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RESEARCH ARTICLE

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Cloning and prokaryotic expression in *TaCaM2-3* of wheat and preparation of antiserum

Abstract Multiple calmodulin (CaM) isoforms exist in plant organisms and vary by their primary structures of 148 amino acids. They have different expression patterns and/or target enzyme activation abilities. To further understand the biological significance of TaCaM isoforms, total RNA was isolated from mature leaves of wheat and then *TaCaM2-3* gene was amplified by PCR after reverse transcription. The PCR product was generated into T-easy vector to subsequently sequence. Then the recombinant expression vector (pET28a-*TaCaM2-3*) was constructed and transformed into *E. coli* strain BL21 to obtain a high level expression vector of CaM. SDS-PAGE analysis showed that the recombinant *E. coli* could express an approximate 20 kD protein. A western blotting analysis showed an anti-CaM monoclonal antibody specifically bound to the 20 kD band of expressed product. TaCaM-II was purified by Ni-NTA affinity chromatography from recombinant bacterial lysate. TaCaM-II protein was used to immunize New Zealand white rabbits to produce a polyclonal antiserum. The specificity of the anti-TaCaM-II antiserum was successfully verified by western blotting analysis.

Keywords *TaCaM2-3* cloning and expression, western blotting analysis, preparation of antiserum

1 Introduction

The calcium ion is an important component of a diverse array of plant signal transduction pathways (Rudd and Franklin, 2001). In fact, it is the very ubiquity of this intracellular second messenger that has prompted investigations into how specificity is controlled in calcium-based signal systems (McAinsh and Hetherington, 1998). Several Ca^{2+} sensors have been identified in higher plants. The best known one is calmodulin (CaM) which is one of the most conserved eukaryotic proteins. CaM contains four elongation factors (EF) – hand domains for Ca^{2+} binding (Snedden and Fromm, 2001). Higher plant CaM shares a 98% similarity with vertebrate CaM, and except for amidation states, amino acid sequences of CaM are identical among mammals and avian species. However, the family of CaM genes which encode proteins with the same or similar structures were found in a single plant in recent years, and are named CaM isoforms. The Arabidopsis genome has 11 CaM genes encoding at least seven isoforms (Gawienowski et al., 1993). Eight independent CaM genes (*PCaM1-8*) were isolated from potato and divided into five types, of which *PCaM5-8* encoded the same CaM isoforms (Takezawa et al., 1995). In plants, CaM isoforms are differentially expressed in response to numerous environmental stimulus such as touch, heat shock, cold, light and pathogens and activate different target enzymes.

In wheat (*Triticum aestivum* L.), Yang et al. (1996) identified ten CaM genes and distinguished them into four CaM subfamilies (SF1-4) and three different isoforms. Ten different cDNA clone-like proteins were isolated by screening a root-tip cDNA library of common wheat. The cDNAs *TaCaM1-1*, *1-2*, *1-3*, *3-1*, *3-2*, *3-3*, and *4-1* were found to encode a CaM isoform (designated TaCaM-I). The cDNAs *TaCaM2-2* and *TaCaM2-3* encoded a CaM isoform (designated TaCaM-II) that differs from TaCaM-I by only two conservative amino acids (Ile to Val at position 126 and Glu to Asp at position 140). In contrast, *TaCaM2-1* was found to encode a CaM isoform (designated as TaCaM-III) that differs substantially in its N-terminal (residues1-18) from all other reported CaMs. This CaM isoform contains only three of the four conserved calcium-binding sites found in other CaMs. In the course of common wheat development, CaM genes exhibit developmentally regulated organs, tissues, cells and subfamilies specific expression patterns.

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When the wheat-leaf rust fungus interaction system was investigated in our preliminary work, results showed that the differences between compatible and incompatible interactions not only depended on resistant genes, but also depended on the differences of time, space and intensity of expressed resistance genes and defense responses (Wang, 1999). Hypersensitive reaction (HR) is important to resist leaf rust (Heo et al., 1999; Wang and Yang., 1992). In addition, Ca^{2+} was involved in the signal transduction after wheat leaf rust infection, and cytoplast Ca^{2+} rising may be induced by the Ca^{2+} influx (Guan, 2006; Hou et al., 2007; Liu, 2004). Additionally, we have found that the CaMs participated in the defense responses of wheat leaf rust infection via the Calmodulin antagonist (unpublished), but the exact isoforms of CaM involved in this program are still not fully understood. The preparation of antiserum is important to investigate the location, quantity and physiological functions of CaM isoforms in the defense responses of wheat leaf rust infection. In this paper, we cloned a prokaryotic expressed *TaCaM-2-3* in wheat and prepared the antiserum in order to study the involvement of calmodulin in resistance against leaf rust fungus in wheat.

2 Materials and methods

2.1 Materials

E. coli DH5α, BL21 (DE3), and expression vector 'pET28a+' were preserved in our laboratory. T-easy vector, *Hin*dIII, *Bam*HI and T4 ligase were purchased from TAKARA Company. IPTG (Isopropyl-β-D-thiogalactopyranoside) and X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) were purchased from Shanghai Sangon Biotechnology Company.

2.2 RNA extraction

Total RNAs were extracted from 100 mg leaf tissue of wheat cultivar L10 and the extraction method using TIANGEN RNA Plant kit and the isolation of poly(A) RNA using Oligotex mRNA mini kit were operated according to the manufacturer's protocol (Qiagen, Germany). The quality and quantity of RNA were measured by a nucleonic acid and protein detection instrument (Nano-Drop ND-1000, American).

2.3 cDNA synthesis and PCR reaction

RNA was used as template for synthesis of first-strand cDNA described as promega kit (A3500). PCR reaction was carried out in 50 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 30 cycles for 30 s at 94°C, 45 s at an annealing temperature of 66°C and 30 s at 72°C for extension, with a final extension at 72°C for10 min. The primers used in the reaction are listed in the following with the underlined regions noted as restriction enzyme sites for *Bam*HI and *Hin*dIII:

TaCaM2-3: F5′-CGGGATCCATGGCGGACCAGCT-CACCG-3′ R5′-CCCAAGCTTTTACTTGGCCATCAT-GACTTTAAC-3′.

2.4 Products cloning and construction of expression vector

The PCR products were gel-purified and cloned into the T-vector, and then the T-vector was used to transfer the PCR products to competent DH5α cells. Transformed cells were selected on LB medium with X-gal. The positive colonies were picked out separately for further identification by DNA sequencing. The sequence was digested with *Hin*dIII and *Bam*HI and inserted into 'pET28a+' expression vector. After that, to test, the expression vector was digested by *Bam*HI and *Hin*dIII.

2.5 Expression of TaCaM-II protein in *E. coli* BL21

E. coli BL21 cells introduced with the recombinant plasmids above were used for TaCaM-II expression. The confirmed construct was used to chemically transform *E. coli* BL21 for expression of His-TaCaM-II fusion protein. The *E. coli* BL21 cells were cultured in LB solid medium (tryptone 10 $g \cdot L^{-1}$, yeast extract 5 $g \cdot L^{-1}$, NaCl 10 $g \cdot L^{-1}$, agar 15 $g \cdot L^{-1}$ and kanamycin 50 $\mu g \cdot mL^{-1}$). 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 then inoculated into 50 mL of the fresh medium described above. To optimize its expression, the bacterial culture was grown at 37°C until OD600 nm was 0.5–0.8, at which time protein expression was induced by adding 0.8 mM IPTG. The culture was shaken at 200 $r \cdot min^{-1}$ at 37°C in 100 mL erlenmeyer flask. After induction, the samples were taken at 0 h, 2 h, 4 h, 6 h and 8 h. Then the cells were separated from the culture medium by centrifugation at 8000 $r \cdot min^{-1}$ for 15 min at 4°C, and then the harvested cellular pellet was resuspended in ice-cold buffer (10 mM Tris–HCl, pH 8.0, and 100 mM DTT). The suspension was sonicated on ice for 5 min (5S/20S) to disintegrate the cells and centrifuged at 12000 $r \cdot min^{-1}$ for 10 min at 4°C. The pellet was resuspended in 10 mM Tris–HCl buffer. The supernatant and the resuspended pellet were added with 4×sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 100 mM DTT), and analyzed by SDS-PAGE.

2.6 SDS–PAGE and western blotting analysis

The fusion protein was analyzed by SDS–PAGE under

reducing conditions using 15% gels and the Tris–glycine system. The separated proteins were stained by Coomassie brilliant blue R-250 or electroblotted on PVDF membrane for western blotting analysis using a mini-transblot system (Bio-Rad). After electroblotting, proteins were fixed with 0.2% (v/v) glutaraldehyde in TBS (10 mM Tris–HCl pH 7.5, NaCl 150 mM) for 50 min under constant agitation. The membrane was blocked with TBS containing 5% (v/v) BSA for 60 min, and incubated with primary antibody diluted in blocking buffer for 60 min at room temperature. After washing in TBST (0.05% Tween-20 in TBS), the membrane was incubated for 1 h at room temperature with secondary antibody diluted in blocking buffer. After several washes in TBST and a final wash in TBS, immunodetection was performed with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.7 Protein purification and concentration detection

The recombinant His-tagged proteins were purified by Ni-NTA affinity chromatography according to the manufacturer's protocol QIAexpress Ni-NTA Fast Start Handbook (Qiagen), and analyzed by SDS-PAGE. The concentration of purified proteins were detected by Coomassie brilliant blue G250.

2.8 Antiserum preparation using purified TaCaM-II

The purified His-TaCaM-II protein was injected into rabbits to produce a polyclonal TaCaM-II antibody (Xiao et al., 2006; Cao et al., 2007; Huang et al., 2008). Two New Zealand white rabbits were immunized intravenously with 100 μg of the His-TaCaM-II protein per rabbit and followed by a second immunization two weeks later. After the second injection, three additional injections were performed at 3-week intervals. One week after the last injection, sera were collected and used to test the TaCaM-II antibody. The rabbit with the best reactivity toward His-TaCaM-II was sacrificed and the serum was collected and stored at −20°C for the use of ELISA (Huang, 2008) and western blotting.

3 Results

3.1 PCR amplification

Total RNA was obtained from mature leaves and analysed by 1% (w/v) denatured agarose gel electrophoresis (Fig. 1). Synthesized cDNA was amplified with *TaCaM2-3*F and *TaCaM2-3*R, restriction primers and highly protected pfu Taq DNA polymerase to obtain 450 bp fragments. The amplified products were analysed by 1% (w/v) agarose gel electrophoresis (Fig. 2)

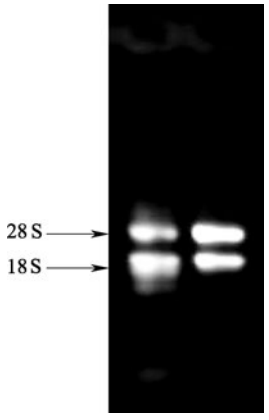

Fig. 1 Agarose gel electrophoresis of total RNA

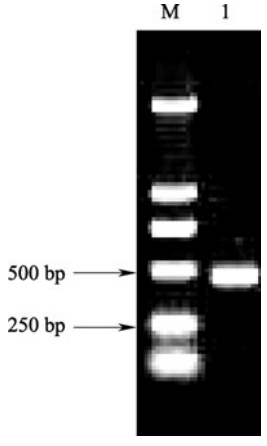

Fig. 2 Amplification of *TaCaM2-3* gene
Note: M stands for DNA marker and 1 for *TaCaM2-3* fragment.

3.2 Construction of recombinant pET28a-*TaCaM2-3* and restriction enzyme digestion

The T-vector and prokaryotic expression vector plasmid were digested by *Bam*HI and *Hin*dIII, respectively. The recovered target segment was reconstructed and its product was transformed into *E. coli* DH5α and positively cloned by anti-kanamycin. As a result, recombinant pET28a-*TaCaM2-3* was detected by *Bam*HI and *Hin*d III digestion and analysed by 1% (w/v) agarose gel electrophoresis (Fig. 3).

3.3 Expression of TaCaM-II protein in *E. coli* BL21 and western blotting analysis

The recombinant pET28a-*TaCaM2-3* was transformed into *E. coli* BL21 (DE3). After induction by 0.8 mM IPTG, we plotted the growth curve (Fig. 4). Recombinant bacteria growth slowed down compared with the uninduced bacteria. The samples detected with 15% SDS–PAGE (Fig. 5) showed an approx 20 kD protein band consistent

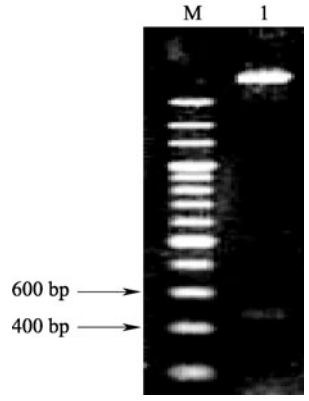

Fig. 3 Identification of pET28a-*TaCaM2-3* recombinant
Note: M stands for DNA marker and 1 for *TaCaM2-3* recombinant.

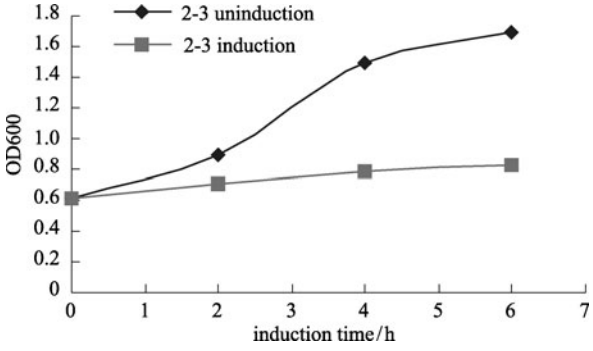

Fig. 4 The growth curve of the strain *E .coli* pET28a-*TaCaM2-3* with IPTG induction

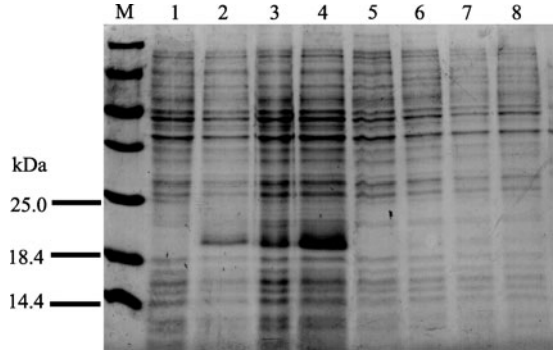

Fig. 5 Induced expression of pET28a-*TaCaM2-3* in *E. coli* BL21
Note: M stands for marker. 1–4 stand for supernatant of *TaCaM2-3* inducted at 0 h, 2 h, 4 h and 6 h. 5–8 stand for pellet of *TaCaM2-3* inducted at 0 h, 2 h, 4 h and 6 h, respectively.

with the predicted size. TOTALLAB2.0 software analysis showed the highest expression after induction by IPTG, and fusion protein accounted for about 25% of the total proteins. In addition, the proteins were mainly in the supernatants, and rarely found in inclusion bodies. This is because CaM is a soluble globulin protein, existing in soluble forms within the cell. The samples were detected with 15% SDS–PAGE.

The identity of the pET28a-*TaCaM2-3* fusion proteins was confirmed by immunodetection using a commercial mAbs against CaM and His (Fig. 6). After electroblotting, western blotting detected an anti-CaM monoclonal antibody and 6×His of Ni^{2+}-NTA-coupled antibody as a primary antibody, and HRP labeled rabbit anti-mouse IgG antibody as secondary antibody. Western blotting analysis showed that TaCaM-II protein specifically binds with anti-CaM antibody (anti-CaM) indicating that the expressed protein is a calmodulin protein (Fig. 6A). In addition, the recombinant vector 'pET28a+' has 6×His Tags, detected with Ni^{2+}-NTA-coupled antibody, which identified 6×His joints of the fusion protein. The results also showed a positive reaction band at the expected size, indicating that the expressed protein has 6 × His (Fig. 6B).

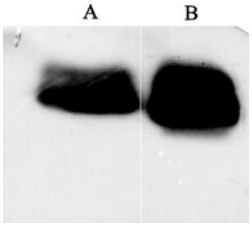

Fig. 6 Western blotting verification of TaCaM-II protein
Note: A and B stand for mAbs against CaM and mAbs against His respectively.

3.4 Protein purification and concentration detection

The recombinant vector 'pET28a+' with 6×His-Tag, fusion protein was purified by a Ni^{2+}-NTA affinity chromatography column purification system according to the non-denaturing protein purification operation guide of the *QIAexpress Ni-NTA Fast Start Handbook*. The purified protein was then detected by SDS-PAGE (Fig. 7). Wash buffer removed most of the hybrid protein. After elution

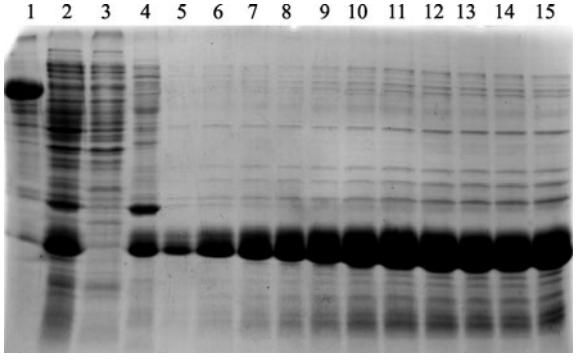

Fig. 7 Purify of TaCaM-II fusion protein
Note: 1–4 stand for 1 mg·mL^{-1}BSA, cell lysate, rflow-through fractions and washed fractions by 15 mM imidazole. 5–15 stand for eluted fractions by 60 mM imidazole, respectively.

with elution buffer for a longer time, a large quantity of the fusion protein was eluted. The concentration of separated protein was 1.5 mg·mL^{-1} measured by Coomassie brilliant blue G-250.

3.5 Preparation and identification of polyclonal antiserum against TaCaM-II

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

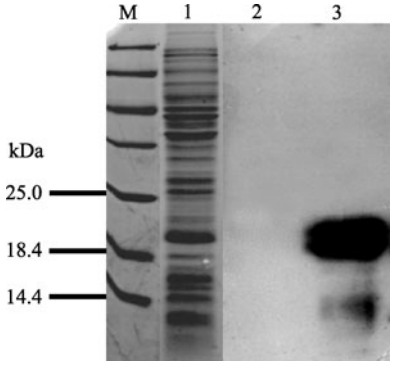

Fig. 8 Western blotting verification of anti-TaCaM-II
Note: M, 1, 2 and 3 stand for marker proteins with molecular masses in kilodaltons, total proteins in induced *E. coli*, pET28a+ empty vector and TaCaM-II fusion protein respectively. M and Lane 1 are from SDS-PAGE, Lane 2 and 3 are from Western blotting.

4 Discussion

Calmodulin, CaM, an ubiquitous Ca^{2+} sensor protein, is sensitive and can respond to the instantaneous rise of Ca^{2+}. Ca^{2+}/CaM is one of the most intensive and widely studied plant cell signal transduction mechanisms. In plants, the presence of multiple CaM isoforms enhances the Ca^{2+} signal-mediated signal network diversity and complexity. Changes in the amino acid composition of CaM subtypes may interact with different target proteins, leading to different biological functions. Meanwhile, CaM was induced to initiate resistance signal transduction in the early stages of wheat-leaf rust interaction (Mao and Wang, 1995). We expect to prepare different isoform antibodies of CaM for further discussion about which isoform of CaM may be involved in the response of defending pathogen infection.

In this study, wheat calmodulin isoform II was expressed in *E. coli* strain BL21, and clear bands were obtained by SDS-PAGE separation and stained with Coomassie blue R250 (Fig. 5). Western blotting also detected TaCaM-II protein, both an anti-CaM monoclonal antibody and 6×His of Ni^{2+}-NTA-coupled antibody (Fig. 6). Based on this, the fusion proteins were purified (Fig. 7). Antiserum was TaCaM-II through the immunization, and the antiserum could specifically bind the fusion protein of prokaryotic expression. This laid the foundation for further analysis of CaM function and the role of the CaM isoforms in the defense responses.

The induction concentration of the IPTG and the induction temperature of this expression system were accessed in the earlier work from our laboratory. By screening with the optimal expression conditions in LB medium, at 37°C, and when the OD600 reached 0.5–0.8, the *E. coli* were induced with 0.8 mM IPTG (final concentration) for 6 h. The fusion proteins of the prokaryotic expression vector 'pET28a+' and the exogenous gene were not only in cells in soluble form, but also the formation of inclusion bodies. Since the end of the vector has a 6 × His tag, it is beneficial for the purification of the fusion proteins. Large quantities of fusion proteins were obtained based on QIAexpress Ni-NTA Fast Start Handbook (Fig. 7).

In this paper, the purified fusion proteins were used as antigen to get the rabbit anti-TaCaM-II antiserum. The western blotting results indicated that the antiserum and the fusion proteins can specifically react with each other. The expressed fusion proteins maintained the TaCaM-II immunogenicity, which means it can specifically recognize the TaCaM-II protein. However, whether the antiserum can distinguish the CaM isoforms still needs further study to identify these two proteins, TaCaM-I and TaCaM-III.

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