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RESEARCH ARTICLE

Cloning and prokaryotic expression of *TaE3* from wheat and preparation of antiserum

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Abstract **The E3 ubiquitin ligase is a multi-functional protein that performs vital roles, particularly in various stress environment. To further understand the biological significance of E3 ubiquitin ligase gene from wheat (*TaE3*), total RNA was isolated from wheat leaves and then *TaE3* gene was amplified by PCR after reverse transcription. The PCR product was cloned into PMD19-T vector to sequence subsequently. And then the recombinant expression vector (pET30a-GST-*TaE3*-His) was constructed and transformed into *E. coli* strain BL21 (DE3). SDS-PAGE analysis showed that the recombinant *E. coli* could express a proximate 43 kDa protein. TaE3 fusion protein was purified by Ni-NTA affinity chromatography from recombinant bacterial lysate and was used to immunize rabbit to produce polyclonal antibody. The titer and specificity of the anti-TaE3 antibody were successfully detected by indirect ELISA and western blot analysis.**

Keywords E3 ubiquitin ligase (E3), wheat, prokaryotic expression, western blot analysis

Introduction

Ubiquitination is a post-translational protein modification widely found in eukaryotic cells (Dye and Schulman, 2007; Hunter, 2007). In higher plants, ubiquitinated proteins are involved in diverse cellular processes, including abiotic or biotic stress responses, circadian rhythms, cell cycles, and differentiation (Moon et al., 2004; Smalle and Viestra, 2004; Dreher and Callis, 2007). Ubiquitin (Ub), a highly conserved 76-amino-acid polypeptide, is first activated by an E1 Ub activating enzyme in an ATP-dependent manner and is transferred to an E2 Ub-conjugating enzyme. The Ub-E2 complex then binds an E3 Ub ligase that catalyzes the formation of an isopeptide bond between a lysine residue of the target proteins and the C-terminal glycine of Ub (Smalle and Viestra, 2004). The Lys-48 and Lys-63 residues of Ub are used to form a poly-Ub chain. The most abundant Lys-48-linked poly-ubiquitinated target proteins are rapidly degraded by the 26S proteasome complex, while Lys-63-linked poly-ubiquitination confers nonproteolytic functions, such as DNA repair and protein trafficking (Jacobson et al., 2009). Interaction with other proteins, lipidation, subcellular localization, and protein activity can also be altered by mono- or multi-ubiquitination (Mukhopadhyay and Riezman, 2007).

E3 Ubiquitin ligases are central components of the ubiquitination pathway. The selection of specific targets in the ubiquitination pathway is governed mainly by a very large and diverse family of E3 ligases (Yang et al., 2005; Yang et al., 2008). The *Arabidopsis* (*A. thaliana*) genome, for example, is predicted to encode for over 1300 E3 ligases, which can be classified into different subgroups based on the presence of the U-box, really interesting new gene (RING), or homologous to E6-AP C-terminus E2 binding domain (Stone et al., 2005).

Currently, lots of functions of the E3 ubiquitin ligase are identified in plants. Evidence suggests that E3 plays different roles in a variety of biological processes and the pathways of plants defense to biostress (Wang, 1999; Wang et al., 2006; Yang et al., 2006) and abiostress, such as fungus (Yaeno and Iba, 2008), drought (Kraft et al., 2005; Cho et al., 2008; Lee et al., 2009), temperature and hormone stress. Hyun et al. (2009) reported that drought stress-induced Rma1H1, a RING membrane-anchor E3 ubiquitin ligase homolog, regulated aquaporin levels via ubiquitination in transgenic *Arabidopsis*

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plants. Prasad and Stone (2010) clarified that *Arabidopsis* RING E3 ligase XBAT32 regulated lateral root production through its role in ethylene biosynthesis. SCF ubiquitin ligase plays a crucial role in the N gene–mediated resistance response to tobacco mosaic virus, which has been reported these years (Liu et al., 2002). And these achievements have laid a good foundation for the further studies of the function of *E3* in plants.

In our laboratory's former study, Yan et al. (2009) obtained a differentially expressed fragment *TaSSH16h508* by the suppression subtractive hybridization in the wheat-leaf rust fungus interaction system. BlastX analysis showed that the deduced amino acid sequence was consistent with the putative wheat E3 ubiquitin protein ligase (GenBank: EEF39625.1). It suggested that *TaE3* may be involved in the wheat defense responses to leaf rust. The preparation of antiserum is important to investigate the location, quantity and physiologic functions of TaE3 in the defense responses to wheat leaf rust infection. In this research, we constructed prokaryotic expression vector, successfully expressed and purified fusion protein, and obtained the polyclonal antibody of TaE3 by injecting the purified fusion protein to immunize rabbits. It provided essential materials for further research of *TaE3*'s functions.

Materials and methods

Materials

E. coli DH5α, BL21 (DE3), and expression vector pET30a-GST were preserved in our laboratory. PMD19-T vector, *Eco*RI, *Hin*dIII and T4 ligase were purchased from TAKARA Company. IPTG (isopropyl-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) were purchased from Shanghai Sangon Biotechnology Company.

RNA extraction

Total RNAs were extracted from leaf tissue of wheat near-isogenic line Tc*Lr19* following instruction of TaKaRa RNAiso^{TM} Plus. The quality and quantity of RNA were measured by nucleonic acid and protein detection instrument (NanoDrop ND-1000, American). The integrity of RNA was detected by 1% agarose gel electrophoresis.

The amplification of *TaE3* gene by PCR

RNA was used as template for synthesis of first-strand cDNA described as reverse transcriptase M-MLV (D2640A). PCR reaction was carried out in 100 μL reaction volume using first-strand cDNA as template at the following conditions: an initial denaturation step at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, 62.1°C for 45 s and 72°C for 2 min. The final extension was at 72°C for 10 min. The primers used in the reaction were listed in the following with the underlined regions noted as restriction enzyme sites for *Eco*RI and *Hin*d III:

TaE3 primers: F5′-CGGAATTCATGCTTGAGAATGACA-TACGTG-3′

R5′-CCCAAGCTTTGTAGATCTCGCCCATTTCC-3′

Construction of expression vector

The PCR products were gel-purified and connected with the PMD19-T vector, and then used to transfer competent cells *E. coli* DH5α. Transformed cells were selected on LB medium with IPTG and X-gal. The positive colonies were picked out separately for the further identification by DNA sequencing. The fragment was digested with *Eco*RI and *Hin*dIII and inserted into pET30a-GST expression vector, designated as PET30a-GST-*TaE3*-His. After that, the expression vector was tested by the digestion of *Eco*RI and *Hin*dIII.

Expression of TaE3 protein in *E. coli* BL21 (DE3)

E. coli BL21 cells introduced with the recombinant plasmids above was used for TaE3 expression. The high expression clone was verified by SDS-PAGE analysis and cultured in LB liquid medium overnight at 37°C for small-scale culture. The overnight culture (500 μL) was inoculated into 50 mL of the fresh medium. To optimize its expression, the bacterial culture was grown at 37°C until 0.5–0.8 OD600 nm, at which time protein expression was induced by adding 0.25 mmol/L IPTG, respectively. The cultures were shaken at 180 r/min at 28°C in an 100 mL erlenmeyer flask. After induction, the samples were taken at different time. Then the cells were separated from the culture medium by centrifugation at 8000 r/min for 15 min at 4°C, after that the harvested cellular pellet was resuspended in ice-cold buffer (10 mmol/L Tris-HCl, pH 8.0, and 100 mmol/L DTT). The suspension was sonicated on ice for 5 min (5 s/10 s) to disintegrate the cells and centrifuged at 12000 r/min for 10 min at 4°C. The supernatant was added with 4 × sample buffer (100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 100 mmol/L DTT), and analyzed by SDS-PAGE.

Protein purification and concentration detection

The recombinant His-tagged protein was purified by Ni-NTA affinity chromatography. Several tubes were added with 100 μL Ni-NTA and 1.8 mL bacterial lysates, respectively, and then incubated overnight at 4°C. The next day, the tubes were centrifuged at 2600 r/min for 5 min at 4°C, in which the supernatant was carefully removed. The Ni-NTA was washed three times with column buffer, and then washed twice with 15 mmol/L imidazole, then eluted with 300 mmol/L imidazole and analyzed by SDS-PAGE. The concentration of purified proteins was detected by Coomassie brilliant blue G-250. The purified protein was added to a final concentra-

tion of 10% glycerol and stored at −80°C.

Antiserum preparation using purified TaE3 fusion protein

The purified fusion protein was injected into rabbits to produce polyclonal TaE3 antibodies. The concrete experimental protocol referred to the method of Cao et al. (2007) and Liu et al. (2010).The serum with the best reactivity toward GST-TaE3-His was collected and stored at −20°C for the use of ELISA, Western blot, and immunohistochemistry assays.

Results

PCR amplification

Total RNA was obtained from mature leaves and analyzed by 1% (w/v) agarose gel electrophoresis (Fig. 1). The ORF of *TaE3* about 800 bp with the addition of *Eco*RI and *Hin*dIII restriction sites on both ends was successfully amplified with RT-PCR. The amplified products were analyzed by 1% (w/v) agarose gel electrophoresis (Fig. 2).

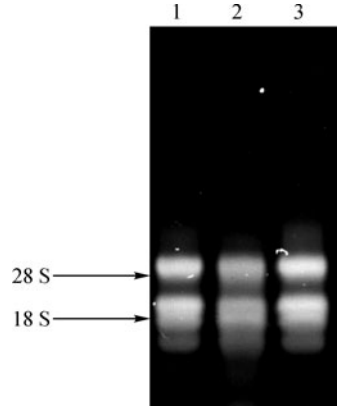

Figure 1 Integrity detection of total RNA. 1, 2 and 3 are the same RNA samples of wheat.

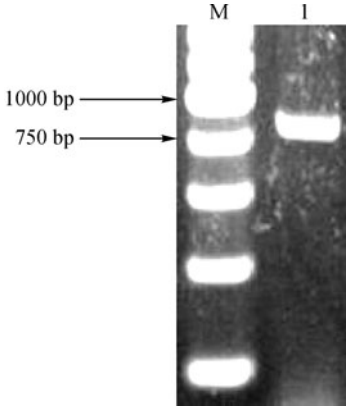

Figure 2 Amplification of *TaE3* gene. M represents DNA marker; 1 represents the amplification products of RT-PCR from wheat *E3* gene.

Construction of recombinant pET30a-GST-*TaE3*-His and restriction enzyme digestion

The gene *TaE3* amplified by RT-PCR was cloned into PMD19-T vector, designated as PMD19-T-*TaE3*. The PMD19-T-*TaE3* and prokaryotic expression vector plasmid were digested by *Eco*RI and *Hin*dIII, respectively. The recovered target segment was reconstructed to obtain the recombinant plasmid pET30a-GST-*TaE3*-His and its product was transformed into *E. coli* DH5α and subsequently sequenced. Sequencing of the recombinant plasmid indicated that the ORF of *TaE3* was in frame inserted into the pET30a vector with the beginning of GST-tag in the N-terminal and His-tag in the C-terminal of the recombinant protein. As a result, the recombinant pET30a-GST-*TaE3*-His was detected by *Eco*RI and *Hin*dIII digestion and analyzed by 1% (w/v) agarose gel electrophoresis (Fig. 3).

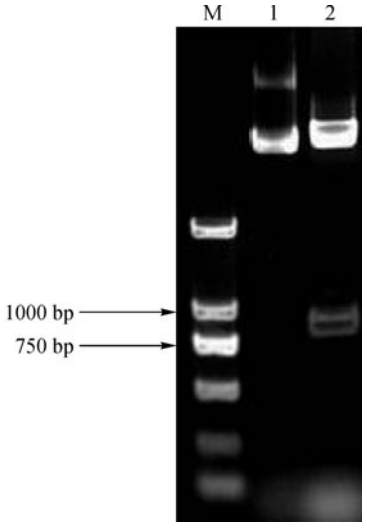

Figure 3 Restriction enzyme analysis of pET30a-GST-*TaE3*-His recombinant plasmid. M represents DNA marker. 1 represents the integrated pET30a-GST-*TaE3*-His plasmid; 2 represents the enzyme digestion fractions of pET30a-GST-*TaE3*-His by *Eco*RI and *Hin*dIII.

Exploration of prokaryotic expression conditions of TaE3 and detection of SDS-PAGE

The recombinant pET30a-GST-*TaE3*-His was transformed into *E. coli* BL21 (DE3). To optimize its expression, the bacterial culture was grown at 37°C until 0.5–0.8 OD600 nm, at which time protein expression was induced by adding 0.5 mmol/L, 0.25 mmol/L, 0.125 mmol/L, 0.1 mmol/L and 0 mmol/L IPTG, respectively. After induction, the samples were taken at 0 h, 2 h, 4 h, 6 h and overnight (20 h), and then the protein expression in the supersonic bacterial lyses supernatant was detected by SDS-PAGE analysis. As shown in Fig. 4, a new band appeared at about 43 kDa, this is consistent with the theoretical size of fusion protein. The

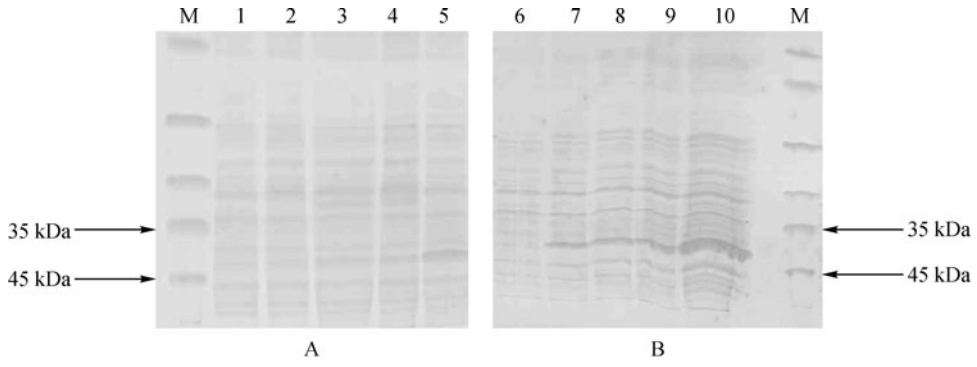

Figure 4 Induced expression of pET30a-GST-*TaE3*-His in *E. coli* BL21. M stands for marker, 1–5 stands for supernatant with 0.25 mmol/L IPTG induced at 0 h, 2 h, 4 h, 6 h and overnight respectively and 6–10 stands for supernatant, induced overnight by 0 mmol/L, 0.5 mmol/L, 0.25 mmol/L, 0.125 mmol/L, 0.1 mmol/L IPTG, respectively.

comprehensive analysis of results of Figs. 4A and 4B, we came to a conclusion that under the conditions of induction at 20 h by 0.1 mmol/L IPTG the highest expression level was identified. Therefore, we induced the large-scale fusion protein expression under this condition.

The identity of the pET30a-TaE3-GST fusion proteins

Considered the His-tag of the fusion protein, the identity of the pET30a-TaE3-GST fusion proteins was confirmed by immunodetection using commercial 6 × His of Ni^{2+}-NTA-coupled antibodies as primary antibodies, and HRP labeled rabbit anti-mouse IgG antibodies as secondary antibodies, with a negative control by its right side. And the clear band in channel 1 (Fig. 5) shows that the fusion protein has a good specification to these his-tagged antibodies, which means it probably, is the TaE3 fusion protein.

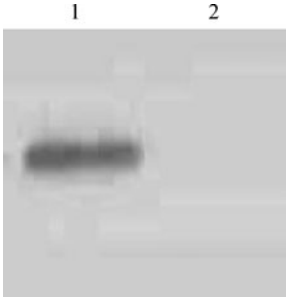

Figure 5 Western blotting verification of TaE3 protein. 1 and 2 stand for His-tag antibody against the supernatant of the bacterial lyses and negative control, respectively.

The purification of TaE3 fusion protein

The Fusion protein was purified by Ni-NTA affinity chromatography. Bacterial lyses supernatant (before purification) and the eluate (after purification) were analyzed by SDS-PAGE (Fig. 6). The purified product had one clear main band and the size of the purified protein was as same as the product

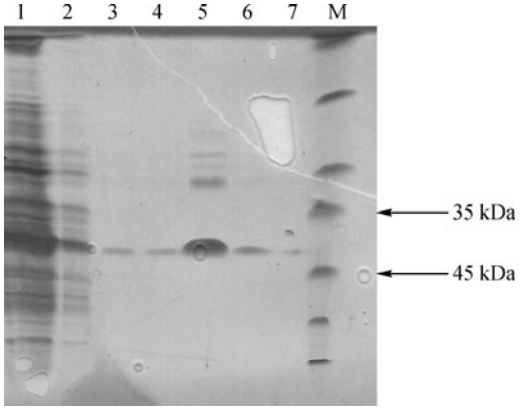

Figure 6 Purify of TaE3 fusion protein. 1 represents the bacterial lyses supernatant, 2 represents the supernatant after purified by Ni-NTA affinity chromatography, 3–4 represent the supernatant was washed twice by the wash buffer (15 mmol/L imidazole), 5–7 represent the supernatant was washed third times by the wash buffer (300 mmol/L imidazole); and M stands for marker.

of the specific expression. Wash buffer removed most of the unspecific proteins. After elution with an elution buffer, a large scale of the fusion protein was washed down. The concentration of separated protein was 1.0 mg/mL, measured by the method of Coomassie brilliant blue G-250.

Preparation and identification of polyclonal antiserum against TaE3

The New Zealand white rabbits were immunized with the purified fusion protein as antigen. The titer of TaE3 antiserum was 1∶768000 detected by indirect ELISA (Fig. 7). Western blotting detected with prepared TaE3 antiserum as primary antibodies, and HRP labeled rabbit anti-rabbit IgG antibodies as secondary antibodies. The results showed that TaE3 rabbit antiserum positively reacted with *E. coli* expressed fusion protein (Fig. 8), indicating that the prepared antiserum had a certain specificity.

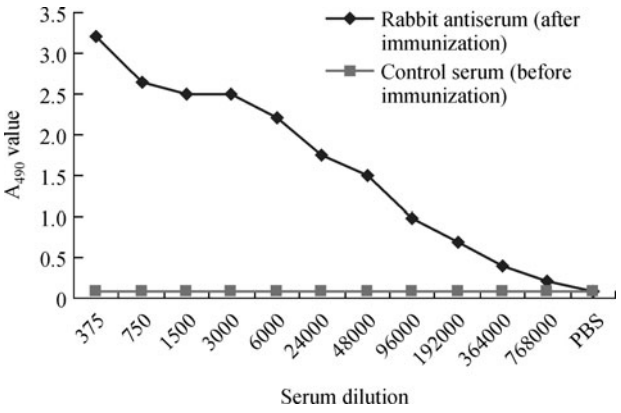

Figure 7 ELISA detection of antiserum titer.

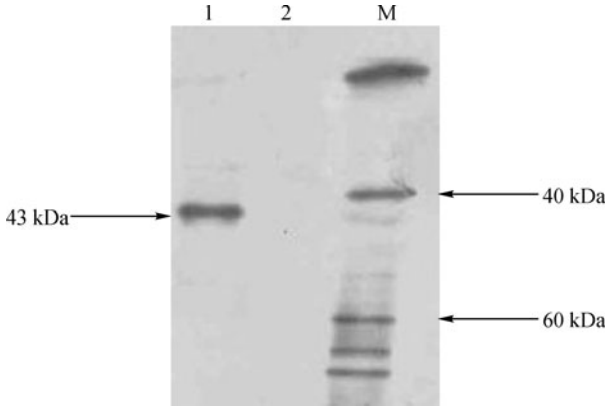

Figure 8 Western blotting verification of anti-TaE3. M stands for marker, 1 stands for TaE3 fusion protein and 2 stands for negative control.

Discussion

Plants E3 ubquitin ligases have recently attracted much interest due to growing evidence that they play important roles in the mediation of cellular responses to environmental stresses. For example, they are known to participate in cold stress signal transduction (Lee et al., 2001; Dong et al., 2006), in the responses to salt and osmotic stress through increased ABA biosynthesis (Ko et al., 2006), and in the ABA-mediated drought signaling pathway (Zhang et al., 2007).

In the former studies in our laboratory, Yan et al. (2009) constructed a SSH library of wheat and leaf rust interaction, and found that in the 16 h library, this putative *E3* gene attained a peak expression, indicated that it may plays crucial roles in the pathway of the interaction between the wheat and the leaf rust. These findings prompted us to construct a fusion vector to express the fusion protein for the further detect of different levels of this E3 ligase in the hours after the fungus inoculated to the wheat leaves.

To facilitate the expression and purification of fusion proteins, in this study, the *TaE3* gene fragment was cloned into pET30a-GST vector with a GST-tag and a His-tag by using genetic recombination. GST-tag promoted the expression of fusion protein in the front of the insert and His-tag was in favor of purified fusion protein in the back-end of the insert. We successfully constructed the prokaryotic expression vector pET30a-GST-*TaE3*-His. TaE3 fusion protein was expressed in *E. coli* BL21 (DE3), and clear bands were obtained by SDS-PAGE separation and stained with Coomassie blue R250 (Fig. 4). We explored the optimal conditions for the prokaryotic expression to obtain the best expression of fusion protein. Fig. 4 shows that the fusion protein was highly expressed in the supernatant at low temperature, low IPTG concentration, therefore we took the condition of induction with 0.1 mmol/L IPTG overnight at 28°C as the best condition to carry out large-scale expression of the fusion protein. In this experiment, we purified fusion protein by Ni-NTA affinity chromatography and obtained a clear main band by SDS-PAGE analysis. We detected antibody titer and specificity by indirect ELISA and Western blotting, respectively. The titer of TaE3 antiserum was 1∶768000 detected by indirect ELISA. Western blot analysis showed that antibody could specifically bind with TaE3 fusion protein, indicating that the specificity of antibody can be used for subsequent functional studies. The preparation of TaE3 antiserum is of great significance on the research of biologic function and complex regulatory network in plants.

Acknowledgements

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