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ORIGINAL ARTICLE

Overexpression Analysis of *emv2* gene coding for Late Embryogenesis Abundant Protein from *Vigna radiata* (Wilczek)

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Abstract - Late embryogenesis abundant (LEA) proteins are speculated to protect against water stress deficit in plants. An over expression system for mungbean late embryogenesis abundant protein, *emv2* was constructed in a pET29a vector, designated pET-*emv2* which is responsible for higher expression under the transcriptional/translational control of T7/lac promoter incorporated in the *Escherichia coli* BL21 (DE3).Induction protocol was optimized for pET recombinants harboring the target gene. Overexpressed EMV2 protein was purified to homogeneity and the protein profile monitored by SDS-PAGE.

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Late embryogenesis abundant (LEA) proteins have been found in a wide range of plant species in response to water deficit resulting from desiccation, cold and osmotic stress. LEA-type proteins fall into a number of families, with diverse structures and functions (Close 1996; Ingram and Bartels 1996; Thomashow 1998). It has been suggested that LEA-type proteins act as water-binding molecules, in ion sequestration and in macromolecule and membrane stabilization (i.e. chaperone-like activity; Dure 1993; Close 1996; Ingram and Bartels 1996; Thomashow 1998, 1999). Group1 LEA proteins posses' 20-mer motif conserved regions in form of repeat regions rich in Glycine residues. In our earlier studies, we had reported the presence of LEA-type proteins in the embryonic axes of the developing seeds of mungbean (*Vigna radiata*) referred to as EMV proteins (Manickam and Carlier, 1980). These proteins are classified into Group 1 LEA protein based on motif analysis and its functions were predicted based on the secondary structures (Rajesh and Manickam, 2006). The discovery and use of new stress-tolerance-associated genes, as well as heterologous genes, to confer plant stress tolerance has been the subject of ongoing efforts to obtain tolerant plants. Recently, we reported sense expression of *emv2* in indica rice plants that shall tolerate limited water situations (Rajesh *et al*., 2008).

Overexpression of a single LEA-type protein is not always sufficient to confer plant stress tolerance. Although some progress of physiological roles of LEA proteins has been made, it is difficult to see the protective effect of over-expression of a single LEA protein gene introduced in transgenic plant, due to expression of the endogenous lea gene families that existed in genomic DNA of the host plant cells. Liu and Zheng, 2005 demonstrated that expression system of *E. coli* is a simple, convenient, and effective model to determine the function of a heterogeneous protein under stress treatments. Some plant genes related with dehydration stresses have been isolated by functional expression screening in

E. coli system. Most of these proteins such as CCTa, XVSAP1, BBC1, LEA3, HSP100 and ubiquitin proteins are generally located in cytoplasm of the plant cells (Miyasaka et al., 2000 and Tanaka et al., 2001 and 2002). In the present study, as a preliminary investigation to asses the overexpression potential of emv2 in prokaryotic system *E coli* and its subsequent assessment for salt tolerance studies, we have constructed a pET based recombinant overexpression system with *emv2* gene, coding for a LEA protein from *Vigna radiata* and the protein expression profile was electrophoretically analyzed.

MATERIALS AND METHODS

Materials

The insert *emv2* gene (NCBI GenBank accession numbers U31211, Fig.1) coding for a Group 1 Late Embryogenesis Abundant- type protein from mung bean (*Vigna radiata*) is a proprietary gene isolated from our laboratory. pET29a vector and host cells *E coli* BL21 (DE3) from Novagen Inc, Germany. All other chemical reagents/fine chemicals otherwise mentioned are purchased from Sigma-Aldrich, Bangalore, India.

Methods

Construction of pET-*emv2* overexpression system

The insert *emv2* was prepared by restriction digestion of plant transformation vector pCEMV2 constructed in our laboratory. Restriction digestion was done as per standard procedures (Sambrook *et al*., 1989). Approximately, 200-250 ng each of plasmid DNA were digested with 1µl of *Bam* HI (10U/µl) and *Hin*d III (20U/µl) (Bangalore Genei, Bangalore, India) in an appropriate buffer at 37ºC for 1 h. The digested products were fractionated on agarose gel (Fig.2). Insert DNA was eluted from the agarose gel following phenol: chloroform method. The gel was visualized over UV transilluminator and the desired band was excised with a sterile scalpel and frozen at -70 ºC for 1 h and macerated into pieces with blunt ended micropipette tip. TE saturated phenol; pH 8.0 was added to this, mixed and incubated at 65 ºC for solubilising gel pieces. The mix was centrifuged, supernatant saved and ethanol precipitated. Eluted DNA was used for subcloning into pET29a at the *Bam* H1 and *Hin*d III cloning/expression region (Fig. 3) under the transcriptional/translational control of T7/lac promoter. The vector, pET29a was prepared following similar procedure and restriction digested. e*mv2* was ligated into the vector following sticky end ligation using T_4 DNA ligase. Fresh competent cells of *E coli* BL21 (DE3) was prepared by treating the cells with 100mM $CaCl_2$ and the pET recombinants harboring *emv2* gene were transformed into highly effective BL21 (DE3) host cells by heat shock treatment at 42 ºC for 90 sec. The transformants were selected using kanamycin, subcultured and used for overexpression analysis.

Overexpression and Purification of the pET-*EMV2*

Recombinant *E. coli*, BL21 (DE3) harbouring pET-*emv2* (pET29a + *emv2) or* pET29a vector alone were grown in LB broth containing kanamycin 50 $mgml^{-1}$ at 37°C till OD_{600} = ~0.6. Isopropyl *b*-D-thio galactopyranoside (IPTG) was added to each flask at a final concentration of 1 mM followed by further cell growth at 37 °C for 6 h (OD_{600} = ~1.3). This was followed by pelleting of cells by centrifugation, washing in sterile distilled water, resuspension in 500 ml of 50 mM Tris-HCl, pH 7.0 and sonication in TE buffer containing 1 mM PMSF, until more than 90% of the cells were lysed. Sonicated cells were centrifuged at 7000 x *g* for 15 min at 4°C. The pellet was suspended in the TE buffer containing 1.0% triton X-100 and washed twice in the same buffer. Finally, the pellet was dissolved in a small volume of sterile double distilled water and protein profile analyzed by SDS-PAGE.

RESULTS

Construction of pET-*emv2* overexpression system

Insert cDNA *emv2* and the cloning vector pET29a was prepared following standard protocols. Sticky end ligation of the insert *emv2* and pET29a was performed using T_4 DNA ligase. The construct was designated as pET-*emv2*. The pET recombinants and also the control, pET29a vector were transformed into competent cells of *E. coli* BL21 (DE3) by heat shock treatment and induced for overexpression.

Overexpression analysis of pET-*emv2*

A 625 base pair cDNA fragment, emv2 encoding the Late embryogenesis abundant protein from mungbean (*Vigna radiata*) was subcloned into the expression vector pET 29a as described above, making the construct pET-*emv2*. *E. coli* BL21 (DE3) cells were transformed with this plasmid or pET29a as a control and induced with IPTG as described above. After induction, cells were lysed and the polypeptide profiles compared by SDS-PAGE. Purity of eluted fractions from recombinant *E. coli* strains monitored by SDS-PAGE analysis showed a prominent band of ~ 12 kDa in case of pET-*emv2*, whereas there was no distinct band of that size in pET29a control (Fig.4). Other prominent protein bands as visualized were similar to the control.

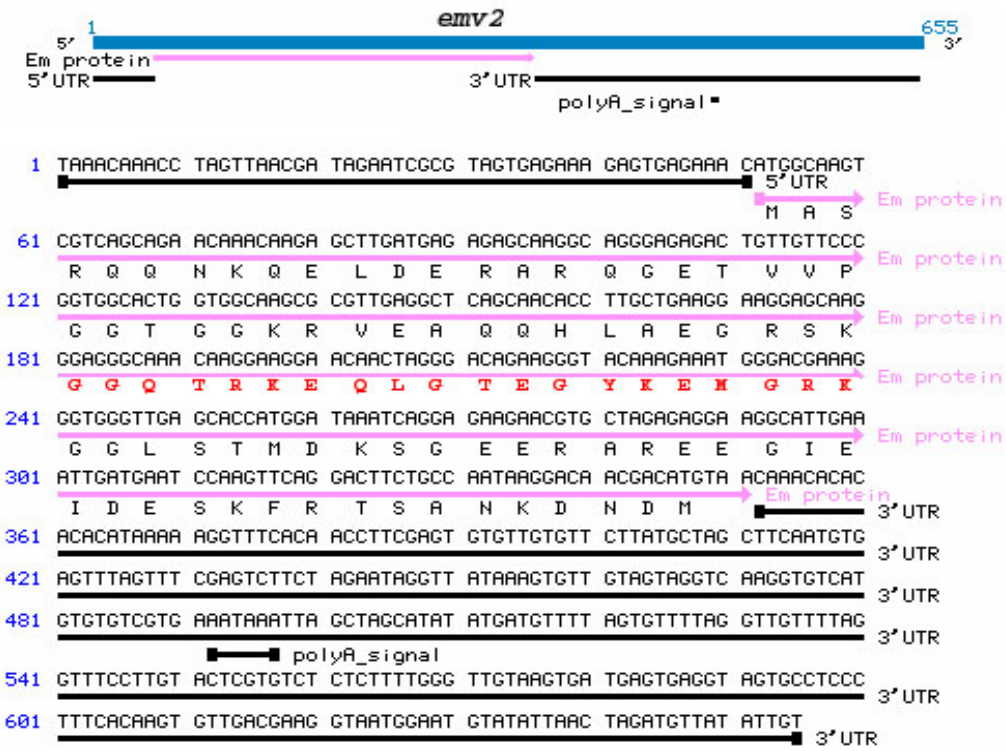

Fig. 1. Organization of *emv2*, a LEA protein coding gene from *Vigna radiata*
20-mer amino acid repeat motif at position 181-240 is presented in red.

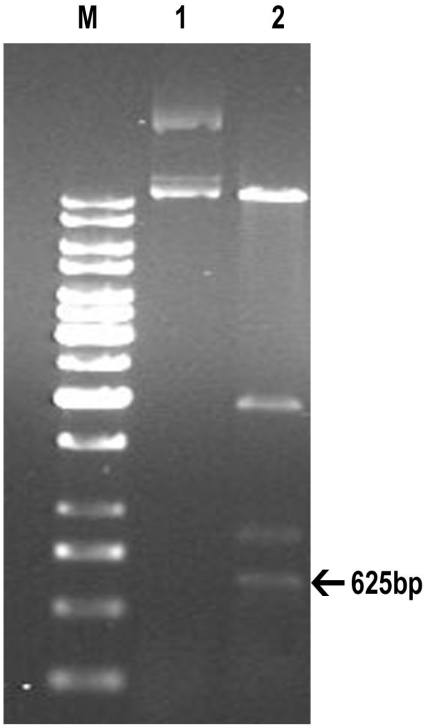

Fig. 2. Preparation of insert by restriction digestion
The figure shows unrestricted plasmid pCEMV2 (*lane 1*), *Bam*H1 and *Hind* III sequentially digest DNA (*lane 2*) and 1kb DNA ladder given in *lane M*.

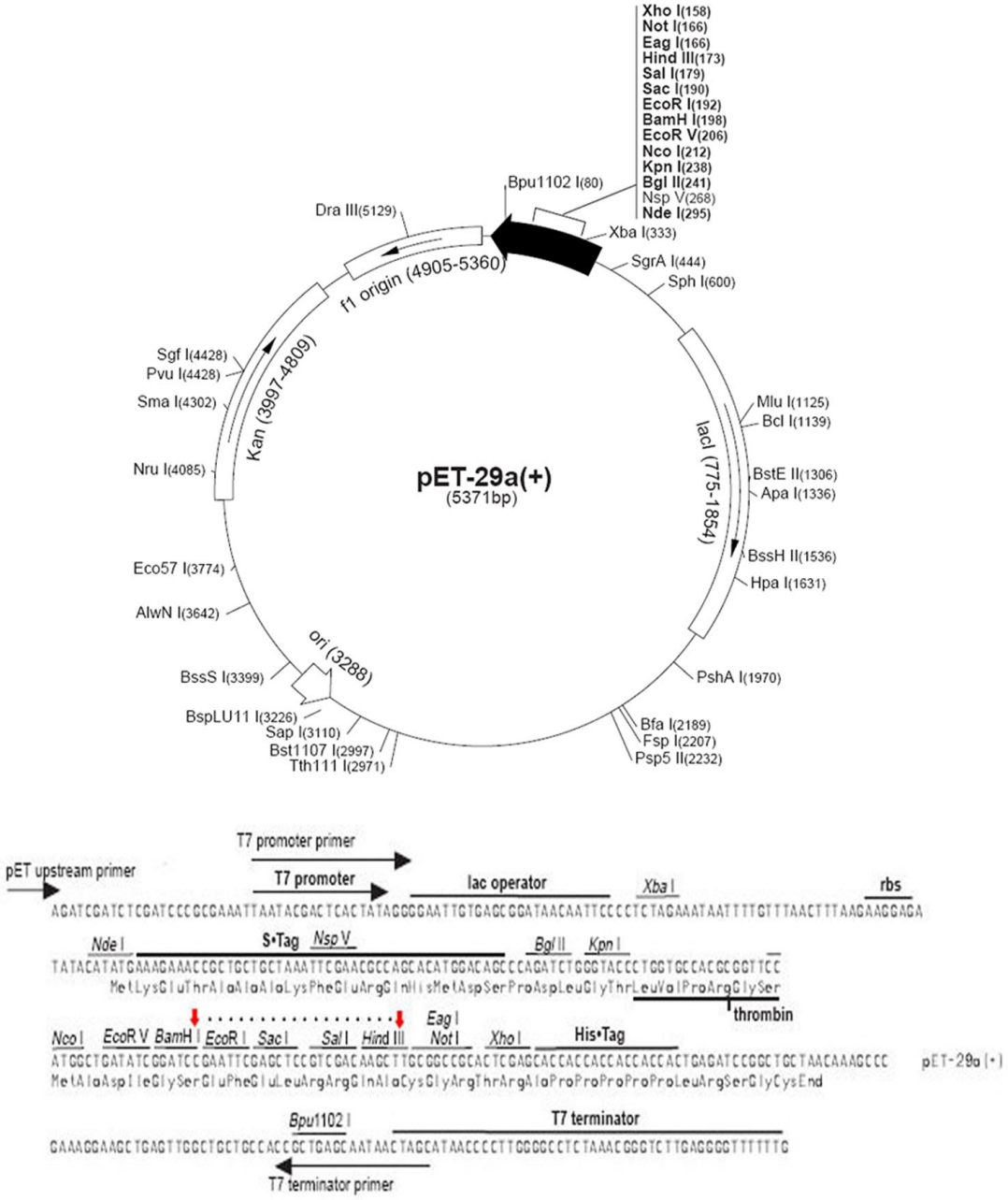

Fig. 3. Physical map and cloning/expression region of pET29 a (+) vector

Over expression system constructed with mungbean late embryogenesis abundant protein gene, *emv2* with flanking *Bam* H1 and *Hind* III sites. Cloning position indicated as red down arrows.

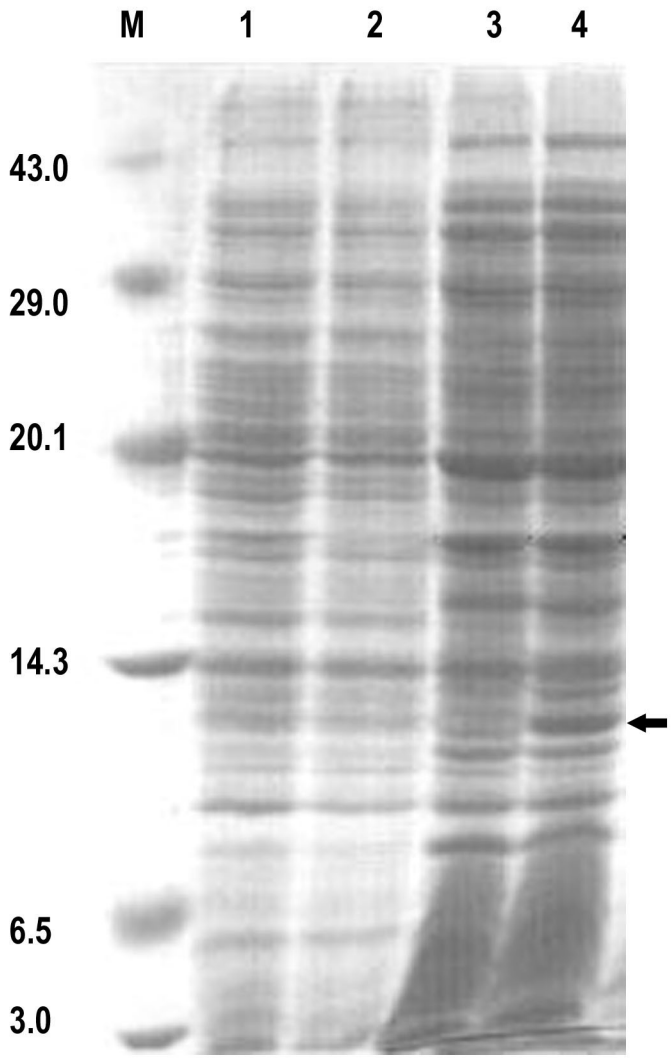

Fig. 4. Expression of pET-*emv2* in *E. coli* BL21 (DE3)

Cultures of *E. coli* BL21 (DE3) containing plasmids were made and supernatants prepared as described under methods. The figure shows samples from host cells BL21 uninduced (*lane 1*), BL21 induced (*lane 2*), pET-*emv2* uninduced (*lane 3*) and pET-*emv2* induced 12kDa EMV2 protein (*lane 4*). Markers are given in *lane M*. Numbers give molecular masses in kilodaltons.

DISCUSSION

LEA proteins and heat-shock proteins have been shown to be involved in protecting macromolecules, such as enzymes and lipids (Shinozaki and Yamaguchi-shinozaki, 2000 and Seki *et al.*, 2002). Although several studies have been extensively carried out documenting the responses of LEA proteins to different abiotic as well as biotic conditions, the true biochemical function of these loosely related proteins is yet to be fully understood. The pET expression system is the most powerful system and is choice for the cloning and expression of recombinant proteins in *E. coli* and the target genes cloned are driven under the transcriptional/translational control of strong bacteriophage T7 signals; expression is induced by providing a source of T7 RNA polymerase in the host cell with an advantage that when fully induced, almost all of the cell's resources are converted to target gene expression and the desired product can comprise more than 50% of the total cell protein within a few hours after induction (pET Manual, 2003). A 625 bp mungbean *emv2* gene, containing a 20-mer amino acid repeat motif (GGQTRKQQLGSEGYHEMGRK) at positions 181-240 and a hydrophilic plant signature (GETVVPGGT) at position 94-128 was cloned in a pET29a vector system and expressed in *E. coli*, and has shown to retain intact by expressing the EMV protein of mass 12,000 Daltons at a relatively higher level than background. These signature repeats in the

LEA proteins of *Vigna radiata* are similar and share identity of about 30% to the GsiB stress protein from *Bacillus subtilis* (Stacy and Aalen, 1998) which is induced by glucose or phosphate starvation, oxygen limitation, heat, oxidation and salinity (Volker *et al.,* 1994). This remarkable conservation suggests an important role of LEA proteins in stress adaptation.

Considering the higher expression level of *emv2* gene, this gene can be overexpressed in plant system either singly or in tandem with our already reported *emv1* gene (NCBI GenBank Acc. Number U31210) which has two such 20-mer repeat motifs at regions 44-63 and 64-83 (Rajesh and Manickam, 2006), to produce dehydration tolerant protein at higher expression level. The principle of functional expression screening is that due to the expression of foreign plant genes carried by plasmid DNA in prokaryote cells, the host *E. coli* cells acquire stress tolerance. Hence at the next level, this protein will be further tested for its salt tolerance capacity by MALDI-TOF and Spot assay experiments. Liu and Zheng, 2005 has demonstrated expression of a 22-mer motif containing Group 3 LEA protein from soybean and has reported to directly contribute to increasing stress tolerance of the bacteria host cells, and also indicated that some of the protective mechanisms might be common in prokaryote and eukaryote under stress conditions (Garray-Arrayo *et al.,* 2000). The transgenic approaches using LEA proteins, so far described were on the basis of insights gained from the physiological and biochemical studies of stress tolerance in different crop plants. Interestingly, any results gained from transgenic studies by overexpression of these dehydration tolerant LEA genes will be miraculous and will help us to better understand the physiological functions of different stress-related genes.

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REFERENCES

Close, T.J. 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. **Physiol. Plant., 97:** 795-803

Dure, L. 1993. Structural motifs in Lea proteins. **In.** Plant Responses to Cellular Dehydration during Environmental Stress. **Current Topics in Plant Physiology**, Vol **10.** TJ Close, EA Bray, (eds). American Society of Plant Physiologists, Rockville, MD, pp 91-103

Garay-Arroyo, A., J.M .Colmenero-Flores, A Garciarrubio and AA Covarrubias. 2000. Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. **J Biol. Chem., 275:** 5668–5674

Ingram ,G and D. Bartels. 1996. The molecular basis of dehydration tolerance in plants. **Annu. Rev. Plant Physiol. Plant Mol. Biol., 47:** 377-403

Lium Y and Y. Zheng. 2005. PM2, a group 3 LEA protein from soybean, and its 22-mer repeating region confer salt tolerance in *Escherichia coli*. **Biochem. Biophy. Res. Comm., 331:** 325–332

Manickam, A and AR Carlier. 1980. Isolation and Function of a Low Molecular Weight Protein of Mung Bean Embryonic Axes. **Planta 149:** 234-240

Miyasaka, H., H. Kanaboshi, K. Ikeda. 2000. Isolation of several antistress genes from the halotolerant green alga Chlamydomonas by simple functional expression screening with *Escherichia coli*. **World J. Microb. Biot**. 16 : 23–29.

pET System Manual, 2003. 10th Edition. www.novagen.com. p 68.

Rajesh, S and A. Manickam, 2006. Prediction of functions for two LEA proteins from mungbean. **Bioinformation** 1(4): 133-138

Rajesh, S., S. Krishnaveni, D. Sudhakar, M. Raveendran, P. Sivakumar, R. Gnanam and A. Manickam. 2008. *Agrobacterium*-Mediated Transformation of *indica* Rice (*Oryza sativa* L.), IR64 with Mungbean LEA Protein Gene for Water-Stress Tolerance. **Amer. J. Plant Physiol.** 3(3): 101-110.

Sambrook ,J., EF. Fritsch and T. Maniatis. 1989. **Molecular cloning. A laboratory manual**. Cold Spring Harbor Laboratory Press, Cold Spring harbor, New York.

Seki, M., M. Narusaka, J. Ishida, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A Enju and T. Sakurai. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. **Plant J., 31:** 279–292

Shinozaki, K and Y. Yamaguchi-Shinozaki. 2000. Molecular responses to dehydration and low temperature differences and cross-talk between two stress signaling pathways. **Plant Biol., 3:** 217-223

Stacy, R and R. Aalen. 1998. Identification of sequence homology between the internal hydrophilic repeated motifs of group 1 late embryogenesis abundant proteins in plants and hydrophilic repeats of the

general stress protein GsiB of *Bacillus Subtilis*. **Planta 206:** 476-478

Tanaka, S., K. Lkeda and M. Ono. 2002. Isolation of several anti-stress genes from a mangrove plant Avicennia marina, World J. Microb. Biot. 18: 801–804.

Tanaka, S., K. Ikeda and Miyasaka. 2001. Enhanced tolerance against salt-stress and freezing-stress of Escherichia coli cells expressing algal bbc1 gene, **Curr. Microbiol**. 42: 173–177.

Thomashow, MF. 1998. Role of cold-responsive genes in plant freezing tolerance. **Plant Physiol., 118:** 1–7

Thomashow, MF. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. **Annu. Rev. Plant Physiol. Plant Mol. Biol., 50:** 571-599

Volker, U., S Engelmann, B Maul, S Riethdorf, A Voelker, R Schmid, H Mach and M Hecker. 1994. Analysis of the induction of general stress proteins of *Bacillus subtilis*. **Microbiology 140:** 741-752