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Research Paper

Abiotic Stress Response of Transgenic *Arabidopsis* Overexpressing *Brassica napus* Group 1 *LEA* Genes

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Group 1 late embryogenesis-abundant (LEA) proteins are highly hydrophilic group of proteins with a characteristic internal 20-mer amino acid sequence. In *Arabidopsis*, group 1 LEA (*AtEm1* and *AtEm6*) are expressed at very high levels in seeds during maturation phase, and are necessary for desiccation tolerance of seeds. These genes are also induced by stress hormone ABA in seedlings during early seedling establishment phase. Since these LEA proteins are stored in seeds and also accumulate during seedling establishment, they may impart desiccation tolerance to the germinating seedlings. To examine whether these proteins play a role during seedling establishment under stress conditions, we cloned group 1 *LEA* genes namely *BnEm6* and *BnEm1* from *Brassica napus* and overexpressed in *Arabidopsis* plants. BnEm6 and BnEM1 LEA proteins possess one and four tandem repeats of "20-mer conserved sequence motif", respectively. A semi-quantitative RT-PCR analysis showed that these genes are expressed in seeds. Abiotic stress tolerance of transgenic *Arabidopsis* overexpressing *BnEm1* and *Em6* under constitutive *CaMV35S* promoter were evaluated during germination and seedling establishment. These transgenic plants showed significantly higher total root length and surface area as compared to wild-type plants under 200 mM mannitol stress. Under salt stress also, root length was higher in transgenic plants as compared to wild-type plants. These results suggest that LEA1 proteins may probably play a role in root growth under osmotic stresses during seedling establishment.

Key Words: *Brassica napus*; LEA1; Osmotic Stress; Root Length

Introduction

Late Embryogenesis Abundant (LEA) proteins are hydrophilic proteins that accumulate to high levels during the desiccation stage of seed maturation. Later, these proteins were also found to express in vegetative tissues in response to ABA and abiotic stresses [1, 2, 3]. LEA proteins are ubiquitous in plants, fungi, microbes, nematodes and shrimp [4]. Based on sequence similarity and conserved domains, LEA proteins have been classified into nine groups [5, 2]. These proteins are diverse in structure and show different sub-cellular localization and tissue-specific expression patterns. Based on their protein structure, physio-chemical and biological properties, several functions have been proposed for LEA proteins. These functions include water binding, cryoprotection, membrane stabilization, osmoprotection and prevention of protein aggregation under stresses [6, 7, 8, 9].

The LEA proteins accumulate primarily during embryo development and seed maturation and are generally associated with desiccation tolerance of seeds. In addition to seeds, some of the *LEA* genes also express in vegetative tissues in response to

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abiotic stresses and ABA. There are about 51 *LEA* genes in *Arabidopsis* genome, of which 22 genes showed high constitutive expression in non-seed organs. Among these, 12 genes showed more than 3 fold induction under stress conditions [10]. Moreover, *LEA* genes of the same group do not show identical expression profile suggesting that these proteins have non-redundant functions, and regulated by different signalling pathways [2]. Their wide occurrence, differential expression, and regulation emphasize the need for a comprehensive analysis for these proteins belonging to different groups. Among the LEA groups, ectopic expression of Group 3 and 4 LEA proteins in vegetative tissues have been shown to confer tolerance to various abiotic stresses in different plant species such as wheat, cabbage, rice and *Arabidopsis* [3, 11, 12, 13, 14], while the role of other groups of LEA proteins in conferring abiotic stress tolerance remains to be examined.

The group 1 LEA proteins, also called as Em (Early methionine labelled) proteins, were first identified in embryo of mature wheat [15]. Since then, several Em proteins have been identified from various plant species. Group 1 LEA proteins are encoded by two Em genes, *AtEm1* and *AtEm6* in *Arabidopsis*. Both of these genes express during later stages of seed development [16, 17], but not in mature leaf, stem or floral buds [16]. However, they express during germination and are induced by plant stress hormone ABA during early seedling establishment [18]. In seeds the *Em* gene expression is regulated by ABA-dependent transcription factors *ABI3* and *ABI5* [19, 20]. An insertional mutant of *AtEm6* gene of *Arabidopsis* displayed premature dehydration of seeds at the distal end of the silique. It was suggested that *AtEm6* is probably involved in water retention/ controlled desiccation during embryo maturation, and thus required for normal seed development and desiccation tolerance [21, 22]. Since, group 1 LEA proteins are expressed during seed maturation and stored in seeds, and also expressed in developing seedling; we hypothesized that they may play a role in abiotic stress tolerance during germination and seedlings establishment. Therefore, to analyze the role of group 1 LEAs in abiotic stress tolerance, we developed transgenic *Arabidopsis* plants ectopically expressing *Brassica napus* group 1 *LEA* genes and evaluated their osmotic stress tolerance. Our study suggests that group 1 LEAs may play a role in root growth under osmotic stress during seedling establishment.

Material and Methods

Plant Material and Treatments

For expression analysis of *LEA1* genes, seedlings of *B. napus* cv. Elect, were grown in soil medium in natural field conditions. One-month-old seedlings were irrigated with half strength Hoagland solution supplemented with 200 mM NaCl or 200 mM mannitol for imposing salt and osmotic stresses, respectively. For ABA treatment, 100 μM ABA in 0.02% Tween-20 was sprayed on the seedlings. For cold treatment, one-month-old seedlings in soil medium were incubated at 4°C under continuous illumination of 100 μmol m^{-2} s^{-1} PAR. For all abiotic stress treatments, leaf samples were harvested after 6 h of treatment. To study the expression of *LEA1* genes in flowers, the stem of the inflorescence from field grown plants of *B. napus* were excised and base of the stem was immersed in half-strength Hoagland solution containing 200 mM NaCl and incubated in a growth chamber at 24°C and 100 μmol m^{-2} s^{-1} PAR for 6 h. For all the experiments, seedlings or inflorescence treated with half strength Hoagland solutions were used as control.

Isolation and Sequence Characterization of BnEm1 and BnEm6

Based on the nucleotide sequence similarity of *AtEm1* (At3g51810) and *AtEm6* (At2g40170), homologous ESTs from *B. napus* (CD830410 and CD834253) were identified. To clone *Em* genes from *B. napus*, the following primers were designed and used: BnEm6-F: 5′-CGGGATCCATGGCTTCTCAA CAGGAGAAG-3′ and BnEm6-R: 5′-CCGA GCTCTTAAGTC CTGGTCCTGGATGTG-3′ and BnEm1-F 5′-CGGGATCCATGGCGTCAAAGC AACAAAGC-3′ and BnEm1-R: 5′-CCGAGC TCTCACTTGTTGGTGAACTTGGAC-3′. The forward and reverse primers had *BamHI* and *SacI* restriction sites at their 5′ end (underlined). Total RNA

was extracted from the seed tissues by using RNeasy plant mini kit (Qiagen, Germany). One μg total RNA was used for RT-PCR by using Qiagen one-step RT-PCR kit (Qiagen, Germany) following the manufacturer's protocol. The 276 bp and 459 bp amplicons obtained by RT-PCR were cloned in pGEMTeasy vector (PROMEGA). These clones were sequenced and the sequences were submitted in GenBank. Pair-wise sequence alignments were examined by using CLATALW [http://www.ebi.ac.uk/Tools/msa/clustalw2/]. For amino acid sequence alignment, Multalin (http://multalin.toulouse.inra.fr/multalin/multalin.html) was used, and for protein localization analysis, PSORT [http://psort.ims.u-tokyo.ac.jp/] program was used. Pfam domain analysis was carried out by using SMART [http://smart.embl-heidelberg.de/smart/set_mode.cgi].

Expression Analysis of BnEm1 and BnEm6 Genes

Total RNA was extracted from mature seeds, control, ABA and stress-treated leaves and flowers using RNeasy plant mini kit (Qiagen, Germany). In RT-PCR reaction, along with 0.6 mM each of *LEA1* forward and reverse primers, 0.2 mM each of the following tubulin-specific primers were used that served as internal control: Tubulin-F: 5′-CAGCAATACAGTG CCTTGAGTG-3′ and Tubulin-R: 5′-CCTGTG TACCAATGAAGGAAAGCC-3′. For expression analysis of *BnEm6*, instead of multiplexing, RT-PCR for tubulin was carried out in separate reaction mixture having same amount of RNA. PCR conditions were as follows, initial denaturation for 4 min at 94°C, 30 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, followed by 72°C for 5 min. The RT-PCR amplicons were analyzed by electrophoresis on 1.5% agarose gel. Expression of *Em* genes in different tissues of *Arabidopsis* was analysed using Genevestigator [23]. Further, ESTs of oil seeds Brassicas were searched for the expression of *Em1* and *Em6* to find if they express during germination.

Over Expression of BnEm1 and BnEm6 in Arabidopsis

BnEm6 and *BnEm1* were PCR-amplified from *pGEMT-BnEm1* and *pGEMT-BnEm6* construct with the gene specific forward and reverse primers, restricted with *Bam*HI and *Sac*I, and cloned into modified binary vector pCAMBIA1200-*P35S*::*GUS*::*NOS* by replacing *GUS* with *BnEm1* and *BnEm6*, respectively. These binary vectors were transformed into *Agrobacterium* strain *LBA4404* and were used for transformation of *Arabidopsis thaliana* ecotype Columbia by floral dip transformation method [24]. The T1 plants were selected on hygromycin (30 μg ml^{-1}). The T1 plants were confirmed by PCR. T2 lines which showed 3:1 segregation for hygromycin resistance were carried forward to T3 generation. Presence of the transgene in transgenic *Arabidopsis* was confirmed by PCR and RT-PCR using gene specific forward primers and reverse primers. The confirmed homozygous T3 and T4 lines were used for functional validation of *BnEm1* and *BnEm6*. To assess osmotic and cold stress tolerance of transgenic *Arabidopsis* overexpressing *BnEm1* and *BnEm6*, the transgenic and wild type seedlings were transplanted on MS media supplemented with 150 mM NaCl or 200 mM mannitol, and grown on this media for three weeks in culture room under a continuous illumination of 100 μmol m^{-2} s^{-1} light intensity. Photographs were taken after two weeks to record the differences in growth. The roots were scanned by using a root scanner and the root traits were measured by using WinRhizo software (Regent Instruments Inc., Canada). The root trait data were subjected to T-test.

Results and Discussion

Isolation and Sequence Characterization of BnEm1 and BnEm6

The homologs of *AtEm1* and *AtEm6* cloned from *B. napus* in this study are named as *BnEm1* (*BnLEA1b*, GenBank accession No. DQ178983) and *BnEm6* (*BnLEA1a*, GenBank accession No. DQ178982). The coding sequences of *BnEm1* and *BnEm6* consist of 459 bp and 276 bp, respectively. *BnEm1* and *BnEm6* show highest similarity with *Arabidopsis* Group 1 LEA, *AtEm* 1 (93%) and *AtEm* 6 (83%), respectively (Figs. 1a and b). The deduced amino acid sequences of *BnEm1 and BnEm6* share 59% similarity with each other, and shares about 71 and 63 % similarity with

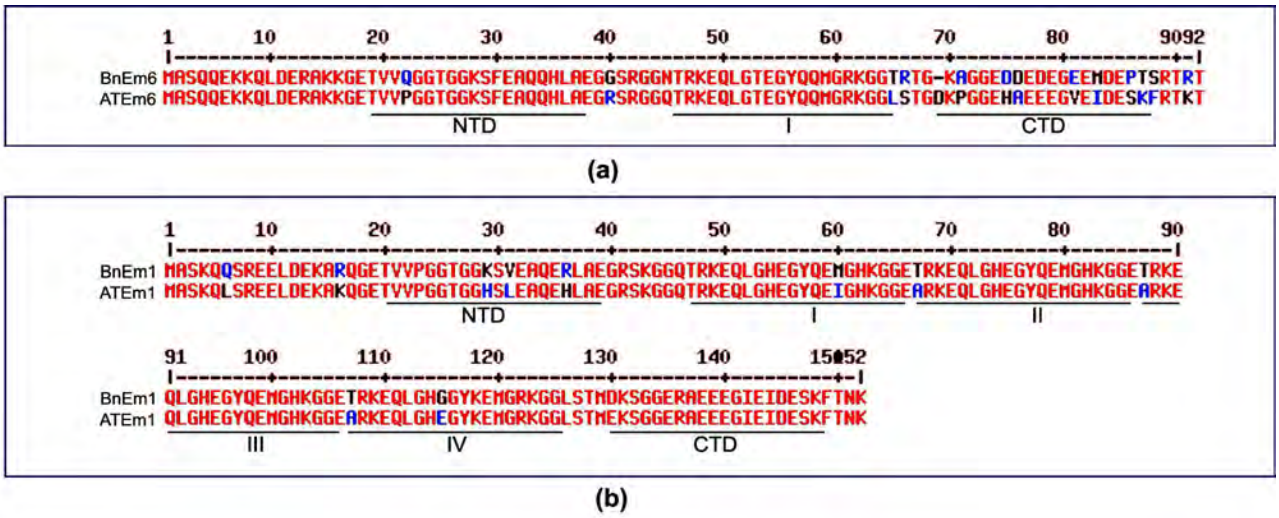

Fig. 1: Sequence alignment of group 1 LEA proteins from *Brassica napus*. Comparison of deduced amino acid sequences of (A) *BnEm6* with *ATEm6* and (B) *BnEm1* with *ATEm1*, group 1 LEA from *A. thaliana*. NTD; N terminal domain motif, CTD; C terminal domain motif, Roman numerals I, II, III, and IV denote 20 amino acid motifs in LEA protein

soybean Group 1 LEA (GmD-19) protein, respectively. *BnEm6* encodes a polypeptide (91 amino acids) with a predicted molecular mass of 9.77 kDa and p*I* of 5.23, while *BnEm1* encodes a polypeptide (152 amino acids) with a predicted molecular mass of 16.67 kDa and p*I* of 6.02. The domain analysis confirmed the presence of LEA_5 domain (pfam00477) in both the proteins. *BnEm6* contains one LEA_5 domain (from 1 to 87 amino acids), while *BnEm1* contains two LEA_5 domains (from 1 to 86, and 82 to 149 amino acids). Group 1 LEAs are characterized by the presence of a conserved hydrophilic 20 amino acid signature motif (TRKEQ[L/M]G[T/E]EGY[Q/K]EMGRKGG[L/E]) (4). Analysis of protein sequence of these BnEm proteins revealed that BnEm6 has one such 20 amino acid motif, while BnEm1 has four tandem repeats of 20-amino acid motif. Both of these proteins also contain two other conserved motifs, an N-terminal motif (TVVPGGTGGKSLEAQE[H/N]LAE) located just upstream of the 20-mer and a C-terminal motif (D[K/ E]SGGERA[A/E][E/R]EGI[E/D]IDESK[F/Y] as described by Battaglia *et al*. [4]. Another characteristic of group 1 LEA proteins is their high glycine content (~20%) (4). Both of these proteins have a high proportion of Glu and Gly residues. BnEm6 consists of 14.3% and 19.8% Glu and Gly residues, while BnEm1 consists of 17.8% and 21.1% Glu and Gly residues, respectively. Thus, both of these proteins conform to the characteristics of group 1 LEA proteins. PSORT analysis predicted cytoplasmic localization for both of these proteins. The hydrophilic and high degree of random coil structure of group 1 LEA proteins suggested that these proteins may serve as water-binding proteins and act as hydration buffers to regulate water status, and thus may contribute to seed viability by preventing total cellular desiccation [22, 25]. The Grand average of hydropathicity (GRAVY) value for BnEm6 and BnEm1 was –1.607 and –1.577, respectively. This showed that BnEm proteins are highly hydrophilic in nature.

1.1 Expression of BnEm1 and BnEm6

In plants, group 1 LEA proteins preferentially accumulate during embryo development and seed maturation [16, 17, 26]. In contrast to *LEA* genes of other groups, group 1 *LEA* genes are mainly expressed in embryonic tissues, but not induced in vegetative tissues treated with ABA [26]. In our study also it was found that both the group 1 LEA genes of *B. napus* expressed exclusively in mature seeds (Fig. 2). Expression of these genes was undetectable in rosette leaves (30 days after germination) and flower

neither in control nor in osmotic stress conditions (data not shown). Previous studies showed that ABA induces expression of *Em* genes in the roots and shoots of developing seedlings [17, 27]. Hence, we also examined the expression of *AtEm* genes in Arabidopsis by using Genevestigator [23]. It was found that both of the *AtEm* genes are expressed at highest level in seeds, while a medium level of expression was observed in developing seedlings (Fig. 2C). In BLAST search of *B. napus* ESTs, we found the ESTs of *Em1* and *Em6* in germinating seeds. These results suggest that these genes express in seedlings and play a role during seedling establishment.

3.3. Ectopic Overexpression of BnEm1 and BnEm6 Enhances Root Traits Under Osmotic Stresses in Transgenic Arabidopsis

Since ABA induces *AtEm* genes during seedling development phase in *Arabidopsis*, and heterologous expression of wheat *Em* gene conferred osmotic stress tolerance in yeast [7], we studied the role of group *Em* genes in abiotic stress tolerance of transgenic *Arabidopsis* plants overexpressing *BnEm6* and *BnEm1* (Fig. 3). RT-PCR analysis showed that both *BnEm1* and *BnEm6* express constitutively in transgenic *Arabidopsis* seedlings (Figs. 3d & 3e). These transgenic plants were compared with wild type *Arabidopsis* under osmotic and cold stresses. The stress tolerance was assessed at both germination as well as early seedling development stages. There was no difference in germination percentage between wild type and transgenic seeds on MS, 100 mM NaCl or 150 mM mannitol. Previous study on *Atem6* mutant also showed that loss of function of *AtEm6* did not affect seed germination percentage either in normal or PEG-imposed osmotic stress conditions [22]. Our results also suggest that BnEm proteins do not contribute to germination under osmotic stress conditions. For evaluation of abiotic stress tolerance during seedling establishment, wild type and transgenic seeds were germinated on MS medium. The 6 day old seedlings were then transplanted on MS medium (control), MS + 150 mM NaCl, and MS + 200 mM mannitol and allowed to grow for 3 weeks. Transgenic plants exhibited better root growth as compared to wild type plants under osmotic stress (Fig. 4). At 200 mM mannitol stress, there was a significant increase in both total root length (sum of length of all roots of a plant) and surface area of both *Em1* and *Em6* transgenic plants as compared to the wild type plants (Figs. 5 & 6). Whereas under salt stress, root length of the transgenic plants exhibited significant increase but surface area remained similar

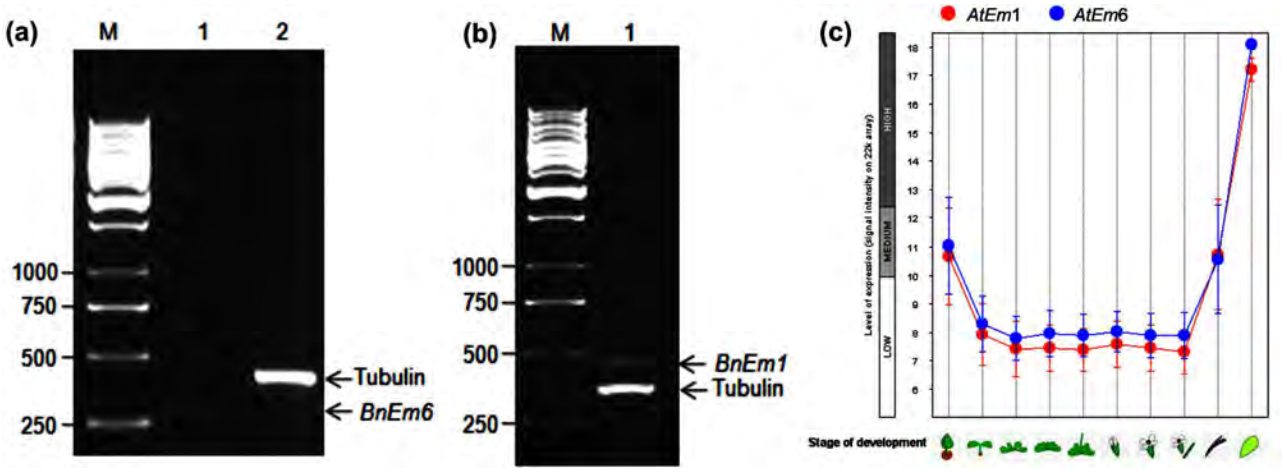

Fig. 2: Expression analysis of *BnLEA1* genes. (A) Expression of *BnEm6* and (B) *BnEm1* in mature seeds of *Brassica napus* (Tubulin was used as reaction control. M, 1kb molecular weight marker). (C). Expression pattern of *AtEm1* and *AtEm6* during development in *Arabidopsis* (Expression data were obtained by using Genevestigator)

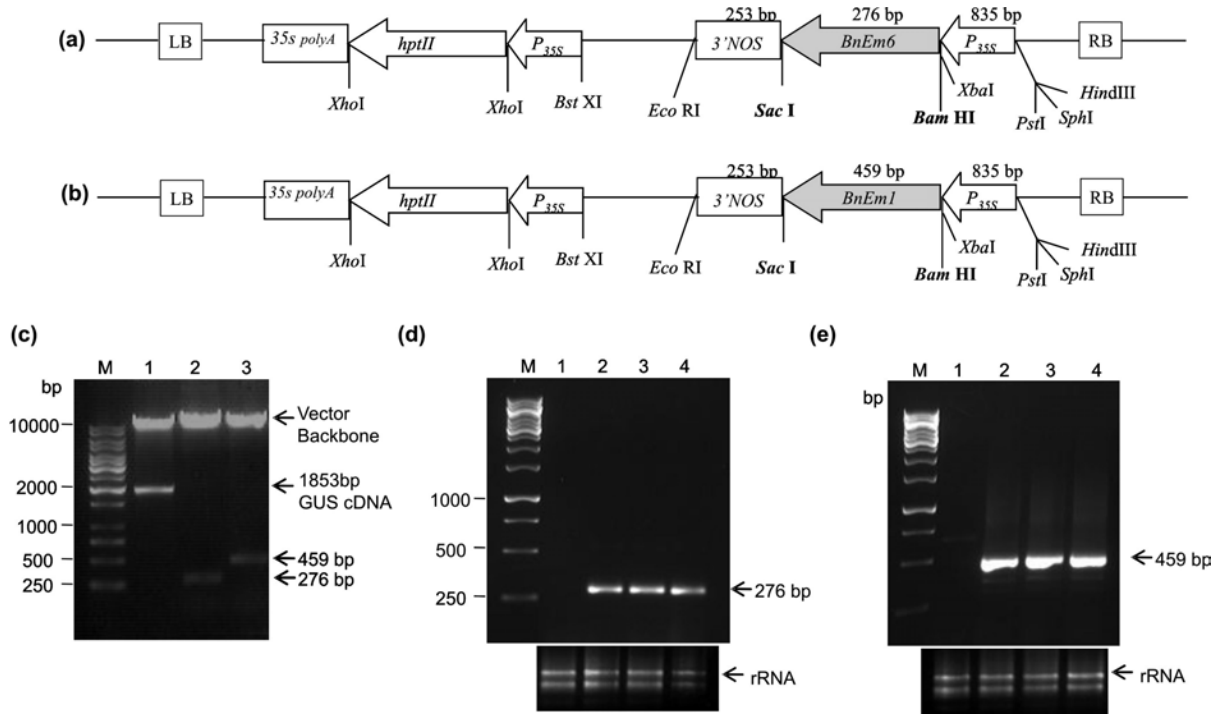

Fig. 3: Development and confirmation of *BnEm6* and *BnEm1* overexpressing *Arabidopsis* transgenic plants. Diagram of the T-DNA region of the binary vectors used for transformation (A) pCAMBIA1200-*P35S*::*BnEm6*::*NOS* and (B) pCAMBIA1200-*P35S*:: *BnEm1*::*NOS*. RB and LB are the right and left border sequences, respectively; P35S, CaMV35S promoter; *BnEm6* and *BnEm1*, coding region of *BnEm6* and *BnEm1* respectively. NOS, nopaline synthase terminator; *HPT*II, hygromycin phosphotransferase gene II; 35S polyA, CaMV35S terminator; (C) Restriction confirmation of the recombinant binary vectors. Lanes 1-3 are pC1200*P35S*:*GUS*:*NOS*, pC1200*P35S*:*BnEm6:NOS* and pC1200*PP35S*: *BnEm1:NOS* plasmids, respectively, restricted with *Bam*HI and *Sac*I. (D) Constitutive expression of *BnEm6 cDNA* in transgenic plants harboring pC1200*P35S*: *BnEm6*. M, marker; lane 1, Wild type plant (negative control); lanes 2-4, transgenic plants ox-1, ox-2 and ox-9 overexpressing *BnEm6* cDNA. (E) Constitutive expression of *BnEm1 cDNA* in transgenic plants harboring pC1200*P35S*: *BnEm1*. M, marker; lane 1, Wild type plant (negative control); lanes 2–4, transgenic plants ox-2, -ox-4, and ox-8 overexpressing *BnEm1* cDNA. Lower panel shows RNA used for RT-PCR

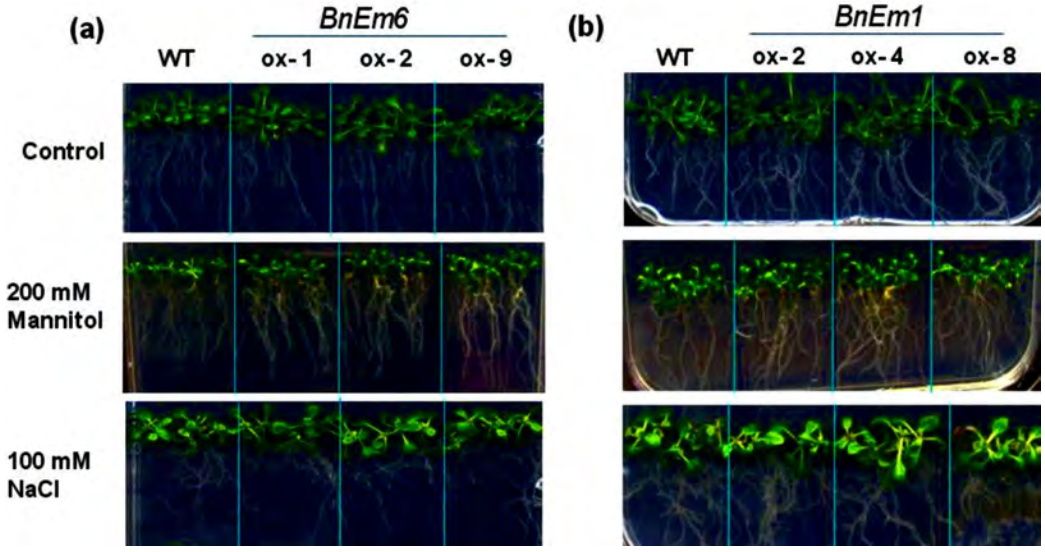

Fig. 4: Effect of osmotic stresses on vegetative growth of transgenic *Arabidopsis* seedlings overexpressing *BnEm6* and *BnEm1* after 2 weeks of stress treatment. Six days old Wild Type and transgenic *Arabidopsis* plants overexpressing *LEA1* were transplanted on MS media (control), MS + 200 mM mannitol, MS + 100 mM NaCl. (A) WT, wild type plants; ox-1, ox-2 and ox-9, transgenic plants overexpressing *BnEm6* under CaMV35S promoter. (B) WT, wild type plants; ox-2, ox-4, and ox-8, transgenic plants overexpressing *BnEm1* under CaMV35S promoter

to that of the wild type plants (Fig. 5 and 6). The root surface area of transgenic plants overexpressing *BnEm6* was significantly higher as compared to that of wild type plants under cold stress (Fig. 6). Total root length of *BnEm1* and *BnEm6* transgenic plants (two out of three lines) was significantly higher than that of wild type plants under cold stress (Figs. 5 & 6). These results show that overexpression of *BnEm1* and *BnEm6* genes resulted in enhancement of root traits in the seedlings. The fact that both the members of group 1 *LEA* genes exhibit similar phenotype on overexpression in *Arabidopsis* transgenic plants suggests functional redundancy. In *Arabidopsis* functional redundancy between the two members of group 1 *LEA* was suggested based on the increased expression levels of *AtEm1* to compensate for the loss of function of *AtEm6* in *Atem*6-1 mutant [22].

In conclusion, the present work isolated and characterized group 1 *LEA* genes namely *Em1* and *Em6* from *B. napus*. The results from this study

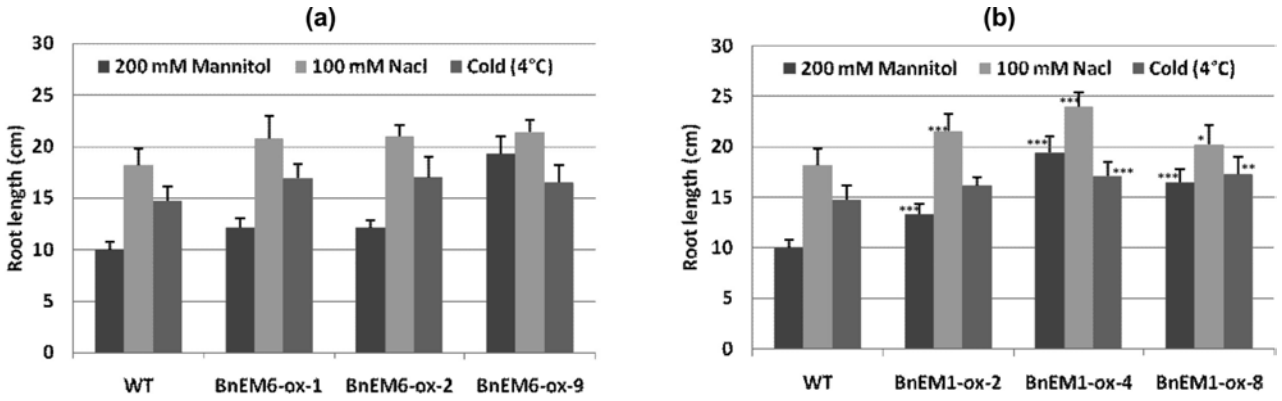

Fig. 5: Effect of osmotic and cold stresses on root lengths of transgenic *Arabidopsis* seedlings overexpressing group 1 *LEA* from *Brassica* species. A) *BnEm6*-ox-1, *BnEm6*-ox-2 and *BnEm6*-ox-9, transgenic plants overexpressing *BnEm6* and B) *BnEm1*-ox-2, *BnEm1*-ox-4, *BnEm1*-ox-8, transgenic plants overexpressing *BnEm1* with wild type plants as control. Six days old Wild Type and transgenic *Arabidopsis* plants overexpressing *LEA1 cDNAs* were transplanted on MS media (for control and cold 4°C), MS + 200 mM mannitol, MS + 100 mM NaCl. Observations were recorded after 2 weeks. Here, stars denote p value, ***, $p \leq 0.001$, **, $p \leq 0.01$, and *, $p \leq 0.05$

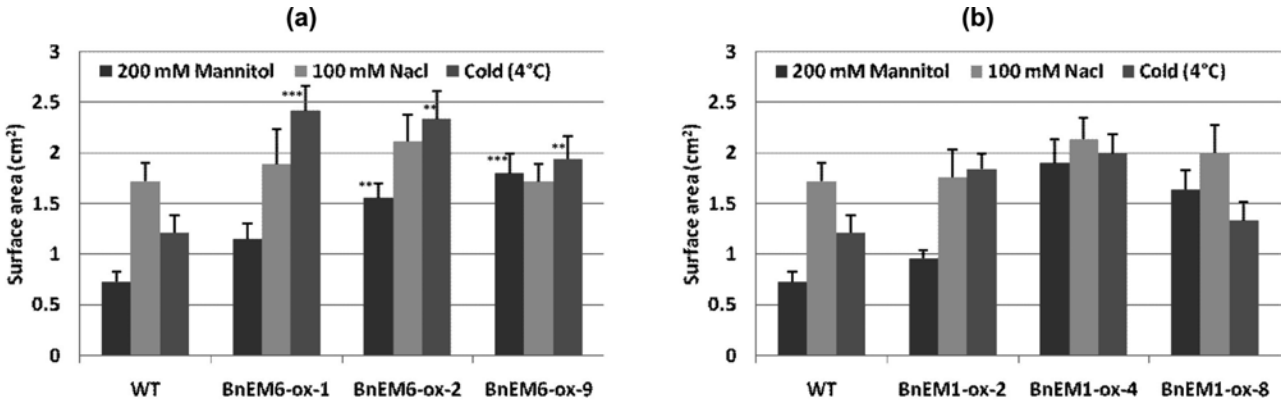

Fig. 6: Effect of osmotic and cold stresses on surface area of roots from transgenic *Arabidopsis* seedlings overexpressing group 1 *LEA* from *Brassica* species. A) *BnEm6*-ox-1, *BnEm6*-ox-2 and *BnEm6*-ox-9, transgenic plants overexpressing *BnEm6* and B) *BnEm1*-ox-2, *BnEm1*-ox-4, *BnEm1*-ox-8, transgenic plants overexpressing *BnEm1* with wild type plants as control. Six days old Wild Type and transgenic *Arabidopsis* plants overexpressing *LEA1 cDNAs* were transplanted on MS media (for control and cold 4°C), MS + 200 mM mannitol, MS + 100 mM NaCl. Observations were recorded after 2 weeks. Here stars denote p value, ***, $p \leq 0.001$, **, $p \leq 0.01$, and *, $p \leq 0.05$

showed that expression of *BnEm1* and *BnEm6* are highly expressed in seeds during maturation phase. Although, these genes are not expressed in the mature leaves of one month old *Brassica* plants, it was found that their homologs in *Arabidopsis* express during early seedling development stage. Constitutive ectopic overexpression of *BnEm1* and *BnEm6* enhanced the root traits (by enhancing root branching) under osmotic stress conditions in *Arabidopsis* suggesting that these proteins may play a positive role in promoting root growth under stress during early phase of seedling establishment. The study also highlights the need to validate the role of individual family members of *LEA* genes for their exploitation for enhancement of abiotic stress tolerance.

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References

1. Tunnacliffe A and Wise MJ *Naturwissenschaften* **94** (2007) 791-812
2. Bies-Etheve N, Gaubier-Comella P, Debures A, Lasserre E, Jobet E, Raynal M, Cooke R and Delseny M *Plant Mol Biol* **67** (2008) 107-124
3. Dalal M, Tayal D, Chinnusamy V and Bansal KC *J Biotechnol* **139** (2009) 137-145
4. Battaglia M, Olvera-Carrillo Y, Garciarrubio A, Camposv F and Covarrubias AA *Plant Physiol* **148** (2008) 6-24
5. Wise MJ *BMC Bioinform* **4** (2003) 52
6. Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N and Sarhan F *Plant Cell* **10** (1998) 623-638
7. Swire-Clark GA and Marcotte WR Jr *Plant Mol Biol* **39** (1999) 117-128
8. Goyal K, Walton LJ and Tunnacliffe A *Biochem J* **388** (2005) 151-157
9. Grelet J, Benamar A, Teyssier E, Avelange-Macherel M-H, Grunwald D and Macherel D *Plant Physiol* **137** (2005) 157-167
10. Hundertmark M and Hincha DK *BMC Genomics* 9 (2008) 118-139
11. Olvera-Carrillo Y, Campos F, Reyes JL, Garciarrubio A and Covarrubias AA *Plant Physiology* **154** (2010) 373-390
12. Bahieldin A, Mahfouz HT, Eissa HF, Saleh OM, Ramadan AM, Ahmed IA, Dyer WE, El-Itriby HA and Madkour MA *Physiol Plant* **123** (2005) 421-427
13. Park BJ, Liu ZC, Kanno A and Kameya T *Plant Sci* **169** (2005) 553-558
14. Xiao B, Huang Y, Tang N and Xiong L *Theor Appl Genet* **115** (2007) 35-46
15. Cuming AC and Lane BG *Eur J Biochem* **99** (1979) 217-224
16. Gaubier P, Raynal M, Hull G, Huestis G, Gellet F, Arenas C, Pages M and Delseny M *Mol Gen Genet* **238** (1993) 409-418
17. Vicient CM, Hull G, Guilleminot J, Devic M and Delseny M *J Exp Bot* **51**(2000) 1211-1220
18. Manickam A, Van Damme EJM, Kalaiselvi K, Verhaert P and Peumans WJ *Physiologia Plantarum* **97** (1996) 524-530
19. Parcy F, Valon C, Raynal M, Gaubier-Comella P, Delseny M and Giraudat J *Plant Cell* **6** (1994) 1567-1582
20. Carles C, Bies-Etheve N, Aspart L, Leaon-Kloosterziel K M, Koornneef M, Echeverria M and Delseny M *Plant J* **30** (2002) 373-383
21. Manfre AJ, Lanni LM and Marcotte WR Jr *Plant Physiol* **140** (2006) 140-149
22. Manfre AJ, LaHatte GA, Climer CR and Marcotte WR Jr *Plant Cell Physiol* **50** (2009) 243-253
23. Hruz T, Laule O, Szabo G, Wessendrop F, Bleuler S, Oertle L, Widmayer P, Gruissem W and Zimmermann P *Adv Bioinformatics* (2008) Article ID 420747, 5 pages
24. Clough SJ and Bent AF *Plant J* **16** (1998) 735-743
25. McCubbin WD, Kay CM and Lane BG *Can J Biochem Cell Biol* **63** (1985) 803-811
26. Bies N, Aspart L, Carles C, Gallois P and Delseny M *J Exp Bot* **49** (1998) 1925-1933
27. Jiang W and Yu D *BMC Plant Biol* **9** (2009) 96.