliana* protein database; their sequence identity was 49% over the entire length. Accordingly, PnFL2-006_H01 was inferred to encode an LEA_1 family protein and designated as PnLEA1. The GRAVY score of PnLEA1 was −0.832, and thus PnLEA1 was expected to be a hydrophilic protein, similar to other LEA proteins (Battaglia et al. 2008, Hand et al. 2011).

PnFL2-020_A03 was deduced to code for a protein of 314 aa. The hmmscan program demonstrated that the N-terminal domain from 73 to 168 and the C-terminal domain from 198 to 293 of the deduced PnFL2-020_A03 protein were identified as belonging to the LEA_2 family (PF03168) in Pfam. The

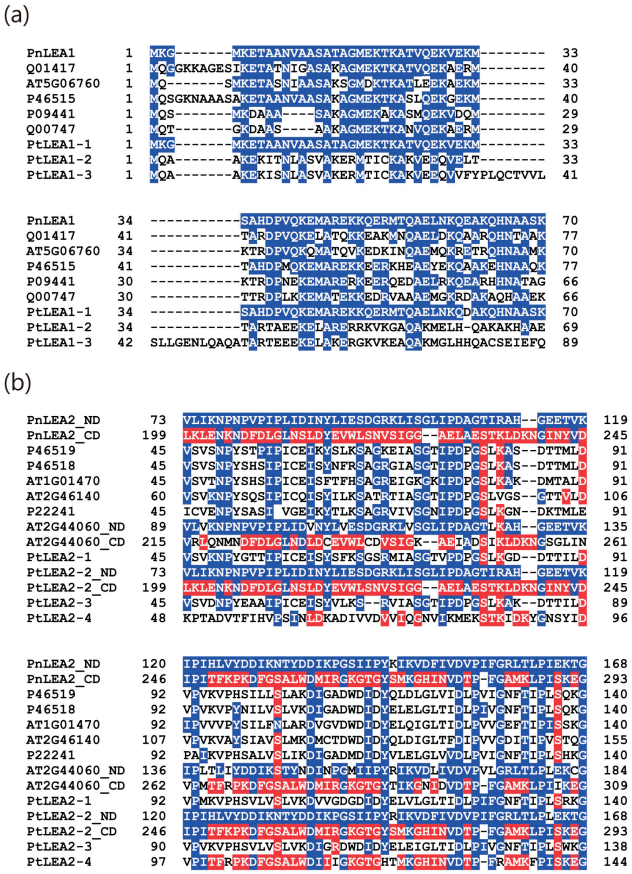

Fig. 1. Alignment of characteristic domain sequences of LEA proteins.

(a) Comparison of LEA_1 domains between *Populus nigra* PnLEA1 (PnFL2-006_H01) and other LEA_1 family proteins: Q01417, *Glycine max* 18 kDa seed maturation protein; AT5G06760, *Arabidopsis thaliana* LEA protein 46 (UniProt accession number: Q9FG31); P46515, *Helianthus annuus* 11 kDa LEA protein; P09441, *Gossypium hirsutum* LEA protein D-113; Q00747, *Solanum lycopersicum* protein LE25; and PtLEA1-1–PtLEA1-3, *Populus trichocarpa* LEA_1 family proteins. Identical amino acid residues with PnLEA1 are shaded blue. Numbers are amino acid positions. (b) Comparison of LEA_2 domains between PnLEA2 (PnFL2-020_A03) and other LEA_2 family proteins: PnLEA2_ND, N-terminal LEA_2 domain of PnLEA2; PnLEA2_CD, C-terminal LEA_2 domain of PnLEA2; P46519, *G. max* desiccation protectant protein Lea14 homolog; P46518, *G. hirsutum* Lea14-A; AT1G01470, *A. thaliana* probable desiccation-related protein LEA14 (O03983); AT2G46140, *A. thaliana* desiccation-related protein (O82355); P22241, *Craterostigma plantagineum* desiccation-related protein PCC27-45; AT2G44060_ND, N-terminal LEA_2 domain of *A. thaliana* LEA26; AT2G44060_CD, C-terminal LEA_2 domain of *A. thaliana* LEA26; PtLEA2-1–PtLEA2-4, *P. trichocarpa* LEA_2 family proteins; PtLEA2-2_ND, N-terminal LEA_2 domain of PtLEA2-2; and PtLEA2-2_CD, C-terminal LEA_2 domain of PtLEA2-2. Amino acid residues identical with PnLEA2_ND and with PnLEA2_CD are shaded blue and red, respectively.

i-Evalue of the N-terminal LEA_2 domain and the C-terminal LEA_2 domain was 1.9e-14 and 2.1e-11, respectively. Although both the N-terminal and the C-terminal LEA_2 domains were identified as the LEA_2 family, the sequence identity between these two domains was only 25% (Fig. 1b). Five orthologs of the PnFL2-020_A03 protein were found in the UniProtKB/Swiss-Prot database: *G. max* desiccation protectant protein Lea14 homolog (P46519); *G. hirsutum* LEA protein Lea14-A (P46518); *A. thaliana* probable desiccation-related protein LEA14 (O03983, AT1G01470); *A. thaliana* desiccation-related protein (O82355, AT2G46140); and *Craterostigma plantagineum* desiccation-related protein PCC27-45 (P22241). These five proteins belong to the LEA_2 family in UniProtKB and have homology with the N-terminal LEA_2 domain of PnFL2-020_A03 (Fig. 1b). However, they possess only one LEA_2 domain, not two domains. In addition to AT1G01470 and AT2G46140, AT2G44060 was newly discovered from the *A. thaliana* protein database as an orthologue. The deduced AT2G44060 protein (325 aa) possesses two LEA_2 domains similar to PnFL2-020_A03 (Fig. 1b) and has been reported as LEA26 of the LEA_2 family (Hundertmark and Hincha 2008). The protein sequence identities of AT2G44060, AT1G01470 (151 aa), and AT2G46140 (166 aa) with PnFL2-020_A03 was 71%, 15%, and 16%, respectively, over the entire length. Consequently, PnFL2-020_A03 was thought to encode an LEA_2 family protein and was designated as PnLEA2. The GRAVY score of PnLEA2 is −0.385 that implies lower hydrophilicity than PnLEA1. This is probably because PnLEA2 includes more hydrophobic amino acid residues such as Ile (37 aa), Leu (22 aa), and Phe (13 aa).

P. trichocarpa, which is in the same genus as *P. nigra*, has been reported to possess 53 LEA protein genes in the genome (Lan et al. 2013). The 53 deduced LEA proteins have been separated into eight groups (PtLEA1–PtLEA8) according to the Pfam nomenclature and renaming. Phylogenetic analysis was performed to clarify the relationship between PnLEA1, PnLEA2, the *P. trichocarpa* LEA proteins, and the above-mentioned ortholog proteins (Fig. 2). PnLEA1 and three *P. trichocarpa* LEA1 group proteins (PtLEA1-1 to PtLEA1-3) formed a clade; PtLEA1-1 was the closest to PnLEA1 in that clade. PtLEA1-1, PtLEA1-2 and PtLEA1-3 showed protein sequence homology to PnLEA1 (Fig. 1a). The amino acid sequence identity of PtLEA1-1(175 aa) with PnLEA1 showed a high score at 95%, while those of PtLEA1-2 (124 aa) and PtLEA1-3 (162 aa) were low at 21% and 16%, respectively. The five orthologs of PnLEA1 from UniProtKB/Swiss-Pro were also included in the same clade as the *P. trichocarpa* LEA1 group proteins. PnLEA2 and four *P. trichocarpa* LEA2 group proteins (PtLEA2-1 to PtLEA2-4) formed a different clade from the LEA1 group clade. PnLEA2 configured a small

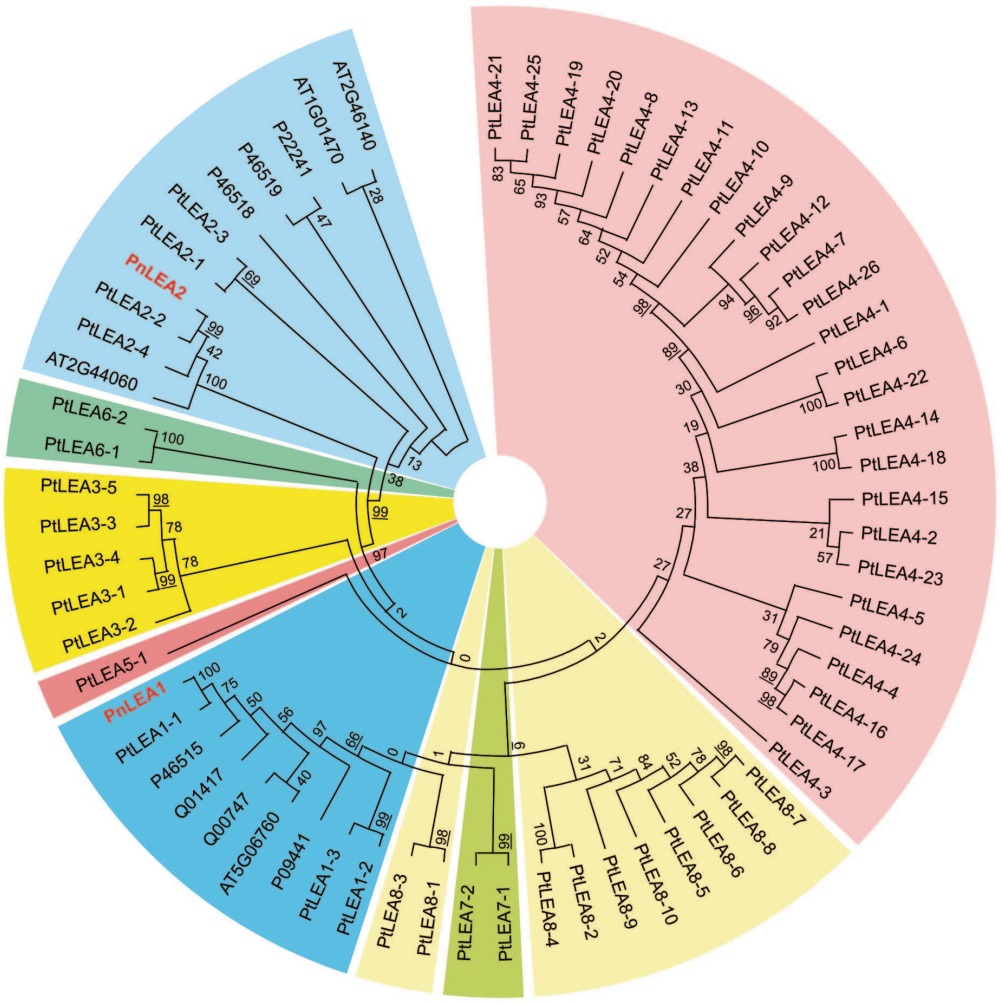

Fig. 2. Phylogenetic analysis of PnLEA1, PnLEA2, *P. trichocarpa*, and other LEA proteins.
The sequences of deduced LEA proteins were aligned by ClustalW, and phylogenetic relationship was calculated by the maximum parsimony method. Phylogenetic tree is colored differently for each of eight LEA protein groups (PtLEA1–PtLEA8) of *P. trichocarpa*. Other LEA proteins, except *P. nigra* and *P. trichocarpa*, are described in the Fig. 1 legend. Bootstrap values are shown as the percentage of 1,000 replications. Confusing numbers are underlined.

clade with PtLEA2-2, PtLEA2-4, and AT2G44060. PtLEA2-1, PtLEA2-3, and the above-mentioned five PnLEA2 orthologs located in close clades to the PnLEA2 clade. PtLEA2-2 (314 aa) had two LEA_2 domains, similar to PnLEA2, and their sequence identity was 99% (Fig. 1b). PtLEA2-1 (151 aa), PtLEA2-3 (149 aa), and PtLEA2-4 (163 aa) had only one LEA2 domain. PtLEA2-1 and PtLEA2-3 were slightly similar to the N-terminal domain of PnLEA2, while PtLEA2-4 was similar to the C-terminal domain of PnLEA2 (Fig. 1b). PnLEA2 was matched to 38% of the amino acid residues of PtLEA2-4, 12% of PtLEA2-1, and 12% of PtLEA2-3 over the entire length.

3.2 Effect of environmental stress on the expression of LEA protein genes

To compare the expression of *PnLEA1* and *PnLEA2* in underground and aboveground organs of *P. nigra* under normal growth conditions, total RNA was isolated from roots and leaves of *P. nigra* trees and analyzed using RT-qPCR. Both genes were expressed in roots and leaves (Fig. S1). The expression of *PnLEA1* tended to be higher in leaves than in roots, though the difference was not significant. *PnLEA2* showed a significantly higher expression (~2.2-fold) in leaves than in roots.

Some LEA proteins and their gene expressions have been reported to be induced by dehydration (Ried and Walker-Simmons 1993, Blackman et al. 1995). To confirm whether the *PnLEA1* and *PnLEA2* genes were induced by drought stress, we ceased watering of the poplar plantlets. The water content of vermiculites in the pots began to decrease at 1 d after watering cessation and decreased to ~4% after 8 d (Fig. 3a). Leaves of the plantlets drooped after 8 d and appeared to wilt (Fig. S2). The expression of *PnLEA1* in leaves was induced by drought stress and increased considerably (Fig. 3b), although

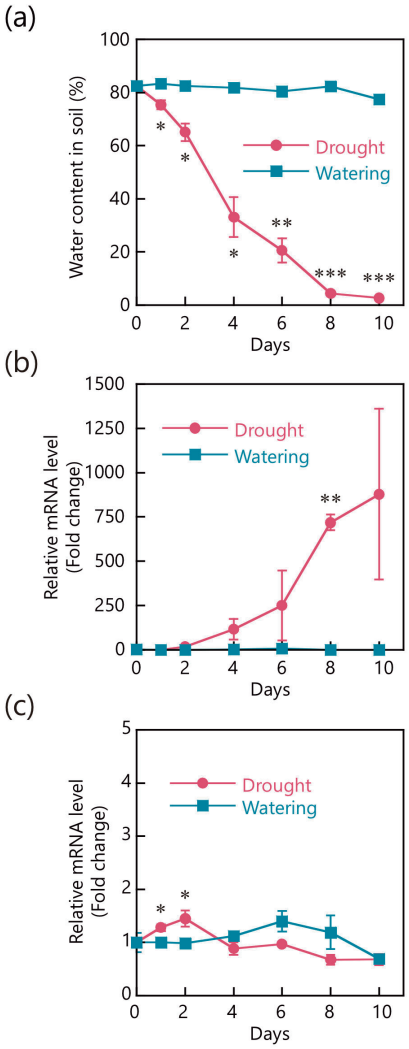

Fig. 3. Effect of drought stress on gene expression of *PnLEA1* and *PnLEA2*.
(a) Soil water content measured during watering (squares) and at days after watering cessation (circles). Expression of *PnLEA1* (b) and *PnLEA2* (c) in leaves was analyzed by RT-qPCR. mRNA levels of each gene at day 0 were defined as 1.0. Error bars represent ±SD (n = 3). Asterisks indicate significant differences between drought treatment and control with watering (Welch's t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

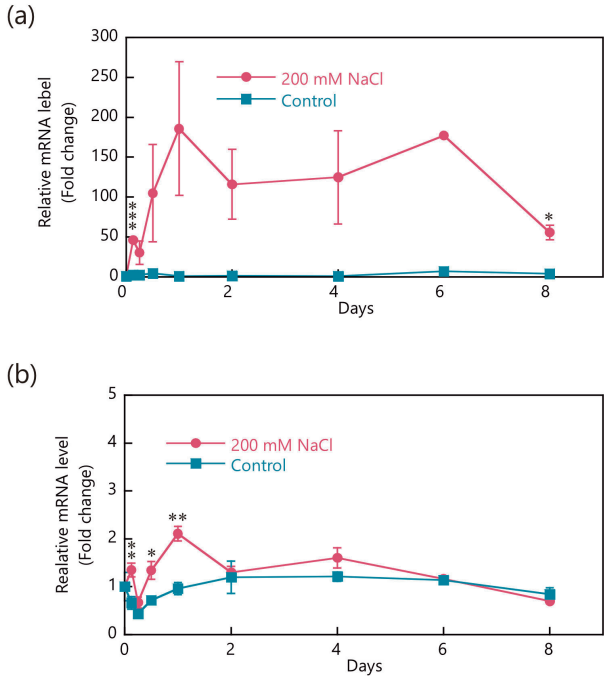

Fig. 4. Effect of salinity stress on gene expression of *PnLEA1* and *PnLEA2*.
Hydroponic solution of 200 mM NaCl (circles) or no NaCl (squares) was added to pots with poplar plantlets. mRNA levels of *PnLEA1* (a) and *PnLEA2* (b) in leaves at day 0 were defined as 1.0. Error bars represent ±SD (n = 3). Only mean values are shown at 6 d because n = 2. Asterisks indicate significant differences between NaCl treatment and control without NaCl (Welch's t-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

the increases were not always significant because the measured values were dispersed. The relative mRNA level at 8 d after watering cessation was significant at ~700-fold higher than at day 0. Under well-watered conditions, *PnLEA1* expression remained low for 10 d. *PnLEA2* expression increased ~1.5-fold at 2 d after watering cessation and subsequently returned the original level at 4 d (Fig. 3c).

We exposed poplar plantlets to salinity stress to investigate whether other environmental stresses affect the gene expression of *PnLEA1* and *PnLEA2*. A hydroponic solution containing 200 mM NaCl was added to the plantlet pot once per day; therefore, the roots were likely exposed to NaCl at approximately the same concentration. Although the effect of NaCl treatment on the poplar plantlets was not observed until days 6 to 8, after that leaf abscission commenced (Fig. S3). *PnLEA1* showed an expression pattern induced by salinity stress in leaves, though their mRNA levels were also dispersed (Fig. 4a). The mRNA of *PnLEA1* increased significantly and rapidly, approximately 46-fold at 3 h after NaCl treatment. The elevated expression reached approximately 186-fold after 24 h but was not significant (p = 0.09). The up-regulated *PnLEA1* expression level was maintained higher than that of the control plants for 8 d. *PnLEA2* also showed increased gene expression with the NaCl treatment, but it's up-regulated level was considerably lower than *PnLEA1* (Fig. 4b). The *PnLEA2* mRNA level increased significantly (~two-fold) 24 h after NaCl treatment. The high expression at 24 h was not maintained and returned to the same level as that of the control after 2 d.

Some LEA protein genes have been reported to be induced by temperature stress (Cai et al. 1995, Colmenero-Flores et al. 1997). We investigated the effects of heat and cold stress on *PnLEA1* and *PnLEA2* gene expression. Some of the poplar plantlets were transferred from an incubator at 25℃ to other incubators for stress treatment and kept for 48 h at

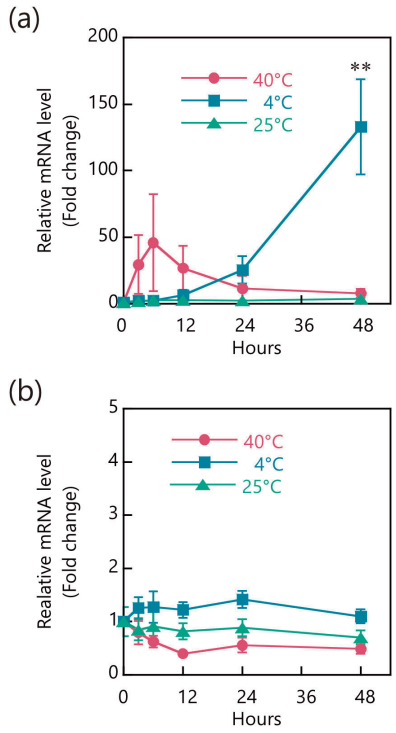

Fig. 5. Effect of temperature stress on gene expression of *PnLEA1* and *PnLEA2*.
Poplar plantlets were incubated for 48 h at 40 ℃ (circles), 4℃ (squares), and 25℃ (triangles). mRNA levels of *PnLEA1* (a) and *PnLEA2* (b) in leaves at 0 h were defined as 1.0. Error bars represent ±SD (n = 3). Asterisks indicate significant differences between control at 25℃ and other temperature treatments (Dunnett's test; $^{**}p < 0.01$).

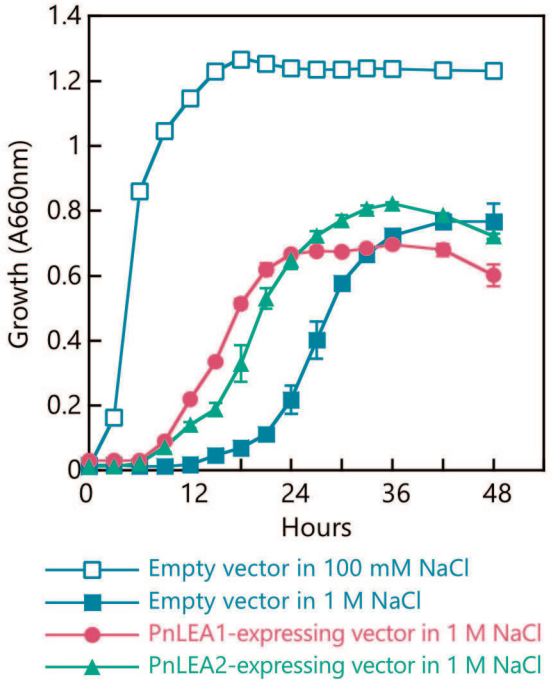

Fig. 6. Growth curves of *E. coli* under salinity stress conditions.
E. coli BL21 (DE3) strains harboring indicated vectors were cultured in media of 100 mM NaCl or 1 M NaCl for 48 h. Growth of *E. coli* is represented by absorbance at 660 nm. Empty vector in a 100 mM NaCl medium (open squares), empty vector in a 1 M NaCl medium (closed squares), PnLEA1-expressing vector in a 1 M NaCl medium (circles), and PnLEA2-expressing vector in a 1 M NaCl medium (triangles). Error bars show ± SD (n = 3).

high temperature (40℃) or low temperature (4℃). Heat or cold stress did not influence the appearance of the poplar plantlets. However, heat stress tended to raise the *PnLEA1* expression in leaves, and the expression level was ~46-fold after 24 h, but the raised transcription levels were not statistically significant (Fig. 5a). Under cold stress conditions, *PnLEA1* expression was low until 12 h after treatment and thereafter significantly increased ~130-fold at 48 h after treatment. On the other hand, neither heat nor cold stress significantly changed the expression level of *PnLEA2* (Fig. 5b).

3.3 Acquisition of salinity tolerance in *E. coli* by PnLEA proteins

To elucidate the function of PnLEA1 and PnLEA2 proteins, their genes were separately overexpressed in *E. coli*, and its growth was measured in culture media, including low or high concentrations of NaCl. Common media such as L-broth contain 0.5–1% (~86–171 mM) NaCl and are generally used for *E. coli* proliferation. Therefore, we used media containing 100 mM NaCl as a control. In the presence of 100 mM NaCl, the early growth of the empty vector-harboring *E. coli* reached 0.86 AU at 660 nm at 6 h; thereafter, the stationary phase growth reached more than 1.27 AU after 18 h (Fig. 6). In contrast, high concentrations (1 M) of NaCl delayed and arrested the growth of *E. coli*. In the media containing 1 M NaCl, *E. coli* growth was 0.014 AU at 6 h and 0.069 AU after 18 h (Fig. 6). Subsequently, *E. coli* started to proliferate and attained 0.77 AU of growth in 1 M NaCl at 48 h, which was ~63% of 1.23 AU in 100 mM NaCl.

The PnLEA1-overexpressing *E. coli* grew faster than the empty vector-harboring *E. coli* in the presence of 1 M NaCl (Fig. 6). The growth of PnLEA1 reached 0.090 AU after 9 h compared with 0.013 AU of the empty vector. Subsequently, the PnLEA1-overexpressing *E. coli* entered a logarithmic growth phase, and its growth was 0.67 AU after 24 h, significantly higher than 0.22 AU of the empty vector-harboring *E.coli*. However, the growth of PnLEA1-overexpressing *E. coli* did not exceed 0.7 AU and showed a lower growth (0.60 AU) than the empty vector-harboring *E.coli* after 48 h. The PnLEA2-overexpressing *E. coli* also showed faster cell proliferation than

the empty vector-harboring *E. coli* in 1 M NaCl media (Fig. 6). The growth of PnLEA2-overexpressing *E. coli* was 0.072 AU after 9 h, 0.65 AU after 24 h, and 0.72 AU after 48 h. PnLEA2 demonstrated significantly higher growth than PnLEA1 at 30 h to 48 h. Eventually, the growth of *E. coli* overexpressing PnLEA1 and PnLEA2 were ~49% and ~59%, respectively, in 1 M NaCl media after 48 h, compared to the empty vector-harboring *E. coli* in 100 mM NaCl media.

4. Discussion

Woody plants have been exposed to various environmental stressors for a long time; however, the understanding of their response and tolerance mechanisms is not as advanced as herbaceous plants. It is important to elucidate the molecular mechanisms involved in maintaining cellular activity during environmental changes in woody plants. LEA proteins have been considered to be important molecules involved in stress response for some plant and animal species. In this study, we isolated the cDNAs of two LEA proteins, PnLEA1 and PnLEA2, from black poplar. Sequence analysis and comparison with other plant LEA proteins revealed that PnLEA1 and PnLEA2 had different LEA domains and that PnLEA1 and PnLEA2 were categorized into the LEA_1 family of the Pfam protein families database and into the LEA_2 family, respectively (Figs. 1 and 2). Additionally, both PnLEA1 and PnLEA2 were deduced to be hydrophilic proteins characteristic of typical LEA proteins. In this study, we referred mainly to the Pfam database for annotation, because LEA protein genes from *P. trichocarpa* and many other plant species were classified based on the Pfam database. However, the taxonomy of plant LEA proteins has not been completely unified; thus, in some cases, different names for LEA families or LEA proteins have been used. For example, *Arabidopsis* AT5G06760, which is the most similar ortholog of PnLEA1, named LEA46, belongs to the LEA_1 family of Pfam (Hundertmark and Hincha 2008) and is also named AtLEA4-5, categorized to LEA group 4 (Bies-Ethève et al. 2008, Olvera-Carrillo et al. 2010). Similarly, the LEA_2 family in plants is equivalent to the LEA group 7 proteins in *Arabidopsis* (Bies-Ethève et al. 2008), or the LEA group 5C proteins in plants (Battaglia et al. 2008). In a database for LEA proteins (LEAPdb), the term “class” has been used for classification instead of family and group (Hunault and Jaspard 2010). Thus, LEA proteins have ambiguous classification and nomenclature, probably because they have a wide variety of origins and structures, leaving many unanswered questions concerning their molecular characterization and physiological function.

PnLEA1 gene expression was greatly increased by drought, salinity, and cold stress in poplar leaves (Figs. 3–5). These results suggest that *PnLEA1* plays an important role in plant response to external environmental changes. *PnLEA1*-homologous genes from other plant species show a partially similar expression pattern to that of *PnLEA1*. In *P. trichocarpa, PtLEA1-1* is expressed in leaves, shoots, roots, buds, and phloem under normal, drought stress, and salt stress conditions (Lan et al. 2013). Although the expression of AT5G06760 is low in *Arabidopsis* leaves under normal conditions, it is induced by NaCl, drought, cold, and ABA treatment (Hundertmark and Hincha 2008). Similarly, the *Brassica napus LEA4-1 (BnLEA4-1)* gene, a homolog of AT5G06760, is rarely expressed in leaves under normal conditions and is induced by ABA, NaCl, and cold stress (Dalal et al. 2009). Since *PnLEA1* was induced by both drought stress and salinity stress, its gene expression is likely to be involved in osmotic stress. There is also the possibility that the difference in gene expression patterns of *PnLEA1* between drought and salinity stress reflects the state of osmotic stress in poplar plants. AT5G06760 shows low expression in seedlings, but the high expression is elicited not only by ABA and NaCl but also polyethylene glycol (high osmolality) (Olvera-Carrillo et al. 2010). The transcription factor AtMYB44 suppresses the gene expression of AT5G06760 induced by NaCl (salt stress) or mannitol (osmotic stress), and AtMYB44 binds to the promoter of AT5G06760 under normal conditions and separates from it by mannitol treatment (Nguyen et al. 2019). Accordingly, *PnLEA1* might be repressed by similar transcription factors under normal conditions and induced by multiple stress responses under unsuitable environmental conditions including osmotic stress.

PnLEA2 also showed expression inducible by environmental stress; however, the degree of expression was different from that of *PnLEA1*. The expression of *PnLEA2* in leaves was ~1.5–2-fold up-regulated by drought and salinity stress, but not by temperature stress (Figs. 3–5). The inducible expression of *PnLEA2* is thought to be weaker than that of *PnLEA1* in the investigated stress treatments. Similarly, *PtLEA2-2* is expressed under normal, drought, and salt stress conditions in *P. trichocarpa* (Lan et al. 2013). AT2G44060 is the most similar *Arabidopsis* ortholog and likewise shows ~2.2- and ~4.7-fold gene expression induced by drought and 100 mM NaCl treatment, respectively (Hundertmark and Hincha 2008). Therefore, it is suggested that PnLEA2-homologous proteins with two LEA_2 domains have similar gene expression characteristics and are involved in responses to similar environmental changes. Interestingly, LEA proteins with only one LEA_2 domain show versatile gene expression patterns. For example, *PtLEA2-1* is expressed in the investigated samples similarly to *PtLEA2-2* in *P. trichocarpa*; however, *PtLEA2-3* and *PtLEA2-4* are not expressed at all (Lan et al. 2013). AT1G01470 is strongly induced by salts, cold, and ABA in *Arabidopsis*, while AT2G46140 is slightly induced only by

drought conditions (Dunaeva and Adamska 2001, Hundertmark and Hincha 2008). Accordingly, even if LEA proteins have homologous domain structures, they likely play different roles in maintaining cellular function. In *P. trichocarpa*, 20 of the 53 LEA protein genes were not expressed in any investigated tissue or response to drought and salinity stress (Lan et al. 2013). It is important to clarify how the gene expression of poplar LEA proteins is regulated in response to exogenous environmental changes.

With both PnLEA1- and PnLEA2-overexpressing, *E. coli* acquired early tolerance to high salinity (Fig. 6). When *E. coli* is in high osmolality, it first induces the influx of K^+, and glutamate synthesis is increased. Subsequently, compatible solutes (osmoprotectants) such as glycine betaine, proline, and trehalose are transported into the cells from outside or synthesized endogenously (Kempf and Bremer 1998). These compatible solutes are needed for the resistance of *E. coli* to conditions of high osmolality, including high concentrations of NaCl. Soybean LEA proteins have been reported to possibly function as osmoprotectants in vitro (Shih et al. 2010). The *E. coli* expression system has also shown that other plant LEA proteins can provide stress tolerance in *E. coli* (Lan et al. 2005, Liu and Zheng 2005, Ling et al. 2016). Although PnLEA1 and PnLEA2 are different in structure and gene expression, it appears that both LEA proteins help *E. coli* cell proliferation in the early stage under high salinity conditions, due to their characteristic properties as compatible solutes. The results of PnLEA1- or PnLEA2-overexpression in *E. coli* support a putative function of PnLEA proteins as a substitute for compatible solutes. In addition, some LEA proteins can protect other enzyme activity under stress conditions in vitro. Dehydrin DHN-5, a wheat LEA protein, preserves β-glucosidase and glucose oxidase/peroxidase activities from heat inactivation (Brini et al. 2010). The AT2G44060 protein can protect lactate dehydrogenase from inactivation during freeze-thaw cycles (Dang et al. 2014). Considering the induction of expression of *PnLEA1* and *PnLEA2* by environmental stress, both genes are thought to be involved in the early response to environmental stress and might safeguard other proteins in *P. nigra* cells against inactivation by severe environmental stress.

Because some LEA protein genes are expressed to correlate with physiological and environmental stressors, and their translated LEA proteins accumulate in plant bodies, they have been used to produce transgenic plants with higher tolerance to environmental stress for over 20 years (Xu et al. 1996). It has been reported that the overexpression of BnLEA4-1 provides better growth in transgenic *A. thaliana* than non-transgenic plants under 150 mM NaCl or drought conditions (Dalal et al. 2009). Transgenic tobacco plants overexpressing maize ZmLEA5C, an LEA_4 family protein, show tolerance to osmotic and cold stress (Liu et al. 2014). The overexpression of rice OsLEA14-A, an ortholog to AT1G01470, confers better growth than non-transgenic rice under stress conditions such as drought, 200 mM NaCl, 1 mM $CuSO_4$, or 1 mM $HgCl_2$ (Hu et al. 2019). In woody plants, transgenic poplar trees (*Populus simonii* × *P. nigra*) overexpressing *Tamarix androssowii* TaLEA, an LEA_3 family protein, have lower damage under 200 mM NaCl or drought stress conditions compared to non-transgenic poplars (Gao et al. 2013). TaLEA and ThbZIP (a basic leucine zipper protein from *Tamarix hispida*) were co-overexpressed in birch (*Betula platyphylla*) and partially mitigated the influence of salt stress in transgenic birch (Zhao et al. 2016). Those transgenic plants may be able to survive under mild stress conditions such as a short dry period or a moderate concentration of salts (for example, 200 mM NaCl = approximately 1.2% NaCl); however, it might be hard for them to survive under severe stress conditions such as a long dry season or seawater (~3.5% NaCl). If PnLEA1 or PnLEA2 is overexpressed in *P. nigra*, it is not deduced to contribute to a significant improvement in stress tolerance, because PnLEA1 and PnLEA2 might only play pioneering and partial roles in stress response, and in particular, the endogenous *PnLEA1* is primarily induced at a high level when poplar plants are exposed to environmental stresses. It is strongly required that other stress response factors harboring different functions from LEA proteins cooperate with LEA proteins to confer enhanced stress tolerance.

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Supplementary data legends

Supplementary data can be found at
https://www.ffpri.affrc.go.jp/pubs/bulletin/460/460toc-en.html

Fig. S1

Gene expression of *PnLEA1* and *PnLEA2* in *Populus nigra*.

Total RNA was isolated from roots (R) and 10th leaf (L) from apex of ~1.5 m poplar trees. mRNA level of roots was defined as 1.0. Error bars represent ±SD ($n = 3$). Asterisks indicate significant differences between roots and leaves (Welch's *t*-test; ***$p < 0.001$).

Fig. S2

Poplar plantlets under drought stress conditions.

Dates show days after end of watering. The plantlets in each photo are not the same individuals, because they have been discarded after collecting the leaves in order to avoid the effects of leaf detachment.

Fig. S3

Poplar plantlets under salinity stress conditions.

Dates show days after exposing poplar plantlets to hydroponic solution with 200 mM NaCl. The plantlets in the photos are not equal to the leaves-collected plantlets for the reason as described in the Fig. S2 legend.

Table S1

Synthetic DNA primers used for RT-qPCR and vector construction.

ポプラの環境ストレス応答に関与する LEA タンパク質の性質

西口 満 [1)*]

要旨

後期胚発生蓄積 (late embryogenesis abundant, LEA) タンパク質は、種子発達過程の後期および環境ストレスを受けた栄養組織で蓄積することが知られている。LEA タンパク質は植物だけでなく、動物、真菌類、細菌類にも存在する。LEA タンパク質は多様な構造と遺伝子発現様式を持ち、環境条件の不利な変化から細胞を保護していると考えられている。本研究では、ポプラ (*Populus nigra* L.) の LEA タンパク質の性質を明らかにするため、２つの LEA タンパク質、PnLEA1 と PnLEA2 の cDNA を単離し、解析を行った。予想される PnLEA1 タンパク質と PnLEA2 タンパク質は構造的に異なっており、PnLEA1 は 1 つの LEA_1 ドメインを持つ LEA_1 ファミリータンパク質、PnLEA2 は２つの LEA_2 ドメインを持つ LEA_2 ファミリータンパク質と同定された。*PnLEA1* 遺伝子と *PnLEA2* 遺伝子は、根および葉で発現していた。*PnLEA1* の遺伝子発現は、乾燥、高塩、および低温ストレスで増加した。*PnLEA2* も乾燥および高塩ストレスで遺伝子発現を増加させたが、増加の程度は *PnLEA1* よりも小さかった。PnLEA1 または PnLEA2 を過剰発現した大腸菌は初期の耐塩性が向上した。これらの結果は、ポプラにおいて PnLEA1 および PnLEA2 が環境ストレス耐性機構に関与することを示唆している。

キーワード：環境ストレス、大腸菌、遺伝子発現、LEA タンパク質、メッセンジャー RNA、ポプラ

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