

# Soybean PM2 Protein (LEA3) Confers the Tolerance of *Escherichia coli* and Stabilization of Enzyme Activity Under Diverse Stresses

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**Abstract** Late embryogenesis abundant (LEA) proteins are closely associated with the tolerance of diverse stresses in organisms. To elucidate the function of group 3 LEA proteins, the soybean PM2 protein (LEA3) was expressed in *E. coli* and the protective function of the PM2 protein was assayed both in vivo and in vitro. The results of a spot assay and survival ratio demonstrated that the expression of the PM2 protein conferred the tolerance to the *E. coli* recombinant for different temperature conditions (4, –20 or 50°C) or high-salinity stresses (120 mmol/l MgCl<sub>2</sub> or 120 mmol/l CaCl<sub>2</sub>). In addition, it was demonstrated that the in vitro addition of the PM2 protein could prevent the lactate dehydrogenase (LDH) inactivation normally induced by freeze–thaw. In the 62°C condition, the PM2 protein (1:5 mass ratio to LDH) effectively prevented the LDH thermo-denaturation by acting synergistically with trehalose (62.5 µg/ml), although the PM2 protein alone at this concentration showed little protective effect on LDH activity. Furthermore, the results showed that the PM2 protein could partially prevent the thermo-denaturation of the bacterial proteome after boiling for 2 min. Based on these results, we propose that the PM2 protein itself, or together with trehalose, conferred the tolerance to the *E. coli* recombinant against diverse stresses by protecting proteins and enzyme activity under low- or high- temperature conditions.

## Introduction

Late embryogenesis abundant (LEA) proteins are associated with the acquisition of resistance to various environmental stresses in organisms [1, 2]. More than 20 years ago, the LEA protein was first characterized in cottonseed during the maturation phase of embryogenesis [3]. Later, more LEA proteins were discovered to accumulate in plant vegetative tissues exposed to various stresses, such as chilling, drought and high salinity [1, 2]. The LEA proteins belong to a multigene family, which is classified into 5–9 groups based on expression patterns and sequences [1, 2]. Among them, the Group 3 LEA (LEA3) proteins are characterized by a repeated 11-mer amino acid motif (TAQAAKEKAGE) [4]. Recently, LEA3-like proteins were also found to be related with stresses in some non-plant organisms, such as invertebrates, green alga, and even some bacteria [5–7]. There are extensive correlative data linking the expression of LEA3 proteins with tolerance toward diverse stresses in organisms [1, 8, 9]. However, there was exception: the expression of certain LEA3 proteins (barley HVA1, *Cratogeomys* pcC3-06) could not enhance the heat- or drought-tolerance of yeast or tobacco [10, 11].

Earlier studies suggested various biochemical mechanisms for LEA3 proteins, such as the repair of improperly folded proteins as a chaperone, the binding of metal ions, the stabilization of membrane structure, and the increase of cellular mechanical strength through the generation of filaments [4, 12–14]. Recently, LEA3 proteins were experimentally demonstrated to protect small unilamellar vesicles (SUVs) or cell membranes against desiccation [8, 13], and to prevent the protein aggregation and enzyme inactivation caused by desiccation, freeze–thaw or heat stresses [15, 16]. Current in vitro data have provided support for the multifunctional activity of LEA3 proteins, however, the

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activity remains to be tested in different assays using the same protein [14].

The soybean PM2 protein is a member of the LEA3 family [17]. Previously, we demonstrated that the over-expression of the PM2 protein enhanced the tolerance of *E. coli* to a high concentration of NaCl and KCl [18]. In the present study, we also demonstrated that the PM2 protein could improve the tolerance of recombinant *E. coli* under temperature (4, -20, 50°C) or salinity (MgCl<sub>2</sub> or CaCl<sub>2</sub>) stress. Additionally, the in vitro protective function of the PM2 protein on LDH activity was evaluated under diverse stresses, such as freeze-thaw, high temperature or high salinity.

## Materials and Methods

### Strains and Plasmids

The *E. coli* BL/pET and BL/PM2 strains were constructed and kept in the Key Laboratory of Microorganism and Genetic Engineering of Shenzhen City, P.R. China [18]. The soybean PM2 gene (Genebank accession no. M80664) was inserted into the pET28a vector and then transformed into *E. coli* BL21 Star cells to create the BL/PM2 recombinant. There is a 6× His-tag constructed at the N-terminal of the PM2 fusion protein. The empty vector of pET28a was introduced into *E. coli* to create BL/pET as the control.

### The Spot Assay and Survival Ratio of Recombinant *E. coli* Under Diverse Stresses

To assay the temperature tolerance of the *E. coli* recombinant, the cultures were induced with IPTG at a final concentration of 1 mmol/l for 4 h and exposed to 4 and -20°C for 24 h, or 50°C for 45 min, respectively. The concentration of these cultures was identified at OD<sub>600</sub> = 0.8 and then diluted serially (1:10 and 1:100). Ten microliters of each sample were spotted onto LB plates with 1 mmol/l IPTG before incubation overnight at 37°C. In addition, 100 µl of stressed and unstressed samples were spread on the LB plates, respectively. The number of colonies on the plates was calculated to determine the survival ratios [18].

For the assay of the salinity tolerance of the recombinants, *E. coli* cells were spread on LB plates containing 120, 250 or 500 mmol/l MgCl<sub>2</sub> or CaCl<sub>2</sub>, respectively. After incubation for 48 h at 37°C, the survival ratios of *E. coli* were calculated [18].

### LDH Activity Measurement

LDH from hog muscle was purchased from Roche (Indianapolis, IN, USA). The LDH was diluted in 100 mmol/l sodium phosphate buffer (pH 7.0) to a final concentration of

50 mg/l. Test proteins were added to equal volumes of LDH at mass ratios of 1:5, 1:1, 5:1 or 10:1 (test protein: LDH). In the freeze-thaw assay, the enzyme mixture was flash-frozen in liquid N<sub>2</sub> for 1 min and then thawed for 5 min at 25°C. This freeze-thaw cycle was repeated up to 10 times. For the thermo-denaturation assay, the LDH mixture was incubated at 62°C for 5 min and then kept on ice for 20 min. For the high-salinity assay, the LDH mixture was supplemented with 1 mol/l NaCl and kept at 25°C for 20 min.

To determine the LDH activity, 5 µl of the LDH mixture was made up to 2 ml with the assay buffer (100 mmol/l PBS buffer containing 7.5 mmol/l pyruvate and 0.1 mmol/l NADH), or the assay buffer supplemented with 1 mol/l NaCl for the high-salinity treatment. The LDH activity was monitored by the absorbance at 340 nm for 2 min due to the conversion of NADH into NAD at 37°C. All of the values given were expressed as the percentage of the rate of the reaction measured for the untreated samples.

### Thermo-Stability of Soluble Proteome of *E. coli*

The soluble proteins were extracted from *E. coli* cells and the amount of soluble protein was quantified by the Bradford protein assay and diluted to 1.54 g/l. The proteome was kept at 100°C for 2 min with 0.5 g/l PM2 or BSA. And the soluble protein extraction was kept boiling and used as control. The samples were centrifuged at 10,000 rpm for 20 min at 4°C. The amount of protein in the supernatant was quantified again by the Bradford protein assay and the protein fractions were detected by SDS-PAGE.

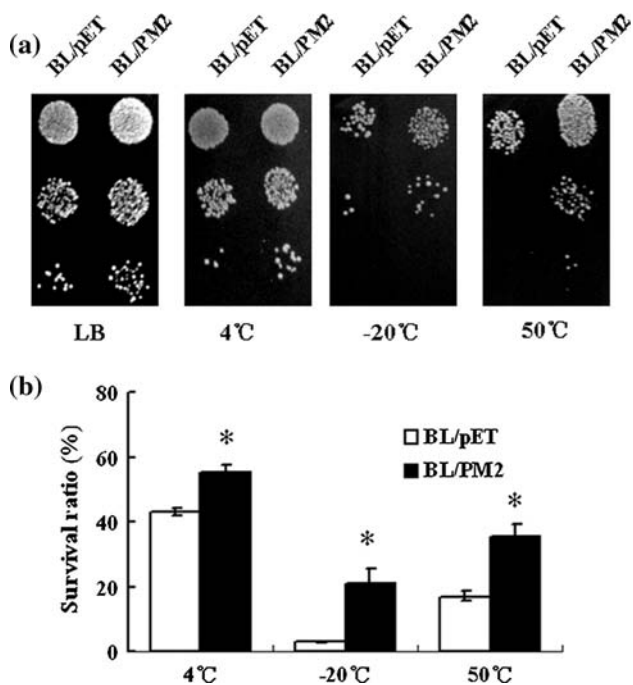
### Statistical Analysis

The survival ratio assay and LDH activity measurement were carried out in triplicate. The values shown in the table and figures were mean value ± SD. The statistical significance was determined by the Student's *t* test.

## Results

### The Temperature Tolerance of *E. coli* Recombinants Expressing the PM2 Fusion Protein

To determine the effect of the over-expression of the PM2 protein on the growth of *E. coli* recombinants under different temperature stresses, cultures of BL/pET and BL/PM2 recombinants were induced by IPTG, and then the spot assay and survival ratio were performed. The results of the spot assay showed that there was no obvious difference in growth behaviors between BL/pET and BL/PM2 at 37°C, indicating that expression of the PM2 protein did not inhibit the growth of the *E. coli* recombinant (Fig. 1a).



**Fig. 1** The growth performance of BL/PM2 and BL/pET cells. The *E. coli* cells were subjected to 4 or  $-20^{\circ}\text{C}$  for 24 h, or  $50^{\circ}\text{C}$  for 45 min, and were then spotted (a) or spread (b) on LB plates containing 1 mmol/l IPTG. The survival ratio was calculated according to [18]. Significant differences in the growth performance between BL/PM2 and BL/pET cells are indicated as \*  $P < 0.05$ , evaluated with the Student's *t* test

When the cells were subjected to the temperatures of 4,  $-20$ , and  $50^{\circ}\text{C}$ , however, the numbers of BL/PM2 colonies were much greater than those of BL/pET (Fig. 1a). Furthermore, under the stresses of 4,  $-20$ , and  $50^{\circ}\text{C}$ , the survival ratios of BL/PM2 were about 55, 21, and 35%, respectively, which were much higher than those of the control BL/pET (Fig. 1b).

#### The High-Salinity Tolerance of *E. coli* Recombinants Expressing the PM2 Fusion Protein

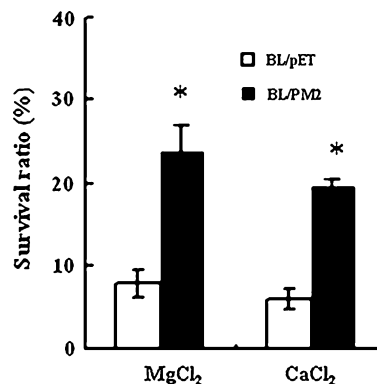
Previously, we demonstrated that the expression of the PM2 protein contributes to *E. coli* cell tolerance to 500 mmol/l NaCl or 500 mmol/l KCl [18]. In the present study, neither BL/pET nor BL/PM2 could survive on the medium supplemented with 500 mmol/l or 250 mmol/l  $\text{MgCl}_2$  or  $\text{CaCl}_2$  (Table 1). When the concentration of  $\text{MgCl}_2$  or  $\text{CaCl}_2$  in the plates was reduced to 120 mmol/l, the survival ratio of BL/PM2 was 24 or 20%, respectively, much higher than that of the BL/pET control (Fig. 2).

These results indicated that the over-expression of the PM2 protein could enhance the tolerance of *E. coli* recombinants under low- and high-temperature and high-salinity conditions. These results are in contrast to those from our previous studies of the soybean ZLDE-2 and

**Table 1** The effect of different concentrations of  $\text{MgCl}_2$  or  $\text{CaCl}_2$  on the growth of the *E. coli* recombinants

<i>E. coli</i>	Colony number on the LB plate with high salinity					
	$\text{MgCl}_2$ concentration (mmol/l)			$\text{CaCl}_2$ concentration (mmol/l)		
	50	120	250/500	50	120	250/500
BL/pET	>1000	30–150	–	>1000	20–70	–
BL/PM2	>1000	50–300	–	>1000	50–200	–

– means no or few colony



**Fig. 2** The survival ratios of BL/PM2 and BL/pET cells under high-salinity conditions. *E. coli* cells were spread on LB plates containing 120 mmol/l  $\text{MgCl}_2$  or 120 mmol/l  $\text{CaCl}_2$ , respectively, and then incubated at  $37^{\circ}\text{C}$  for 48 h. Significant differences in the survival ratio between BL/PM2 and BL/pET cells are indicated as \*  $P < 0.05$ , evaluated with the Student's *t* test

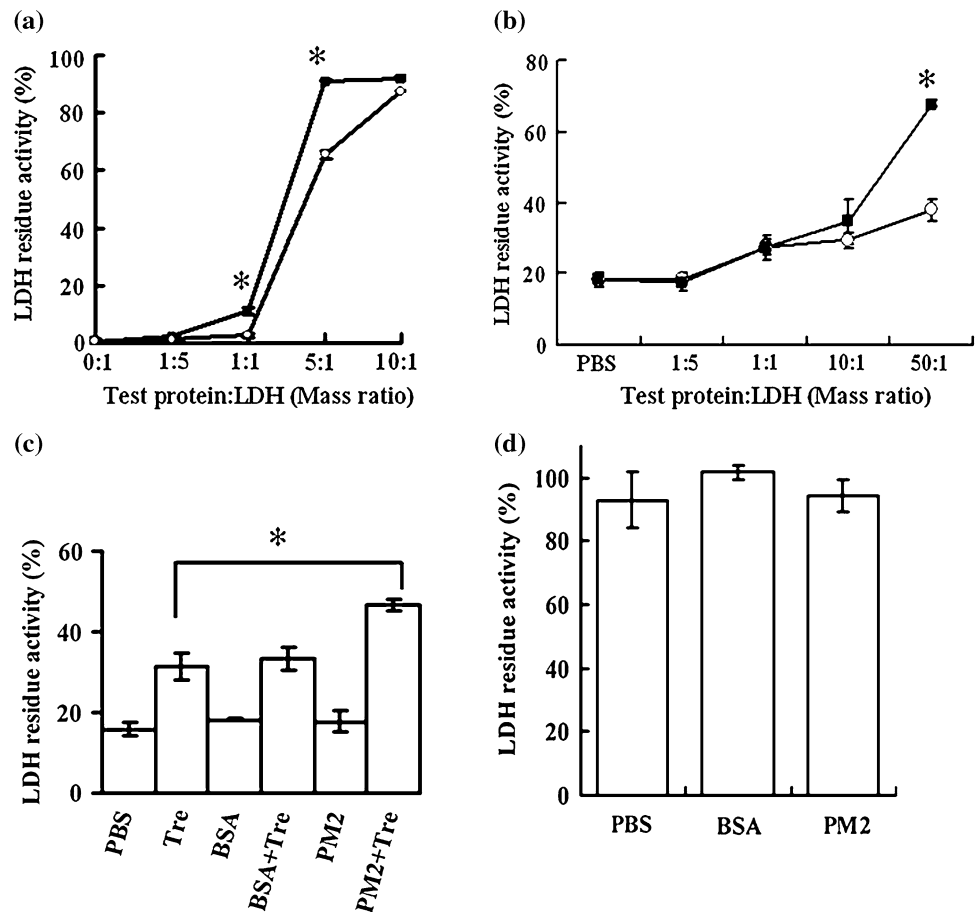
Sali3-2 proteins [19, 20]. Thus, the possibility could be ruled out that any heterogeneous protein expressed in *E. coli* could protect the recombinants from stresses as was observed for the soybean PM2 protein.

#### The Protective Effect of the PM2 Fusion Protein on LDH Activity Under Different Stresses In Vitro

LDH is known to be sensitive to water limitation upon freeze–thaw [15]. The protective effect of the PM2 protein on LDH activity after a freeze–thaw treatment was assessed. After 10 cycles of freeze–thaw treatments, the residual LDH activity was reduced to 1% (Fig. 3a). In the presence of PM2, the LDH activity remained at 11% even at the mass ratio of 1:1 (PM2: LDH). This result indicated that the PM2 protein prevented the LDH inactivation that is normally induced by a freeze–thaw. Furthermore, the protective effect of PM2 on LDH activity was manifested in a dose-dependant manner in the range from a 1:1 to 10:1 mass ratio (PM2: LDH), as shown in Fig. 3a.

Then, the thermo-protective action of PM2 on LDH activity was evaluated. Under the stress of  $62^{\circ}\text{C}$ , the residual LDH activity decreased to 18% at a 1:5 mass ratio

**Fig. 3** The protective effect of the PM2 protein on LDH activity. The LDH residual activity after 10 freeze–thaw cycles was determined with PM2 (■) or BSA (○) protein (a). Significant differences in the residual activity of LDH between PM2 protein group and control group are indicated as \*  $P < 0.05$ , evaluated with the Student's  $t$  test. The residual activity of LDH at 62°C was detected in the presence of different concentrations of PM2 (■) or BSA (○) (b). Trehalose showed a synergistic protective effect with PM2, but not with BSA at a 1:5 mass ratio of tested protein: LDH (c). 1 mol/l NaCl showed no deleterious effects on LDH activity with or without PM2 or BSA (d). The data are the averages  $\pm$  standard deviations of three independent experiments. \*  $P < 0.05$



of PM2: LDH. Upon increasing the PM2: LDH mass ratio to 50:1, the residual LDH activity rose to 68% of the original activity (Fig. 3b). It was noteworthy that the LDH residual activity remained at 47% when 62.5  $\mu\text{g}/\text{ml}$  trehalose was added to the PM2 mixture (1:5 mass ratio to LDH), much higher than that with trehalose alone (Fig. 3c). Obviously, the addition of the PM2 protein could enhance the protective effect of trehalose against LDH thermo-inactivation in a synergistic fashion. In contrast, such a synergistic effect was not observed for the combination of BSA and trehalose (Fig. 3c).

In the presence of 1 mol/l NaCl, however, the residual LDH activity remained at 100%, indicating that the LDH activity was not sensitive to a high concentration of ions. The LDH activity also retained the original level when BSA or the PM2 protein was supplemented under the high-saline condition (Fig. 3d).

#### The Thermo-Protective Effect of the PM2 Protein on the *E. coli* Soluble Proteome

To examine whether the PM2 protein can protect other proteins beside LDH, the proteome of *E. coli* was extracted

and kept at 100°C with or without the PM2 protein. The amount of soluble protein remaining after centrifugation was measured. The results showed that only 0.6% of the proteome remained stable in the supernatant after boiling (Table 2), whereas 18.6% of the proteins remained stable in the presence of PM2, which was more than the sum of the proteome (0.6%) and PM2 protein (8.75%). Furthermore, the SDS-PAGE pattern of the thermostable proteins was similar in the absence or presence of the PM2 protein, indicating that the PM2 protein protected the *E. coli* proteome non-specifically (Fig. 4).

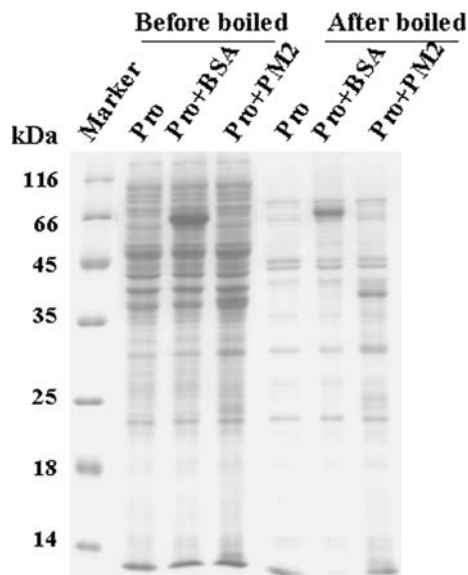
#### Discussion

There are extensive correlative data linking the expression of LEA3 proteins with stress resistance in plants, and LEA3 proteins have been proposed to play important roles in protecting plants from abiotic stresses [1, 8, 9]. However, it is still unclear whether the same LEA3 protein could contribute to protecting organisms from diverse stresses. In this and our previous paper, we demonstrated that the over-expression of the same PM2 protein enhanced

**Table 2** The protective effect of the PM2 protein on the soluble proteome of *E. coli*

	Proteome	BSA	PM2	Proteome + BSA	Proteome + PM2
Percentage of thermo-stable protein (%)	0.60 ± 0.47	1.9 ± 0.63	8.75 ± 0.69	3.5 ± 0.71	18.6 ± 2.56*

Significant differences in the percentage of thermo-stable protein of proteome + PM2 and the sum of the proteome and PM2 protein is indicated in the table as \*  $P < 0.05$ , evaluated with the Student's *t* test



**Fig. 4** The effect of the PM2 protein on the *E. coli* soluble proteome. The soluble proteome of *E. coli* was boiled at 100°C for 2 min in the presence of BSA or the PM2 protein. The supernatant after centrifugation was subjected to SDS-PAGE and stained with Coomassie blue

the tolerance of *E. coli* recombinants to diverse stresses: low or high temperatures, and high salinities of NaCl, KCl, MgCl<sub>2</sub> or CaCl<sub>2</sub> [18].

In general, the denaturation and dysfunction of enzymes (or other protein) occur when cells are subjected to dehydration, or low- or high-temperature stress [15, 16, 21]. In the present study, the *in vitro* data showed that the PM2 protein protected LDH against the inactivation induced by freeze-thaw cycles. Thus, we hypothesized that the PM2 protein would play an important role in protecting the proteins and enzymes of *E. coli* cells *in vivo* under low-temperature stress.

It has been reported that *in vitro* LEA proteins protect the firefly luciferase enzyme at high temperatures [22]. It has been hypothesized that LEA proteins may play protective roles for proteins by acting as chaperone molecules [22]. Alternatively, as described by Goyal et al., LEA proteins could function as a kind of “molecular shield” by forming a physical barrier between neighboring LDH molecules [15]. In the present study, the PM2 protein alone could protect LDH activity to some extent *in vitro* under a high-

temperature stress. In comparison, the PM2 protein, by acting synergistically with trehalose, protected LDH activity more effectively. Furthermore, the presence of the PM2 protein could non-specifically prevent the proteome of *E. coli* from thermo-denaturation. These results are consistent with those for the AavLEA1 protein [15]. As trehalose synthesis could be induced dramatically upon the exposure of *E. coli* to stress conditions [23], it is reasonable to speculate that the PM2 protein, together with trehalose, could prevent enzymes (or proteins) from thermo-denaturation and enhance the thermo-tolerance of *E. coli* cells. In addition, the results showed that the protective effect of PM2 at a 50:1 mass ratio to LDH was much higher than that at the 10:1 mass ratio. Thus, it is still unclear how LEA proteins exactly function and further investigation will be required on the behaviors of LEA proteins under stress conditions.

Generally, a high concentration of ions may be toxic to proteins (enzymes), organelles, and membranes in the cells [8, 24]. In the present study, the *in vitro* results showed that the LDH activity remained at 100% even in 1 mol/l NaCl, indicating that LDH was not sensitive to high salinity. In previous papers and the current study, the *in vivo* results showed that the expression of the PM2 protein could directly contribute to the enhanced high-salt tolerance of *E. coli* [18]. Some reports suggest that the LEA3 protein could sequester or bind excess ions in cells subjected to high salinity [4, 10]. For this reason, we hypothesized that the expression of the soybean PM2 protein might alleviate deleterious effect of ions on *E. coli*.

There are examples in the literature where multiple functions of the same LEA protein have been assayed. For instance, it has been demonstrated that the *Citrus* CuCOR15 (dehydrin) may act as an antioxidant, protein stabilizer, and water buffer, and even may bind ions or nucleic acids [25]. Our results herein showed that the PM2 protein functioned as a protein stabilizer to maintain LDH activity and to alleviate ion toxicity in host cells. Thus, we speculate that the soybean PM2 protein has potential multiple protective functions, similar to CuCOR15. Further experimental evidence is required to provide advanced knowledge on the diverse functions of the LEA proteins.

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