Doi: 10.31276/VJSTE.61(1).23-29

Expression and purification of thioredoxin-his$_6$-ZmDREB2.7 fusion protein in *Escherichia coli* for raising antibodies

Thuy Linh Nguyen[1, 2], Thuy Duong Nguyen[1], Van Hai Nong[1], Thi Thu Hue Huynh[1*]

[1]*Institute of Genome Research, Vietnam Academy of Science and Technology*
[2]*University of Science, Vietnam National University, Hanoi*

Received 3 August 2018; accepted 23 November 2018

Abstract:

Dehydration-responsive element-binding (DREB) proteins play a critical role in the plant's drought-tolerance mechanism despite their presence in minor amounts in the cell. In this study, a maize-derived transcription factor protein, ZmDREB2.7, was overexpressed in the *Escherichia coli* strain Rosetta 1. The interested gene conjugating with the thioredoxin gene (*TrxA*) and his$_6$ tag in the pET-32a vector encoded a 55.7 kDa fusion protein. The optimum condition for inducing the thioredoxin-his$_6$-ZmDREB2.7 expression was five hours of induction with 0.05 mM IPTG at 30^{0}C. The Tris-HCl 20 mM pH 8.0 lysis buffer was harnessed to extract the recombinant protein for the purification process. Using the immobilized-metal affinity chromatography column, the recombinant protein was purified and then injected into rabbits. The antisera containing polyclonal antibodies (pAbs) could specifically recognize the ZmDREB2.7 fusion protein. This study represents updated data on the bacterial expression of the recombinant ZmDREB2.7 protein and the production of anti-ZmDREB2.7 pAbs.

Keywords: ***E. coli*****, fusion expression, recombinant protein, ZmDREB2.7 protein.**

Classification number: **3.1**

Introduction

The ZmDREB2.7 protein belongs to the DREBs transcription factor family that involved in the plant abiotic resistance mechanism. The DREB transcription factors can be classified into two groups based on the protein structure: DREB1, and DREB2, despite the fact that they both contain an AP2 DNA-binding domain. In fact, DREB proteins bind specifically to the dehydration-responsive element (DRE) which contains a core motif of A/GCCGAC locating in the promoter region of many genes induced by drought and/or cold [1]. The DREB2 proteins and their coding genes were characterized in different species. In *Arabidopsis thaliana*, DREB2A and DREB2B are induced by osmotic stress and high temperature. Transgenic *A. thaliana* plants overexpressing *AtDREB2A CA*, which was *AtDREB2A* with a deletion of the negative regulatory domain, showed an improved stress tolerance to drought and heat-shock stresses [2, 3]. An *OsDREB2B* gene isolated from rice enhanced drought and cold tolerance in transgenic plants without any phenotypic changes [4]. Meanwhile, a *PeDREB2* gene from the desert-grown tree (*Populus euphratica*) was reported to be induced by cold, drought, and high salinity conditions and PeDREB2 could specifically bind to the DRE element in the promoter region of many stress-driven genes [5]. In addition, the transient expression of PeDREB2 in onion epidermis cells showed that the protein localized to the nucleus which confirmed that DREB proteins act as a transcription factor [5]. Pandey and colleagues [6] built a model of a wheat DREB2 protein (*Triticum aestivum* L.) and reported that the protein interacts with the major DNA grove through its β-sheets.

In maize (*Zea mays* L.), a genome-wide analysis [1] successfully identified and cloned 18 *ZmDREB* genes (10 Zm*DREB1* genes and 8 *ZmDREB2* genes). Among them,

**Corresponding author: Email: hthue@igr.ac.vn*

ZmDREB2.7 was reported as the most potential gene for crop improvement by marker-assisted breeding and genetic engineering. *ZmDREB2.7-Tv* is a gene isolated from Tevang 1 maize cultivar which exhibits a good tolerance with drought and cold conditions. Originally, the DREB transcription factors are present in a small amount in the plant cells. In addition, the ZmDREB2.7 protein is induced mainly when a plant confronts osmotic stress [1]. In order to clarify the role of ZmDREB2.7 protein involvement in drought tolerance, it is necessary to obtain the protein in a high quantity with good quality. Therefore, the heterologous expression of the DREB2.7 in bacteria brings advantages. A high amount of the ZmDREB2.7 protein could be used for understanding the characteristics of the protein. In addition, anti-ZmDREB2.7 polyclonal serum can be employed to detect the presence of the specific ZmDREB2.7 protein.

Heterologous protein expression in other host systems has been harnessed for the production of many plant proteins [7]. Due to the fact that the protein isolation from the plant is high-cost, labor-intensive, time-consuming, and low-quantity, bacterial expression systems offer a promising alternative. In fact, the plant proteins produced by bacteria were widely employed for research, therapy, and industrial applications. The popularity of using *E. coli* as a workhorse for synthesizing plant protein is a result of its rapid growth at high-cell density on an inexpensive carbon source, well-known genetics, and the commercial availability of enormous expression vectors and strains. However, challenges faced when using bacterial systems to express eukaryotic proteins are lack of post-translation modification and formation of inclusion bodies containing inactive proteins [8]. These causes can be classified into two categories: those that are in the gene sequences and those that are the limitations of the *E. coli* [8]. Fortunately, a number of literature reviews provided comprehensive knowledge to optimize the procedures and parameters involved in the bacterial heterologous protein expression and the purification process [7-11]. In order to troubleshoot the aforementioned problems, the recombinant host, the strain, the expression vector, the inducing conditions, and the approach to modifying the coding sequence of the interested protein should be carefully considered.

The immunization of animals to induce an immune response is a procedure performed routinely worldwide. The process produces antibodies against a specific antigen in laboratory animals such as mice, rabbits, and chickens. Among them, mice and rabbits are the most frequent species used for antibody production. Depending on the desired application and the availability of time and money, scientists may choose between generating monoclonal antibodies (mAbs) or polyclonal antibodies (pAbs). Production of mAbs is a labor-intensive and time-consuming work. In addition, generation of mAbs comprises cell culture which requires high financial investment, but a low titer of mAbs can be obtained. Meanwhile, the induction of pAbs usually takes 4-8 weeks with high titer. In fact, polyclonal antiserum can be obtained with inexpensive procedures and instruments. Therefore, the pAbs is suitable for many applications and is favored by many scientists [12].

In this study, we introduced the *ZmDREB2.7* gene into the pET-32a expression vector to generate the thioredoxin-his$_6$-ZmDREB2.7 fusion protein. The recombinant ZmDREB2.7 fusion protein was overexpressed, purified and used for raising polyclonal antibodies.

Materials and methods

Construction of the recombinant expression vector

The coding sequence of *ZmDREB2.7* originated from maize was cloned into the pJET1.2 vector at Genome Biodiversity Laboratory, Institute of Genome Research. In order to enable cloning the gene into the pET-32a expression vector, two primers (Zm2.7 BamHI F: 5' - TAGTCGGATCCGATCGGGTGCCGC - 3', Zm2.7 EcoRI R: 5'- CGACGAGAATTCTAAAGAGGGACGACGA - 3') were designed with *Eco*RI and *Bam*HI restriction sites at the 5' and 3' end, respectively. The PCR reaction using the primer pair was conducted with the total volume of 25 µl which contains 12.5 µl 2X Thermo Scientific Dream*Taq* PCR Master Mix, 1 µl of 10 µM each primer, 1 µl of 10 µg/µl pJET1.2+ZmDREB2.7 plasmid, 0.8 µl of DMSO, and 8.7 µl ddH_2O. The temperature conditions were as follows: 4 min at 94°C followed by 35 cycles of 45 sec at 94°C, 45 sec at 56°C and 1 min 10 sec at 72°C, then a final extension of 3 min at 72°C. The PCR product of approximately 1.1 kb long was digested with *Eco*RI and *Bam*HI restriction enzymes, and the same to the pET-32a expression vector. The two digested fragments, one of *ZmDREB2.7* and one of the linearized pET-32a plasmid in which both flanked by *Eco*RI and *Bam*HI restriction sites, were ligated using standard molecular biology techniques [13]. The identity of clones harboring the pET-32a+*ZmDREB2.7* plasmid was identified by restriction enzymes-based screening and confirmed by sequencing.

Expression of thioredoxin-his$_6$-ZmDREB2.7 in E. coli

The pET-32a+*ZmDREB2.7* expression vector was transformed into the *E. coli* strain Rosetta 1. A transformed colony was used to optimize the heterologous protein expression as followed the isopropyl-β-D-thio-galactopyranoside (IPTG)-induce protocol [13]. A colony was inoculated in 3 ml of LB medium supplied with 50 mg/l ampicillin with 200 rpm shaking overnight at 37°C.

The 16-hour culture was transferred into fresh 25 ml of LB medium containing 50 mg/l ampicillin to achieve the final OD_{600} of 0.1. The culture was incubated with 200 rpm shaking at 37°C. When the culture's OD_{600} reached 0.6-0.8, the transformant was induced by adding 0.1M IPTG with an appropriate final concentration. After five hours, cells in 1 ml of the induced culture were collected by centrifuging at 5,000 rpm for 5 min. The cell pellets were suspended with the same volume of lysis buffer and stored at -20°C until further processing.

Cell extract was prepared using the freeze-thaw protocol with Qsonica Q55-220 Sonicator Ultrasonic Processor (Cole-Parmer®) on ice [13]. The condition for sonication step was as follows: five cycles of 1 min 30 sec with a rest period of 2 min between cycles. One hundred µl of lysate was transferred into a new tube as the total protein sample. The cell lysate was separated by centrifuging at 10,000 rpm for 5 min at 4°C. The soluble protein fraction as the supernatant was collected. The bacterial cell debris was resuspended in 900 µl lysis buffer and treated as the insoluble protein fraction.

Purification of the fusion protein

The large-scale soluble protein fraction was prepared as described above then added with 500 mM NaCl and filtered through a 0.45 µm syringe filter. The his_6-tag protein was purified using the 5 ml HisTrap™ HP columns (GE Healthcare, Piscataway, NJ, USA) by following the manufacturer's instruction. The solution flowed through the column at the speed of 0.5 ml/min. The protein sample was loaded on the column and washed with 25 ml washing buffer (20 mM Tris HCl, 100 mM NaCl, 50 mM Imidazole, pH 8.0). The protein was eluted by applying 10 ml elution buffer (20 mM Tris HCl, 100 mM NaCl, 250 mM Imidazole, pH 8.0). All fractions containing the fusion protein were analyzed by SDS-PAGE. The eluted fractions then were applied with Microcon® centrifugal filter (Millipore, MA, USA) for desalting and concentrating, and then used as an antigen for injection into rabbits.

Raising of polyclonal antibodies

Two healthy 3-month-old rabbits used for immunization were provided by Vetvaco National Veterinary Joint-Stock Company (VETVACO., JSC), and weighed about 2.5-3.0 kg at the time of acquisition. The pAbs production procedure and laboratory animals care were adopted from the CCAC guidelines on antibody production by the Canadian Council on Animal Care (CCAC) with some modifications (https://www.ccac.ca/Documents/Standards/Guidelines/Antibody_production.pdf). Rabbits were given intramuscular injections at one site on their limbs and subcutaneous injection at five sites on their backs. The first priming injection was performed with a low dose of 0.25 mg/ml purified recombinant ZmDREB2.7 protein (Antigen-Ag) emulsified in Freund's Complete Adjuvant (FCA). After that, the rabbits received three additional injections with raising concentrations of Ag 0.5 mg/ml, 0.75 mg/ml, and 0.1 mg/ml in Freund's Incomplete Adjuvant (FIA), respectively. Each additional injection was administered at 10-day intervals.

Bleeding was implemented from ear veins three times, seven days after each administered day and the last time at day 10 of the final injection. Rabbit blood was collected into a sterile 15 ml centrifuge tube and placed at room temperature for 30 min followed by incubating at 4°C for one hour. The antiserum was collected by centrifuging the blood tubes at 5,000 rpm for 10 min at 4°C then pipetting the supernatants into new tubes and stored at 4°C.

Agglutination test was conducted by mixing 20 µl of antiserum and Ag on a sterile plate. The plate was placed at room temperature for 10 min. After that, if the collected serum contained pAbs of a specific Ag, white clumps could be observed.

SDS-PAGE and dot blot analysis

The SDS-PAGE analysis was conducted using Tris-Glycine Gel, including a separate gel of 12.6% and a stacking gel of 5%, with the Bio-Rad system according to the manufacturer's instructions. Protein was then electrophoresed using a Bio-Rad PowerPac Basic Mini Electrophoresis system (Bio-Rad), for 35 min at 200 V. Protein was visualized by Coomassie blue staining.

For dot blot analysis, a range of concentration of the purified recombinant ZmDREB2.7 protein (from 1 mg/ml to 5 mg/ml) was loaded onto nitrocellulose membrane by pipetting. The membrane was dried at room temperature for about 20 min and incubated with a 1:8 dilution of rabbit serum containing anti-ZmDREB2.7 antibodies. After that, the primary antibody was recognized by the secondary antibody Goat Anti-Rabbit IgG (whole molecule)-Alkaline Phosphatase (Sigma-Aldrich), and the membrane was exposed to 1-Step™ NBT/BCIP substrate solution (Thermo Fisher Scientific).

Results

Construction of the recombinant expression vector

The recognition sites of restriction enzyme *Eco*RI and *Bam*HI were introduced at the 5' and 3' ends of *ZmDREB2.7* gene, respectively, in order to clone the gene into the pET-32a plasmid (Fig. 1A). The *ZmDREB2.7* gene was designed to be in frame with *TrxA* (thioredoxin) gene and fused with

his_6 tag sequence for a further purification experiment (Fig. 1A). Thus, the expected thioredoxin-his_6-ZmDREB2.7 fusion protein contains 522 amino acids and has a theoretical weight of 55.7 kDa.

By mean of PCR with two specific primers, Zm2.7 BamHIF and Zm2.7 EcoRIR, the approximately 1089 bp-long coding sequence of *ZmDREB2.7* was successfully amplified from the cloning vector pJET1.2+*ZmDREB2.7* (Fig. 1B). The PCR product was double digested with two mentioned restriction enzymes, and the vector pET-32a was linearized using the same enzymes (Fig. 1C). The ligation product of the two digested samples was transformed into the DH10β competent cells. We isolated plasmids from six random colonies and characterized by electrophoresis on 1% agarose gel. As shown in Fig. 1D, all plasmid bands obtained from putative recombinant clones were higher than the empty vector pET-32a. The recombinant vectors were verified by restriction enzyme-based screening (Fig. 1E) and confirmed by sequencing (data not shown). Taken together, we successfully constructed the pET-32a+*ZmDREB2.7* bacterial expression vector.

Expression of thioredoxin-his_6-ZmDREB2.7 in E. coli

The pET-32a+*ZmDREB2.7* recombinant vector was transformed into the competent *E. coli* strain Rosetta 1, and a number of recombinant colonies were obtained. At first, we accessed the solubility of four different lysis buffers based on Tris buffer and Phosphate buffer to the heterologous protein (data not shown). The result showed that most of the proteins produced by the recombinant strain (about 90-95%) were in the soluble protein fraction. The Tris-HCl pH 8.0 buffer was chosen for subsequent experiments due to the highest solubility to the fusion protein.

Other factors affecting protein expression-including time of induction, temperature, and IPTG's concentration - were examined (Figs. 2A-2C). As expected, the expression of the recombinant protein increased over time and reached the highest level after five hours (Fig. 2A). However, the production of the fusion protein was not influenced by the tested concentration of IPTG as the intensity of bands representing the interested protein was nearly the same in all lanes on the SDS-PAGE gel (Fig. 2C). The same situation was observed when inducing protein expression at 30°C

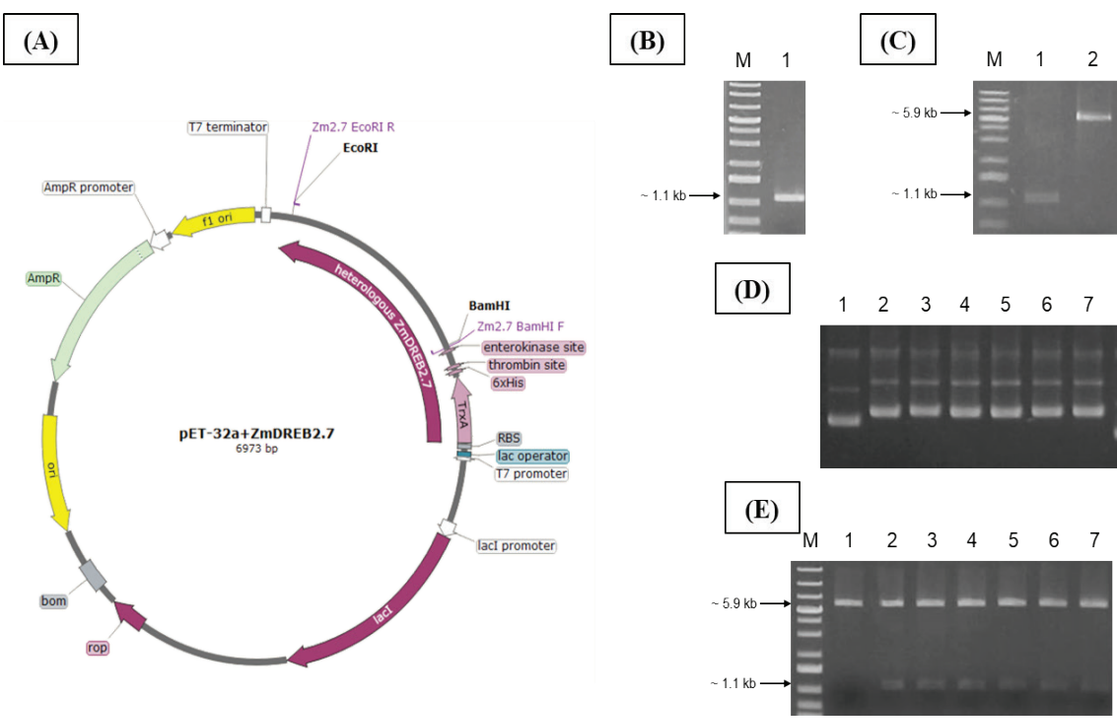

Fig. 1. Construction of the pET-32a+*ZmDREB2.7* expression vector. (**A**) schematic illustration of the pET-32a+*ZmDREB2.7* expression vector; (**B**) PCR amplification of *ZmDREB2.7* fragment flanked by *Eco*RI and *Bam*HI recognition sites. 1: product of PCR using pJET1.2+*ZmDREB2.7* as the template; (**C**) double digestion of DNA with *Eco*RI and *Bam*HI, 1: PCR products amplifying *ZmDREB2.7* gene from pJET1.2+*ZmDREB2.7*, 2: the pET-32a plasmid; (**D**) plasmid isolation from bacteria colonies. 1: the pET-32a vector, 2-7: plasmids isolated from six putative recombinant colonies, respectively; (**E**) restriction enzyme-based screening of putative recombinant colonies. 1: the pET-32a vector, 2-7: plasmids isolated from six putative recombinant colonies, respectively. M: marker 1 kb plus (Thermo Fisher Scientific).

and 37°C (Fig. 2B). Taken together, the optimum condition established to produce the thioredoxin-his$_6$-ZmDREB2.7 fusion protein was five hours of induction using 0.05 mM IPTG at 30°C (Fig. 2D).

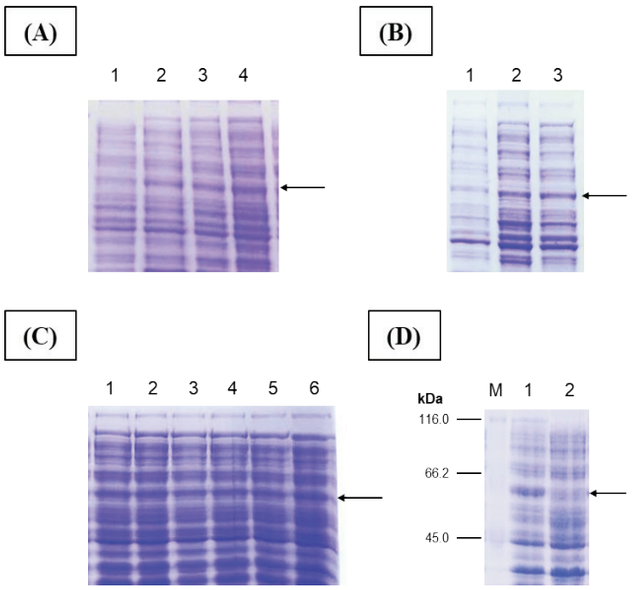

Fig. 2. Expression of the thioredoxin-his$_6$-ZmDREB2.7 fusion protein. (**A**) effect of induction period on the expression of the fusion protein. 1-4: 0h, 1h, 3h, 5h after adding IPTG, respectively; (**B**) effect of temperature on the expression of the fusion protein. 1: 25^{0}C, 2: 30^{0}C, 3: 37^{0}C; (**C**) effect of IPTG's concentration on the expression of the fusion protein. 1-6: IPTG of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0 mM, respectively; (**D**) overexpression of the recombinant protein in the *E. coli* strain Rosetta 1 harboring the pET-32a+*ZmDREB2.7* vector. 1: optimized induction conditions. 2: without IPTG. M: Thermo Scientific™ Pierce™ Unstained Protein Molecular Weight Marker. The arrow indicates the interested protein.

Purification of the thioredoxin-his$_6$-ZmDREB2.7 fusion protein

We took advantage of the fact that the DREB2.7 fusion protein contains a his$_6$ sequence at N-terminal to purify the fusion protein by immobilized-metal affinity chromatography (IMAC). The fusion protein was large-scale overexpressed with optimized conditions and utilized for the purification process. Fig. 3 showed the SDS-PAGE analysis of the recombinant protein purified through the IMAC column. Most of the proteins of the host strain were in the unbound fraction (lane 2) and the wash fraction (lane 3). Lanes 4-10 showed the protein fractions after applying the elution buffer containing 250 mM Imidazole. The arrow pointed to the expected full-length protein. The interested protein eluted with the high amount as judged by Coomassie staining. Thus, we concentrated and desalted the elution fractions for antibodies production.

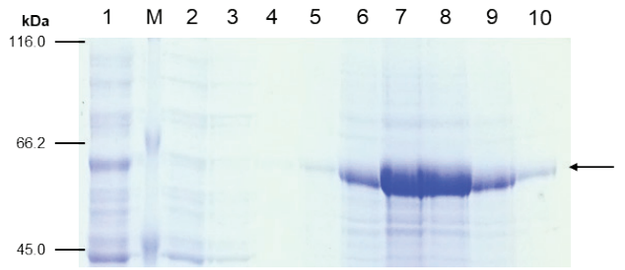

Fig. 3. Purification of the thioredoxin-his$_6$-ZmDREB2.7 fusion protein using HisTrap™ HP columns. M: Thermo Scientific™ Pierce™ Unstained Protein Molecular Weight Marker. 1: the soluble fraction of the recombinant *E. coli* strain. 2: the flow through from column. 3: elute after washing with 50 mM Imidazole. 4-10: fractions after applying the elution buffer containing 250 nM Imidazole. The arrow indicates the interested protein.

Raising of anti-ZmDREB2.7 fusion protein polyclonal antibodies

The protein after the purification step was used for injection into two rabbits via the procedure described above. The agglutination test was implemented using sera from the two rabbits against the purified recombinant ZmDREB2.7 fusion protein. The assays were conducted seven days after each injection to monitor antibody response during the immunization process. We obtained the positive result of the agglutination test immediately after the priming injection. In addition, the intensity of reactions rose as more injections were given. As shown in Fig. 4A, there were visible white clumps after 30 minutes combining the serum of the last bleeding with the antigen. Moreover, it was obvious that serum originated from the first rabbit exhibited higher

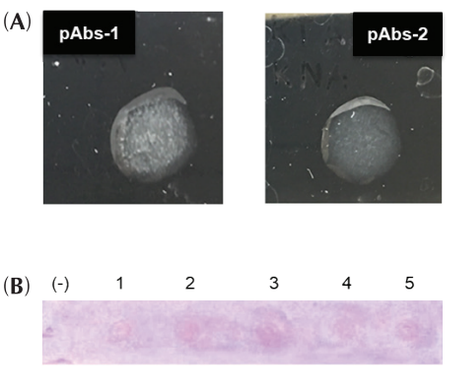

Fig. 4. Agglutination test and dot blot analysis using rabbit anti-fusion protein sera. (**A**) agglutination test of rabbit sera (the last bleeding) to the antigen. pAbs-1, pAbs2: the antisera from the first and the second immunization rabbit, respectively. (**B**) dot blot analysis of the rabbit sera to the antigen. (-): H_2O. 1-5: serial dilutions of the ZmDREB2.7 fusion protein ranging from 1 mg/ml to 5 mg/ml, respectively.

response than that from the second one. It was supposed that immunized rabbits produced pAbs to the thioredoxin-his_6-ZmDREB2.7 fusion protein. Therefore, we harnessed the anti-fusion protein serum from the first rabbit for dot blot analysis to confirm the specificity of the serum. As the result shows, the pAbs generated in rabbit serum can efficiently recognize the recombinant ZmDREB2.7 fusion protein (Fig. 4B).

Discussion

The *E. coli* expression system has been exploited for the production of a variety of proteins. Even though there are some drawbacks, such as lack of post-translation modification, the bacterial expression system remains faster and cheaper for producing eukaryote proteins. Therefore, we adopted an *E. coli* expression system and did optimization of components involved in the protein expression process to obtain the high expression level of the ZmDREB2.7 fusion protein.

Even though the *ZmDREB2.7* gene contains few rare codons to *E. coli*, it has a high GC content (70%). Meanwhile, the GC content in the *E. coli* genome was about 50.5% [14]. Additionally, the maize-derived gene shows a low codon adaptation index in *E. coli* which is 0.68 (the acceptable figure is from 0.8 to 1.0). The difference in codon bias between maize and *E. coli* normally causes early termination and produces truncated versions of the heterologous protein. Due to such limitations in the gene sequence, we harnessed the *E. coli* strain Rosetta and the protocol which gradually induces the heterologous protein expression. The Rosetta 1 strain has many advantages for enhanced protein expression [15]. The strain as a BL21 derivation is deficient in protease Lon and OmpT which could increase the stability of expressed recombinant proteins. In addition, Rosetta 1 harbors a compatible plasmid which produces tRNAs for rare codons AUA, AGG, AGA, CGG, CUA, CCC, and GGA. Then, the tRNA pool can compensate for the difference in codon bias between *E. coli* and the original source of the interested gene. Therefore, it was not necessary to optimize codon usage of the *ZmDREB2.7* gene to ensure that the heterologous protein was expressed in full length.

When a eukaryote protein expresses at a high level in the bacteria cell, it may be found in inclusion bodies due to inappropriate folding. To overcome this issue, the *ZmDREB2.7* gene was conjugated with the *TrxA* in the vector pET-32a. TrxA normally located in *E. coli* cytoplasm is a compact, highly soluble, and thermally stable protein. These properties allow trxA to serve as a molecular chaperone. Therefore, when ZmDREB2.7 N-terminally fused with trxA protein, the recombinant protein could avoid forming an inclusion body [16]. Additionally, theoretically, slowing down the production rate can help the newly synthesized proteins fold more properly [10]. It is also reported that sometimes inducing at low temperature facilitates soluble thioredoxin-fused protein [17]. In the study, we induced the fusion protein at 30°C for five hours as there was no significant difference of the protein expression level between 30°C and 37°C (Fig. 2B). Because the expression levels of the fusion protein were nearly the same at a range of inducer concentration (Fig. 2C), the lowest concentration of inducer (0.05 mM IPTG) was the optimum choice to increase the protein production.

In addition, the ZmDREB2.7 was fused with the his_6 sequence to enable purification using IMAC system. The his_6 tag at N-terminal guarantees that the translation process initiates in the correct position. As expected, the induced protein bound to the Ni column was the full-length one with the molecular weight of approximately 55.7 kDa.

There are several factors to consider when raising pAbs in laboratory animals. In fact, rabbits are commonly used for reasons of cost-effectiveness, ease of handling, and high amount of serum compared to mice. We used the young rabbits because immune function peaks at puberty and declines with age [12]. There were several reports of batch-to-batch variants when producing pAbs by immunizing animals, so two rabbits per antigen are recommended. In our study, two rabbits responded differently as the agglutination test exhibited more white precipitations with the antiserum from the first one (Fig. 4A). The number of injections and the amount of the ZmDREB2.7 fusion protein were tightly controlled. We used three booster doses that were double, triple, and four times the priming dose, respectively. In addition, the adjuvant was added to induce a high titer of antibodies without any side effects to the animal. A high quantity of anti-ZmDREB2.7 fusion protein serum was obtained from the raising pAbs experiment.

Conclusions

In conclusion, we successfully cloned the *ZmDREB2.7* gene into the pET-32a vector. The expression vector worked well in the *E. coli* Rosetta 1 that the thioredoxin-his_6-ZmDREB2.7 fusion protein was overexpressed. The optimized conditions for the production of the interested protein were five hours at 30°C using 0.05 mM of IPTG. The fusion protein was purified by IMAC column and used to raise pAbs in the rabbit. The obtained antiserum can specifically bind to the ZmDREB2.7 fusion protein.

ACKNOWLEDGEMENTS

The present research was supported by a grant from the Vietnam Ministry of Agriculture and Rural Development (MARD) named "Isolating genes related to drought

tolerance and constructing vectors for maize improvement".

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] S. Liu, X. Wang, H. Wang, H. Xin, X. Yang, J. Yan, F. Qin (2013), "Genome-wide analysis of *ZmDREB* genes and their association with natural variation in drought tolerance at seedling stage of *Zea mays* L", *PLOS Genet.*, **9(9)**, e1003790.

[2] Y. Sakuma, K. Maruyama, Y. Osakabe, F. Qin, M. Seki, K. Shinozaki, K. Yamaguchi-Shinozaki (2006a), "Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression", *Plant Cell*, **18**, pp.1292-1309.

[3] Y. Sakuma, K. Maruyama, F. Qin, Y. Osakabe, K. Shinozaki, K. Yamaguchi-Shinozaki (2006b), "Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression", *Proc. Natl. Acad. Sci. USA*, **103**, pp.18822-18827.

[4] J.Q. Chen, X.P. Meng, Y. Zhang, M. Xia, X.P. Wang (2008), "Over-expression of *OsDREB* genes lead to enhanced drought tolerance in rice", *Biotechnol. Let.*, **30(12)**, pp.2191-2198.

[5] J. Chen, X. Xia, W. Yin (2009), "Expression profiling and functional characterization of a DREB2-type gene from *Populus euphratica*", *Biochem. Biophys. Res. Commun.,* **378(3)**, pp.483-487.

[6] B. Pandey, P. Sharma, M. Saini, D.M. Pandey, I. Sharma (2014), "Isolation and characterization of dehydration-responsive element-binding factor 2 (DREB2) from Indian wheat (Triticum aestivum L.) cultivars, *Aust. J. Crop Sci.*, **8(1)**, p.44.

[7] F. Yesilirmak, Z. Sayers (2009), "Heterelogous expression of plant genes", *Int. J. Plant Genomics*, **2009**, ID 296482.

[8] S. Sahdev, S.K. Khattar, K.S. Saini (2008), "Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies", *Mol. Cell. Biochem.,* **307(1-2),** pp.249-264.

[9] D.M. Francis, R. Page (2010), "Strategies to optimize protein expression in *E. coli*", *Curr. Protoc Protein Sci.*, **61(1)**, pp.5-24.

[10] G.L. Rosano, E.A. Ceccarelli (2014), "Recombinant protein expression in *Escherichia coli*: advances and challenges", *Front. Microbiol.*, **5**, p.172.

[11] A.R. Gomes, S.M. Byregowda, B.M. Veeregowda, V. Balamurugan (2016), "An overview of heterologous expression host systems for the production of recombinant proteins", *Adv. Anim. Vet. Sci*., **4(7)**, pp.346-356.

[12] H.F. Stills (2012), "Polyclonal antibody production", *The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents,* pp.259-274.

[13] J. Sambrook, D.W. Russell (2001), *Molecular cloning: a laboratory manual*, Cold Spring Harb Lab Press Cold Spring Harb NY, 999.

[14] S. Mann, Y.P.P. Chen (2010), "Bacterial genomic G+C composition-eliciting environmental adaptation", *Genomics*, **95(1)**, pp.7-15.

[15] R. Novy, D. Drott, K. Yeager, R. Mierendorf (2001), "Overcoming the codon bias of *E. coli* for enhanced protein expression", *Innovations*, **12**, pp.1-3.

[16] Y. Li (2009), "Carrier proteins for fusion expression of antimicrobial peptides in *Escherichia coli*", *Biotechnol. Appl. Biochem.*, **54(1)**, pp.1-9.

[17] X. Xu, F. Jin, X. Yu, S. Ji, J. Wang, H. Cheng, W. Zhang (2007), "Expression and purification of a recombinant antibacterial peptide, cecropin, from *Escherichia coli*", *Protein Expr. Purif.*, **53(2)**, pp.293-301.