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MOLECULAR CHARACTERIZATION AND PROKARYOTIC EXPRESSION OF *ORF507* STERILITY-ASSOCIATED GENE IN CHILLI PEPPER (*CAPSICUM ANNUM* L.) CYTOPLASMIC MALE STERILITY

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Abstract

9704A is a cytoplasmic male sterility (CMS) of chilli pepper (*Capsicum annuum* L) that has been commercially used in chilli pepper hybrid production in China. However, little is known about the CMS-associated gene in chilli pepper 9704A.In this study, according to the sequence of relative references, a fragment of 593 bp including a 507-bp single-coding exon, *Orf507* (GenBank accession No. GU357841) was cloned from leaves of chilli pepper CMS-9704A. Results revealed that the *orf456-2* had one nucleotide deleted in codon 150 (nt450delC) and consists of a 507-bp single-coding exon which encodes 168 amino acid compared with the original sequence data of *orf456*. RT-PCR results showed that the *Orf507* was transcribed in buds and leaves at all developmental stages of CMS-9704A and a hybrid F_1, while no band was detected in the maintainer line. The nuclear restorer gene may restore fertility by post-transcriptional processing or translational/post-translational modification, but did not interfere with transcription of *Orf507* directly. The entire coding region of the *Orf507* was inserted into an expression vector pET32α(+) and transformed into *Escherichia coli* Rosetta. Expression of the fusion protein was successfully induced by isopropyl-β-D-thiogalactopyranoside. This study could establish a basis for understanding the molecular mechanism of chilli pepper CMS.

Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait resulting in the inability to produce or release functional pollen, has been identified and characterized in many crop species (Geiger & Schnell, 1970; Kaul, 1988; Bonhomme, 1992; Khan *et al*., 2008; Saxena *et al*., 2010; Mei *et al*., 2011; Nasreen *et al*., 2011). Discovery and introgression of CMS cytoplasm in crop plants enables the production of high purity F_1 seed without laborious emasculation (Hanson & Bentolila, 2004). At the same time, the studying of the sterility-associated gene is helpful for studying the interaction between nuclear and mitochondrial genetic systems and the regulation of mitochrondrial gene expression. Numerous studies have shown that CMS is related to abnormal mitochondrial gene reorganization (Touzet & Budar, 2004; Linke & Börner, 2005).

CMS in chilli pepper (*Capsicum annuum* L.) was first documented by Martin & Grawford (1951) and has been utilized worldwide for the production of F_1 hybrids. However, little attention has been paid to the molecular analysis of CMS genes in chilli pepper, and during recent decades only some CMS-related fragments have been isolated and characterized in chilli pepper (Kim & Kim, 2005; Kim *et al*., 2007; Deng *et al*., 2010a-b; Jo *et al*., 2009).

CMS is restored by the specific dominant nuclear gene restorer-of-fertility (*Rf*), which is often able to alter the expression of the CMS-associated gene (Hanson & Bentolila, 2004). Recently, several *Rf* genes have been isolated (Brown *et al*., 2003; Kazama & Toriyama, 2003; Komori *et al*., 2004). Most *Rf* genes encode pentatricopeptide repeat proteins which have a repeated motif composed of a degenerative array of 35 amino acids that binds RNA and edits CMS-associated genes (Liu *et al*., 2001; Small & Peeters, 2000; Kotera *et al*., 2005; Wang *et al*., 2006). The first *Rf* gene was reported in the chilli pepper CMS line by Peterson (1958). Many attempts have been made both to determine the number of *Rf* genes and to find these genes. Dominant restorer alleles have been found in several hot and small-fruited chilli pepper genotypes (Woong, 1990; Zhang *et al*., 2000; Wang *et al*., 2004; Jo *et al*., 2010).

The line CMS-9704A bred and selected from a natural chilli pepper mutant of CMS by Institute of Vegetable Crops, Hunan Academy of Agricultural Science, has been used extensively in hybrid chilli pepper production in China (Zou, 2002). However, little is known about the CMS-associated gene in chilli pepper 9704A.

In the present study characterization of CMS-9704A *Orf507* DNA and cDNA sequences is reported. RT-PCR was conducted in different developmental stages of buds to verify the transcription of *Orf507* and to elucidate the effect of modifying *Orf507* expression of the restorer gene. At the same time, *Orf507* was cloned into pET32α (+) and the recombination proteins of *Orf507* were effectively expressed in *Escherichia coli* Rosetta (DE3).

Materials and Methods

Plant materials: A typical CMS line (9704A), a maintainer line (9704B) and a F_1 hybrid line were grown in experimental fields of Yunnan Agricultural University. Leaves and buds for DNA and RNA isolations were frozen in liquid nitrogen and stored at –80°C until further use.

Plasmid and chemical reagents: Vector pET32α(+), His·Bind® Purification Kits, and *E. coli* Rosetta (DE3) used as the host were purchased from Novagen, USA. The *E. coli* DH5α, vector pMD18-T, Genomic DNA Extraction Kit, Trizol, High Fidelity PrimeScrip® RT-PCR Kit, *Hind* III, *EcoR* I, dNTPs and T4 DNA ligase were purchased from TaKaRa, China. Ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma, USA. The other reagents were purchased from Chinese companies.

Nucleic acid isolation and PCR amplification: Genomic DNA was extracted using a Universal Genomic DNA Extraction Kit. A CMS-associated gene fragment was amplified with the forward primer: P1, 5'-CCG**GAATTC**CAGCCTAGCTCGACCCAA-3' (*EcoR* I restriction site in bold) and reverse primer: P2, 5'-CCC**AAGCTT**GCCTCCATCCTCCGTTAT-3' (*Hind* III restriction site in bold) designed according to Kim et al. (2007). The 20μl reaction system was: 1.5μl of (25 ng/μl) DNA, 1μl of 2.5mM mixed dNTPs, 2μl of 10 × Taq DNA polymerase buffer ($MgCl_2$ plus), 0.4μl of 10μM forward and reverse primer, 0.3μl of 5 U/μl Taq DNA polymerase and 13.4μl of sterile water. The PCR program initially started with 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 57°C for 45 s, 72°C for 90 s, and then 72°C extension for 10 min, and finally 4°C to terminate the reaction.

mRNA isolation and CMS-associated gene transcription by semi-quantitative PCR assays: Total RNA from buds and leaves of CMS-9704A, 9704B and F_1, was extracted by the Trizol procedure and cDNA was synthesized using a High Fidelity PrimeScrip® RT-PCR Kit according to the manufacturer's protocol. A pair of primers (P3: 5'-ATGCCCAAAAGTCCCATGT-3'; P4: 5'-TTAAAAAGCGCTAAACAAATTG-3') designed according to the chilli pepper *Orf507* was used to amplify the full-length cDNA and to assay *Orf507* expression levels. We selected the housekeeping gene *Actin* as the control. The control primers used were: 5'-TGCAGGAATCCACGAGACTAC-3' (Actin-F) and 5'-TACCACCACTGAGCACAATGTT-3' (Actin-R). The 20-μl reaction system was 4 μl of 5 × PrimeSTAR PCR Buffer, 0.4 μl dNTP Mixture (10 mM each), 0.2μl of forward and reverse primer (10 μM), 0.2 μl of PrimeSTAR HS DNA polymerase (2.5 U/μl), 2μl of cDNA and 13μl of RNase-free dH_2O. The products of amplification were checked on a 1.5% agarose gel and visualized with ethidium bromide. After the densitometric analysis of three independent gels, the relative transcript amount was expressed as a percentage of control (CMS). A pair of primers for the *Apx* gene (P5, 5'-TCCTATTATGCTCCGTCTCG-3'; and P6, 5'-AACAGGTGGTTCTGGCTTG-3') was designed according to DQ002888 and AY078080 to exclude the possibility of DNA pollution in the transcription profile of *Orf507*.

Construction of the expression vector pET32α(+)–*Orf507*: The amplified product (593 bp) was cloned into the plasmid pET32α(+). Then the positive clones were transformed into a competent *E. coli* strain DH5α according to the manufacturer's instructions.

Expression of recombinant Orf507 protein in *E. coli* Rosetta (DE3): Expression of the recombinant Orf507 protein in *E. coli* Rosetta (DE3) was performed according to Zhao *et al*. (2011). Protein expression was induced by addition of 0.5 mM IPTG. After induction for 1.5, 3.0 and 4.5 h at 37°C, the cells were collected by centrifugation.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western-blotting analysis: SDS-PAGE was performed according to Laemmli (1970) with 15% separating gel, and western blot according to Towbin *et al*. (1979) with 1:5000 dilution of each antibody.

Sequence and bioinformation analysis: The products were cloned into pMD18-T vector and sequenced bidirectionally. At least 10 independent clones were sequenced for each PCR product. Sequencing data were edited and aligned using DNASTAR software (DNASTAR Inc, Madison, USA). The DNA sequence prediction was conducted using GenScan software (http://genes.mit.edu/GENSCAN.html). The theoretical isoelectric point (pI) and molecular weight (Mw) of proteins was computed using Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html).The secondary structure of the deduced amino acid sequences was predicted with SOPMA (http://npsa-pbil.ibcp.fr/).

Results and Discussion

PCR result and sequence analysis: PCR was used to clone a fragment of *orf456* using total DNA extracted from CMS-9704A and 9704B. A single PCR product of the expected size (about 600 bp) was obtained in CMS-9704A, while no band was detected in 9704B. The PCR products were cloned and sequenced. The sequence analysis and comparison with *orf456* revealed the presence of a single nucleotide deletion mutation in the coding regions of *orf456*. The mutation "C" deleted in codon 150(nt450delC) caused a frameshift mutation, which made the original stop codon changed to asparagine (N), so the translated fragment was extended, ending with a new termination codon, giving a total length of 507 bp (Fig. 1). The newly predicted ORF, *orf507* which was homologous to *orf456* in chilli pepper, has been deposited in GenBank with accession numbers GU357841. The RT-PCR for the full-length cDNA confirmed that the *orf456-2* was transcripted in the CMS and F_1 as both of these carried the S-cytoplasm, while no band was detected in the maintainer line. The theoretical isoelectric point and molecular weight of the putative proteins were 5.42 and 19555.52 respectively computed using the compute pI/Mw Tool. The secondary structure predicted with SOPMA(Combet et al. 2000) indicates that the deduced *orf507* contains 70 α-helices, 52 extended strands, 15 β-turns and 31 random coils.

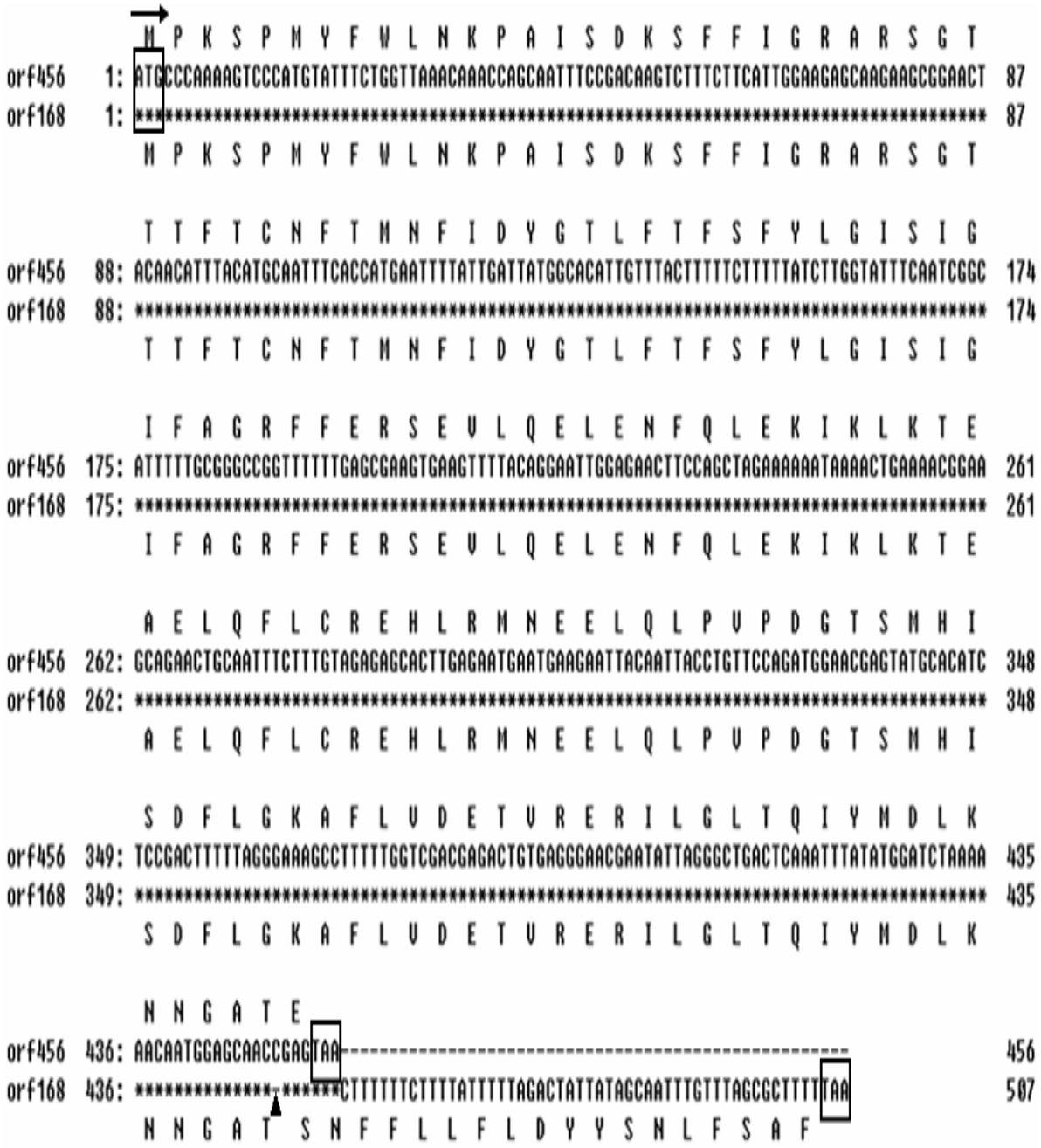

Fig. 1. Alignment of the *orf456* (top line) and *orf507* (bottom line) sequences. *Asterisks* represent identical nucleotides. *boxed nucleotides* indicate the position of the initiation (*horizontal arrow*) and termination codons. The mutation "C" deleted at the +450nt(*with black arrowhead*) caused a frameshift mutation, which made the original stop codon changed to asparagine (N), so the translated fragment was extended, ending with a new termination codon, giving a total length of 507 bp.

Transcription profile of *Orf507* by semi-quantitative PCR assays: To verify the transcription of *Orf507* and to elucidate the effect of the *Rf* gene, RT-PCR was conducted using cDNA from the leaves and buds in various developmental stages of CMS-9704A, maintainer lines and F_1 as templates (Fig. 2). A 507-bp band was amplified at all stages for the CMS line and the F_1, as both of these carried the Sterility-cytoplasm, while no band was observed in any stages for the maintainer line. At the same time, there was little difference in the banding patterns of the *Orf507* transcripts between the CMS and F_1. Amplification of the *APX* gene verified that the cDNA was not polluted by DNA. This suggested that the *Rf* gene did not interfere with transcription of *Orf507* genes. Nuclear restoration by the *Rf* gene may be mediated at the post-transcriptional or translational/post-translational level (Bentolila *et al*., 2002; Kazama & Toriyama, 2003; Komori *et al*., 2004). Due to the complexity of such processes, a more intense study is required to clarify the interaction between *Rf* genes and the *Orf507* transcript.

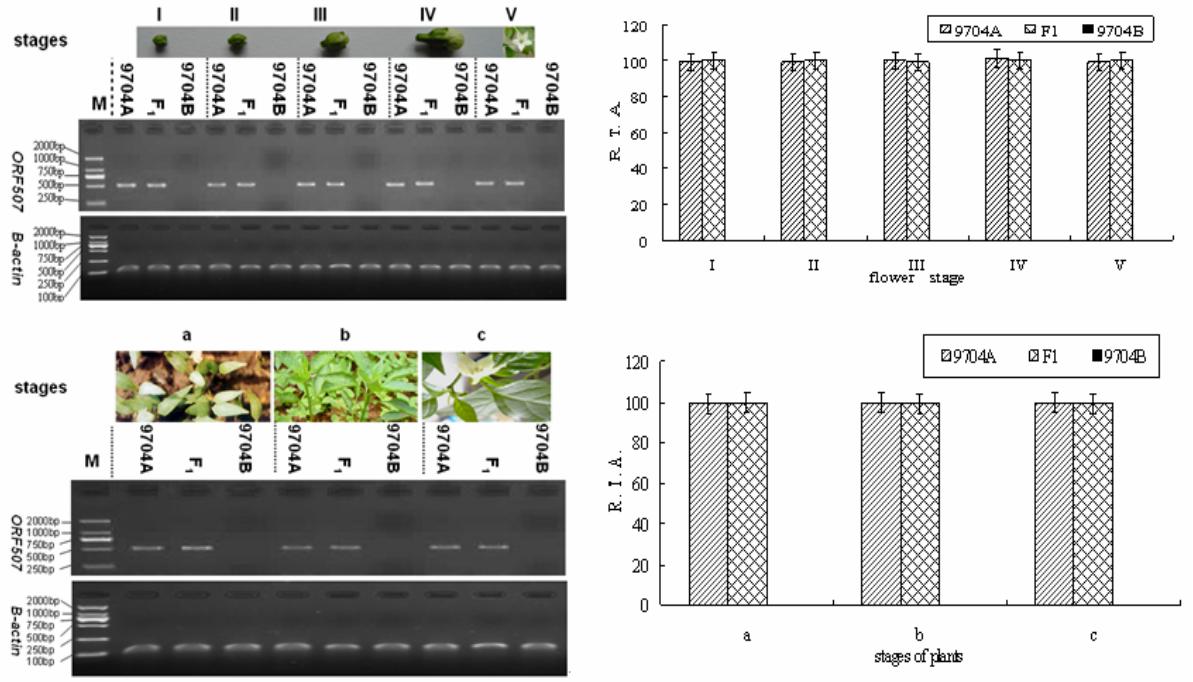

Fig. 2. Transcription of *Orf507* in five bud developmental stages (I: sporogenous cell division stage; II: pollen mother cell meiosis stage; III: uninucleate microspore stage; IV: mature pollen stage; V: Open buds) and in three leaves developmental stages (a: cotyledon period; b: 7-to 9- full leaf stage; c: full buding stage). Band was detected in CMS-9704A and F_1 as both of these carried the sterile (S) cytoplasm in all stages and tissues, while appeared no band in 9704B(Mt). There was little difference in the banding patterns of the *Orf507* transcripts between the CMS and F_1. Representative gel from 3 independent experiments and their densitometric analysis. Relative transcription amount (R.T.A.) is expressed as a percentage of control (CMS-9704A(I or a)) that represents 100%. Mean values ± SD (n=3).

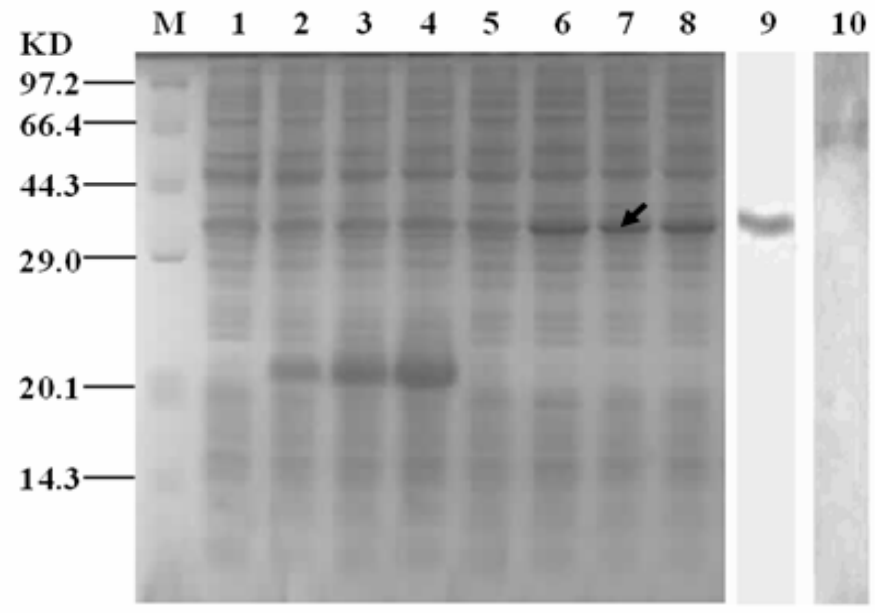

Fig. 3. SDS-PAGE and Western blot of the pET32-Orf507 proteins.
M, Marker proteins with molecular masses in kilodaltons; lane 1, Total protein of E. coli Rosetta (DE3) harboring pET32α(+) before IPTG induction; lane 2-4, Total protein of E. coli Rosetta (DE3) harboring pET32α(+) induced by 0.5 mM IPTG at 37°C for 1.5, 3.0 and 4.5 h, respectively; lane 5, Total protein of E. coli Rosetta (DE3) harboring pET32α(+)-Orf507 before IPTG induction; lane 5-8, Total protein of E. coli Rosetta (DE3) harboring pET32α(+)-Orf507 induced by 0.5 mM IPTG at 37°C for 1.5, 3.0 and 4.5 h, respectively; lane 9, Detection of recombinant protein 6xHis-Orf507(pET32α(+)-Orf507 induced by 0.5 mM IPTG at 37°C for 3.0 h) by Western blotting assay; lane 10, Detection of recombinant protein 6xHis-Orf507(empty vector PET32α(+) induced by 0.5 mM IPTG at 37°C for 3.0 h) by Western blotting assay.

Expression of recombinant chilli pepper CMS-9704A *Orf507* in *E. coli* Rosetta (DE3): The PCR product was purified by kits. After purification, the PCR product was ligated into the pMD18-T vector, and the recombinant plasmid pMD18–*Orf507* was constructed and then transformed into *E. coli* DH5α for identifying the recombinants.

After digestion with *EcoR* I and *Hind* III and purification, the *Orf507* and pET32α (+) plasmid were ligated into a recombinant pET32–*Orf507* with T4 DNA ligase. The recombinant was identified by restriction enzyme and PCR analysis. The products of double restriction enzyme digestion were a pET32α linear fragment of about 5.8 kb and an inserted fragment of about 580 bp. A 593-bp band was detected by PCR analysis. The sequencing results showed that the ORF of *Orf507* was completely inosculated to the coding frame of pET32α (+) protein, and no reading frame displacement was found; and the identity of PCR products and *Orf507* was 100%. The detection result showed that the prokaryotic expression recombinant plasmid of *Orf507* had been successfully constructed.

The *E. coli* Rosetta (DE3), which contained recombinant plasmid pET32α–*Orf507*, was induced by 0.5 mM IPTG for 0, 1.5, 3 and 4.5 h at 37°C, and the recombinant protein expressed (Fig. 3). The protein of the host strain, which contained the pET32α–*Orf507* recombinant plasmid, had a band of Mw of about 38 kDa compared with the control strain into which a blank vector was transferred. This protein Mw was basically the same as the theoretical value of 38 kDa for the *Orf507* recombinant protein (Mw of *Orf507* is 18 kDa, and of His label protein is 20 kDa). After transformation of *E. coli* Rosetta (DE3) and induction by 0.5 mM IPTG for 3 h, proteins separated by SDS-PAGE were electrotransferred onto nitrocellulose membranes. The results of western blot demonstrated that the fusion protein (6x–His–Orf507) could be recognized by anti-6x–His monoclonal antibody (Fig. 3).

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