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Arabidopsis thaliana SEPALLATA3 protein prokaryotic expression and purification

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Abstract

*SEPALLATA*3 (SEP3) can be attributed to E class gene of the ABCE model of floral organ development. In order to reveal how SEP3 proteins form polymers, and the relationship between the polymers and their biological functions, the experiments of *Arabidopsis thaliana* AtSEP3 protein soluble expression in vitro were performed to construct a vector of prokaryotic expression, and investigate induced expression of recombinant proteins in *Escherichia coli* cells. The protein soluble expression was analyzed through the aspects of different protein domains, induction time, induction temperature, etc. Different structural domains and expression conditions were screened, and 0.1% IPTG inducing at 22 °C for 15 h was estimated as an optimal expression strategy. The nickel chelating resin was used to purify the protein in size exclusion chromatography (SEC) and the results indicated that AtSEP3 protein was present in the form of tetramer.

Key words: Botany, SEP3 protein, prokaryotic system, protein purification, polymer.

Introduction

The research on floral organ has been a hot topic in the study of plant development (1,2). In 1991, Coen et al (3) first proposed an 'ABC' model based on genetic research on floral homeotic mutants of *Arabidopsis thaliana* and *Antirrhinum majus*. Whereafter, Theiben et al (4) proposed new models about plant floral development, namely 'ABCD' model, 'ABCDE' model and quaternary model. Currently, the studies about transcription factors of *yeast* MCMI, *Arabidopsis thaliana* AG, *Antirrhinum majus* DEFA, animal serum response factor SRF (MADS) mainly focus on their genetic functions (5) and regulations of plant metabolism (6). By the investigation of *Arabidopsis thaliana* mutants (7), *SEPALLATA-like* gene has been demonstrated to play an important role in formation of pollen and to mature pollen tubes (8). *SEP-like* gene is transcription factor of coding MADS protein, the functions of the gene in dicotyledonous plants show various degrees of redundancy and sub-functionalization (8), the homologous protein of E blossom gene is more important than other A/B/C transcription factors in the formation of floral organ development (9,10). *SEP3* belongs to E class *Arabidopsis thaliana* AGL9-type gene, coding protein is very important to the formation of tetramer complex from MADS box family (11). In the process of blossom, MADS protein participate in many processes for regulation of blossom (11) through interacting with other proteins (12). However, it is unclear that whether the SEP3 protein only functions the formation of heteromeric complexes (13) after combining other transcription factors, or it still contains biological functions after the formation of homologous polymers. Previous studies have demonstrated that MADS protein combines with the specific DNA to form homologous polymers and performs their functions (14). However, the formation mechanisms of homologous polymers from MADS protein and the relationship between their functions are still unclear. This study attempts to analyze the methods for purification of soluble SEP3 protein by in vitro expression, clone the *AtSEP3* gene in different domains to the prokaryotic expression vector pET-15b, screen the soluble expressions followed by induced expression, and obtain high quality *AtSEP3* protein by purification. This work provides an important foundation for expression and purification of plant protein in heterologous prokaryotic expression system.

Materials and methods

Materials

Columbia wild *Arabidopsis thaliana* were grown in Key University Laboratory of Biotechnology and Utilization of Bio-resource of Shandong, College of Life Science, Dezhou University. *Escherichia coli* DH5α competent cell, expression strain *E. coli* 'Rosetta' and expression plasmid pET-15b were preserved in Key University Laboratory of Biotechnology and Utilization of Bio-resource of Shandong, College of Life Science, Dezhou University. DNA extraction kit was purchased from Beijing DingGuoChangSheng Co Ltd. DNA polymerases, various DNA restriction enzyme and DNA were purchased from BaoSheng Biotechnology (Dalian) Co Ltd. DNA ligase was purchased from New England company. DNA purification kit and gel recovery kit were purchased from TianGen Biotechnology (Dalian) Co Ltd. Other analytical reagents were made in China.

Construction of AtSEP3 prokaryotic expression vector

Extract the flower bud tissue RNA of *Arabidopsis thaliana* with Trizol method, cDNA is obtained by re-

verse transcription. Design the primers of two different structural domains (including MIKC domain, IKC) based on conserved sequence of cDNA of *Arabidopsis thaliana* SEP3 gene, sample and sequence after connecting the products obtained by amplification from polymerase chain reaction (PCR) with pMD18-T vector and transformed into *Escherichia coli* DH5α. After extracting plasmid from the correct strain verified by sequencing, the plasmid obtained by *Nco* I and *BamH* I double-enzyme cleavage extraction and pMD18-T vector were loaded into DNA gel recovery kit to recover enzyme cleavage products, the fragments were connected with vector overnight at 16°C then transformed into *Escherichia coli* DH5α and coated on LB plate with ampicillin resistance, PCR-test bacteria liquid followed by bacterial strain selection, determine base sequence of strain samples, clone to obtain AtSEP3 prokaryotic expression vector of fragments in different structural domains.

Soluble conditions screening for recombinant proteins

The induction temperature is closely related to the solubility of prokaryotic expression protein. Under the condition of high temperature, strain rapid expressing target protein is easy to make the protein form incorrect folding and finally present in the form of inclusion body, which is not favorable for obtaining soluble protein. Correct recombinant plasmid verified by sequencing is transformed in expression host strain *E. coli* 'Rosetta', cultured 15.0 h at 37°C, single colony is selected and transferred to 100 mL LB liquid nutrient medium containing 50.0 mg.L^{-1} kanamycin, cultured 3.0 h at 37°C. When the concentration of bacteria is 0.4-0.6 at absorbance D(λ) of 600, isopropyl thiogalactoside (IPTG) at final concentration of 0.1 mmol.L^{-1} is added, the strains are cultured at 37°C, 5 h, and 22°C, 15 h, respectively. Determine the induction temperature for train culture.

Validation of peptide fingerprinting of AtSEP3 recombinant protein

The purified recombinant protein was obtained by polyacrylamide gel electrophoresis (SDSPAGE) separation and detection. Followed by Coomassie brilliant blue staining, the target strips were carefully cut using operation knife and put in 1.5 mL centrifuge tube (with a small amount of double distilled water ddH_2O). Peptide mass fingerprint was identified and analyzed by Beijing XinKeAoDa Technology Co Ltd.

Purification of recombinant AtSEP3 protein

Soluble fusion protein was purified using gel filtration chromatography column followed by preliminary purification with Ni-TNA resin. The specific steps in details were described as follows: 10.0 mL 0.5 mol.L^{-1} NaCl and 20.0 mmol.L^{-1} pH 8.0 lysate of trihydroxymethyl aminomethane - hydrochloric acid (Tris-HCl) were added into 5000 g bacteria liquid precipitation which was obtained by centrifugal collection. The solution was placed in ice water bath, ultrasonic processing for 10 min with a power of 200 W. After 30.0 min, the solution was centrifuged for 40.0 min with 17,000 r.min^{-1} at 4°C. The supernatant was collected, and loaded into the balanced Ni-NTA column. After slowly binding for 30 min using a table concentrator on ice, the hybrid protein which was not specifically bound with Ni-NTA column

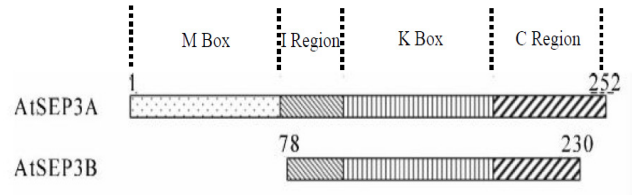

Figure 1. AtSEP3 protein domain construction.

was collected. The buffer solution of different iminazole concentration gradient which has 10 times volume of column was used (4/20/80 mmol. L^{-1} iminazole, 0.2 mol.L^{-1} NaCl, 20.0 mmol.L^{-1} pH 8.0 Tris-HCl) to elute the hybrid protein, the solution with 10 times volume of elution solution was used (200.0 mmol. L^{-1} iminazole, 0.2 mol.L^{-1} NaCl, 20.0 mmol.L^{-1} pH 8.0 Tris-HCl) to wash and collect the target protein. The target protein was then purified using gel filtration chromatography column. The eluent was collected and concentrated to 500.0 μL using ultrafiltration column 4000 g, the buffer solution (0.2 mol.L^{-1} NaCl, 20.0 mmol.L^{-1} pH 8.0 Tris-HCl) was then removed with gel filtration chromatography column using a flow velocity of 0.5 mL.min^{-1} to purify the target protein. A small amount of protein sample was taken and two-fold protein buffer was added, boiled the sample for 10.0 min. After precipitated by centrifugation and ultrasonic processing, 10.0 μL sample with 10.0 μL buffer was detected by SDS-PAGE followed by boiling for 10.0 min.

Results

Construction of pET15-AtSEP3 prokaryotic expression vector

The sequence of AtSEP3 domain in 2 different domains was obtained by sequencing and vector in domains of different size was constructed (see Figure 1) (14). To those successfully transformed strains, the plasmids were extracted for PCR and double-enzyme cleavage identification to obtain target fragments, and pET15-AtSEP3 prokaryotic expression vector in different domains was obtained by successful construction.

Optimization of solubility conditions of recombinant AtSEP3 protein

The proteins were dissolved in lysate followed by

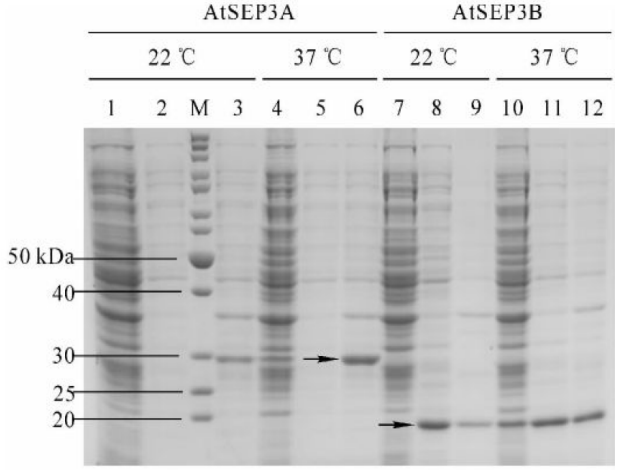

Figure 2. Screening for soluble AtSEP3 protein expression. 1, 4, 7, 10 – total proteins, M – protein labels, 2, 5, 8, 11 – supernatant samples, 3, 6, 9, 12 –precipitation samples.

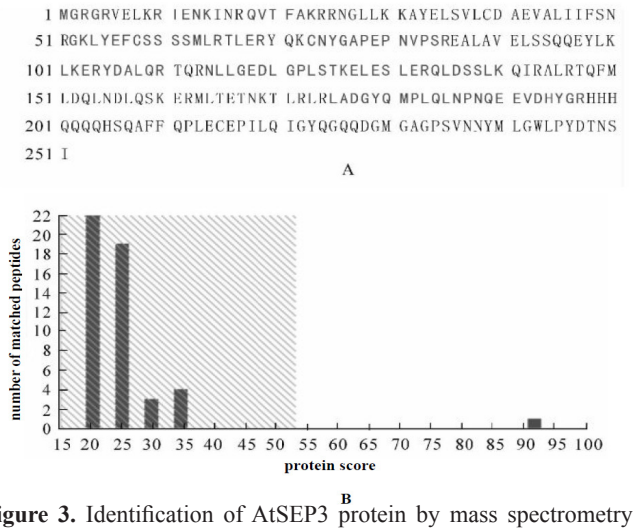

Figure 3. Identification of AtSEP3 protein by mass spectrometry (A) and Mascot Score histogram of mass fingerprinting (B)

enlargement culturing of the constructed prokaryotic expression strains. In SDS electrophoresis diagram (Figure 2), the arrows indicate target proteins. The detection results show that AtSEP3A (30 kDa) present in the form of inclusion body expression and AtSEP3B (18 kDa) is a soluble protein. These experiments suggest that pET-15b is prokaryotic expression vector of AtSEP3B which contains part of IKC region. The soluble protein with high expression is obtained by induction expression of IPTG for 15.0 h at 22°C with a final concentration of 0.1 mmol.L^{-1}.

Identification of recombinant protein by peptide mass fingerprinting

To detect the expression of prokaryotic protein, the target proteins were identified by peptide mass fingerprinting followed by SDS-PAGE Coomassie brilliant blue staining. The initial results (the used program is Mascot: http://www.matrixscience.com, the database is SwissProt) provided by Beijing XinKeAoDa Technology Co Ltd show that identification score is 92 for target protein and the protein NP_850953.1, 18 peptides match with the sequence of target protein (Figure 3A), the gray parts indicate the polypeptide fragments which match with the target proteins (42% of the target protein). Mascot Score histogram (Figure 3B) shows that the proteins with score above 53 have significant difference ($P < 0.05$), the results located in shadow region are not trusted. The gray bar out of the shadow region indicates the matching results, which is compared with the database for verification. Mascot Score histogram shows that the peptides matched with the left small fragments in Figure 3B do not have significant difference, the right gray bar indicates the peptides matched with the protein database, and have significant differences, suggesting that the purified fusion protein is AtSEP3 protein.

Purification of the soluble protein AtSEP3B

The results of electrophoresis of the purified product from each stage are shown in figure 4A. The bovine serum albumin (BSA) solution is used as a reference and present in the forms of monomer and dimer, with molecular weight of 67 kDa and 134 kDa, respectively. The black solid line indicates AtSEP3B, the peak position is close to 67 kDa of the BSA monomer. In figure

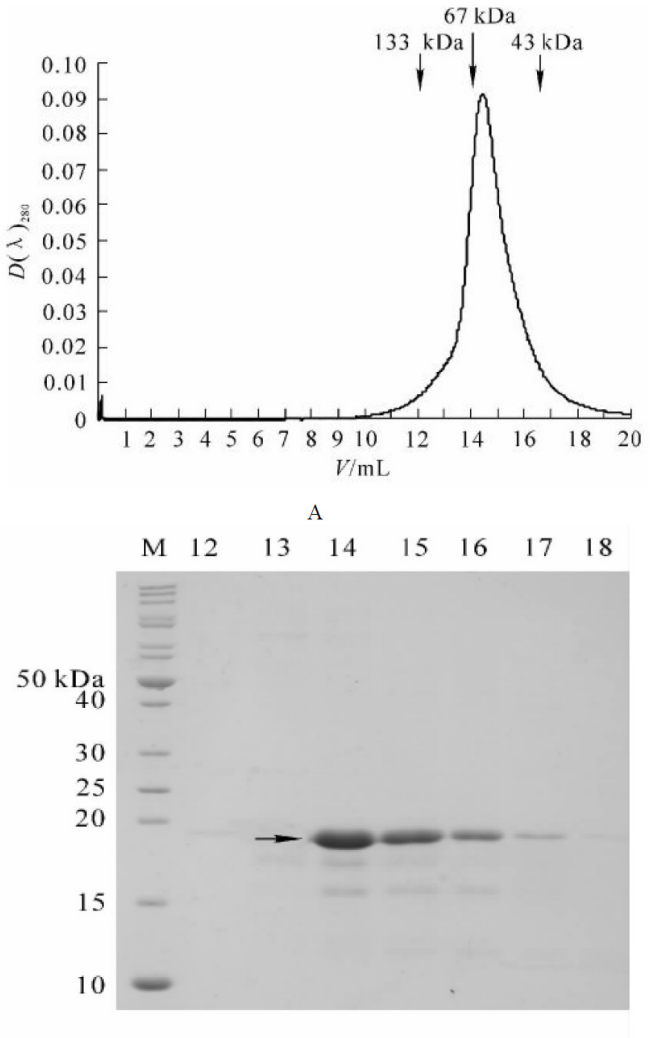

Figure 4. Gel filtration of AtSEP3B proteins. **A**: the chromatogram of AtSEP3B protein precipitation with a buffer solution of 0.2 mol.L^{-1} NaCl, 20.0 mmol.L^{-1} trihydroxymethyl aminomethane - hydrochloric acid (Tris-HCl), pH 8.0. **B**: polyacrylamide gel electrophoresis detection of AtSEP3B protein, 12-18 represent the volume numbers corresponding to A, respectively.

4B, SDS-PAGE detects sample purity after the gel filtration chromatography of protein. The arrow indicates target protein, suggesting that highly purified AtSEP3B protein was obtained. The molecular weight of a single peptide chain is 18 kDa, and the peak in figure 4A is approximately 67 kDa, suggesting that the purified AtSEP3B is present in the form of tetramer.

Discussion

The studies of molecular mechanisms of blossom plants have made significant progress with the model plants. However, recently further research highlights a number of new problems, for example, the molecular mechanisms in plant-controlled floral organ formation, the structure of the MADS box protein and its functional mechanisms are still undefined. This study took an important regulatory gene SEP3 during blossom of *Arabidopsis thaliana* as an example, the vector fragments in different domains were designed and soluble AtSEP3 protein was obtained by purification.

SEP3 gene presents in the form of advanced complex, it plays an important role in different stages of

blossom (15), and it is indispensable in each steps of formation of the ABCE model. After combining with specific DNA fragments, SEP3 protein functions as its transcription factors, it also interacts with other specific proteins to activate or inhibit the downstream gene proteins (16). In 2009, Immink et al (9) proposed a SEP3 quaternary model, in this model the M structural domain in MADS box combines DNA and stabilizes tetramer. Through the analysis of AtSEP3 protein structural domains, this study suggests that the M structural domain is not necessary in the formation of tetramer from SEP3 protein. In short, DNA combination is not necessary to the formation of tetramer. Prokaryotic expression system of *Escherichia coli* is adaptable to a wide variety of prokaryotic and eukaryotic gene expression. However, the expression of exogenous proteins and the solubility is associated with a variety of factors including the expression temperature, prokaryotic expression vector, construction of structural domains and expression strains. Temperature has an important effect on the solubility of SEP3 expression. Decreasing induction temperature and increasing expression time significantly favor the folding of exogenous SEP3 protein and the expression yield. The solubility is also closely related to the protein properties. The uniformity of polarity affects soluble expression of protein. Bioinformatics prediction shows that the M region of SEP3 protein is hydrophilic, and other IKC regions are hydrophobic.

In this paper, different fragments and prokaryotic expression system were screened, the solubility expression of fragments of AtSEP3B structural domains were successfully obtained. These domains present in the form of tetramer, the hydrophobic regions might construct an interaction interface which stabilizes the tetramer. The hydrophilic surface of the tetramer is constructed by the rest of domains, which improve the protein solubility. Further study may investigate the formation of SEP3 protein tetramer and the relationship between the spatial structure and function through the methods of structural biology.

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