Purification and Optimization of Prokaryotic Expression of CSN5B Protein of *Arabidopsis thaliana*

Xu Niu 1,2, Lili Wang 1,2, Shenkui Liu 3, Yuanyuan Bu 1,2

1 Key Laboratory of Saline-Alkali Vegetation Ecology Restoration (Northeast Forestry University), Ministry of Education, Harbin 150040, China
2 College of Life Science, Northeast Forestry University, Harbin 150040, China
3 The State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, Lin’An, Zhejiang 311300, China

Corresponding author email: yuanyuanbu@nefu.edu.cn

Molecular Soil Biology, 2020, Vol.11, No.2 doi: 10.5376/msb.2020.11.0002

Copyright © 2020 Niu et al., This is an open access article published under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract CSN5B is a subunit of the COP9 signalosome (CSN) and plays physiological functions in the form of monomer or complex in plants. The CSN5B of *Arabidopsis thaliana* was cloned by RT-PCR technology and constructed into the prokaryotic expression vector pET-32a (+), which was introduced into *E.coli* BL21 host bacteria. Isopropylβ-D-1-thiogalactopyranoside (IPTG) was used to induce expression, and the cultural temperature and time was optimized. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to detect the induced recombinant protein, and the protein purification was conducted. It was shown that the recombinant CSN5B introduced into *E.coli* could be induced to express, and the induction was relatively effective in the presence of 1mmol/L of IPTG at 30°C, 4h induction effect is better. The molecular weight of the induced recombinant protein was basically consistent with the theoretical molecular weight. This study provided a theoretical basis for in-depth further exploration of the functions of this gene in the future.

Keywords CSN5B protein; Prokaryotic expression; Protein purification

Background

The COP9 signalosome (CSN) is a multi-protein complex which was originally defined as a suppressor of photomorphogenesis in plants. Its plays an important role in plant growth and development (Serino et al., 2003). The CSN is acidic, binds to heparin and localizes in the nucleus (Chamovitz et al., 1996). It is a highly conserved multi-protein complex composed of 8 subunits and affects the key developmental pathways by regulating protein stability (Tuller et al., 2019). The pleiotropic and eukaryotic conserved CSN is the catalytic reaction center in the NEDD pathway, and its mainly exists in the form of complexes and monomers when it functions (Giovanna et al., 2003). When CSN is present as holocomplex, it can regulate the transcription of downstream genes, while the monomeric forms or small subcomplexes can increase protein stability(Wei et al., 2008). The CSN is an evolutionarily conserved multiprotein complex which controls many developmental processes of plant by regulating the activity of CULLIN-RING E3 ubiquitin ligases (CRLs) (Pacurar et al., 2017).

The pivot protein CSN5 is the fifth subunit of the CSN, and it is a regulatory component of the ubiquitin/proteasome system (Caroline et al., 2018). It is known that the CSN5 has two homologous, which are called CSN5A gene and CSN5B gene respectively in *Arabidopsis thaliana*. Previous studies had shown that VTC1 and CSN5B can interact with each other to influence the content of AsA in plants. Under the dark conditions, CSN5B can degrade VTC1 through the 26S proteasome pathway regulating the AsA content negatively (Wang et al., 2013). These studies indicate that CSN5B plays an important role in the growth and development of plants. Therefore, in this study, based on the cloned *Arabidopsis thaliana CSN5B* gene, the recombinant CSN5B protein was further expressed and purified prokaryotically, laying a foundation for indepth researches on the functions of CSN5B.
Materials and methods

1.1 Strains, vectors and main reagents

pET-32a (+) vector, *Escherichia coli* DH5α, BL21 (DE3) competent cells were prepared and stored in this laboratory. pEASYTM-T5 Zero was purchased from TransGen Biotech. The high-fidelity ExTaq enzyme series, pMD18-T Vector, dNTP Mix, T4 DNA ligase, and the reverse transcription kit were obtained from TaKaRa. The DNA restriction enzymes was purchased from Thermo scientific. The plasmid mini-extraction kit was received from CWBIO. The gel recovery kit was product of MBI(Fermentas). The sheep anti-mouse IgG (peroxidase covalent binding), eECL Western Blot and primers was purchased from CWBIO. Low molecular weight protein Marker (TransGen Biotech).

1.2 The extraction of total RNA and protein

The fresh leaves of *Arabidopsis thaliana* were collected, and the total RNA was extracted by the TRizol method according to the reagent instructions. The total protein of the leaves was also extracted. The extracted RNA and protein was stored in a refrigerator at -80°C for later use.

1.3 Prokaryotic expression vector construction

The upstream primer CSN5B-F (5'GGTACCATGGAGGGTTCGTCGTA-3, synthetic primer contains KpnI restriction site) and the downstream primer CSN5B-R (5'-GAATTCTCAATATGTAATCATAGGGTCTGGA-3, synthetic primer contains EcoRI cleavage site) was designed based on the CSN5B sequence (accession number: AT1G71230) in the NCBI database. Then, the total extracted RNA as a template and the oligo (dT) as a reverse transcription primer, the cDNA was obtained through the reverse transcription according to the TaKaRa's kit instructions. PCR reaction was conducted in total volume of 20µL including 1~50 ng cDNA with the final concentration of more than 10µmol/L as a template and 1µL each of the upstream and downstream primers. The reaction was performed under the condition of 94°C/30 s, 55°C/40s, and 72°C/90s for 30 cycles. The amplified product was identified on a 1.5% agarose gel. After the electrophoresis, it was purified using a Thermo Scientific kit, ligated to the pMD18-T vector and transformed into *E. coli* DH5α strain. The pMD18-T vector containing the target gene was identified by colony PCR and other methods. The target gene was double-digested with EcoRI and KpnI and purified. Then, it was ligated into prokaryotic expression vector pET-32a (+), which was double-digested with same restriction enzymes, and then transformed into *E. coli* DH5α. After that, throughout the identification by colony PCR and sequencing, the expression vector pET-32a (+)-CSN5B vector containing the target gene was successfully constructed.

1.4 Induced expression and detection of the foreign gene

The recombinant expression vector pET-32a (+)-CSN5B was transformed into *E. coli* BL21(DE3) plysS strain, and a single colony was picked and inoculated into LB medium with ampicillin (Amp). The plasmid was extracted and identified through the double enzyme digestion, the strains were preserved for the subsequent experiments. Then the correctly transfected strains was cultured at 37°Covernight, diluted in the fresh LB medium containing Amp on the second day and continued to grow until the OD$_{600}$ reached from 0.6 to 0.8. At this moment, IPTG was added, and the shaking culture was continued at 30°Cfor 4h. Next, the cells were collected by centrifugation at 13000 r/min for 1min, and the total protein was extracted.

1.5 Refolding of recombinant protein inclusion bodies

5ml inclusion body lysis solution was added to dissolve the His-CSN5B protein inclusion bodies at room temperature for 4h. Then the sonication was employed for 15min at 50HZ (sonicate for 3S and stop for 5S in a cycle) followed by centrifugation at 13000rpm for 30min at 25°C, and the supernatant was collected. This supernatant was transferred out to obtain the renatured His-CSN5B fusion protein. Phosphate buffer: 0.2M NaH$_2$PO$_4$, 0.2M Na$_2$HPO$_4$; Inclusion body solution: 50ml phosphate buffer, 0.5M NaCl, 10mM imidazole, 8M urea.
1.6 Purification of the recombinant protein

First, 200µl Ni-NTA purification resin was loaded on the protein purification column and 400µl equilibration solution for the Ni-NTA resin equilibration was added. Otherwise, the refolded His-CSN5B protein was added, and 2ml His protein washing buffer was added to wash the miscellaneous proteins for three times. Finally, 200µl His-protein elution buffer was added. After mixing together thoroughly and placing on ice for 15min, the purified pET-32a(+)-CSN5B protein which crossed the column was collected. Protein washing buffer: 50mmol / L Na₂HPO₄, 300 mmol / L NaCl, 20 mmol / L NaCl imidazole; Protein elution buffer: 50mmol / L Na₂HPO₄, 300 mmol / L NaCl, 200mmol / L NaCl imidazole.

2 Results and analysis

2.1 Construction of the prokaryotic expression vector pET-32a(+)-CSN5B

First, the total cDNA of the model plant in Arabidopsis thaliana as a template, CSN5B was cloned by using pair of primers (CSN5B-F/CSN5B-R) through the PCR amplification, and the agarose gel electrophoresis was employed for detection. The results showed that the amplified fragment size was approximately 1074 bp as expected. After the purification from the gel, it was ligated into the pMD18-T cloning vector and transformed into E. coli DH5α. After the colony PCR and double-enzyme digestion identification, pMD18-T-CSN5B, the recombinant cloning vector containing the target gene fragment, was successfully obtained. In order to construct a prokaryotic expression vector of CSN5B protein, the target fragment from pMD18-T-CSN5B was cut by double digestion, cloned into the expression vector pET-32a (+), which was double-digested in the same way, and then transformed into E. coli DH5α strain. The constructed pET-32a(+)-CSN5B was double-digested again to ensure the correct size of the vector and target gene (Figure 1). Through these experiments, pET-32a(+) CSN5B, the recombinant prokaryotic expression vector containing the target gene, was successfully obtained (Figure 1).

2.2 Small amount of induced expression of fusion protein and the optimization of induction conditions

The recombinant expression vector pET-32a(+) CSN5B was transformed into E. coli BL21(DE3) plysS, and a single clone was selected and grown on LB culture medium (Amp+). After the induced expression by IPTG, SDS-PAGE detection was performed. The high concentrations of protein band existed in the host bacteria sample containing the recombinant plasmid pET-32a (+)-CSN5B. The target protein size is about 40kDa and the tag size such as His-tag in the pET-32a (+) vector is approximately 15kDa. Therefore, the total relative molecular weight of the fusion protein is about 55kDa, which was basically consistent with SDS-PAGE results. For large amounts of

![Figure 1 Enzymatic digestion identification of pET-32a (+)-CSN5B](image)
induced expression of the target protein, the optimization of the induction conditions were conducted, and it was shown that the addition of IPTG with the final concentration of 1.0 mmol / L when the bacterial solution was grown to an OD$_{600}$ of 0.6 was best, and the cultivation was most efficient at 30°C for 4h after induction. These optimal conditions are used in all the subsequent experiments. As shown in Figure 2, the induced protein band can be seen from the second lane between 45-55kDa, and the bands gradually thickens with time (the position of the arrow in the figure). The size of the His-CSN5B fusion protein was consistent with the band position of the map, indicating that the expression of His-CSN5B fusion protein was successfully induced.

2.3 Large amounts of induced expression of the fusion protein

Based on the optimized induction conditions, the BL21 bacterial solution introduced with the recombinant plasmid was cultured in 100ml LB medium. When the OD$_{600}$ value reached 0.6, 0.5 mol/L IPTG 100µL with a final concentration of 1mmol/L was added at 30°C. After 4h of induction, the bacteria cells were sonicated and the supernatant and precipitates were collected respectively as shown in Figure 3. The results showed that a large amount of His-CSN5B fusion protein were successfully induced. However, as it can be seen from the figure, the His-CSN5B fusion protein between 50-70kDa was expressed largely in the form of precipitates, and therefore, there naturation process of inclusion bodies were needed for the preparation of protein purification.

2.4 Comparison of the soluble expression with the expression of inclusion bodies of pET-32a (+)-CSN5B fusion protein

The soluble expression of the isolated pET-32a (+)-CSN5B and the reconstituted inclusion bodies were analyzed by SDS-PAGE electrophoresis (separation gel concentration 12%), as shown in Figure 4. It can be seen from the Figure 4, after the induction of the pET-32a (+)-CSN5B fusion protein by IPTG, the amount of inclusion bodies extracted from E. coli was significantly higher than that of soluble expression in the bacterial solution. The renatured protein of inclusion bodies was detected by Western Blot (Figure 5), proving that the inclusion bodies of His-CSN5B fusion protein was successfully renatured.

2.5 Purification of the fusion protein

In order to purify the target protein, the 6 × His tag fused to the recombinant CSN5B protein was used, and the Ni-NTA His Bind Resins affinity chromatography was employed for the purification. The purified protein was detected as a single band in the SDS-PAGE and the size was consistent with the above results (Figure 6). The size

![Figure 2 SDS-PAGE analysis of the effect of 1mmol/L IPTG concentration on the expression of His-CSN5B fusion protein at different times](image)

Note: M: Low molecular weight standard proteins; 1: Not induced; 2-5: His-CSN5B protein induced at 30 ° C, 1 mmol / l IPTG for 1 h, 2 h, 3 h, 4 h
Figure 3: SDS-PAGE analysis of a large number of induced His-CSN5B proteins
Note: M: low molecular weight standard proteins; 1: Expression of His-CSN5B fusion protein after small amount induction; 2: Expression of His-CSN5B fusion protein after large amounts of induction; 3: Supernatant of total lysate after a large amount of induction of His-CSN5B fusion protein; 4: Precipitates of total lysate after a large amount of induction of His-CSN5B fusion protein.

Figure 4: SDS-PAGE analysis of a large number of induced and renatured His-CSN5B proteins
Note: M: low molecular weight standard proteins; 1: Not induced; 2: Expression of His-CSN5B fusion protein after large amounts of induction; 3: Supernatant of total lysate after a large amount of induction of His-CSN5B fusion protein; 4: Precipitates of total lysate after a large amount of induction of His-CSN5B fusion protein; 5: Renatured protein of His-CSN5B inclusion bodies.

Figure 5: Western Blot analysis of protein induction and renaturation
Note: 1: Expression of His-CSN5B fusion protein after large amounts of induction; 2: Renatured protein of His-CSN5B inclusion body;
3 Discussion

Ascorbic acid is an important plant-derived antioxidant, which constitutes the main source of human dietary vitamin C. AsA plays an important role in plant growth and development (Smirnoff et al., 2000; Hemavathi et al., 2010; Zhou et al., 2012). On the one hand, AsA is synthesized through a variety of biological pathways including D-glucose (Loewus et al., 1999), D-galacturonic acid (Davey et al., 1999), and D-man/L-Gal pathway (Wheeler et al., 1998). Among them, the last pathway is particularly important in plants. On the other hand, the environmental signals such as light stress can also induce ASA synthesis (Smirnoff et al., 2000). Light affects the accumulation of AsA in Arabidopsis leaves, therefore, the content of AsA in leaves are influenced by light. AsA content in Arabidopsis leaves under the appropriate high light intensity is higher than that in low light conditions (Yabuta et al., 2007).

The CSN5B subunit in the COP9 signaling complex (CSN) can affect the AsA biosynthesis and regulate the plant responses to oxygen and salt stress. Furthermore, the CSN5B participates in the 26S protease system indicating the regulative function of CSN5B as a photomorphic factor in AsA synthesis (Wang et al., 2013).

Due to the low cost of the prokaryotic expression system, this study tried to achieve the expression and optimization of CSN5B recombinant protein in E. coli. In order to improve the stability of the target protein, through the fusion expression with tag protein, the optimal conditions including a suitable induction temperature were selected. As a result, the expression level of CSN5B in prokaryotic cells was increased and a large amount of purified CSN5B protein was obtained. It was found that the induction temperature, for example, 30°C promoted the expression of CSN5B fusion protein enough, but the expression level of total protein decreased significantly.

The fusion expression with soluble tag protein not only promoted the expression of CSN5B in inclusion bodies, but also it did not affect the expression level of total protein.

In summary, the optimal expression of CSN5B recombinant protein in the prokaryotic system is still a low-cost, fast and effective method. Through the indepth research and optimization of this study, it is expected that the production level of CSN5B recombinant protein can be improved and provide a foundation for further analysis of the functions of CSN5B.
Acknowledgements
This work was supported by the Fundamental Research Funds for the Central Universities (2572016CA14), Heilongjiang Province Government Postdoctoral Science Foundation (LBH-Q18008), and State Key Laboratory of Subtropical Silviculture (KF201707) awarded to Yuanyuan Bu. Further supported by the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT17R99) awarded to Shenkui Liu. The funders had no role in study design.

References

Caroline B., FranOisXavier G., Murad A.M., Emmanuel B., Albuquerque natio E.V .S., and GrossideSa M.F., 2018, Meloidogyne incognita passe-muraille (mipm) gene encodes a cell-penetrating protein that interacts with the CSN5 subunit of the COP9 signalosome, Frontiers in Plant Science, 9: 904
https://doi.org/10.3389/fpls.2018.00904
PMid:29997646 PMCid:PMC6029430

Chamovitz D.A., Wei N., Osterlund M.T., Arnim A.G.V ., and Deng X.W., 1996, The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch, Cell, 86 (1): 115-121
https://doi.org/10.1016/S0092-8674(00)80082-3

Davey M.W., Gilot C., Persiau G., Stergaard J., Han Y ., and Montagu B., 1999, Ascorbate biosynthesis in Arabidopsis cell suspension culture, Plant Physiology, 121 (2): 535-543
https://doi.org/10.1104/pp.121.2.535
PMid:10517845 PMCid:PMC59416

Giovanna S., and Deng X.W., 2003, The COP9 signalosome: regulating plant development through the control of proteolysis, Annual review of plant biology, 54(1): 165-182
https://doi.org/10.1146/annurev.arplant.54.031902.134847
PMid:14502989

Hemavathi, Upadhyaya C.P., Akula N., Young K., Echun S.C., and Kim D.H., 2010, Enhanced ascorbic acid accumulation in transgenic potato confers tolerance to various abiotic stresses, Biotechnology Letters, 32(2): 321-330
https://doi.org/10.1007/s10529-009-0140-0
PMid:19821071

Loewus F.A., 1999, Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi, Phytochemistry (Oxford), 52(2): 193-210
https://doi.org/10.1016/S0031-9422(99)00145-4

Pacurar D.I., Pacurar M.L., Lakheal A., Pacurar A.M., Ranjan A., and Bellini C., 2017, The Arabidopsis COP9 signalosome subunit 4 (csn4) is involved in adventitious root formation, Scientific Reports, 7(1): 628
https://doi.org/10.1038/s41598-017-00744-1
PMid:28377589 PMCid:PMC5429640

Serino G., and Deng X.W., 2003, The COP9 Signalosome: Regulating Plant Development Through the Control of Proteolysis, Annual review of plant biology, 54(1): 165-182
https://doi.org/10.1146/annurev.arplant.54.031902.134847
PMid:14502989

Smirnoff N., 2000, Ascorbate biosynthesis and function in photoprotection, Philosophical Transactions of the Royal Society of London, 355(1402): 1455-1464
https://doi.org/10.1098/rstb.2000.0706
PMid:11127999 PMCid:PMC1692873

Smirnoff, N., and Wheeler G.L., 2000, Ascorbic acid in plants: biosynthesis and function, CRC Critical Reviews in Biochemistry, 35(4), 291-314
https://doi.org/10.1080/10409230008984166
PMid:11005203

Tuller T., Diament A., and Yahalom A., 2019, The COP9 signalosome influences the epigenetic landscape of Arabidopsis thaliana, Bioinformatics (Oxford, England), 35(16)
https://doi.org/10.1093/bioinformatics/bty1053
PMid:30596896

Wang J., Yu Y., Zhang Z., Quan R., Zhang H., and Ma L., 2013, Arabidopsis CSN5B interacts with VTC1 and modulates ascorbic acid synthesis, The Plant Cell, 25(2): 625-636
https://doi.org/10.1105/tpc.112.106880
PMid:23424245 PMCid:PMC3608782

Wei N., Serino G., and Deng X.W., 2008, The COP9 signalosome: more than a protease, Trends in Biochemical Sciences, 33(12): 592-600
https://doi.org/10.1016/j.tibs.2008.09.004
PMid:18926707

Wheeler G.L., Jones M.A., and Smirnoff N., 1998, The biosynthetic pathway of vitamin C in higher plants, Nature, 393(6683): 365-369
https://doi.org/10.1038/30728
PMid:9620799

Yabuta, Ymieda T., Rapolu M., Nakamura A., Motoki T., and Maruta T., 2007, Light regulation of ascorbate biosynthesis is dependent on the photosynthetic
electron transport chain but independent of sugars in *Arabidopsis*, Journal of Experimental Botany, 58(10): 2661-2671

https://doi.org/10.1093/jxb/erm124
PMid:17586607

Zhou Z., Wang Y., Cai G., and He Q., 2012, Neurospora COP9 signalosome integrity plays major roles for hyphal growth conidial development and circadian function, PLoS Genetics, 8(5): e1002712

https://doi.org/10.1371/journal.pgen.1002712
PMid:22589747 PMCID:PMC3349749