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Three tandemly aligned *LEA* genes from *Medicago truncatula* confer differential protection to *Escherichia coli* against abiotic stresses

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Abstract

Late embryogenesis abundant (LEA) proteins are important for abiotic stress tolerance in diverse organisms. Within the LEA protein superfamily, group 4 members are characterized by a conserved N-terminal region and a structurally disordered C-terminal region that varies regarding length and amino acid content. Previous *in vitro* assays have suggested that the conserved N-terminal region shared by group 4 LEA proteins is critical for forming an amphipathic α -helix and protecting enzymatic activities from the adverse effects of desiccation or freezing. However, the cellular roles of the varying C-terminal region remain largely to be characterized. *Medicago truncatula* contains five subgroup LEA4B proteins encoding loci of which three are tandemly arranged on chromosome 7 due to local gene duplication events. In this study, abiotic stresses and addition of abscisic acid (ABA) induced the transcription of the four *LEA4B* genes. *Escherichia coli* cells overexpressing the three tandemly aligned *LEA* genes indicated significantly increased tolerance to salt, osmotic, heat, and freezing stresses. However, the extent of the protective effects on the survival and growth of bacterial cells differed among the LEA proteins, potentially because of variations in the C-terminal region. This possibility was further supported by the observation that the protective effects of the native truncated MtLEA3140, which only contains a conserved N-terminal region, were inferior to the effects of the full-length mutant version. The results suggest that the structurally flexible C-terminal region of group 4 LEA proteins plays roles in protecting cells from damages caused by various abiotic stresses, and provide clues for elucidating the mechanisms underlying the intracellular functions of these proteins.

Additional key words: abscisic acid, barrel medic, cold, C-terminal region, *Escherichia coli*, heat, late embryogenesis abundant protein, salinity.

Introduction

Late embryogenesis abundant (LEA) proteins were originally found during the late developmental stages of cotton seeds undergoing desiccation (Dure *et al.* 1981, 1989), but were subsequently observed to accumulate in plant vegetative organs in response to dehydration-induced stresses, such as drought, salinity, and cold, or to the addition of abscisic acid (ABA) (Battaglia *et al.* 2008, Hundertmark and Hincha 2008). Further identification of LEA suggested that these proteins are widely distributed

across archaeal, eubacterial, and eukaryotic domains, and are not exclusive to plants. Considerable evidence has suggested that LEA proteins are important for tolerance of diverse organisms to abiotic stresses (Battaglia *et al.* 2008, Hundertmark and Hincha 2008). Moreover, several lines of evidence proposed involvement of LEA protein in biotic stress responses (Salleh *et al.* 2012, Yang *et al.* 2012, Liu *et al.* 2013, Mota *et al.* 2019).

The LEA proteins have been divided into several groups based on their sequence similarities and conserved motifs, although different groupings and nomenclatures have been

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Abbreviations: ABA - abscisic acid; cDNA - complementary DNA; CFU - colony forming unit; GRAVY - grand average of hydropathy; IPTG - isopropyl β -D-1-thiogalactopyranoside; LB medium - Luria-Bertani medium; LEA - late embryogenesis abundant; PCR - polymerase chain reaction; PTC - premature termination codon; RT-PCR - reverse transcription PCR; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNP - single nucleotide polymorphism.

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used (Dure *et al.* 1989, Bray 1993, Dure 1993, Wise 2003, Battaglia *et al.* 2008). The classification of LEA proteins proposed by Battaglia *et al.* (2008) categorized LEA proteins into seven groups, of which group 4 comprises members characterized by a conserved N-terminal region with 70 - 80 amino acids tending to form an amphipathic α -helix as well as a structurally disordered C-terminal region that varies in length and amino acid content (Battaglia *et al.* 2008, Olvera-Carrillo *et al.* 2010, Cuevas-Velazquez *et al.* 2016). The group 4 LEA proteins were further divided into subgroup 4A and subgroup 4B. In *Arabidopsis thaliana*, there are three LEA 4 proteins, of which one belongs to subgroup 4A and two are members of subgroup 4B. The phenotyping of *Arabidopsis thaliana* mutants deficient in the group 4 LEA proteins and *LEA*-overexpressing transgenic plants indicated that these LEA proteins contribute to plant tolerance to water stress (Olvera-Carrillo *et al.* 2010). *In vitro* assays have revealed multiple functions for the group 4 LEA proteins, including alleviating dehydration- or freezing-induced membrane damage and protein inactivation, binding of sugar or metals, and scavenging of radicals (Reyes *et al.* 2005, 2008, Hundertmark *et al.* 2011, Liu *et al.* 2011, Dang *et al.* 2014). The results of *in vitro* assays suggest that the conserved N-terminal region of group 4 LEA proteins is critical for the conformational transitions induced by water deficit stress and for the protection of enzyme activities (Reyes *et al.* 2008, Cuevas-Velazquez *et al.* 2016). However, the potential roles of the variable C-terminal region *in vivo* remain largely unknown. Previous identification and our search against *Medicago truncatula* genome revealed five loci that encode group 4 LEA proteins. Of the five LEA proteins, three showing a highly conserved N-terminal region and a varying C-terminal region are encoded by tandemly aligned loci on chromosome 7 of *Medicago truncatula*, offering advantages for characterization of roles played by the C-terminal region of group 4 LEA proteins.

In this study, the three tandemly aligned group 4 LEA protein-encoding genes (*MtLEA3140*, *MtLEA3160*, and *MtLEA370*) in the *Medicago truncatula* genotype R108 will be isolated and overexpressed in *Escherichia coli* cells. The data presented herein may be useful for elucidating the effects of the structurally disordered C-terminal region of group 4 LEA proteins on intracellular functions in response to abiotic stresses.

Materials and methods

Plants, growth conditions, and treatments: Barrel medic (*Medicago truncatula* Gaertn.) genotypes Jemalong A17 (A17) and R108 were used for transcription profiling, while R108 was also used for the cloning of *LEA* genes. Seeds were treated with concentrated sulfuric acid for 10 min and then rinsed thoroughly with water. The scarified seeds were placed on wet filter paper in Petri dishes and incubated at a temperature of 4 °C under darkness for 3 d. The seeds were then incubated in a growth chamber at 21 °C, a 16-h photoperiod, an irradiance of

120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and a relative humidity of 70–75 % to induce germination. After 1 week, the seedlings were transplanted to *Vermiculite* and watered once a week with a solution containing 2 % *NutriSmart 302* fertilizer (CK *Life Sciences*, Hong Kong, China).

Seedlings grown in *Vermiculite* for 2 weeks were subjected to abiotic stresses and ABA treatments. For cold treatment, the seedlings were subjected to 4 °C. For dehydration, the seedlings were removed from the *Vermiculite* and dehydrated in air at 21 °C. Salinity and ABA treatments were carried out by transferring the seedlings to 200 mM NaCl solution or spraying with 50 μM ABA solution, respectively. Samples were collected at appropriate time points and immediately frozen in liquid nitrogen for extraction of total RNAs.

Reverse transcription PCR and transcriptional analysis of *MtLEA* genes: Total RNAs were extracted with *TRIzol* reagent (Gibco, Grand Island, NY, USA), followed by treatment with RNase-free DNase I (Takara, Dalian, China). First-strand cDNAs were synthesized using *M-MLV* reverse transcriptase (Sangon, Shanghai, China). The *MtLEA3140*, *3160*, and *3170* coding sequences were amplified by PCR using first-strand cDNAs from seedlings of R108 that underwent a 24-h cold treatment and the primer sets listed in Table 1 Suppl. with high-fidelity *Pyrobest* DNA polymerase (Takara). Amplicons were cloned into the *Sma*I site of the *pBluescript II SK(+)* vector (Stratagene, La Jolla, CA, USA) for sequencing confirmation. At least three independent clones for each of the genes were randomly chosen for sequencing. Sequence analysis of the *MtLEAs* was carried out with *DNAMAN* software (Lynnon Biosoft, Quebec, Canada).

To examine the effects of abiotic stresses and ABA on the transcription of *MtLEA* genes, first-strand cDNAs from genotype R108 and A17 were synthesized with the *PrimeScript* RT reagent kit (Takara). The subsequent PCR amplification was completed using the primer sets as described above. An actin gene (*Medtr7g026230*; Table 1 Suppl., Zhang *et al.* 2011) was included as an internal control. The PCR products were detected by 0.7 % (m/v) agarose gel electrophoresis.

Site-directed mutagenesis of *MtLEA3140* and heterologous expression of *MtLEAs* in *Escherichia coli*: *MtLEA3140* from genotype R108 cloned in *pBluescript II SK(+)* was used as a template for site-directed mutagenesis with the primer set listed in Table 1 Suppl. and the *QuikChange Site-Directed* mutagenesis kit (Stratagene). The mutagenesis replaced the codon responsible for the PTC (TAG) with CAG based on the corresponding full-length gene sequence in genotype A17. The resulting mutant sequence was named *MtLEA3140M*.

For the heterologous expression of *MtLEA* genes in *Escherichia coli* cells, the open reading frames of the three *MtLEA* genes and the *MtLEA3140M* (mutant version of *MtLEA3140*) were ligated into the *Bam*HI/*Sac*I sites of *pET-30a* (Novagen, town, Germany). The resulting plasmids were introduced into *E. coli* BL21 (DE3) cells (Novagen). Recombinant protein production was induced

by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG; final concentration of 0.5 mM) into bacterial cultures at the exponential growth stage. The cultures were subsequently incubated at 37 °C for 3 h, after which 1 cm³ of culture aliquots were centrifuged at 12 000 g for 1 min. The pellets were resuspended in 0.1 cm³ of 1 $\times$ sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer followed by incubation in boiling water for 10 min. The boiled samples were centrifuged at 12 000 g for 10 min at room temperature, and the supernatants were analyzed by 12 % (m/v) SDS-PAGE. To examine the heat tolerance of MtLEA proteins, the pellets of IPTG-induced cultures were resuspended in 0.08 cm³ of double-distilled water instead of SDS-PAGE

sample buffer and then incubated in boiling water for 30 min. After centrifugation at 12 000 g for 10 min, the supernatants of the boiling water extracted samples were collected for preparation of SDS-PAGE samples. The identities of the recombinant MtLEA proteins were detected by Western blotting using an *anti-His* $\times$ 6 antibody (Boster, Wuhan, China) and an enhanced chemiluminescence kit (Yeasen, Shanghai, China).

Analysis of the abiotic stress tolerance of *Escherichia coli* cells overexpressing MtLEAs: To determine the effects of MtLEA proteins on the tolerance of *E. coli* cells to abiotic stresses, IPTG-induced cultures were diluted with liquid Luria-Bertani (LB) medium to an absorbance

Fig. 1. Sequence analysis of group 4 late embryogenesis abundant (LEA) proteins from *Medicago truncatula* and *Arabidopsis thaliana*. **A** - Alignment of amino acid sequences of LEA proteins. *M. truncatula* LEA protein sequences were deduced from the genotype A17 reference genome. The conserved N-terminal regions of group 4 LEA proteins are *underlined*. The conserved amino acid residues in the C-terminal regions of MtLEA3170 and AtLEA4-5 are *framed*. **B** - Phylogenetic tree presenting the two subgroups of group 4 LEA proteins.

LEA3140-A17	MLGGAKKTIGESIKETAANIGASAKSGMEKTKATI QEKTERMTAHDEIQKEMATCKKKEERV	60
LEA3140-R108	MLGGAKKTIGESIKETAANIGASAKSGMEKTKATI QEKTERMTAHDEIQKEMATCKKKEERV	60
LEA3140M-R108	MLGGAKKTIGESIKETAANIGASAKSGMEKTKATI QEKTERMTAHDEIQKEMATCKKKEERV	60
LEA3160-A17	MQGGAKKTIGESIKETAANIGASAKSGMEKTKATE QEKTERMTAHDEIQKEMATCKKKEERV	60
LEA3160-R108	MQGGAKKTIGESIKETAANIGASAKSGMEKTKATE QEKTERMTAHDEIQKEMATCKKKEERV	60
LEA3170-A17	MQGAKKAGETIKETAANIGASAKSGMEKTKATI QEKTERMTARDEVQKEMATEKKEAKM	59
LEA3170-R108	MQGAKKAGETIKETAANIGASAKSGMEKTKATI QEKTERMTARDEVQKEMATEKKEAKM	59
←		
LEA3140-A17	NQAELDKIAAREHNAAATTGHQLGQ ...GGHHTSGTGGAQTRRQFGL.....	105
LEA3140-R108	NQAELDKIAAREHNAAATTGHQLG*	84
LEA3140M-R108	NQAELDKIAAREHNAAATTGHQLGQ ...GGHHTSGTGGAQTRRQFGL.....	105
LEA3160-A17	NQAELDKIAAREHNAAASAGHQLGV ...GGHHTSGTGGAQTRRQFGL.....	101
LEA3160-R108	NQAELDKIAAREHNAAASAGHQLGV ...GGHHTSGTGGAQTRRQFGL.....	101
LEA3170-A17	NQAELDKIAAREHNAAVKQTTAAAGHMGQPHHTTGTTGTGTATYSTTGNYGHTGAHQCM	119
LEA3170-R108	NQAELDKIAAREHNAAVKQTTAAAGHMGQPHHTTGTTGTGTATYSTTGNYGHTGAHQCM	119
→		
LEA3140-A17		105
LEA3140-R108		84
LEA3140M-R108		105
LEA3160-A17		101
LEA3160-R108		101
LEA3170-A17	SAMPGHGTGQPTGHVVDGVVGSHPFIGINRGTDGTATAHNTRVGCNENATGYTTGGTYK	177
LEA3170-R108	SAMPGHGTGQPTGHVVDGVVGSHPFIGINRGTDGTATAHNTRVGCNENATGYTTGGTYK	177

Fig. 2. Sequences of three MtLEA4B proteins encoded by tandemly aligned loci in *Medicago truncatula* genotypes A17 and R108. The conserved N-terminal regions are indicated with a double-arrowed line. The premature termination codon introduced by a single nucleotide polymorphism is indicated by an asterisk, while amino acid substitutions are framed. *MtLEA3140M-R108*, which is the site-directed mutant version of *MtLEA3140* from R108, is included.

(A_{600}) of 0.9. The cultures were then serially diluted 10-fold in liquid LB medium. For salinity and dehydration treatments, 10 mm³ of serially diluted cultures were dotted to kanamycin (50 mg dm⁻³) and IPTG (0.5 mM) contained agar-solidified LB plates with addition of 500 mM NaCl or KCl (salinity), or 500 mM mannitol (osmotic stress). After incubation at 37 °C for 48 h, the colony growth on the plate was photographed with a digital camera. Heat and freezing treatments were performed by incubating the serially diluted IPTG-induced cultures at 55 °C for 30 min and freezing at -20 °C for 12 h, respectively, followed by dotting 10-mm³ aliquots of the heat or freezing treated cultures to kanamycin contained LB plates for incubation at 37 °C overnight. To quantify the colony growth, the serially diluted cultures were spread over LB plates, and the resulting colonies were counted. Colony survival or growth was determined by calculating the relative colony-forming units (CFUs) [%] = (colony number in stressed cultures / colony number in unstressed cultures) × 100. Data for each of abiotic stress treatments were analyzed with a one-way ANOVA followed by Duncan's multiple test at $P < 0.01$.

Results

A previous genome-wide survey revealed four *M. truncatula* group 4 LEA members that were divided

into two subgroups (Battaglia and Covarrubias 2013). One member (locus *Medtr4g098480*) belonging to subgroup 4A encodes a relatively varying amino acid sequence (Fig. 1). The remaining four members of subgroup 4B, encode amino acid sequences with a highly conserved N-terminal region and a C-terminal region that varies regarding amino acid sequence or length (Fig. 1). Here, further search against genome of *Medicago truncatula* found an additional subgroup 4B member encoded by the locus of *Medtr1g053970* (Fig. 1). Of the four subgroup 4B member loci, there are three occurring as tandemly aligned loci on chromosome 7, *Medtr7g093140*, *Medtr7g093160*, and *Medtr7g093170* that are denoted herein as *MtLEA3140*, *MtLEA3160*, and *MtLEA3170*, respectively.

Three tandemly arranged LEA genes from genotype R108 were cloned and sequenced. In comparisons with the corresponding gene sequences in the genotype A17 reference genome, three, two, and one SNPs were detected in *MtLEA3140*, *MtLEA3160*, and *MtLEA3170*, respectively (Fig. 1 Suppl.). In *MtLEA3140*, one of the SNPs (C-to-T transition) generates a premature termination codon (PTC), while the remaining two transversions (*i.e.*, A-to-C and T-to-A) result in amino acid substitutions (*i.e.*, Thr to Pro and Ser to Thr, respectively) (Fig. 2 and Fig. 1 Suppl.). Additionally, one of the two G-to-A transitions in *MtLEA3160* is responsible for an amino acid substitution (Ala to Thr), while the other transitions (G-to-A in *MtLEA3160* as well as C-to-T in *MtLEA3170*) do not

Fig. 3. The reverse transcription PCR detection of transcription of *MtLEA4B* genes in *Medicago truncatula* genotype A17 and R108 in response to abiotic stresses and abscisic acid.

result in amino acid substitutions because of their in-frame synonymous substitutions (Fig. 2 and Fig. 1 Suppl.).

The deduced amino acid sequences indicated that the estimated molecular masses for MtLEA3160 and MtLEA3170 were 10.6 and 18.1 kDa, respectively, whereas the grand average of hydropathy (GRAVY) indices were -1.067 and -0.864, respectively. Because of the presence of a PTC, *MtLEA3140* from genotype R108 encodes a truncated protein consisting of 84 amino acids, with an estimated molecular mass of 9.0 kDa. This is in contrast to the corresponding full-length mutant version, which comprises 105 amino acids, with an estimated molecular mass of 10.0 kDa. The GRAVY indices of the native truncated MtLEA3140 from R108 and its full length mutant version MtLEA3140M (Fig. 2) are both -1.036.

To determine the transcriptional responses of *MtLEA3140*, *MtLEA3160*, *MtLEA3170* and the locus of *Medtr1g053970* to abiotic stresses, *M. truncatula* seedlings were treated with salinity, desiccation, heat, cold, and ABA. The resulting transcription patterns of the four *LEA4B* genes were analyzed by RT-PCR. The transcription of these four genes from both genotypes A17 and R108 was apparently induced by the abiotic stresses and ABA in a time-dependent manner (Fig. 3), implying that the genes are involved in responses to various abiotic stresses. Moreover, no diurnal transcription oscillations of

the *MtLEA* genes were detected under normal condition (Fig. 2 Suppl.), eliminating the effect of photoperiod or circadian rhythm on their expression patterns. Most LEA proteins remained soluble after being heated because they are intrinsically unstructured (Receveur-Bréchet *et al.* 2006, Rivera-Najera *et al.* 2014). To assess the stability of heat-treated LEA proteins, the three *MtLEA* proteins encoded by the tandemly aligned loci on chromosome 7 from genotype R108 and a full-length mutant version of *LEA3140*, *LEA3140M* (Fig. 2), were overexpressed in *E. coli* cells. The IPTG treatment of bacterial cultures with cells carrying the *LEA*-expression vectors resulted in an apparently extra band in the SDS-PAGE gels while a weak band corresponding to each of the recombinant proteins was also detected in the cultures without addition of IPTG due to leaking expression of the *MtLEAs* (Fig. 4A). The apparent molecular masses of the recombinant LEA proteins were higher than the estimated molecular masses (Fig. 4) because of the high hydrophilicity or disordered structure characterized by most hydrophilins, including LEA proteins (Receveur-Bréchet *et al.* 2006, Rivera-Najera *et al.* 2014). However, LEA3160 exhibited slightly greater retarded migration during the SDS-PAGE analysis than LEA3140M (Fig. 4), even though its estimated molecular mass is lower than that of LEA3140M. Sequencing of *LEA3160* and *LEA3140M* in pET-30a plasmid eliminated

Fig. 4. Heterologous expression of *MtLEAs* in *Escherichia coli* and the heat tolerance of the recombinant proteins. *A* - Total proteins from *E. coli* cultures without (N) and with the addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) (I). The bands of the recombinant *MtLEAs* were indicated by white or black asteroids, and further detected with Western blotting (WB) by using of an anti-His $\times$ 6 antibody. *B* - SDS-PAGE sample buffer (C) and boiling water (B) extracted crude proteins from IPTG induced *E. coli* cultures.

the possible experimental mistakes resulting in the unexpected electrophoretic migration. Western-blotting detection of the His-tag fusing at the N-terminal ends of the recombinant LEA proteins further confirmed the identities of the recombinant proteins (Fig. 4A). Additionally, a weak band with an apparent molecular mass of 21.3 kD was also detected in IPTG-induced LEA3170 sample by Western blotting (Fig. 4A), implying presence of other structurally differed forms of the LEA protein. As expected, incubation of the *E. coli* cultures in boiling water for 30 min did not result in apparent decrease in the abundance of soluble recombinant proteins, but did cause the abundance of *E. coli* endogenous proteins to decrease (Fig. 4B).

Bacterial and yeast cells have been widely used to determine the effects of LEA proteins on abiotic stress tolerance (e.g., Hu *et al.* 2016, Saucedo *et al.* 2017). In this study, we investigated the survival and growth of *E. coli* cells overexpressing *MtLEA* proteins under abiotic stresses. The dot assay and CFU analysis indicated that all the tested *MtLEA* proteins significantly enhanced the tolerance of *E. coli* cells to salt, osmotic, heat, and freezing stresses (Fig. 5). However, the extent of the

tolerance differed among the *MtLEA* proteins (Fig. 5B), with LEA3160 providing the greatest protection against abiotic stresses (Fig. 5B). The survival and growth rates were higher for the *E. coli* cells producing the mutated full-length LEA3140M than that of its corresponding native truncated LEA3140 of genotype R108 under abiotic stresses (Fig. 5B). Thus, the 21 amino acids at the C-terminal of LEA3140M (Fig. 2) are important for the protection of *E. coli* against abiotic stresses. Moreover, cells producing LEA3170, which has the longest C-terminal region of the tested LEA proteins (Fig. 2), exhibited similar tolerance to abiotic stresses as those of cells producing LEA3140, with the exception of an insignificantly higher relative CFUs under NaCl and freezing stresses (Fig. 5B).

Discussion

The group 4 LEA proteins have been phylogenetically divided into subgroups 4A and 4B (Battaglia *et al.* 2008, Olvera-Carrillo *et al.* 2010). In *A. thaliana*, the varying lengths of the C-terminal region and the partially

Fig. 5. Abiotic stress tolerance of *Escherichia coli* cells overexpressing *MtLEAs*. A - Dot assays. B - Colony-forming unit investigations. Triplicates were averaged and bars indicate standard errors. Different upper case letters indicate statistically significant differences of protective effects among *MtLEAs* under each of the abiotic stress treatments according to the Duncan's multiple test ($n = 3$, $P < 0.01$).

overlapping transcription patterns of the members of subgroups 4A and 4B in response to abiotic stresses suggest their function diversification (Olvera-Carrillo *et al.* 2010). The *M. truncatula* genome includes one subgroup 4A member as well as four subgroup 4B members. Of the subgroup 4B, three members are located at tandemly aligned loci on chromosome 7, as described previously (Battaglia *et al.* 2008) and in this study (Fig. 1). The *M. truncatula* subgroup 4A member, encoded by the *Medtr4g098480* locus, differs somewhat from the *A. thaliana* LEA 4A proteins (Fig. 1), implying the likelihood of species- or genus-specific diversification during evolution that needs to be confirmed in future studies. However, the *A. thaliana* and *M. truncatula* subgroup 4B proteins possess a highly conserved N-terminal region, which is considered to be a signature of group 4 LEA proteins (Fig. 1A). MtLEA3170 and AtLEA4-5 share a relatively long and conserved C-terminal region that differs from the very short C-terminal region of MtLEA3140 and MtLEA3160 (Fig. 1A). Therefore, MtLEA3170 represents an ancient subgroup 4B member of *M. truncatula*, from which MtLEA3140, MtLEA3160, and Medtrlg053970 arose through gene duplication events. The diverse abiotic stresses and ABA induced expression patterns of the four *M. truncatula* subgroup 4B LEA genes (Fig. 3) suggest their roles in response to various abiotic stresses. However, the transcript of the *M. truncatula* subgroup 4A member encoded by the *Medtr4g098480* locus accumulated in response to high salinity and dehydration, while cold stress and ABA did not appear to affect its transcription (Fig. 3 Suppl.). These results further support the previously suggested functional divergence between the two LEA4 subgroups in plants (Battaglia *et al.* 2008, Olvera-Carrillo *et al.* 2010).

The heterologous expression of LEA genes in bacterial or yeast cells is an alternative to the time-consuming and labour-intensive generation of transgenic plants for evaluating the effects of LEA proteins on abiotic stress tolerance. Heterologous expression systems may be more useful than the relatively simple *in vitro* assays for clarifying the cellular functions of LEA proteins. *In vitro* assays have confirmed that the conserved N-terminal region of Group 4 LEA proteins is necessary and sufficient for the formation of an amphipathic α -helix and protection of enzymatic activities under water deficit or freezing (Cuevas-Velazquez *et al.* 2016, Reyes *et al.* 2008). However, the evolutionary meaning and *in vivo* functions of the varying C-terminal region remain relatively uncharacterized. Researchers have speculated that the disordered C-terminal region or terminus may expose some amino acids, such as histidine, which bind to metal ions (Cuevas-Velazquez *et al.* 2016, 2017). A recent biophysical characterization of metal ion binding properties suggested that the C-terminal region of AtLEA4-5 could function as an antioxidant and a metal ion scavenger under abiotic stresses (French-Pacheco *et al.* 2018). In this study, we compared protective effects of the three subgroup 4B LEA proteins from *M. truncatula* on survival and growth of *E. coli* cells under salt, osmotic, heat, and freezing stresses. Despite a previous report describing the bacterial growth inhibition associated

with some *A. thaliana* LEA proteins, including LEA4-5 (LEA-D113) (Campos *et al.* 2006), the overexpression of MtLEA proteins did not obviously affect bacterial cell growth under normal conditions in this study (Fig. 5A). This discrepancy is likely because of differences in the *E. coli* genotypes used in the two studies, although subtle variations in the proteins, such as the extra 7–8 amino acid residues in the C-terminal region of MtLEAs compared to AtLEA4-5, may contribute to the differences in bacterial growth. Bacterial cells overexpressing MtLEA proteins were more viable and survived better than the control cells harbouring an empty vector under abiotic stress treatments (*i.e.*, hyper-osmolarity, high salinity, freezing, and heat) (Fig. 5). Nevertheless, bacterial cells producing MtLEA proteins with different C-terminal regions exhibited varying degrees of tolerance against abiotic stresses (Fig. 5), which was in contrast to the results of previous *in vitro* assays (Reyes *et al.* 2008, Cuevas-Velazquez *et al.* 2016). Although MtLEA3170 possesses the longest C-terminal region (Figs. 1 and 2) among the three LEA proteins tested here, its protective effect was similar to that of the native truncated MtLEA3140 from genotype R108, which contains the shortest C-terminal region besides with the conserved N-terminal region. However, the highest abiotic stress tolerance observed in this study was conferred by MtLEA3160, which has a very short C-terminal region (Fig. 5). These results suggest the varying C-terminal region is important in protecting organisms against abiotic stresses whereas the possible effects of the variation of the several amino acid residues needs to be confirmed. This suggestion was further supported by the superior protective effects of the full-length mutant MtLEA3140M over the truncated native MtLEA3140 (Fig. 5). Moreover, the results obtained in this study also imply more complicated mechanisms than what have been proposed previously (Cuevas-Velazquez *et al.* 2016, 2017) in case of the cellular roles played by the C-terminal regions of group LEA4 proteins. Additionally, it is worthy to be noted here that MtLEA3160 exhibited slightly slower electrophoretic migration than LEA3140M (Fig. 4), even though its estimated molecular mass was lower than that of LEA3140M. This result might be due to LEA3160 having a higher hydropathicity or a more flexible structure than LEA3140M. Whether the speculated reasons for the unexpected electrophoretic migration of LEA3160 are associated with the fact that LEA3160 provided the greatest protection against abiotic stresses here (Fig. 5) should be elucidated in future studies.

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