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Biochemical and Biophysical Research Communications 331 (2005) 325–332
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PM2, a group 3 LEA protein from soybean, and its 22-mer repeating region confer salt tolerance in *Escherichia coli*

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Received 7 March 2005
Available online 1 April 2005

Abstract

To have knowledge of the effect of soybean PM2 protein in protecting dehydrated cells and its functional region, *PM2* cDNA was isolated from soybean immature seeds. The recombinants expressing full-length PM2, truncated polypeptides of PM2A (aa 1–262) or PM2B (aa 129–262, 22-mer repeating region), or artificial polypeptide PM2C (duplication of 22-mer repeating region) were constructed. By using SDS–PAGE and mass spectrometry approaches, these fusion polypeptides were identified and proved to be hydrophilic and heat-stable. Spot assays of BL/PM2 and BL/pET28 (as control) showed that protein PM2 increased salt tolerance (500 mM NaCl or 500 mM KCl) of *Escherichia coli*, rather than osmotic tolerance (1100 mM sorbitol). In addition, comparing the survival ratios of the transformants under 500 mM NaCl or 500 mM KCl stresses, the results showed that: (1) the survival ratios of BL/PM2 and BL/PM2B were quite similar, both showing much higher values than those of BL/pET28. (2) The survival ratios of BL/PM2C were much higher than those of BL/PM2, BL/PM2A, and BL/PM2B. This provides the first experimental evidence that PM2 polypeptide enhances salt tolerance of *E. coli* cells, and the 22-mer repeat region is an important functional region.

Keywords: LEA3 protein; 22-aa repeating region; High salt tolerance; Hydrophilic protein; Heat-stable protein; Soybean

Late embryogenesis abundant (LEA) proteins are supposed to be one of the proteins to involve in coping with unfavorable conditions for plants [1–3]. Usually, LEA proteins are accumulated in abundance in seed, pollen, and also induced by dehydration, osmotic stress, cold or exogenous abscisic acid [4,5]. Up to now, more and more LEA proteins are found in various plants, in some algae (*Chlamydomonas* W80), bacteria (*Deinococcus radiodurans*), and nematode [5–9].

According to the common amino acid sequence motifs and the conserved structural features, LEA proteins are classified into at least seven groups [10]. There are many literatures about the roles of group 3 LEA (LEA3) proteins in protecting stressed cells [3,11–14]. LEA3 proteins are characterized by a highly conserved 11-amino-acid repeating motif of "TAQAAKEKAGE." The motif was predicted to form a putative amphiphilic α-helical structure. When plants are exposed to dehydration conditions, LEA3 proteins are proposed to protect cellular structures from the effect of water loss and to attenuate the damage caused by high concentration of ions in dehydrating cells in such ways as sequestering or scavenging excess ions in cytoplasm of stressed cells, or as forming cytoskeleton or a tight hydrophilic cytoplasm network or keeping proteins and membrane structures stable [3,15–19]. Based on the predicted structure of LEA3 proteins and on the results of the correlation of *lea*3 gene expression with physiological and environmental stresses, it is hypothesized that LEA3 proteins would play a protective role in plant cells under stressed conditions. Recent years, there are some experimental

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doi:10.1016/j.bbrc.2005.03.165

evidences supporting this hypothesis. For example, Xu et al. [13], Sivamani et al. [11], and Zhang et al. [10] demonstrated that expression of *HVA1* (*lea*3) gene from barley could confer salt and osmotic tolerance in transgenic rice, wheat, and yeast recombinants as well. In vitro, HIC6 and HIC12 (LEA3) proteins from *Chlorella vulgaris* diminished freeze damage of the enzyme lactate dehydrogenase [17].

Although some progress of physiological roles of LEA3 proteins was made as reported above, it is difficult to see the protective effect of over-expression of a *single* LEA3 protein introduced in transgenic plant, due to endogenous *lea*3 gene families that existed in genomic DNA of the host plant cells. In addition, multigene family of *lea* brings difficulty in knocking out the specific *lea* gene [10]. Lan et al. [20] in our laboratory and Yamada et al. [21] provided experimental evidences that soybean PM30 (LEA3) and mangrove CCTα proteins could enhance salt tolerance when expressed in *Escherichia coli* recombinant [20,21]. Meanwhile, the results proved that expression system of *E. coli* is a simple, convenient, and effective model to determine the function of a heterogeneous protein in *E. coli* cells under stress treatments.

Soybean PM2 protein (Accession No. M80664) is one of LEA3 protein families. As reported by Hsing et al. [22], six copies of 22-mer amino acid repeating motif of VNKMGEYKDYAAEKAKEGKDAT are present besides six copies of 11-mer amino acid motif in PM2 protein sequence [22]. As shown above, the sequence of 11-mer and 22-mer repeating motif is completely different. After searching in GenBank database of NCBI, other six embryonic or LEA3 proteins containing 22-mer repeating motifs were found out (listed in Table 1). To our knowledge, little information about the physiological roles of these 22-mer repeats in plant cells has been reported until now. More and more interest rises on the effect and mechanism of LEA3 proteins on protecting dehydrated cells.

In this study, we constructed the recombinant plasmids expressing the full-length of soybean PM2, two truncated PM2, and one artificial sequence of duplication of 22-mer repeating motif (Fig. 1A). These polypeptides were identified by mass spectrometry and proved to be hydrophilic and heat-stable. By spot assay and comparing the survival ratios of the constructed recombinants, the effects of the full-length PM2 and the 22-mer repeating region in protecting transformed bacterial cells from high salt conditions were determined. To our knowledge, this is the first experimental evidence that soybean PM2 protein and its 22-mer repeating region contribute to protecting *E. coli* cells under salt conditions.

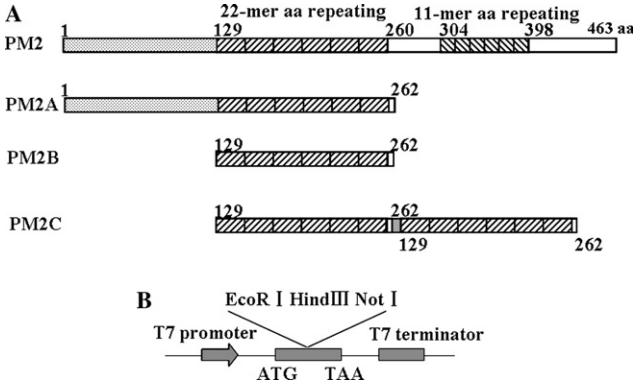

Fig. 1. Schematic representation of the polypeptides and pET28a vector. Polypeptides of full-length PM2 protein, the truncated and artificial polypeptides, namely PM2A, PM2B, and PM2C (A) were expressed in pET28a vector carried by recombinants of *E. coli* BL21 Star as described under Materials and methods. The number of upper frames represents the positions of amino acids in PM2 sequence. The grey box located in the middle of PM2C as a linker represents the amino acid sequence (Thr-Arg) introduced by restriction enzyme sites. The nucleotide sequence of *PM2* was cloned into the *Eco*RI/*Not*I sites of pET28a vector, and *PM2A*, *PM2B*, and *PM2C* were ligated to the *Eco*RI and *Hin*dIII digested pET28a vector (B).

Materials and methods

Materials. Soybean (*Glycine max* L. Merr.cv Bainong 6[#]) seeds were kindly provided by Institute of Agriculture Science in Baicheng City (Jilin Province, PR China). *E. coli* JM109 (recA$^-$) and BL21 Star (DE3) strains were kept in Key Laboratory of Microorganism and Genetic Engineering of Shenzhen City. All chemical reagents were purchased from Sigma–Aldrich Company.

In 2003, the plants were grown in a field of Baicheng City of Jilin Province, PR China. In summer, the immature seeds were collected from the pods 35–45 days after flowering and then used for RNA extraction immediately.

Table 1
The proteins containing 22-mer repeating motif found in GenBank database

Accession No. in GenBank	Name of polypeptide	Source	Category	Length of protein (aa)	Copy number of 22-mer motif
X16131	DC-8 [23]	*Daucus carota*	Embryonic protein	555	10
M80664	PM2 [22]	*Glycine max*	LEA3	463	6
CAA79329	BP8 [24]	*Betula pendula*	Embryonic protein	474	4
T47561	Lea-like	*Arabidopsis thaliana*	Putative LEA,	479	4
AAD01431	PM18[25]	*Glycine max*	LEA3	316	3
AAD20140	AtECP63	*Arabidopsis thaliana*	LEA3	448	3
AAG01035	IAP1 [26]	*Phaseolus vulgaris*	IAA-protein conjugate	318	2

Isolation of soybean PM2 cDNA. Total RNA was isolated from soybean immature seeds (35–45 days after flowering) by RNAgents Total RNA Isolation System (Promega, USA). The reverse transcription reactions were performed using the 3′-Full Core Set (Takara, Japan) according to the manufacturer's directions. To get the nucleotide sequence of full-length *PM2*, a pair of primers was designed according to *PM2* cDNA sequence. The primer sequences were Forward 1: 5′-atggcgtccaagaaacaaga-3′, Reverse 1: 5′-tgcgtctatatatactaata-3′. The amplified *PM2* cDNA was cloned into pGEM-T easy vector (Promega, USA) to create recombinant plasmid pGEM:PM2. The recombinant plasmid was introduced into *E. coli* JM109.

Construction of full-length PM2 and several deletion clones. The *PM2* sequence was cloned into the *Eco*RI/*Not*I sites of pET28a vector to create plasmid pET:PM2.

After studying the plasmid pGEM:PM2 sequence, *Eco*RI site in the vector ahead of *PM2* sequence and *Hin*dIII site in middle of the *PM2* sequence were found. Therefore, the recombinant plasmid pGEM:PM2 was digested with both *Eco*RI and *Hin*dIII so as to produce a 786 bp fragment of *PM2A* (Fig. 1A). In order to get the sequence of *PM2B* (Fig. 1A) encoding six copies of 22-mer repeats, PCR was performed by using the template of plasmid pGEM:PM2 and the following primers, i.e., Forward 2: 5′-caccgaattctctagaggttccaaggtc ggagagtac-3′ and Reverse 2: 5′-gtccaagcttcctagcactagtccccaacgtcgtatc ttt-3′ (restriction enzyme sites underlined). Then, the amplified fragment was treated with *Eco*RI plus *Hin*dIII. Finally, the fragments of *PM2A* and *PM2B* with sticky *Eco*RI/*Hin*dIII ends were ligated into *Eco*RI/*Hin*dIII digested pET28a vector, respectively, so as to create plasmids pET:PM2A and pET:PM2B (Fig. 1B).

To get the plasmid pET:PM2C, the amplified *PM2B* fragment was treated with *Xba*I/*Hin*dIII and cloned into plasmid pET:PM2B which was digested with *Spe*I/*Hin*dIII.

All the recombinant plasmids pET:PM2, pET:PM2A, pET:PM2B, pET:PM2C, and the empty pET28a vector were introduced into *E. coli* BL21 Star to create transformed strains of BL/PM2, BL/PM2A, BL/PM2B, BL/PM2C, and BL/pET28 (control), respectively.

Expression and identification of protein. When bacterial cultures were incubated to reach exponential growth phase, isopropylthio-β-galactoside (IPTG) was added into the cell cultures to a final concentration of 1 mM to induce expression of the inserted gene in recombinants. After incubated for 4 h at 34 °C, the *E. coli* cells were boiled for 5 min in the sample buffer according to the standard method [27] and loaded on SDS–PAGE gels. The bands in gels were stained with Coomassie brilliant blue.

Preparing the protein sample for identification, protein in-gel digestion with trypsin was performed by the standard method [28]. The system of HPLC-MS/MS (Agilent 1100 Series LC/MSD) was operated with the electrospray ionization (ESI) source in the positive ion mode. Agilent Zorba × 300sb-C_{18} (2.1 × 150 mm, 5 μm), flow rate of 0.25 mL/min. The mobile phase consisted of A = 0.1% HCOOH and B = CH_3CN (0.1% HCOOH). The gradient program was that in 10 min 0% B was increased to 20% B and in another 10 min increased to 80% B. The temperature of the drying gas was 350 °C. The flow rate of the drying gas was 9 L/min. The nebulizer pressure was 40 psi. The spray voltage was 4000 V. MS/MS was set at the automatic mode when the relative abundance of precursor ion was above 20%. The MS/MS spectra were submitted to a database search on MASCOT (Matrix Science, UK). MALDI-TOF-MS was performed on a Bruker Autoflex MALDI-TOF mass spectrometer (Bruker Daltonic, Germany) fitted with a 337-nm nitrogen laser, using pulsed extraction, and operating in positive reflective mode, α-cyano-4-hydroxycinnamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid was used as matrix. The peptide mixture was cleaned up using C_{18} Zip Tip (Millipore, USA).

Solubility and heat-stability of expressed protein in E. coli. To study the solubility of expressed heterogeneous polypeptides in recombinant *E. coli*, the cell cultures were incubated in LB liquid medium with IPTG for 4 h, and then the cell pellet after centrifugation was collected and treated with 100 mg/L lysozyme for 15 min. The supernatant after re-centrifugation was electrophoresed on a 15% SDS–polyacrylamide gel.

To test the heat-stability of the expressed protein, bacterial cells were induced by IPTG for 4 h and centrifuged for 10 min at 3000 rpm. The cell pellet was re-suspended in 100 μl PBS buffer. The cell suspension was boiled for 10 min and centrifuged again. The heat-stable proteins in supernatant were separated on a 15% SDS–polyacrylamide gel.

Spot assay of recombinant E. coli on stressed medium. To test stress tolerance of BL/PM2, the cell cultures of BL/PM2 and BL/pET28 were adjusted to OD_{600} 0.8 in spectrophotometer (Pharmacia, USA) and then diluted serially (1:10 and 1:100, respectively). Ten microliters of each sample was spotted onto the LB plates containing 171 mM NaCl and the LB plates with additional 500 mM NaCl, 500 mM KCl or 1100 mM sorbitol, respectively. Then the plates were incubated at 37 °C. Each test was done three times.

Survival ratio determination of E. coli under salt conditions. To determine the effect of 22-mer repeating region on salt tolerance of recombinant *E. coli*, BL/PM2, BL/PM2A, BL/PM2B, and BL/PM2C were diluted serially as above and spread onto the LB plates containing 171 mM NaCl and the LB plates with additional 500 mM NaCl or 500 mM KCl, respectively. After the plates were incubated for 1–2 day(s) at 37 °C, the colony number on each plate was recorded. Survival ratio of the transformants under salt conditions was calculated according to the following formula:

$$\text{Survival ratio} = (\text{mean of colony number on stressed plate}/\text{mean of colony number on LB plate}) \times 100\%.$$

The experiments were repeated five times and each time with three repeated plates. The survival ratios and standard deviation are shown in column diagram.

Results

SDS–PAGE and mass spectrometry (MS) analysis of proteins from recombinant E. coli

As described by Hsing et al. [22], soybean *PM2* gene encodes a protein of 463 amino acids, in which sequence there are six copies of 22-mer repeating motif from aa 129 to aa 260. In order to determine the effect of expressed soybean PM2 protein on salt tolerance of recombinant *E. coli*, the recombined plasmid carrying *PM2* gene (pET:PM2) was constructed (Fig. 1). The *PM2* gene on pET:PM2 vector is translated to a fusion protein with an N-terminus of 36 amino acids of pET28a vector sequence. When this 36 aa sequence was blasted in GenBank database, no homology related with salt tolerance is found. So it seemed unlikely that the N-terminal part of the fusion protein contributed to increasing the stress tolerance of transformed *E. coli* cells. Then according to the structural characteristic of the PM2 sequence, three recombinants carrying two shortened (BL/PM2A and BL/PM2B) and one artificial fragment (BL/PM2C) were designed and obtained, respectively (Fig. 1). These three nucleotide sequences encode the fusion proteins with the same N-terminus of pET:PM2 plasmid as the PM2 fusion protein and

with C-terminus of 11 aa encoded by pET28a vector sequence.

To examine the expression of the foreign gene/fragments in recombinant *E. coli*, total proteins of bacteria were electrophoresed on SDS–polyacrylamide gel. The specific bands of 50 kDa (BL/PM2), 35 kDa (BL/PM2A), and 22 kDa (BL/PM2B) were detected by SDS–PAGE (Fig. 2), respectively. For BL/PM2C, among three bands of 38, 25, and 20 kDa (Fig. 2), the polypeptide of 38 kDa was similar to the theoretical molecular weight (MW) (≈35.97 kDa) of PM2C polypeptide. In MS identification, we focused on the analysis of these four aim proteins.

The protein mass fingerprinting (PMF) of the 50 kDa polypeptide expressed by BL/PM2 is shown in Fig. 3A. The search result on Mascot showed that this polypeptide was PM2 protein (Score108, Fig. 3B). Similarly, HPLC-MS/MS analysis showed that BL/PM2A, BL/PM2B, and BL/PM2C matched PM2 protein too, and the scores in Mascot database were 56, 116, and 213, respectively (Figs. 3C–E).

Determination of solubility and heat-stability of expressed polypeptides

The supernatant of cell lysis was electrophoresed on SDS–polyacrylamide gels. The aim protein bands of PM2, PM2A, PM2B, and PM2C in supernatant of *E. coli* lysis were observed as shown in Fig. 4A. In addition, even after these *E. coli* cells were treated in boiling water, the aim proteins still existed in the supernatant (Fig. 4B), but most of the proteins of the *E. coli* cells disappeared from the supernatant at the same time. These results indicated that these aim polypeptides of PM2, PM2A, PM2B, and PM2C exhibited well hydrophilicity and heat-stability in *E. coli*.

Fig. 2 shows that other bands with the smaller MW besides the specific aim bands from BL/PM2 and BL/PM2C were detected by SDS–PAGE analysis. They also

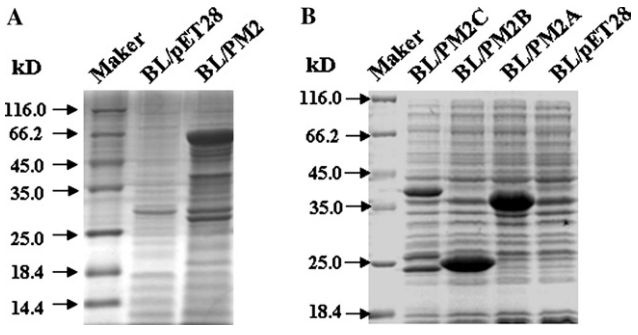

Fig. 2. SDS–PAGE analysis of total proteins from *E. coli*. Total proteins from BL/pET28 and BL/PM2, BL/PM2A, BL/PM2B, and BL/PM2C were separated by SDS–PAGE. The differential protein bands near the calculated molecular mass of polypeptides expressed by BL/PM2(A), BL/PM2A, BL/PM2B, and BL/PM2C (B) were about 50, 35, 22, and 38 kDa.

behaved as soluble and heat-stable proteins (Fig. 4). The two smaller proteins (about 20 and 25 kDa) expressed by BL/PM2C strain excised from Fig. 2B were identified as part of PM2 polypeptide by HPLC-MS/MS (data not shown). We speculated that the shorter polypeptides might be the products of aim proteins degraded in the special sites, but the mechanism of degradation is unclear.

Enhancement of salt-tolerance of recombinant E. coli cells carrying soybean PM2 gene

In order to determine the function of expressed PM2 fusion protein in stress conditions, the effects of high concentrations of NaCl, KCl, and sorbitol on the growth of BL/PM2 and BL/pET28 were examined in advance (Table 2). The diluted cultures of $OD_{600} = 0.8$ were spread on the LB plates with additional 300–700 mM NaCl and KCl. No colony of BL/pET28 was observed on the plates supplemented with 700 mM NaCl and 700 mM KCl. As a result, the concentrations of 500 mM NaCl and 500 mM KCl as stress conditions were used further for testing salt tolerance of host cells. The concentration of 1100 mM sorbitol was determined in the same way.

The aliquots of BL/pET28 and BL/PM2 cultures were spotted onto LB plates and the LB plates with supplementary 500 mM NaCl, 500 mM KCl or 1100 mM sorbitol, respectively. On LB plate and the plate with 1100 mM sorbitol, no significant difference was observed between the colony numbers of these two strains. On the plate supplemented with 500 mM NaCl and 500 mM KCl, the colony number of BL/PM2 was much more than that of the control strain BL/pET28 when compared with the same cell density (Fig. 5). All these results showed that expressed soybean PM2 fusion polypeptide conferred salt tolerance on the host cells, but it had little effect on increasing osmotic tolerance caused by sorbitol. Therefore, effects of soybean PM2 polypeptide expression on growth of the recombinants under high salt treatments were studied in more detail.

Enhancement of salt tolerance of the recombinant E. coli harboring PM2, PM2A, PM2B, and PM2C fragments

Aliquots of the recombinants (BL/PM2, BL/PM2A, BL/PM2B, and BL/PM2C) and the control strain (BL/pET28) were spread on the LB plates and the LB plates supplemented with 500 mM NaCl or 500 mM KCl, respectively. After incubating for 1–2 day(s), the number of colonies on the plate was counted. And the survival ratios of recombinants were calculated. Fig. 6 showed that the survival ratios of BL/PM2 were 2- to 3-fold higher than those of the control strain BL/pET28 under the conditions of high NaCl or KCl. The results indicated that full-length PM2 obviously

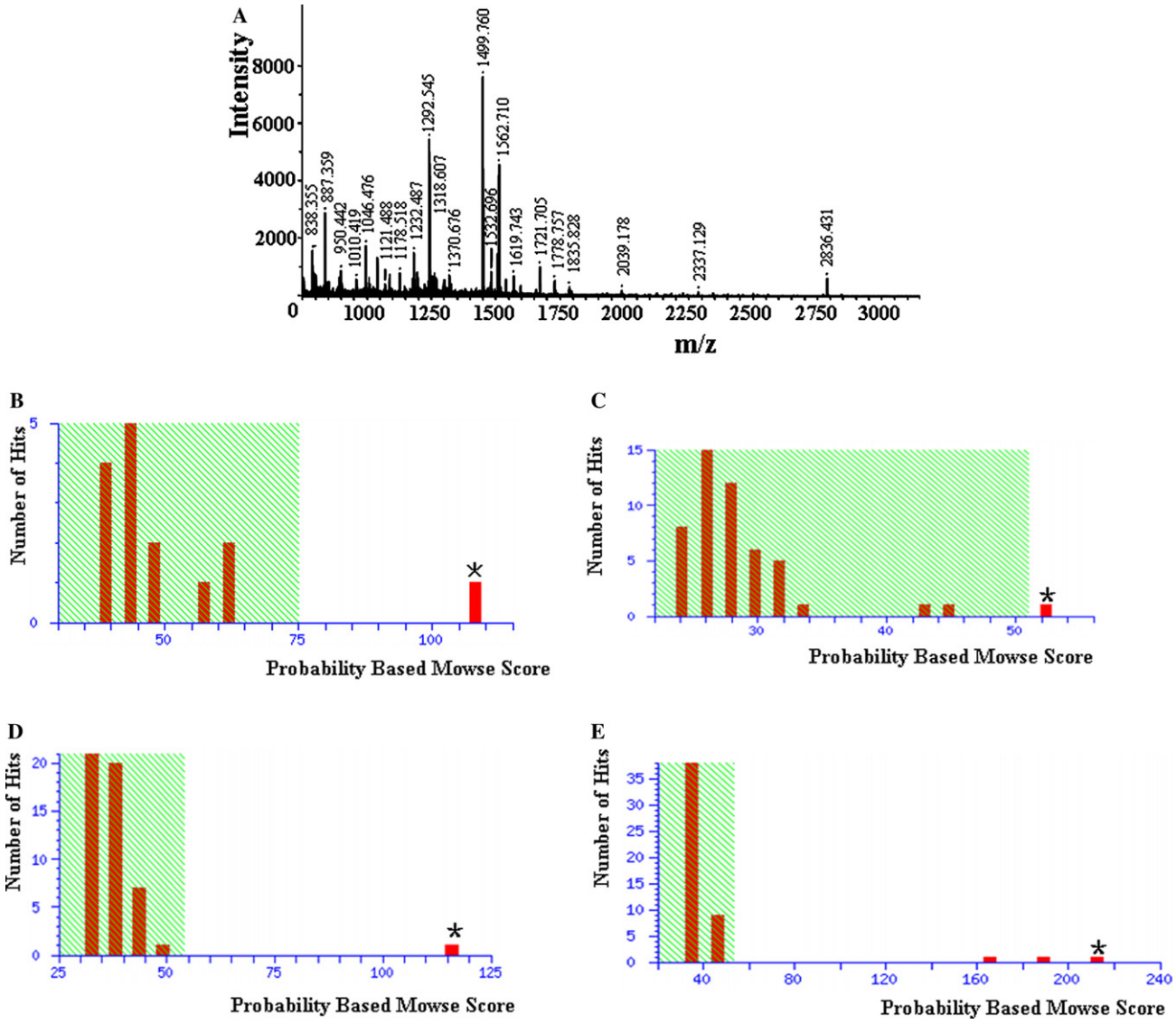

Fig. 3. Determination of aim proteins expressed in recombinant *E. coli* by mass spectrometry. The tryptic polypeptide PM2 expressed by BL/PM2 was analyzed by MALDI/TOF-MS to get the protein mass fingerprinting (A). The search result (B) on Mascot showed that the polypeptide was PM2 protein, whose molecular weight is 59 kDa, and the score was 108 (star mark above the column represents the score of aim protein matched to PM2 protein sequence). The polypeptides of PM2A, PM2B, and PM2C were sequenced by HPLC-MS/MS, and the results were submitted to Mascot database. The scores were 52 (C), 116 (D), and 213 (E), respectively.

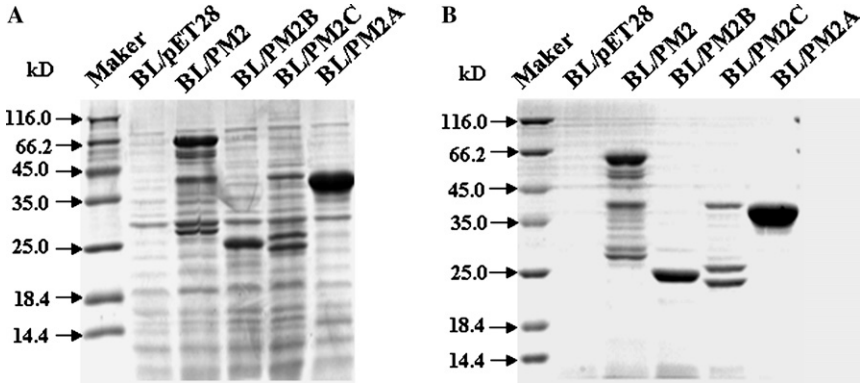

Fig. 4. Solubility and heat-stability analysis of the polypeptides. The soluble and heat-stable polypeptides of recombinants were separated by SDS–PAGE. The molecular masses of aim polypeptides expressed by BL/PM2, BL/PM2A, BL/PM2B, and BL/PM2C were about 50, 35, 22, and 38 kDa.

Table 2
Colony number of BL/pET28 and BL/PM2 on the plates supplemented with high concentration of NaCl and KCl

Strain	Colony number on LB plate with supplementary					
	NaCl concentration			KCl concentration		
	300 mM	500 mM	700 mM	300 mM	500 mM	700 mM
BL/pET28	>1000	10–100	0	>1000	10–50	0
BL/PM2	>1000	10–500	10–50	>1000	50–100	10–50

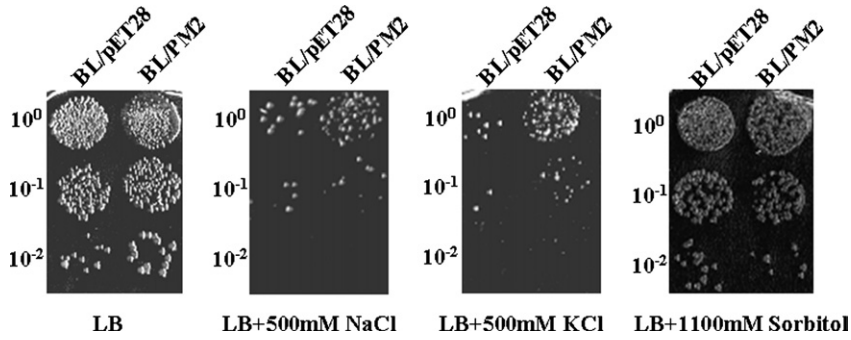

Fig. 5. Spot assay of BL/pET28 and BL/PM2 on the LB plates and the plates with high salt or sorbitol. IPTG was added to the cultures of BL/pET28 and BL/PM2 to induce the recombinants expressing aimed protein. The cultures were adjusted to $OD_{600} = 0.8$. Ten microliters of the serially diluted bacterial suspension was spotted onto the LB plates containing 171 mM NaCl and the plates with additional 500 mM NaCl, 500 mM KCl, and 1100 mM sorbitol.

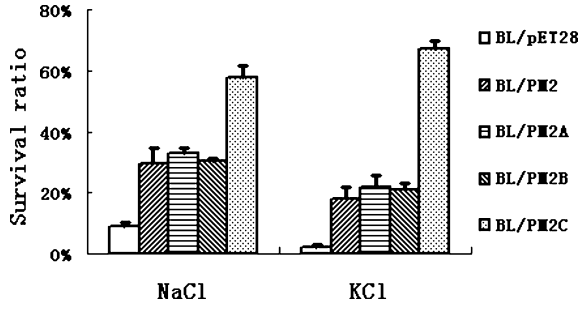

Fig. 6. Survival ratios of the recombinants under high salt conditions. The cultures of BL/pET28, BL/PM2, BL/PM2A, BL/PM2B, and BL/PM2C were spread on LB plates and the plates with additional 500 mM NaCl or 500 mM KCl. The colony numbers appearing on the plates were counted and used for calculating the survival ratios as described under Materials and methods.

enhanced host cells' salt tolerance, which were consistent with the results from the above spot assay in the present paper.

Compared with BL/PM2, the survival ratios of BL/PM2A and BL/PM2B in high NaCl and KCl stresses were quite similar to those of BL/ PM2. It was noted that the survival ratios of BL/PM2C were much higher than those of the other three recombinant strains.

Discussion

Recently, some plant genes related with dehydration stresses have been isolated by functional expression screening in *E. coli*. Most of these proteins such as CCTα, XVSAP1, BBC1, LEA3, HSP100, and ubiquitin proteins are usually located in cytoplasm of the plant cells [21,29–32]. 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. The results show that expression of these plant genes directly contributes to increasing stress tolerance of the bacteria host cells, and also indicate that some of the protective mechanisms might be common in prokaryote and eukaryote under stress conditions [33].

High salt and osmotic stresses would cause the intracellular dehydration and damage of both proteins and cellular membrane as well [1,2,20]. Herein, our results of enhancement of salt tolerance of the recombinant bacteria cells can indicate that expression of soybean *PM2* gene in host cells could confer protective function against damage of proteins, cellular membrane, and cells. The enhanced salt-tolerance of PM2 proteins functioned in *E. coli* is consistent with that of other LEA3 proteins described in transgenic wheat [11], rice [12,13], onion [34], in yeast transformants [10], and in bacteria cells [20]. Houde et al. [35] suggested that hydrophilic and heat-stable proteins may modify the structure of proteins and bind water directly to attenuate the damage caused by desiccation [35]. Also, it was supposed that LEA3 proteins were involved in protecting cells directly or by interacting with other cellular components (i.e., non-reducing sugar or proteins) [15,19,36]. In this present paper, no signal peptide was found in the N terminus

of PM2, the truncated polypeptides, and the artificial polypeptide (data not shown). And these polypeptides are also hydrophilic and heat-stable proteins. For this reason, we speculated that the soybean PM2 proteins, the truncated polypeptides, and the artificial polypeptides with both the specific conserved regions and characteristics of hydrophilicity should be distributed and function in cytoplasm of *E. coli* cell. When high salt stress was applied, these proteins or polypeptides in the cells might be interacting with common or similar components existing in prokaryotes or in plants, might be sequestering excess ions in cytoplasm, or forming a tight hydrogen bonding network in the dehydrating cytoplasm. In such ways, they could protect the host cells' survival from excess ion toxicant by stabilizing the structure of protein and cellular membrane in stressed cells. However, from the preliminary result presented in this study, we can see the specifically protective roles of LEA3 protein to the cells under high salt condition rather than those under osmotic stress by sorbitol. Based on the results of protective functional similarity of LEA3 proteins in prokaryote and plant cells under dehydrations, it is reasonable to speculate that the protective mechanisms of LEA3 proteins in prokaryote might be similar to those in plants.

Some LEA3 proteins and embryonic proteins contain a highly conserved 22-mer repeating motif (listed in Table 1). Between the conserved 22-mer repeating motifs there is more than 60% homology, indicating that this kind of sequence would have an important physiological role in development of seeds. From Fig. 6, we can see that the survival ratios of BL/PM2 and BL/PM2B are quite similar under salt conditions, both being much higher value than that of BL/pET28 with an empty vector as control, although polypeptide PM2B is only 1/3 of PM2 sequence in length. This provides the first experimental evidence for a hypothesis that *only* PM2B region composed with six copies of 22-mer repeating motif is an important functional region to enhance salt tolerance of the host cells.

In additional, the survival ratio of BL/PM2C in high salt conditions was 1–2 times high than that of BL/PM2B as PM2C polypeptide was a duplication of PM2B. Besides, although the sequences of PM2C (286aa) and PM2A (262aa) are similar in length, the survival ratio of the former is much higher than that of the latter (Fig. 6). Based on the results, we speculated that the front part (aa 1–128) of the PM2A sequence seems to have no obvious influence on enhancement of salt tolerance to host cells. These results collectively suggested 22-mer repeating motifs as the functional region to protect host cells under salt conditions. The sequence of 22-mer motif is different from 11-mer motif in amino acid composition and the secondary structure (data not shown). For this reason, the mechanism of 22-mer region in protecting *E. coli* cells from salt stress might show some difference from that of 11-mer region. The more details in protective mechanism as for the functional region of six copies of 22-mer repeating motif and the effect of "non-functional" front part (aa 1–128) in the PM2A sequence on salt tolerance of cells are under study.

Acknowledgments

This work was supported by the National Nature Science Foundation of China (30470107) and the National Special Program for Research and Industrialization of Transgenic Plants (JY03A18). We thank Dr. Ying Lan, Qingguo Han, Yong Wang, Ranhui Li, and Zhanhui Ye at Shenzhen University for their kind help in experiments.

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