CLONING, EXPRESSION AND PURIFICATION OF *ABO3* GENE INVOLVED IN DROUGHT STRESS TOLERANCE FROM *Arabidopsis thaliana* in *Escherichia coli*

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ABSTRACT

Drought is one of the most severe environmental stress factors that affects the growth and development of plants. The effects of drought are foreseen to increase with climate change and growing water scarcity. In many parts of the world, including Vietnam, plants may frequently encounter drought stress. Plants have evolved specific mechanisms to respond to drought stresses. Studying these protective mechanisms will contribute to our knowledge of tolerance and resistance to stress. Recently, some studies indicated that the *ABO3* gene coding for WRKY63 is a key gene in stress signalling pathways related to drought tolerance, of which the functions and interactions are currently unknown. Therefore, investigating its functions and interactions are considered effective strategies to control a plant's resistance against drought. In this study, we show the results of expression and purification of the ABO3 protein coding for WRKY63 in *E. coli,* a solid step towards studying about the protein's interactions and functions. The *ABO3* gene was amplified from cDNA of *Arabidopsis thaliana* by PCR, cloned into T-Blunt vectors, and sequenced. This gene then was subcloned into the expression vector pQE-30 and expressed in *E. coli* strain M15. Finally, the ABO3 recombinant protein was initially purified by affinity chromatography for further studies.

Keywords: Arabidopsis, transcription factor, *ABO3*, resistance, *E. coli*, protein expression.

Nghiên cứu tách dòng, biểu hiện và tinh sạch protein ABO3 từ cây *Arabidopsis thaliana* trong vi khuẩn *Escherichia coli*

TÓM TẮT

Hạn hán là một trong những nhân tố gây ảnh hưởng nghiêm trọng nhất tới sự sinh trưởng và phát triển của thực vật. Những ảnh hưởng của hạn hán được dự đoán là sẽgia tăng cùng với sự thay đổi khí hậu và làm trầm trọng hơn vấn đề khan hiếm nước sạch. Ở nhiều nơi trên thế giới bao gồm cả Việt Nam, thực vật thường xuyên phải đối mặt với vấn đề hạn hán. Do đó chúng đã sử dụng những cơ chế, cách thức khác nhau nhằm đáp ứng lại điều kiện thiếu nước để tồn tại và phát triển. Các nghiên cứu về cơ chế tự bảo vệ của thực vật sẽ góp phần xây dựng nền tảng kiến thức cơ bản về khả năng chống chịu và kháng stress ở thực vật. Gen *ABO3* mã hóa cho protein WRKY63 là một gen chủ chốt trong con đường dẫn truyền tín hiệu kháng hạn. Tuy nhiên chức năng và cách thức tương tác của nó hiện tại vẫn chưa được làm sáng tỏ. Dó đó, việc khảo sát vai trò, chức năng và sự tương tác của gene này sẽ góp phần nâng cao sự hiểu biết về khả năng chịu hạn của thực vât. Trong nghiên cứu này, chúng tôi đã thực hiện các nội dung để biểu hiện và tinh sạch protein ABO3 từ cây *A. thaliana* trong *E. coli,* tạo tiền đề cho những nghiên cứu về chức năng và sự tương tác của protein ABO3. Gen *ABO3* được khuếch đại từ nguồn cDNA của *Arabidopsis thaliana* bằng phương pháp PCR, sau đó được tách dòng và đọc trình tự. Cuối cùng gen được chuyển vào vector biểu hiện pQE-30 và biểu hiện trong vi khuẩn *E. coli* chủng M15. Protein tái tổ hợp ABO3 được tinh sạch nhờ phương pháp sắc kí ái lực.

Từ khóa: Arabidopsis, *ABO3*, WRKY63, hạn hán, *E. coli*, biểu hiện protein.

1. INTRODUCTION

Plants face a wide range of stresses, both biological and environmental. Of which, drought is a major environmental stress factor that affects the growth and development of plants. Drought, or soil water deficit, can be chronic in climatic regions with low water availability, or random and unpredictable due to changes in weather conditions during the period of plant growth. The effects of drought are foreseen to increase with climate change and growing water scarcity. Water deficit is a significant challenge to the future of crop production. About 15 million km^2 of the planet's land surface is covered by cropland (Ramankutty *et al.*, 2008), and about 16% of this area is equipped for irrigation (Siebert *et al.*, 2005). Thus, in many parts of the world, including Vietnam, plants may frequently encounter drought stress. Rainfall is very seasonal, and periodic drought occurs regularly. The effect of drought is more prominent in sandy soils with a low water holding capacity. On such soils, some plants may experience drought stress after only a few days without water.

Plants have evolved specific acclimation and adaptation mechanisms to respond to and survive short- and long-term drought stresses. In response to drought brought by soil water deficit, plants can exhibit either drought escape or drought resistance mechanisms, with resistance further classified into drought avoidance (maintenance of tissue water potential) and drought tolerance (Price *et al.*, 2002). Drought tolerance is the ability to withstand a water deficit with low tissue water potential (Ingram and Bartels, 1996). Among other mechanisms, plants can survive under drought stress by maintaining cell turgor and reducing evaporative water loss by accumulating compatible solutes (Yancey *et al.*, 1982).

In recent years, a lot of molecular information has been generated on the responses of plants to environmental stresses. Many studies have indicated that the *ABO3* gene coding for WRKY63 is a key gene in stress signalling pathways related to drought tolerance. According to the TAIR website (https://www.arabidopsis.org/), ABO3 includes 726 nucleotides coding for a 241 aa protein (~27 kDa). Analysis of T-DNA insertion mutants of AtWRKY63 (*ABO3*) indicated that AtWRKY63 plays an important role in plant responses to ABA and drought stress. AtWRKY63 was induced by ABA treatment, and mutations of AtWRKY63 rendered the mutants less drought tolerant and more sensitive to ABA in both seedling establishment and seedling growth. EMSAs (Electrophoretic mobility shift assays) showed that the W-box sequence upstream of the AtABF2 promoter could be bound by AtABO3, supporting its repressed expression in the AtABO3 mutant plants. However, over-expression of AtABO3 did not result in drought tolerance, thus, AtABO3 needs either co-factors or some post-translational modifications to activate the downstream genes for stress tolerance (Ren *et al.*, 2010). Disruption of WRKY63/ABO3 in Arabidopsis enhanced ABA sensitivity but reduced tolerance to drought stress due to impaired ABA-induced stomatal closure in the mutant. In addition, as revealed by gene expression analysis, the *ABF2/AREB1* (abscisic acid responsive elements-binding factor 2/ abscisic acid-responsive element binding protein 1) level was lower in the *ABO3* mutant than in the wild type, which was consistent with the binding ability of WRKY63 to the promoter of ABF2/AREB1 *in vitro*. In summary, WRKY63 plays an important role in the complex network of ABA-dependent gene expression and drought stress response (Ren *et al.*, 2010). Genome-wide expression analysis following high-light stress in transgenic lines with perturbed AtWRKY40 and AtWRKY63 functions revealed that these factors are involved in regulating stress-responsive genes encoding mitochondrial and chloroplast proteins but have little effect on more constitutively expressed genes encoding organellar proteins. Furthermore, it appears that AtWRKY40 and AtWRKY63 are particularly involved in regulating the expression of genes responding commonly to both mitochondrial and chloroplast dysfunction but not of genes responding to either mitochondrial or chloroplast perturbation (Olivier Van Aken *et al.*, 2013).

The functions and interactions of ABO3 in response to drought stress are currently unknown. It is necessary to investigate the protein's functions and interactions that contribute to the plant's resistance against drought. Therefore, this study was conducted to successfully express the ABO3 protein *in vitro*, which will serve as the experimental materials for future studies.

2. MATERIALS AND METHODS

2.1. Materials

cDNA of *Arabidopsis thaliana* WT (wild type) and *E. coli* strains (XL1-Blue and M15) were contributed by Prof. Chung Woo Sik from the Laboratory of Molecular and Cellular Biochemistry, Gyeongsang National University, Korea. The cloning vector (T-Blunt) and expression vector (pQE-30) were products of SolGent company. The restriction enzymes, buffers, and chemicals were supplied by Fermentas, Promega, and Invitrogen companies.

2.2. Methods

2.2.1. Amplification of the ABO3 gene from Arabidopsis cDNA

Based on the information about the sequence of the *ABO3* gene on the TAIR website (https://www.arabidopsis.org/), a specific pair of primers was designed to amplify the full length gene from cDNA. The PCR reaction was carried out with the following components: forward primer (5'-GGATCCATGTTTTCAAACATCGATCA-3'): 1 µl (10 pmol); reverse primer (5'-CCCGGGTCAAAACAACATCAGGTCTT-3'): 1 µl (10 pmol); 12.5 µl Master Mix (2X); 1 µl cDNA; and 9.5 µl H_2O for a final volume of 25 µl. The PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles at 94 °C for 50 s, 56 °C for 40 s, and 72 °C for 1 min, followed by 72 °C for 8 min. The PCR products were examined on a 1% agarose gel. The remaining PCR product was stored at 4 °C for further experiments.

2.2.2. Cloning of ABO3 into T-Blunt vectors

The purified PCR products were inserted into T-Blunt vectors following the instructions of company. The ligation products were transformed into *E. coli* XL1-Blue competent cells by the heat-shock method and then placed on selective medium containing 50 µg/ml ampicillin, 50 µg/ml X-Gal, and 0.5 mM IPTG. Cloned T-Blunt vectors were extracted and purified using the GeneJET™ Plasmid Miniprep Kit of Fermentas, which digested the vectors using the *Bam*H I and *Sma* I restriction enzymes. An electrophoresis gel was run to inspect the products. The sequence of the *ABO3* gene in the T-Blunt vectors was sequenced by the laboratory of 1st BASE sequencing INT Company using the M13 pair of primers. The data were analyzed by BioEdit software.

2.2.3. Subcloning of the ABO3 gene from the T-Blunt vectors into the pQE-30 vectors

The *ABO3* gene was digested from T-Blunt vectors by *Bam*H I and *Sma* I enzymes, and the contemporaneous expression vector pQE-30 was also digested with these enzymes. Then, the *ABO3* gene was inserted into the opened pQE-30 vectors by T4 ligase. The plasmids containing the *ABO3* gene were digested with *Bam*H I and *Sma* I for examination before being transformed into *E. coli* strain M15 to express the target gene.

2.2.4. Expression and purification of the ABO3 recombinant protein

The transformation cells were cultured with shaking on a selective medium with 50 µg/ml ampicillin at 37°C overnight. 500 µl of each overnight culture was transferred to an appropriately labeled flask containing 10 ml LB medium. The flasks were incubated at 37°C with shaking at 250 rpm until OD_{600} reached 0.6. Each tube containing 10 ml of the culture was split into six 5 ml tubes. Five tubes were induced by 0.5 mM of isopropyl β-D-thiogalactopyranoside (IPTG) under different temperature and induction durations; and the remaining tube was not induced to be used as the control. All the tubes were incubated at

37°C with shaking at 250 rpm. Cell extraction was denatured by sonication to perform SDS-PAGE. The recombinant protein expressed in soluble form also had a 6 histidine tag. Therefore, it was purified by an affinity column of Ni-NTA agarose (Qiagen) following the instructions of company.

3. RESULTS AND DISCUSSIONS

3.1. Cloning of the *ABO3* gene

3.1.1. Amplification of the ABO3 gene

The *ABO3* gene is located on the first chromosome, and contains 726 nucleotides coding for a 241 amino acid protein. The *ABO3* gene was amplified by PCR using cDNA from Arabidopsis as the template. The total RNA was extracted from Arabidopsis and previously exposed to different stress conditions including ABA, salt (NaCl), salicylic acid (SA), and normal conditions. Then, the RNA was converted to cDNA by RT-PCR. The PCR products were examined on a 1% agarose gel.

The agarose gel (Fig. 1) showed clear bands with the expected size of the *ABO3* gene (726 bp). Therefore, it can be concluded that the *ABO3* gene would be able to be transcribed in the Arabidopsis genome at all examined conditions.

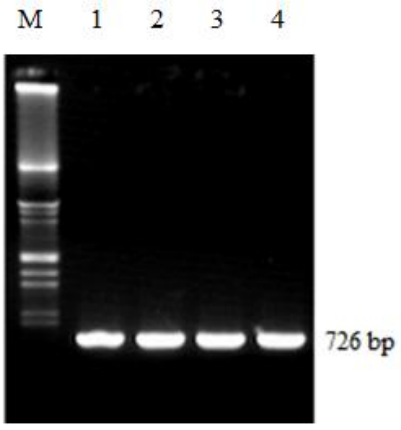

Fig. 1. Agarose gel electrophoresis of PCR products

Note: Lane M: Phage Lambda DNA EcoR I-Hind III Marker; Lanes 1-4: PCR products with cDNA extracted from Arabidopsis in normal (1), ABA stress (2), NaCl stress (3), and SA stress (4) conditions.

3.1.2. Cloning of the ABO3 gene in T-Blunt vectors

The purified PCR products were inserted to T-Blunt vectors following the instruction of company. T-Blunt cloning vectors were extracted and purified using the GeneJET™ Plasmid Miniprep Kit of Fermentas, which utilized the restriction enzymes *Bam*H I and *Sma* I for digestions. An electrophoresis gel was run to evaluate the restricted products (Fig. 2).

As seen in Figure 2, colonies with successful *ABO3* insertions (lanes 1, 3, 6, and 7) and false positives (lanes 2, 4, and 5) both gave clear bands, even though this problem should be impossible. It could be that the high efficiency transformation resulted in a high number of transformed bacteria seeded on a plate, and therefore, degraded the antibiotic. Because of this degradation, satellite colonies without plasmids could grow, too. A greater understanding about why this issue occurred would be beneficial during further PCR studies. With four cultures that appeared to have the expected sizes, ~726 bp, respective to the size of *ABO3* gene, and ~ 4000 bp, respective to the size T-Blunt vector, we can conclude that the *ABO3* gene was inserted in the correct direction in the cloning vector.

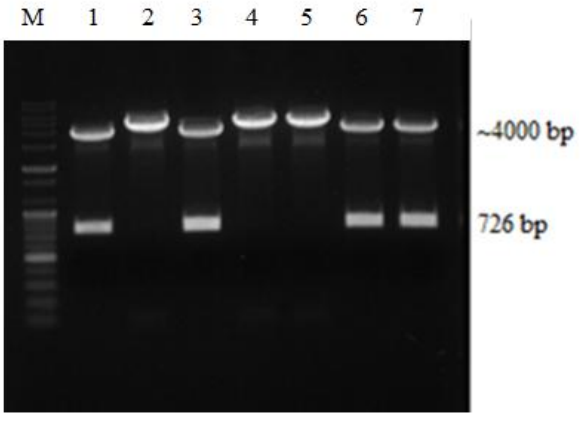

Fig. 2. Agarose electrophoresis of selected T-Blunt plasmids after being digested by *Bam*H I and *Sma* I

Note: Lane M: 100-bp-Plus-DNA-Ladder; Lanes 1-7: Differences in plasmid sizes after digestion by restriction enzymes.

3.2. Sub-cloning of the *ABO3* gene in the expression vector

The *ABO3* gene in the T-Blunt vectors was sequenced by the laboratory of 1st BASE Sequencing INT Company using the M13 pair of primers. The data were analyzed by BioEdit software. The selected correct sequence of the *ABO3* gene was separated from the T-Blunt vectors by the *Bam*H I and *Sma* I enzymes, and the contemporaneous expression vector pQE-30 was also digested with these enzymes. Then, the *ABO3* gene was inserted into the opened pQE-30 vector by T4 ligase. The plasmids containing the *ABO3* gene were selected by digestion with *Bam*H I and *Sma* I for examination before being transformed into *E. coli* strain M15 for protein expression. Expected bands, including the *ABO3* gene and the digested pQE-30 vector, were separated from the gel and purified (Fig. 3).

The agarose gel showed bands of the expected size of the *ABO3* gene (~726 bp) and of the pQE-30 vector (~3.4 kb). The bands are clearly distinct and visible at their respective sizes. These results showed that the *ABO3* gene was successfully ligated into the expression vector pQE-30 and transformed into the *E. coli* M15 strain.

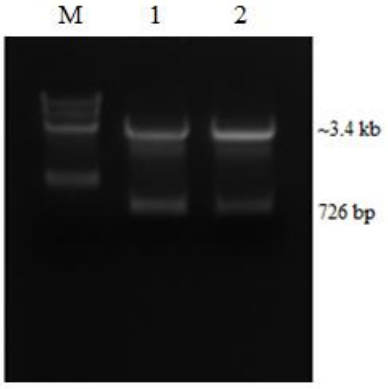

Fig. 3. Agarose electrophoresis of the pQE-30 plasmids after being digested by the *Bam*H I and *Sma* I enzymes

Note: Lane M: Phage Lambda DNA EcoR I-Hind III Marker; Lanes 1 and 2: Digestion products of different plasmid samples.

3.3. Expression of the *ABO3* gene in *E. coli*

Cells were collected by centrifugation, and then sonicated in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 2 mM PMSF, and 0.1% Triton X-100) for protein extraction. The results of the SDS-PAGE showed that the ABO3 protein was expressed in *E. coli* strain M15, and the optimized expression conditions were 30°C after 2 h of induction with 0.5 mM IPTG (Fig. 4).

The comparisons of the molecular weights of the bands with the ladder suggest that the weight of the recombinant protein is nearly 27 kDa, which is the size of the expected recombinant protein. This result clearly shows that the ABO3 recombinant protein could be expressed in *E. coli* bacteria when induced with IPTG and it was not produced when uninduced.

Under different temperature and duration of induction conditions, the ABO3 protein was expressed at different levels. Fig. 4A indicates that the ABO3 protein was well expressed at 20-35°C (lanes 3-6) after 2 hours of induction, however, it was expressed the best at 30°C (lane 5). Fig. 4B shows that the ABO3 protein was well expressed after 2-5 h of induction (lanes 3-6). Therefore, it can be concluded that ABO3 protein expression in *E. coli* strain M15 is possible and its optimized expression conditions are 30°C after 2 of induction with 0.5 mM IPTG.

3.4. Purification of ABO3 recombinant protein

The purified protein was inspected by SDS-PAGE. As a result, we got highly purified ABO3 protein, which was not contaminated with other proteins from *E. coli* (Fig. 5).

This result demonstrated that our purification gave rise to the expected pattern of bands (in accordance with the molecular weight ~27 kDa). The purified protein was stored at -20°C for further studies.

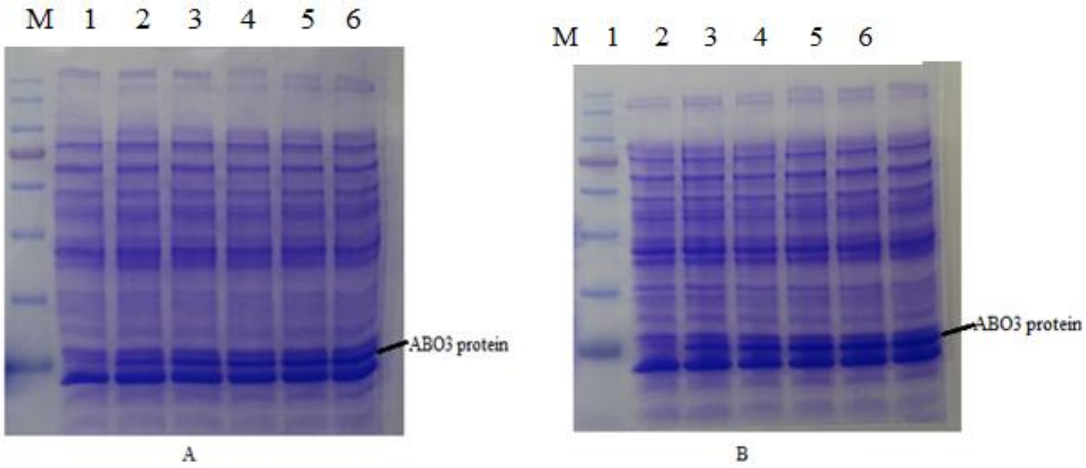

Fig. 4. SDS-PAGE analysis of expressed ABO3 protein in *E. coli* after induction by 0.5 mM IPTG

Note: Figure 4A. Lane M: Protein ladder; Lane 1: Control sample (no induction); Lanes 2-6: Protein samples in different temperatures of induction 15, 20, 25, 30, and 35°C (after 2 hours of induction). Figure 4B. Lane M: Protein ladder; Lane 1: Control sample (no induction); Lanes 2-6: Protein samples in different times of induction 1, 2, 3, 4, and 5 h (at 30°C).

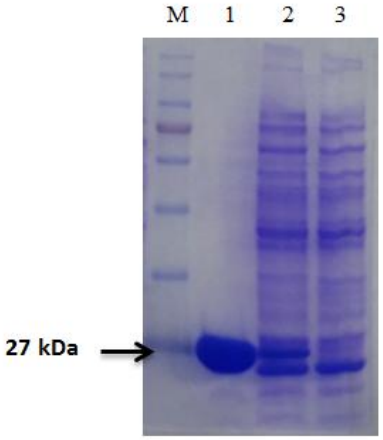

Fig. 5. Purification of ABO3 protein

Note: Lane M: Protein ladder; Lane 1: ABO3 protein after purification; Lane 2: Crude proteins extracted from E. coli before purification; Lane 3: Crude proteins extracted from E. coli before induction.

4. CONCLUSIONS

We successfully conducted the PCR process to amplify the *ABO3* gene using specific primers. The *ABO3* gene was inserted into T-Blunt cloning vectors by T4 ligase. The complex ABO3-T-Blunt vector was then cloned into *E. coli* strain XL1-Blue. ABO3 protein expression in *E. coli* strain M15 is possible and the optimized expression conditions are 30°C after 2 h of induction with 0.5 mM IPTG. The ABO3 recombinant protein was initially purified successful by affinity chromatography to use for further studies.

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