Gene Clone and Bioinformatics Analysis of Subtilisin-Like Protease in *Cucumis sativus*

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Short Research Article

ABSTRACT

Background: Cucumber target leaf spot (TLS), caused by Corynespora cassiicola (C. Cassiicola), is a serious disease in cucumber (Cucumis sativus) production worldwide. Therefore, cultivating new varieties of TLS resistance of C. sativus is an important goal of cucumber breeding. Previous studies have shown that subtilisin-like protease (SUBP) plays an important role in response to C. Cassiicola infection in resistant plants.

Objective: In this study, the full-length cDNA of the CsSUBP gene was cloned, and the prokaryotic expression vector was successfully constructed in order to study the effects of subtilisin. Futhermore, vital clues regarding CsSUBP gene involved in TLS resistance of C. sativus are gained from the bioinformatics assay.

Method: The CsSUBP gene was identified by sequencing with the intermediate vector pMD18 by designing specific primers and PCR amplification techniques. The prokaryotic expression vector pET30a-CsSUBP was further constructed and identified by colony PCR and EcoR V and Sal I double digestion.

Result: The primary structure of CsSUBP was predicted and analyzed by bioinformatics analysis. The results showed that CsSUBP was weakly acidic protein, N-terminal signal peptide region, including a Inhibitor_I9 domain domain.

Conclusion: The pET30a-CsSUBP prokaryotic expression vector was constructed successfully. This study is convenient for the study of prokaryotic expression and its kinase activity.

Keywords: Subtilisin protease; gene clone; bioinformatics assay; prokaryotic expression.

1. INTRODUCTION

Cucumber (*Cucumis sativus*) is one of the largest vegetable crops in China. In recent years, cucumber target leaf spot (TLS), caused by *Corynespora cassiicola* (*C. cassiicola*), is a major disease in cucumber production [1]. At present, the prevention and treatment of TLS are mainly chemical control, chemical pesticide can be used to inhibit the activity of pathogens. However, *C. cassiicola* can easily mutate with environmental changes and develop resistance to various chemical pesticide, so it is difficult to prevent and eliminate the disease efficiently [2]. The best method for controlling the disease in cucumber is the use of resistant cultivars, but few high

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resistant varieties of cucumber can enhance resistance of *C. cassiicola* at present. Therefore, understanding the molecular mechanisms and identifying the molecular components involved in the defense responses of the TLS resistant cucumber cultivars will provide a promising approach to restraining this disease.

In our previous study, a kind of subtilisin-like protease (SUBP) from resistant cucumber plants(Jingyou 38) after the fungus inoculation were explored using iTRAQ quantitative proteomics technique. It is speculated that the SUBP may play an important role in cucumber resistance to early invasion of *C. cassiicola*.

SUBP is a serine protease widely found in plants, bacteria, fungi, and parasites [3-8]. Such proteases have a typical Asp/Ser/His catalytic domain in structure. Studies have shown that it is closely related to the stability of the fungal phenotype and the pathogenicity of the pathogen. It plays an important immune stimulating role in the interaction between plants and pathogens [9]. SUBP is also involved in plant cell programmed cell death (PCD) processes [10]. A number of studies have demonstrated the role of SUBP in plant and pathogen interactions. For example, two subtilisin-like proteins, SAS-1 and SAS-2, in oat (Avena sativa) exhibit specific activity of mammalian caspase apoptosis protease and participate in the signal transduction of PCD during the non-affinity interaction of *oat-Cochliobolus victoriae*, thereby inducing HR production [11-12]. Overexpression of Arabidopsis subtilases gene *AtSBT3.3* activates downstream immune signaling and induces innate immune response in *Arabidopsis* [13].

In this study, the cDNA sequence of *CsSUBP* gene was cloned and the prokaryotic expression vector was constructed. The primary structure of CsSUBP was predicted and analyzed using bioinformatics analysis methods. On this basis, we can further explore the defense function and molecular mechanisms of SUBP against *C. cassiicola* in cucumber.

2. MATERIALS AND METHODS

2.1 Plant Materials

The cucumber varieties used in the experiments were Jinyou 38 (preliminary experiments showed that this variety was resistant to TLS), which were planted in a greenhouse at 28℃ under 16:8 light/dark cycles.

2.2 Full-length cDNA Cloning

Two candidate genes were screened through preliminary test on the basis of the proteomics analysis. The cDNA sequences of *CsSUBP* (A0A0A0K993) from resistant cucumber leaves were clone. The sequence of *CsSUBP* were aligned with cucumber genome database using the service provided byhttp://cucurbitgenomics.org/BLAST. The primers (Table 1) were designed by using the DNAMAN. The PCR fragments amplified from the cDNAs were cloned into TA vectors pMD18-T and were sequenced.

2.3 Construction of Recombinant Prokaryotic Expression Vector pET30a-*CsSUBP*

Recombinant plasmid pMD18-T-*CsSUBP* and expression vector pET30a were digested by *EcoR* V and *Sal* respectively. The double-digested DNA fragment had the same sticky ends as the pET30a vector backbone, and the two fragments were ligated into a complete recombinant plasmid using T4 ligase and ligated overnight at 16℃ and then trans-formed into E. coli DH5α competent cells, blue and white screening. Recombinant prokaryotic expression vector was identified by direct colony PCR. Recombinant prokaryotic expression vector pET30a-*CsSUBP* plasmid was extracted and the product was digested by *EcoR V* and *Sal* respectively. It was digested for 3 h at 37℃ in a constant temperature metal bath and electrophoresed on a 1.5% agarose gel. Plasmids with positive clones were sent for bioinformatics sequencing.

2.4 Bioinformatics Analysis

The specific bioinformatics [14-17] analysis contents and tools used are as follows see Table 2.

Table 1. *CsSUBP* gene primer sequences

Gene name	Primer sequence (5' to 3 ')

CsSUBP-F	GATATCATGTCCGGCCAATCCACAGCCCTG
CsSUBP-R	GTCGACCTAATGAAGCCTTGCTGCTCCTCCT

Table 2. Bioinformatics analysis content and Tools website

Analyze the content	Name of software	Bioinformatics Analysis Tools Website
Primary structure	ProtParam	http://web.expasy.org/protparam/
Secondary structure	SOPMA	http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_sopma.html
Hydrophobiciy	ProtScale	http://web.expasy.org/protscale/
Signal peptide	SignalP 4.0	http://www.cbs.dtu.dk/services/SignalP/
Functional domains	SMART	http://smart.embl-heidelberg.de/

3. RESULTS

3.1 Amplification of *CsSUBP*

The reverse transcription of cDNA was used as the template. PCR amplification product was subjected to 1.5% agarose gel electrophoresis and a fragment of approximately 282 bp was obtained as expected (Fig. 1).

3.2 Construction of Prokaryotic Expression Vector

To construct the prokaryotic expression vector, we recycle the small fragments of pMD18-T-*CsSUBP* (Figs. 2, 3) and large fragments of pET30a plasmid after double digest with *EcoR* V and *Sal*. The colony PCR products of recombinant plasmid pET30a-*CsSUBP* via agarose gel electrophoresis analysis showed 282 bp of the target gene band (Figs. 4, 5). Sequencing results showed that the gene fragments was consistent with the target gene sequence in GenBank (Fig. 6).

3.3 Bioinformatics Analysis of CsSUBP proteins

Some information about the CsSUBP are obtained by some online tools (shown in table2). The protein molecular weight is 23146.02, chemical formula is $C_{834}H_{1388}N_{282}O_{348}S_{69}$, isoelectric point (pI) is 5.28. It is weakly acidic and hydrophobic average coefficient is 0.794. It is speculated that CsSUBP may be a hydrophobic protein (shown in Fig 8). Neither signal peptide nor transmembrane region is found in the CsSUBP (shown in Fig 9).

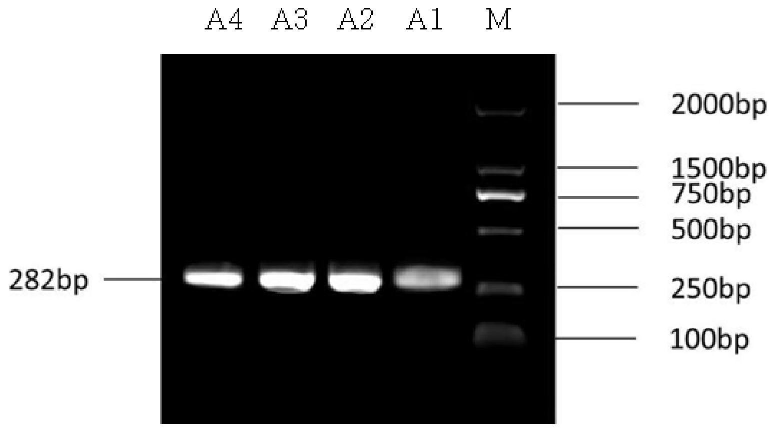

Fig. 1. Electrophoresis profile of PCR products of CsSUBP Gene. A1-A4: *CsSUBP* PCR amplification products; M: DL2000 DNA Marker

Fig. 2. The colony PCR of recombinant plasmid pMD18-CsSUBP. B1-B3,B5-B8: Colony PCR of *CsSUBP* gene; B4: water; M: DNA marker DL2000

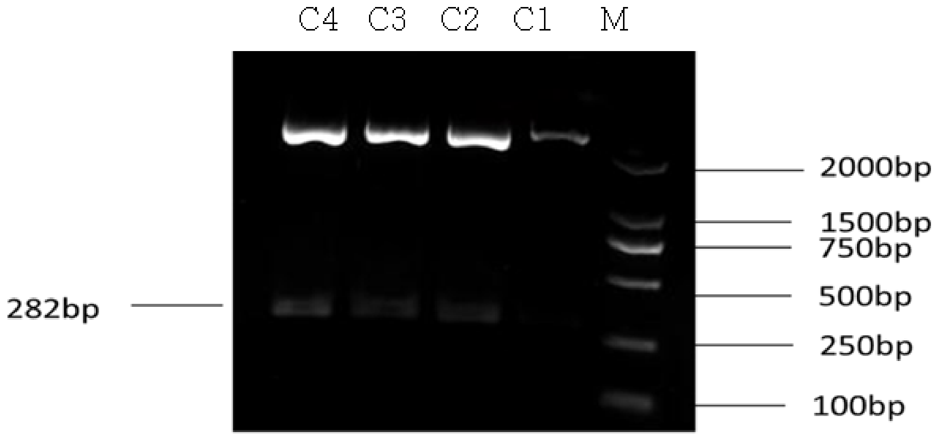

Fig. 3. Double digestion of recombined vector pMD18-*CsSUBP* with restriction enzymes.C1-C4: Double digestion products; M: DNA marker DL2000

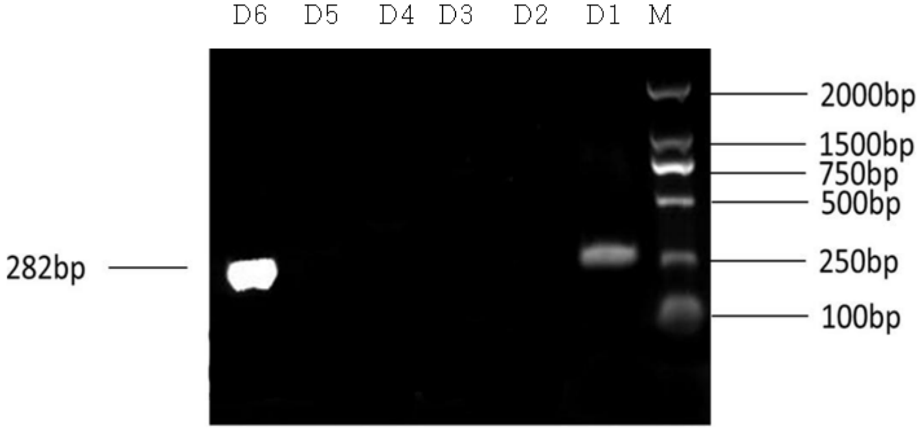

Fig. 4. The colony PCR of recombinant plasmid of pET30a-*CsSUBP*. D1-D3, D6: Colony PCR products of *CsSUBP* gene; D4, D5: Water control; M: DNA marker DL200

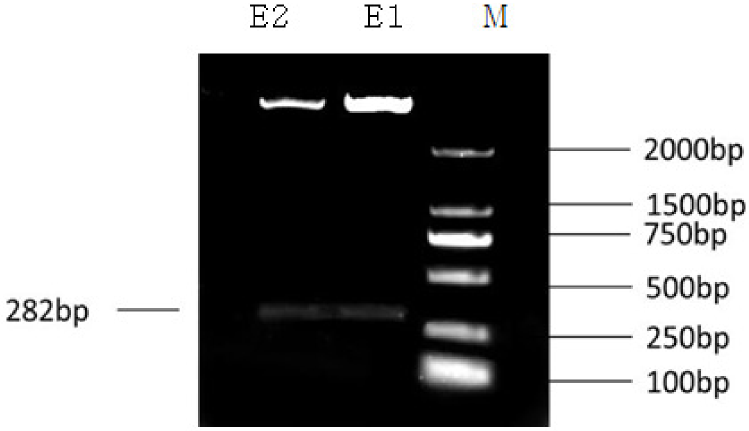

Fig. 5. Double digestion of recombined vector pET30a-*CsSUBP* with restriction enzymes. E1, E2: Double digestion products; M: DNA marker DL2000

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SUBP.seq                   ....................................................................................................    0
subp-6-M13F_-47__B04.ab1RC GCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGC  800
Consensus

SUBP.seq                   .................................ATGTCCGGCCAATCCACAGCGGTGCACATCGTTTACACTGAGCGGCCTCACAATGAAGAACCCGAGG   67
subp-6-M13F_-47__B04.ab1RC TCGGTACCCGGGGATCCTCTAGAGATTGATATCATGTCCGGCCAATCCACAGCGGTGCACATCGTTTACACTGAGCGGCCTCACAATGAAGAACCCGAGG  900
Consensus                                                   atgtccggccaatccacagcggtgcacatcgtttacactgagcggcctcacaatgaagaacccgagg

SUBP.seq                   CCTATCACATCCGAACCCTAGCTTCTGTTCTTGGCAGTGAAGGCGCTGCTAGGGAGGCGCTTGTGTATAGCTATAAAAATGCTGCGAGTGGCTTCTCTGC  167
subp-6-M13F_-47__B04.ab1RC CCTATCACATCCGAACCCTAGCTTCTGTTCTTGGCAGTGAAGGCGCTGCTAGGGAGGCGCTTGTGTATAGCTATAAAAATGCTGCGAGTGGCTTCTCTGC 1000
Consensus                  cctatcacatccgaaccctagcttctgttcttggcagtgaaggcgctgctagggaggcgcttgtgtatagctataaaaatgctgcgagtggcttctctgc

SUBP.seq                   TCGGCTTACGTCCGATCAGGTTGCGGAGATTACCAAACAACCAGGAGTATTGCAGGTTGTCCAAAGCGGTAATAATAAGCTGCATTCTGGAGGAGGAGCA  267
subp-6-M13F_-47__B04.ab1RC TCGGCTTACGTCCGATCAGGTTGCGGAGATTACCAAACAACCAGGAGTATTGCAGGTTGTCCAAAGCGGTAATAATAAGCTGCATTCTGGAGGAGGAGCA 1100
Consensus                  tcggcttacgtccgatcaggttgcggagattaccaaacaaccaggagtattgcaggttgtccaaagcggtaataataagctgcattctggaggaggagca

SUBP.seq                   GCAAGGCTTCATTAG....................................................                                   282
subp-6-M13F_-47__B04.ab1RC GCAAGGCTTCATTAGGTCGACAATCGTCGACCTGCAGGCATGCAAGCTGGCACTGGCCGTCGTACTC                                  1167
Consensus                  gcaaggcttcattag
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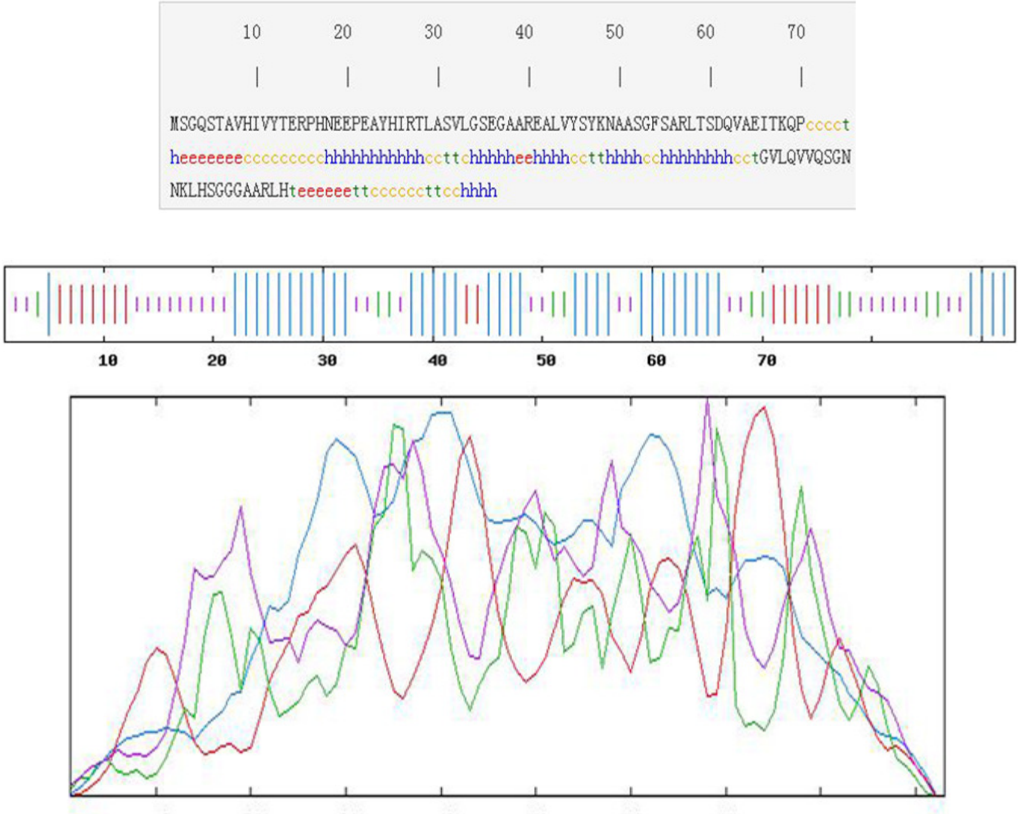
Fig. 6. Sequencing results recombinant vector pET30a-*CsSUBP*

Secondary structure of CsSUBP is analysed by SOPMA. The result showed that CsSUBP consists of 39.78% alpha helix (h), 16.13% extended strand (e), 11.83% beta turn (t) and 32.26% random coil(c) (Fig.7 and Table 3).

A major functional domains of *CsSUBP*-encoded proteins is found by SMART analysis. The results are shown in Table 4.

Inhibitor_I9 domain, which belongs to a member of the MEROPS family of protease inhibitors I9, a protease pro-peptide inhibitor (sometimes referred to as an activated peptide) that regulates the folding and activity of the proenzyme or zymogen. The anterior segment extends into the enzyme moiety and shields the substrate binding site, thereby promotes enzyme inhibition. Although it often has lower sequence identity, several such propeptides have similar topologies. The propeptide region has an open-loop, sandwich-like anti-parallel α/ β, it also has two α-helices and four β-strands with a 2x(β/α/β) topology.



MSGQSTAVHIVYTERPHNEEPEAYHIRTLASVLGSEGAAREALVYSYKNAASGFSARLTSDQVAEITKQPcccct
heeeeeeecccccccccchhhhhhhhhhhccttchhhhheehhhhccttchhhhcchhhhhhhhcctGVLQVVQSGN
NKLHSGGGAARLHteeeeeettccccccttcchhhh

Fig. 7. The secondary structure of CsSUBP protein. h: Alpha helix; t: Beta turn; e: Extended strand; c: *Random coil*

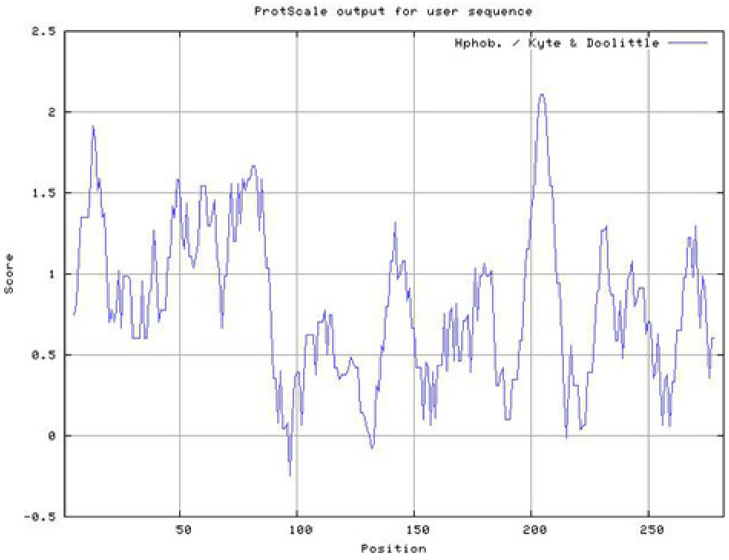


Fig. 8. Prediction the hydrophobicity of CsSUBP protein

Table 3. Prediction the secondary structure of CsSUBP protein

Name	Number	Percentage
Alpha helix	37	39.78%
310 helix	0	0.00%
Pi helix	0	0.00%
Beta bridge	0	0.00%
Extended strand	15	16.13%
Beta turn	11	11.83%
Bend region	0	0.00%
Random coil	30	32.26%
Ambigous states	0	0.00%
Other states	0	0.00%

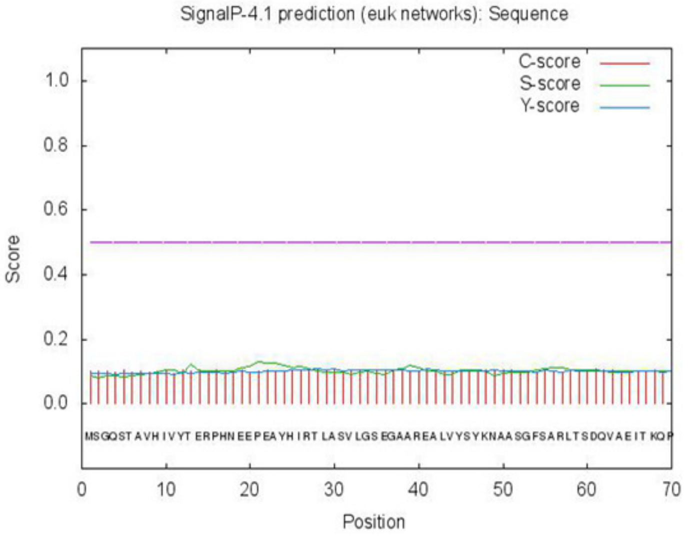


Fig. 9. Prediction the signal peptide of CsSUBP protein

Table 4. Functional domain analysis of *CsSUBP*-encoded proteins

Name	Position	E-value
Inhibitor_I9 domain	8(Starting site)-84(Termination site)	4.8e-15

CsSUBP also belongs to the N-terminal propeptide domain of the meropsis family S8A, a subtilisin peptidase. The use of the subtilisin propeptide as a chaperone helps to fold the mature peptidase. The propeptide is removed by proteolytic cleavage to remove the activating enzyme.

4. DISCUSSION

Cucumber is one of the important vegetables in the world, and it is also the largest greenhouse product in China. Cucumber has a cool taste and medicinal value. However, in the process of production, cucumber will be exposed to a variety of diseases such as blight, powdery mildew, and target leaf spot. Among them, the incidence of target leaf spot is extremely high, which causing serious economic losses [18]. Studies have shown that breeding and using disease-resistant varieties are the most direct, effective, and economical measures for disease control.

Our iTRAQ analysis on the inoculation of cucumber leaves showed the expression level of a subtilisin- like protease (A0A0A0K993) was significantly decreased at 6 h after pathogen infection. In addition, quantitative real-time PCR technology found that its corresponding genes expression were also decreased a downward trend after inoculation of pathogen. We speculated that it may be involved in the early invasion process of cucumber against *C. cassiicola.*

Subtilisin-like protease (SUBP) is a serine protease, which can regulate plant embryogenesis and organ development, fruit ripening and senescence [19-22]. It also plays an important role in the interaction between plant and pathogen [9]. Plant-pathogen interaction begins with the pathogen contact host and ends with a significant disease or susceptibility response [23]. When plant was attacked by pathogen, it will produce two immune defense lines (MTI and ETI). Studies have shown that in plants such as tomato [24], soybean [25], and wheat [26], the subtilisin-like protease can regulate its immune response and participate in intercellular signal transduction during interaction. However, whether it will stimulate the immune response in cucumber is still unclear. In this study, bioinformatics analysis of the subtilisin-like protease showed that it contained a propeptide domain (Inhibitor_I9 domain), which may be the main disease resistance domain of the protein.

E. coli prokaryotic expression system has the characteristics of simple operation, short culture period and high expression level, which is beneficial to the study of protein structure and function [27]. In addition, the prokaryotic expression vector pET-30a has the advantages of strict regulation and exquisite design of multiple cloning sites. In this research, the *SUBP* gene was cloned from Jinyou 38 and the prokaryotic expression vector pET30a-*CsSUBP* was constructed to facilitate the establishment of Cs*SUBP*-encoded recombinant protein. It also lays the foundation for studying the role of protein function and kinase activity in plant disease resistance.

5. CONCLUSION

We have successfully amplified and purified the coding sequence of CsSUBP gene in cucumber, and constructed the recombinant prokaryotic expression vector pET30a-CsSUBP. Bioinformatics analysis shows that CsSUBP belongs to the hydrophobic protein and has no signal peptide at the N- terminus. It contains a functional domain. Its protein chemical formula is $C_{834}H_{1388}N_{282}O_{348}S_{69}$, an isoelectric point (PI) is 5.28 and a molecular weight is 23146.02. Our study is a preparation for revealing the functions and mechanisms of CsSUBP against *C. cassiicola* in cucumber.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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