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## A New *Lea* Gene is Induced During Osmopriming of *Capsicum annuum* L. Seeds

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**Abstract:** The aim of this study was to characterize the transcriptional expression patterns of a new *lea* gene isolated in a previous work from *C. annuum* cv. caballero seeds when osmoprimed with PEG and GA<sub>3</sub>. *Capsicum annuum* is one of the main horticultural crops in México and routinely their seeds have problems when germinating. To correct this problem, osmopriming treatments based on PEG and GA has been used to improve their vigor. Osmopriming is a strategy developed to improve vigor during seed storage, which causes a reduction in germinability and seedling establishment. Osmopriming consists of the pre-imbibition of seeds in a solution containing an inert osmotic agent such as polyethylene glycol (PEG). In combination with PEG, several other compounds such as gibberellic acid (GA) can be used in order to improve the vigor of seeds. Several ESTs with high induced expression in the osmopriming treatment displayed high homology to LEA proteins and one of them corresponded to a complete cDNA coding a new LEA protein of 73 amino acids (*Calea 73* gene). This gene was highly induced in osmoprimed treatments in which KNO<sub>3</sub> instead of GA<sub>3</sub> was used in combination with PEG on *C. annuum* cv. caballero seeds. To our knowledge this is the shortest *lea* gene reported so far.

**Key words:** LEA proteins, *Capsicum annuum*, osmopriming, abscisic acid, water stress

### INTRODUCTION

Pepper (*Capsicum annuum* L.) cv. caballero seeds commonly show problems to germinate vigorously and thus, several osmopriming treatments have been proposed to correct this agronomic deficiency. The use of PEG 6000 alone or in combination with either GA<sub>3</sub> or KNO<sub>3</sub>, has shown to improve the vigor of germinating seeds (Cortez-Baheza *et al.*, 2007). The quality of dry seeds is important in agriculture, since seeds are often the starting material for crop production and crucial for achieving a good harvest. Several aspects of seed quality influence agricultural performance, such as total emergence, the rate and uniformity of emergence,

emergence under suboptimal conditions and seed longevity. To improve these aspects several priming treatments have been developed. During priming treatments seeds are allowed to take up water and partially start their germination-related processes, but emergence of the radicle is prevented to avoid the loss of desiccation tolerance needed for subsequent drying, storage and marketing of the treated seeds. Priming treatments are also used to synchronize the germination of individual seeds (Heydekker *et al.*, 1973). Because priming promotes the initiation of several germination-related processes, it induces causes faster germination and field emergence, especially under adverse field conditions (McDonald, 2000). To prevent radicle protrusion, water uptake may

either be limited by imbibition in an osmotic solution (osmopriming) instead of water or by restricting the period of germination on water and drying the seeds prior the radicle protrusion (Soeda *et al.*, 2005). During osmopriming only a subset of events occurs, in comparison with germination on water, as previously demonstrated at protein level (Gallardo *et al.*, 2001).

The expression of certain genes during maturation and seed processing, as well as osmopriming, results in an altered physiological state and affects seed quality. Therefore, the genes whose expression levels are different among osmopriming treatments might be useful as markers for identifying important processes in the improvement of seed vigor. It has been shown that several late embryogenesis abundant (*lea*) genes are strongly induced in *C. annuum* cv. caballero seeds when osmoprimed with PEG and GA<sub>3</sub> (Cortez-Baheza *et al.*, 2007). LEA proteins cover a number of loosely related groups of proteins whose precise function is unknown (Wise, 2003). LEA proteins were first characterized in cotton plant (Dure *et al.*, 1981) and are produced in abundance during seed development, comprising up to 4% of cellular proteins (Goyal *et al.*, 2005). Their expression is linked to the acquisition of desiccation tolerance in orthodox seeds, pollen and anhydrobiotic plants, but many LEA proteins are induced by exogenous factors such as cold, osmotic stress or ABA and in some cases they are expressed constitutively (Chung *et al.*, 2003; Goyal *et al.*, 2005). LEA proteins have received a special attention due to its apparently important role in desiccation tolerance in several life forms (Berjak, 2006; Tunnacliffe and Wise, 2007). In this study, we report the structural characterization of a complete cDNA corresponding to a new *lea* gene (*Calea 73*) from *C. annuum* cv. caballero seeds osmoprimed with PEG and GA<sub>3</sub>. Expression studies of this gene in seeds and vegetative tissues during four months with different osmotic stresses showed that *Calea 73* was induced in seeds osmoprimed with PEG and KNO<sub>3</sub> and in plants incubated under cold stress and treated with exogenous ABA and slightly induced in some organs during specific drought conditions. Several aspects related to the possible biotechnological applications of this new gene are discussed.

## MATERIALS AND METHODS

**Source of seeds:** The seeds of *Capsicum annuum* cv. caballero were purchased from SAKATA seeds de México (Tesislán, Jalisco, México). This study was conducted from September 2006 to June 2007.

**Chemicals:** Polyethylene glycol (PEG) 6000, Potassium nitrate and gibberellic acid were purchased from Sigma Chemical Co. (St Louis, MO, USA).

**Osmopriming of seeds:** The seeds of *C. annuum* cv. caballero were first dried at 25°C in an incubator (FELISA, Guadalajara, Jalisco, México), then were homogenized in a pneumatic sorter (Manufacturing Company Hoffman, Albany, Or, USA) and finally maintained in flasks at 12% of relative humidity in a growth chamber (Cenviron, Winnipeg, Manitoba, Canada) until their use in osmopriming experiments. Osmopriming treatments were carried out as following: 10 g of seeds were placed in a Petri dish and then the seeds were imbibed in 10 mL of PEG 6000 (500 ppm) + gibberellic acid (500 ppm), or PEG 6000 (500 ppm) both equivalent to -0.01 MPa as measured by thermocouple psychrometry. The time of imbibition was 9 h and then the seeds were washed with distilled water at 25°C. Both treatments were further used in construction of a subtraction suppression hybridization library (CLONTECH PCR-Select™ cDNA subtraction kit; Clontech, Palo Alto, CA, USA) enriched in up-regulated genes for GA<sub>3</sub>.

**Plant material and growth conditions:** Pepper seeds were surface-sterilized in 10% (v/v) sodium hypochlorite for 10 min, rinsed in running tap water for 2 h, sown on water-saturated paper towels and germinated in the dark at 27°C and 100% relative humidity. After 5 days, seedlings were selected for uniform size and transplanted to pots with sterilized soil. Plants obtained by this method were used in ABA and cold stress experiments as following: ABA treatment was carried out by adding 0.1 mM ABA to the irrigating water and by spraying an ABA solution (0.1 mM) to the aerial regions. When collected, tissues were frozen immediately in liquid nitrogen and stored at -80°C until used for RNA extraction. Cold stress experiments were carried out by incubating four-leaf stage plants at 4°C for 3 days and then tissues were collected and processed as in ABA treatments for RNA extraction.

**Drought tolerance experiments:** A soil mixture was prepared (50% fine sand: 50% sandy clay loam) and its field capacity determined according to Daubemire (1974). Sixteen plastic bags (22.2 cm diameter and 35 cm high) were filled with this soil mixture, placing one soil psychrometer (model PCT-55; Wescor, Inc., Logan, Utah) at the half (17.5 cm high) of every bag. The pots were then watered to field capacity, fertilized once with 200 mL of Hoagland's solution (Hoagland and Arnon, 1950) and three seeds were buried in the center of these pots (2 cm depth) and permitted to establish for a month. After the

establishment period, the containers were cleared to one plant per pot selecting even sized plants with experimental homogenization purposes. At this time, the watering was suspended to eight plants (water stress treatment) and the soil water potential monitored every three days in both stressed and non-stressed for the rest of the experiment connecting the thermocouple psychrometers to a microvoltmeter (model HR-33T; Wescor, Inc., Logan, Utah). Leaf water potential of the water-stressed and control plants were measured at 6:00 h am and 13:00 h pm punching and placing round leaf discs in C-52 sample chambers (Wescor, Inc., Logan, Utah) connected to the Wescor HR-33T microvoltmeter. Complete plants were detached extracted from the bags when they reached water potentials of -0.22, -0.47 and -1.5 MPa (value close to the permanent wilting point). A group of plants attaining a water potential of -1.5 MPa were then re-watered to field capacity and permitted to stabilize before they were detached from the bags. All removed plants were stored at -80°C until RNA extraction.

**Isolation of total RNA:** RNeasy plant mini kit (Qiagen, Hilden, Germany) was used to extract the total RNA from 3 g of osmoprimed seeds. RNA purified by RNeasy column was analyzed for integrity and size by formaldehyde agarose gel electrophoresis, while concentration and purity of RNA were determined by OD<sub>260/280</sub> value.

**Slot blot analysis:** The Northern blot analysis was essentially carried out as mentioned in Sambrook *et al.* (1989). For each treatment, 15 µg of total RNA were used.

**DNA sequencing and database comparison:** The nucleotide sequence of *Calea73* gene was determined using the ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Norwalk, CT, USA). The sequence was deposited in Genbank (accession number DQ902577.1). On-line database comparisons were performed using Blastx algorithm (Altschul *et al.*, 1990) from National Center for Biotechnology Information (NCBI). The LEA protein sequences used in the analysis for comparison purposes with *Calea 73* have the following accession numbers: *A. thaliana* (NM\_112632), *G. hirsutum* (P09443), *G. max* (AF117884.1), Dehydrin *C. annuum* (AY225438.1) and *H. annuus* (X59700).

## RESULTS

### Isolation and characterization of *Calea 73* cDNA

**sequence:** A subtracted cDNA library enriched with *C. annuum* cv. caballero genes up-regulated after osmopriming treatment with GA<sub>3</sub> in addition to PEG was generated by suppression subtractive hybridization (Diatchenko *et al.*, 1996). One clone of the library showed high similarity to *lea* gene sequences (Cortez-Baheza *et al.*, 2007). The clone contained an insert of 368 bp, including a poly (A) end which indicated the 3' end of the cDNA. Additionally, this clone showed a complete Open Reading Frame (ORF) encoding a putative LEA protein of 73 aminoacids and 23 bp at 5' end, which was further confirmed by 5'-RACE analysis. Thus, this SSH-library clone corresponds to a full-size cDNA with an ORF of 219 bp and was named as *Calea 73* (Fig. 1A). Phylogenetic analysis utilizing other LEA protein

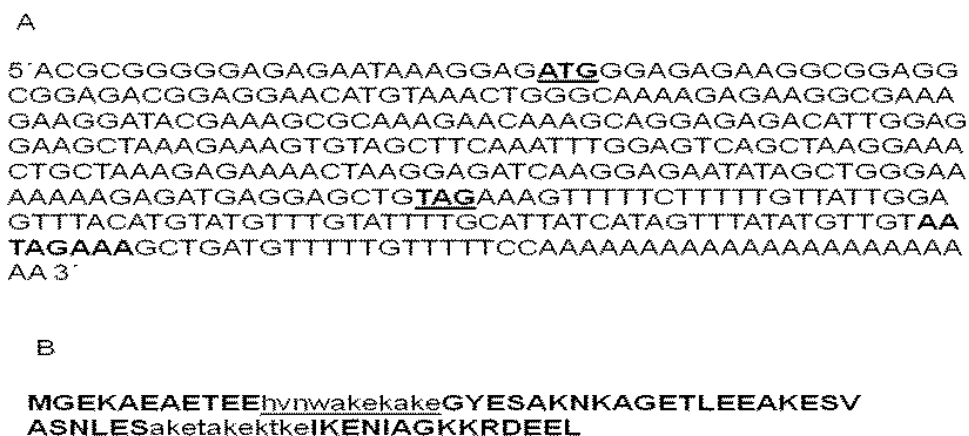


Fig. 1: Nucleotides and amino acids sequence of *Calea 73* gene. Panel A, nucleotide sequence of the complete cDNA isolated from *C. annuum* cv. Caballero plants, indicating in bold and underlined the first and stop codon of the open reading frame and only in bold a putative polyadenylation signal. Panel B, amino acids sequence of the putative LEA protein. Underlined are indicated two putative 11 mer motifs typical in LEA proteins of group 3

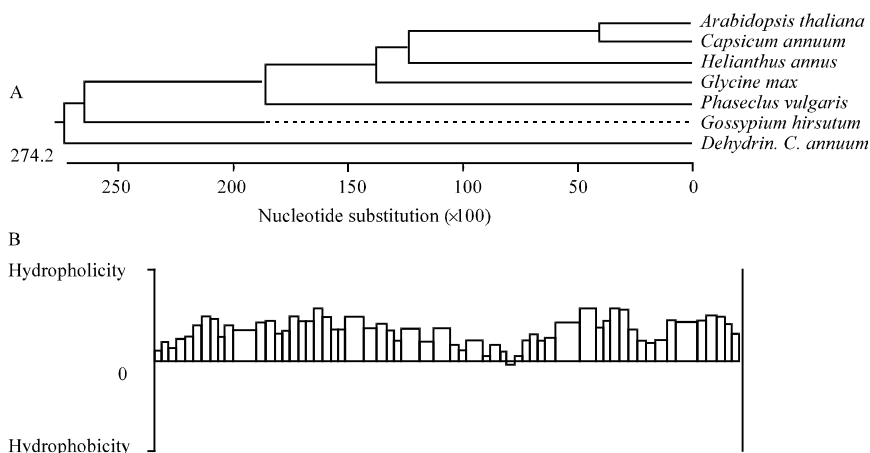


Fig. 2: Phylogenetic tree and aminoacids profile of the putative LEA protein encoded by *Calea 73* gene. Panel A, phylogenetic tree obtained comparing several LEA proteins reported elsewhere. Panel B, amino acids profiles of the putative LEA protein, using PROTEAN tool of DNASTAR

sequences showed that the putative LEA protein encoded by *Calea 73* showed a major similarity with a LEA protein of *Arabidopsis thaliana* (Fig. 2A). Additionally, this protein displayed two putative 11-mer motif previously identified in group 3 LEA proteins (Fig. 1B) and showed a high hidrophilicity profile (Fig. 2B) which is a typical feature in the majority of LEA proteins (Goyal *et al.*, 2005).

**Analysis of expression of *Calea 73* gene in seeds and vegetative tissues:**

Characterization of the *Calea 73* expression in seeds of *C. annuum* cv. caballero under osmopriming treatments indicated that in addition to the induced expression at the transcriptional level under PEG+AG<sub>3</sub>, the treatment with PEG+KNO<sub>3</sub> also induced a similar expression of this gene when these seeds were osmoprimed (Fig. 3).

A feature of *lea* genes is their high transcriptional expression in late embryogenesis stages during seeds development, but once germinating; their expression is highly diminished or even completely disappears (Campos-Alvarez *et al.*, 2002; Grelet *et al.*, 2005). This behavior was displayed by *Calea 73* in this study because its expression was not detected in plants 7 days post-germination (Fig. 4B, lanes 1-4). Expression of *lea* genes often appears to be abscisic acid-dependent (Xiao *et al.*, 2007). Thus, to evaluate whether *Calea 73* is induced by abscisic acid (ABA) in *C. annuum* cv. caballero plants were grown for 4 months and several experiments were carried out to address this question. It was shown that *Calea 73* gene was induced at 1 minute, 16, 24 and 48 h after ectopic application of ABA on leaves of 15 but not on 7 days-old plants of *C. annuum* cv. caballero (Fig. 4A and B, lanes 5-8). Interestingly, plants

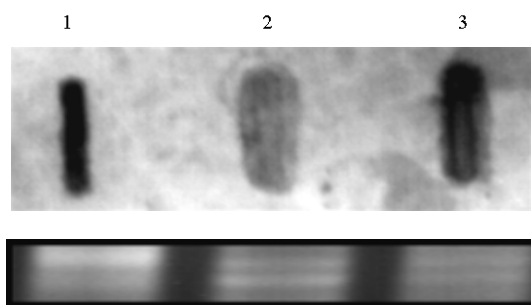


Fig. 3: Expression of *Calea 73* gene on *C. annuum* cv. Caballero seeds treated with several osmopriming solutions. Lane 1, PEG 6000+GA<sub>3</sub>; Lane 2, only PEG 6000; Lane 3, PEG 6000+KNO<sub>3</sub>. The sequence of the *Calea 73* open reading frame was used as a probe

on which no ABA was applied did not display any detectable *Calea 73* expression during these same periods (Fig. 4A and B, lanes 1-4). In addition, it is worth mentioning that the expression of *Calea 73* was detected in low levels in 2 month-old pepper plants, but no during other growing stages on several organs as leaves, root, stem, flowers and fruits regardless of ABA applications (not shown).

Another reported feature of several *lea* genes is their expression under cold stress in plants (Thomashow, 1998; Cumming, 1999; Xiao *et al.*, 2007). In this study, cold stress in 2 months-old *C. annuum* cv. caballero plants displayed an induction of *Calea 73* in root, but not in leaves or stem after 16 h of incubation at 4°C (Fig. 5C). Expression of *Calea 73* was also slightly detected after

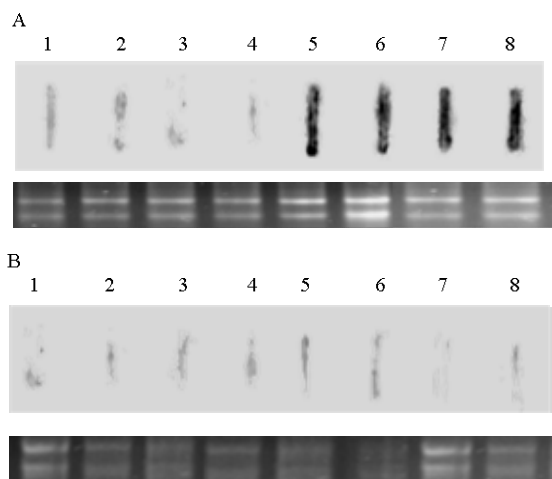


Fig. 4: Expression of *Calea 73* gene on 7 and 15 days-old plants of *C. annuum* cv. Caballero. Panel A, 15 days-old plants. Panel B, 7 days-old plants. On both panels, lanes 1-4 no ABA application (control) and lanes 5-8, RNA extracted from pepper plants in 1 min and 16, 24 and 48 h after ABA application. In control plants, the same times were used. Visualization of rRNA was used as a quantification control. The sequence of the *Calea 73* open reading frame was used as a probe

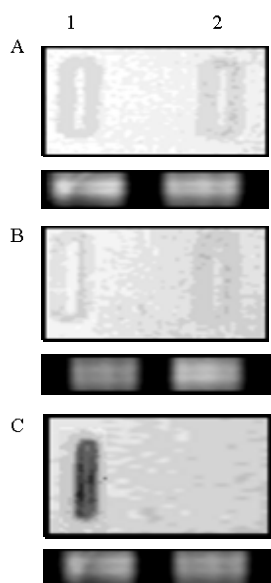


Fig. 5: Expression of *Calea 73* gene in different organs of *C. annuum* cv. Caballero plants during cold stress. Panels A, B and C, RNA extract from leaves, stems and roots, respectively. Visualization of rRNA was used as a quantification control. The sequence of the *Calea 73* open reading frame was used as a probe

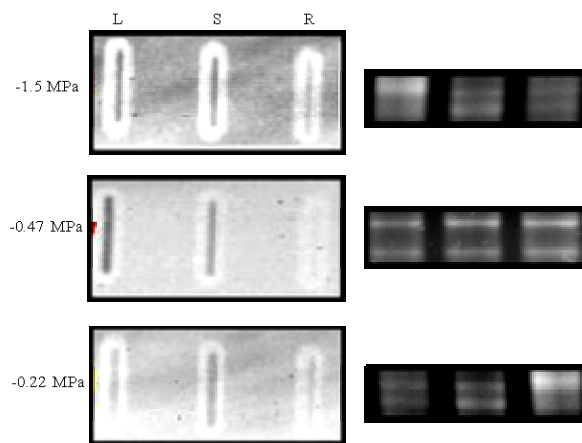


Fig. 6: Expression of *Calea 73* gene in different organs of *C. annuum* cv. Caballero plants during water deficit stress. L, S and R, corresponds to RNA extracted from leaves, stems and roots, respectively. Visualization of rRNA was used as a quantification control. The sequence of the *Calea 73* open reading frame was used as a probe. Water stress experiments were carried out as mentioned in materials and methods

the same incubation time on 3 months-old plants on leaves, stem and root (not shown). No expression of *Calea 73* was detected in other growth stages of the pepper plants evaluated.

**Expression of *Calea 73* on water stress conditions:** As mentioned before, several reports indicated that LEA proteins are implicated in water deficit stress response (Xu *et al.*, 1996; Maqbool *et al.*, 2002; Goyal *et al.*, 2005). Thus, the expression of *Calea 73* was evaluated in *C. annuum* cv. caballero plants on 2 months-old plants. As shown in Fig. 6, *Calea 73* was slightly more induced on leaves than on stem and roots, when water deficit was increased from the control plants (-0.22 MPa) to plants near to the permanent wilting point (-1.5 MPa). Expression of *Calea 73* on stems was low and apparently not induced when increasing the water stress level, while the lowest expression was detected in roots (Fig. 6). No significant change in *Calea 73* expression was detected in rehydrated plants from -1.5 to -0.22 MPa (data not shown).

## DISCUSSION

Osmopriming treatments using PEG in combination with GA<sub>3</sub> or KNO<sub>3</sub>, have shown to be good approaches to re-vigorate pepper seeds for commercial purposes (Cortez-Baheza *et al.*, 2007). Seed maturation is characterized by

a desiccation process, in which, several proteins referred to as LEA accumulate in the embryo (Baker *et al.*, 1988; Hughes and Galau, 1989). As mentioned before *lea* genes are high expressed in developing embryos during seed maturation (Baker *et al.*, 1988; Galau *et al.*, 1986) and environmental stresses (such as cold, salt and water stress) and by application of ABA to the vegetative tissues (Chung *et al.*, 2003; Choi *et al.*, 1999; Cohen and Bray, 1992; Godoy *et al.*, 1990; Aguado-Santacruz, 2006). In this study a structural characterization has been done for a previously isolated complete cDNA encoding a new *lea* gene (*Calea 73*) induced in *C. annuum* cv. caballero seeds during osmopriming with PEG + GA<sub>3</sub> (Cortez-Baheza *et al.*, 2007). The *Calea 73* gene displayed a typical hydrophilic profile and some features reported in group 3 LEA proteins as two putative 11-mer motifs TA (EQ) AAK (EQ) KAXE (Goyal *et al.*, 2005; Tobe *et al.*, 2004). Representatives of this group of genes also occur in prokaryotes (Dure, 2001), protozoans, nematodes and rotifers (Turnacliffe and Lapinsky, 2003).

Based on the data presented here, we propose that *Calea 73* is a new *lea* gene of the group 3 of LEA proteins; to our knowledge it is the shortest one reported so far. On the other hand, our results showed that *Calea 73* was induced on both pepper seeds osmopriming treatments and in some organs in vegetative tissues during some growing stages in response to cold and drought stresses and ABA treatment. Besides of being induced by PEG + GA<sub>3</sub>, the *Calea 73* gene was also stimulated by PEG+KNO<sub>3</sub>, which indicates that this gene is expressed during osmopriming regardless the osmotic solution used. Saline soils contain multiple types of soluble salt components, exerting different effects on the initial growth of plants (Younis and Hatata, 1971; Redmann, 1974; Hardegree and Emmerich, 1990; Tobe *et al.*, 2002, 2003). These salts have effects on cell membranes and cell walls that affect the water potential of the cytosol and cellular extensibility and thus, may affect seed germination and seedling growth (Tobe *et al.*, 2004). In this study, it was evident that the response of *Calea 73* gene to the abiotic stresses evaluated and ABA applications in *C. annuum* cv. caballero plants was stage-growing and organ dependent, especially after 15 days and 2 months post-germination. Although at low levels, even in 2 months-old plants the *Calea 73* expression could be detected without the ectopic application of ABA. These results suggest a highly regulated transcriptional control of *Calea 73*, in which at least: temporal, spatial and hormonal components are involved as in other systems reported elsewhere (Bray, 1997; Vicient *et al.*, 2000). The exact nature of the factors determining this regulation and the functional implications

of the different patterns of expression of *Calea 73* remain to be elucidated. The fact that the rehydration process of pepper plants in our study did not result in a significant change in *Calea 73* gene expression, suggests that the mRNA corresponding to this gene is highly stable under the evaluated conditions, although the importance of this control is unclear so far.

## CONCLUSION

Taking together our results showed that *Calea 73* is a *lea* gene corresponding to group 3, which is induced in vegetative tissues in pepper plants in response to cold, drought and ABA applications, in a growing-stage depending manner. It should be interesting to study the overexpression of this gene in order to evaluate its possible biotechnological application as a new gene conferring drought tolerance in agriculture.

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## REFERENCES

- Aguado-Santacruz, G.A., 2006. Genetic Manipulation of Plants for Increased Drought Tolerance. In: Advances in Agricultural and Food Biotechnology, Guevara-González, R.G. and I. Torres-Pacheco (Eds.). Kerala, India: Research Signpost, pp: 71-98.
- Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215 (3): 403-410.
- Baker, J., C. Steele and L. Dure, 1988. Sequence and characterization of 6 *Lea* proteins and their genes from cotton. Plant Mol. Biol., 11 (3): 277-291.
- Berjak, P., 2006. Unifying perspective of some mechanism basic to desiccation tolerance across life forms. Seed Sci. Res., 16 (1): 1-15.
- Bray, E.A., 1997. Plant responses to water deficit. Trends Plant Sci., 2 (2): 48-54.
- Campos-Álvarez, F., F. Cruz-García, A. Torres-Espinoza, M. Sánchez-Jiménez, J.M. Colmenero-Fuentes, C. Smith-Espinoza, A.A. Covarrubias-Robles and J.M. Vázquez-Ramos, 2002. Expression of late embryogenesis abundant (LEA) protein codifying genes during osmopriming of maize and bean seeds. Agrociencia, 36 (4): 461-470.

- Cohen, A. and E.A. Bray, 1992. Nucleotide sequence of an ABA-induced tomato gene that is expressed in wilted vegetative organs and developing seeds. *Plant Mol. Biol.*, 18 (2): 411-413.
- Cortez-Baheza, E., F. Peraza, M.I. Hernández-Álvarez, G.A. Aguado-Santacruz, I. Torres-Pacheco, M.M. González-Chavira, L. Guevara-Olvera and R.G. Guevara-González, 2007. Profiling the transcriptome in *Capsicum annuum* L. seeds during Osmopriming. *Am. J. Plant Physiol.*, 2 (2): 99-106.
- Cumming, A.C., 1999. LEA Proteins. In: Seed Proteins, Shewry, P.R. and R. Casey (Eds.). Dordrecht. The Netherlands: Kluwer Academic Publishers, pp: 753-780.
- Choi, D.W., B. Zhu and T.J. Close, 1999. The barley (*Hordeum vulgare* L.) dehydrin multigene family: Sequences, allele types, chromosome assignments and expression characteristics of 11 Dhn genes of cv. Dicktoo. *Theor. Applied Genet.*, 98 (8): 1234-1247.
- Chung, E., S.Y. Kim, S.Y. Yi and D. Choi, 2003. *Capsicum annuum* dehydrin, an osmotic-stress gene in hot pepper plants. *Mol. Cells.*, 15 (3): 327-332.
- Daubenmire, R.F., 1974. Plants and Environment. In: A Textbook of Autoecology. John Wiley and Sons Inc., New York, pp: 442.
- Diatchenko, L.Y.F., A.P. Lau, A. Campbell, F. Chenchik, B. Moqadam, S. Huang, K. Lukyanov, N. Lukyanov, E.D. Gurskaya, E. Sverdlov and P.D. Siebert, 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA.*, 93 (12): 6025-6030.
- Dure, L., S.C. Greenway and G.A. Galau, 1981. Developmental biochemistry of cottonseed embryogenesis and germination: Changing messenger ribonucleic acid populations as shown by *in vitro* and *in vivo* protein synthesis. *Biochemistry*, 20 (14): 4162-4168.
- Dure, L., 2001. Occurrence of a repeating 11-mer amino acid sequence motif in diverse organisms. *Prot. Pept. Lett.*, 8 (2): 115-122.
- Galau, G.W., D.W. Hughes and L. Dure, 1986. Abscisic acid induction of cloned cotton late embryogenesis abundant (LEA) messenger RNAs. *Plant Mol. Biol.*, 7 (3): 155-170.
- Gallardo, K., C. Job, S.P.C. Groot, M. Puype, H. Demol, J. Vanderkerckhove and D. Job, 2001. Proteomic analysis of Arabidopsis seed germination and priming. *Plant Physiol.*, 126 (2): 835-848.
- Godoy, J.A., J.M. Pardo and J.A. Pintor-Toro, 1990. A tomato cDNA inducible by salt stress and abscisic acid: Nucleotide sequence and expression pattern. *Plant Mol. Biol.*, 15 (5): 695-705.
- Goyal, K., L.J. Walton and A. Tunnacliffe, 2005. LEA proteins prevent protein aggregation due to water stress. *Biochem. J.*, 388 (1): 151-157.
- Grelet, J., A. Benamar, E. Teyssier, M.H. Avelange-Macherel, D. Grunwald and D. Macherel, 2005. Identification in pea seed mitochondria of a late-embryogenesis abundant protein able to protect enzymes from drying. *Plant Physiol.*, 137 (1): 157-167.
- Hardegree, S.P. and W.E. Emmerich, 1990. Partitioning water potential and specific salt effects on seed germination of four grasses. *Ann. Bot.*, 66 (5): 587-595.
- Heydekker, W., J. Higgins and R.L. Gulliver, 1973. Accelerated germination by osmotic seed treatment. *Nature*, 246 (1): 42-44.
- Hoagland, D.R. and D.I. Arnon, 1950. The water culture method for growing plants without soil. *Circ.* 347. Berkeley, CA: California Agricultural Experiment Station.
- Hughes, D.W. and G.A. Galau, 1989. Temporally modular gene expression during cotyledon development. *Genes Dev.*, 3 (3): 358-369.
- Maqbool, B., H. Zhong, Y. El-Maghraby, A. Ahmad, B. Chai, W. Wang, R. Sabzikar and B. Stcklen, 2002. Competence of oat (*Avena sativa* L.) shoot apical meristems for integrative transformation, inherited expression and osmotic tolerance of transgenic lines containing hva 1. *Theor. Applied Genet.*, 105 (2): 201-208.
- McDonald, M.B., 2000. Seed Priming. In: Seed Technology and its Biological Basis, Black, M. and J.D. Bewley (Eds.). Sheffield Academic Press, Sheffield, UK., pp: 287-325.
- Redmann, R.E., 1974. Osmotic and specific ion effects on the germination of alfalfa. *Can. J. Bot.*, 52 (4): 803-808.
- Sambrook, J., E.F. Fritsch and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.
- Soeda, Y., M.C.J.M. Konings, O. Vorst, A.M.M.L. Van Houwelingen, G.M. Stoopen, C.A. Maliepaard, J. Kodde, R.J. Bino, S.P.C. Groot and A.H.M. Van der Geest, 2005. Gene expression programs during *Brassica oleraceae* seed maturation, osmopriming and germination are indicators of progression of the germination process and the stress tolerance level. *Plant Physiol.*, 137 (1): 354-368.
- Thomashow, M.F., 1998. Role of cold-responsive genes in plant freezing tolerance. *Plant Physiol.*, 118 (1): 1-7.
- Tobe, K., X. Li and K. Omasa, 2002. Effect of sodium, magnesium and calcium salts on seed germination and radical survival of a halophyte, *Kalidium caspicum* (*Chenopodiaceae*). *Aus. J. Bot.*, 50 (2): 163-169.



- Tobe, K., L. Zhang and K. Omasa, 2003. Alleviatory effects of calcium on the toxicity of sodium, potassium and magnesium chlorides to seed germination in three halophytes. *Seed Sci. Res.*, 13 (1): 47-54.
- Tobe, K., L. Xiaoming and K. Omasa, 2004. Effects of five different salts on seed germination and seedling growth of *Haloxylon ammodendron* (*Chenopodiaceae*). *Seed Sci. Res.*, 14 (4): 345-353.
- Tunnacliffe, A. and J. Lapinsky, 2003. Resurrecting Van Leeuwenhoek's rotifers: A reappraisal of the role of disaccharides in anhydrobiosis. *Phil. Trans. Roy. Soc. Biol. Sci.*, 358 (1438): 1755-1771.
- Tunnacliffe, A. and M.J. Wise, 2007. The continuing conundrum of the LEA proteins. *Naturwissenschaften*, 94 (10): 791-812.
- Vicient, C.M., G. Hull, J. Guillemot, M. Devic and M. Delseny, 2000. Differential expression of the *Arabidopsis* genes coding for Em-like proteins. *J. Exp. Bot.*, 51 (348): 1211-1220.
- Wise, M.J., 2003. LEAPing to conclusions: A computational reanalysis of late embryogenesis abundant proteins and their possible roles. *BMC Bioinform.*, 4 (1): 52-70.
- Xiao, B., Y. Huang, N. Tang and L. Xoing, 2007. Over-expression of a LEA gene in rice improves drought resistance under the field conditions. *Theor. Applied Genet.*, 115 (1): 35-46.
- Xu, D., X. Duan, B. Wang, B. Hong, T.H.D. Ho and R. Wu, 1996. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiol.*, 110 (1): 249-257.
- Younis, A.F. and M.A. Hatata, 1971. Studies on the effects of certain salts on germination, on growth of root and on metabolism. I. Effects of chlorides and sulfates of sodium and magnesium on germination of wheat grains. *Plant Soil.*, 34 (1): 183-200.