Cloning and Expression of the Serine Carboxypeptidase Gene in *Zea mays* and Its Antifungal Activity against *Rhizoctonia solani*

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Abstract: The authors cloned and identified a new maize serine carboxypeptidase gene named *ZmSCP* from R15 inbred lines seedlings which were induced by *Rhizoctonia solani* AG1-IA. *ZmSCP* encodes a 332 amino acid protein with a predicted molecular mass of 36.5 kDa and pI of 4.75. Phylogenetic analysis revealed that *ZmSCP* showed closer kinship with *Oryza sativa* and sorghum, which belong to the same evolutionary branch. Amino acid sequence analysis revealed that there are four types of amino acids in *ZmSCP*, the percentages of them are 43.1%, 26.9%, 13.9% and 13.1%. The authors subsequently purified the recombinant protein which expressed in *Escherichia coli* BL21 and analyzed its antimicrobial activities *in vitro*. Results showed that the recombinant protein inhibited hyphal growth of *Rhizoctonia solani*. The study suggests that the expression of *ZmSCP* is closely related to maize sheath blight resistance caused by *Rhizoctonia solani*. Further, the antifungal activity showed that *ZmSCP* may play a role in the disease resistance response.

Key words: Maize, serine carboxypeptidases, banded leaf and sheath blight, *Rhizoctonia solani*.

1. Introduction

SCPs (Serine carboxypeptidases) are proteins belonging to the hydrolase family [1, 2], mainly distributed in lysosomes of animals and vacuoles of plants, and share characteristic structural features, e.g., a signal sequence for intracellular trafficking and/or secretion, multiple N-linked glycosylation sites, and four evolutionarily conserved regions, which consist of a substrate-binding domain and three catalytic sites [3]. At present, SCPs have been isolated from several plants and crop such as *Oryza sativa*, *Arabidopsis thaliana*, *Triticum aestivum*, *Hordeum vulgare*, etc. [4-8]. *Arabidopsis* has at least 51 members [1], while

71 SCPs exist in rice genome [2], indicating functional redundancy for members of the SCP family. SCPs are widely distributed in higher organisms, whereas, some of the members' functions were confirmed, such as hydrolysis of storage protein in seed [9], intracellular components autolysis in programmed cell death [10], seed development [11], trauma reactions [12], resistance to adversity [13]. However, to date, there is little research on SCP in maize.

BLSB (Banded leaf and sheath blight), caused by *Rhizoctonia solani* AG1-IA, is an important disease in maize worldwide. Few genes related to resistance of this pathogen have been isolated in maize, due to its large genome size, complex genome structure, and quantitative trait nature. These characteristics have hampered the ability to conduct detailed studies of

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resistance mechanisms and exploitation of resistance resources in maize. Studies on molecular mechanism of disease resistance mainly concentrated on screening of resistant QTL and cloning of resistant gene [14-16]. In previous studies, the authors have acquired large amount of genes from high tolerance maize inbred line R15 under the induction of *Rhizoctonia solani* using SSH (suppression subtractive hybridization method), and 35 ESTs (expression sequences tags) were identified including serine carboxypeptidases and pathogenesis-related protein [16]. In the present study, in order to investigate the gene function of *ZmSCP*, we isolated the *ZmSCP* from AG1-IA-induced high-resistance inbred maize line R15. Subsequently the authors obtained the prokaryotic expression vector pET32a(+)-*ZmSCP* and purified the recombinant protein which was used for analysis of its antimicrobial activities *in vitro*. The results provide valuable information for comprehensive understanding of *ZmSCP* function.

2. Materials and Methods

2.1 Plant Materials

High-resistance maize inbred line R15 was used in the current study. Seeds were treated with 7% hypochlorite solution for 30 min followed by three washing with sterilized H₂O before being sowed in pots with autoclaved soil. Plants were allowed to grow for 25-35 days and were then inoculated with *Rhizoctonia solani* (kindly provided by the Rice Institute of the Sichuan Agricultural University, Sichuan, China) at 28 °C. *Rhizoctonia solani* AG1-IA was cultured on potato dextrose agar and incubated at 28 °C for 3-5 days before use.

2.2 RNA Extraction and Cloning of *ZmSCP*

Total RNA was extracted using Trizol® reagent (Invitrogen, USA) according to the manufacturer's protocol. Five hundred nanograms of DNase-treated total RNA were reverse-transcribed (TaKaRa, Dalian, China). Primers were designed according to the

ZmSCP sequence (GenBank Accession Number: JF682634): forward primer 5'GAATTCATGCGCGGGTTCGACGGGCC3' (*EcoR* I site is underlined) and reverse primer 5'GCGGCCGCTTCCCCAAGCGTAGGATAGA3' (*Not* I site is underlined). Amplified products were gel-separated on 1.2% agarose and extracted (Omega, China), then cloned into the pMD18-T vector (TaKaRa, Dalian, China) for sequencing. Three positive clones were sequenced. The ExPASy translate tool (<http://au.expasy.org/tools/dna.html>) was used to deduce the amino acid sequence from the cDNA. The amino acid compositions of the *ZmSCP* were carried out using DNA tool 6.0. Cluster analysis was conducted using DNASTar 7.0 to reveal evolutionary relationships of the proteins from different plants.

2.3 Construction of Expression Plasmid and Optimization of Expression Conditions

The pMD18-*ZmSCP* vector and pET32a(+) were digested with *EcoR* I and *Not* I, respectively. The expected fragments were released from pMD18-*ZmSCP* and subcloned into pET32a(+) vector using T₄ DNA ligase (TaKaRa, Dalian, China). The recovered plasmid was transformed into *E. coli* DH5α and positive clones were selected, which were named pET-32a(+)-*ZmSCP*. The resulting plasmid was used to transform *E. coli* BL21 and was verified by sequencing. Bacteria were grown with two temperature levels at 37 °C and 28 °C respectively to an appropriate density ($OD_{600} = 0.6$), and induced with 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM IPTG for 0, 2, 4, 6, 8, and 10 h to identify optimum expression conditions.

2.4 SDS-PAGE and Western Blot

The expressed protein was analyzed by SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis) and western blotting. SDS-PAGE and western blotting were carried out using standard conditions [17]. After SDS-PAGE, proteins were transferred to PVDF membranes incubated in methyl

alcohol for 15 min at room temperature using the Bio-Rad trans-blot apparatus (Bio-Rad, USA) for 1 h at 120 V. The membrane was incubated with mouse anti-His-Tag antibody at a dilution of 1:1,000 at 4 °C overnight, and washed three times for 15 min each in PBS (phosphate-buffered saline). Subsequently, the membrane was incubated with goat anti-mouse IgG antibody conjugated with HRP (horseradish peroxidase) (Boster, China) at a dilution of 1:500 for 3 h at 37 °C. After washing twice in PBST, the membrane was displayed (TIANGEN, China) according to the manufacturer's protocol.

2.5 Purification and Antimicrobial Activity Assays of *ZmSCP* in Vitro

The pET-32a-*ZmSCP* constructs contained a His-tag downstream of the target gene to allow purification of the over-expressed proteins with the His-Bind kit, following the manufacturer's protocol (NovaGen, USA). The induced bacterial cell pellet was collected, lysed, and purified. The recombinant protein in 1× elution buffer was dialysed against 1× PBS (phosphate-buffered saline) with three changes at 4 °C over 24 h. The protein concentration was measured according to Bradford et al. [18] and antimicrobial activity was evaluated *in vitro*.

Rhizoctonia solani was cultured on PDA p (otato dextrose agar) medium at 28 °C. Holes 1 cm in diameter were placed on PDA plates (9.0 cm).

Differing concentrations of *ZmSCP* protein in 1× PBS buffer were added into the three holes (20, 40, and 60 µg), with 1× PBS buffer as control. The plates were incubated at 28 °C for 48 h. In addition, *Rhizoctonia solani* were incubated on PDA (potato dextrose agar) with PBS buffer (CK) or the purified protein at a concentration of 20 µg, 40 µg, and 60 µg (*ZmSCP*) at 28 °C. Germination and growth of *Rhizoctonia solani* were examined at 48 h of incubation under a microscope. The experiment was carried out three times independently, using the same conditions.

3. Results

3.1 Isolation of *ZmSCP* and Sequence Analysis

A specific 999 bp fragment named *ZmSCP* was amplified with gene specific primers. The ExPASy translation tool (<http://au.expasy.org/tools/dna.html>) was used to deduce the amino acid sequence of the cDNA, resulting in a sequence of 332 amino acids (Fig. 1). Further, the results indicated an expected acidic protein of 36.5 kD with an isoelectric point of 4.75. Amino acid sequence analysis revealed that there are four types of amino acids in *ZmSCP*, they are hydrophobic amino acids, hydrophilic amino acids, basic amino acids, acidic amino acids and the percentages are 43.1%, 26.9%, 13.9%, 13.1% respectively (Table 1). Phylogenetic analysis revealed

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1  ATGCGCGGGTTGACGCGGCCACTTCCGTTCTACCTCGAAACCGGGTACGTGGAGGTGGAC
   M R G F D G P L P F Y L E T G Y U E U D
61  GAGCAGCAAGGTGTGACAGCTCTTCTACTACTTCTGCGCGTCCGAGAGCGAGCCCTACGAG
   E Q Q G U Q L F Y V F U R S E S D P Y E
121 GACCGGTGCTGCTGTGCTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
   D P L L L L W L S G G P G C S G I S G L A
181 TACGAGATAGGACCCCTTACAACTTGTATGCCAAGGGCAAGGTGGATTTCCTACTCTTCTC
   Y E I G P L Q F D A Q G Q G G F P T L L
241 TACCGACCGGAGACATGGACCAAGGTGAGCAACATCATATTTGTGGACTCGCCGGTGGGG
   Y R P E T W T K U S N I I F U D S P U G
301 ACTGGGTCTCTGCTACGCGCAAGTCCGAGGAGGGCTCGAGACCTCGCCACCAAGCAAGTG
   T G F S Y A K S E E G L E T C A T K Q U
361 AAGCAGCTAGTCATTTTCTCAGGAAGTGGCTCCAGACCATCCCGGTTCTGATGAAT
   K Q L U I F L R K W L Q D H P R F U M N
421 CCTCTTTATATTGCTGGAGATTATACAGTGGTCTCATCTTCTACTCTTGCATTAGAA
   P L Y I A G D S Y S G L I I P T L A L E
481 ATTGACAGAGCATAGAACTCGGTGAAAGATATTTCCGGTCTCAGGGGTACATTGCT
   I D R S I E L G E K I F S G L K G Y I A
541 GGCACCCGCTGACTGCGCGACAGTTGTATCGGACAGCCAAATTCATACCTTTCTATGCC
   G N P L T G G Q F D T D S Q I P Y F H A
601 ATGGGACTTGTATCCGGTGAACCTTACAGGAATGCTAGGGAGAACTGTGTTGGAAGCAT
   M G L U S G E L Y K N A R E N C G G R H
661 AGTCTCCACTGAATGCTGTATGTGACAGGGCTGTCCAGCTATCAACAATTGCACACGG
   S A P L N A U C A E A U Q A I N N C T R
721 GATATCAATAAGCAATACATCTTGGATCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT
   D I N K Q Y I L D P A C P D D D L L S P
781 AAGACTGTACAGACAGCATGCAAGCAGCGGTTGATGCTAGAGCTGTGAGATTTTCTA
   K T U A E T D G T S R L H L E S A D F L
841 CTTGATTCCAAAATGTGACGCTAAGTTCCAAGATCATATCTTTAACTAATTCATCCCC
   L D S K C A A K F Q R S Y P L T N S I P
901 TTGTCCTTACACTGTCAAGTCTTGAAGTCTTATCTGACCTACACTTTCCTTTGGCCGCA
   L S L H C Q U L D C L S D L H F P L A A
961 GGAAGCTTATACATTCATCTACGCTTGGGGGAATGA
   G S F I H S I L R L G E *

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Fig. 1 Encoding amino acid sequence of *ZmSCP*.

Table 1 Amino acid compositions of *ZmSCP*.

Types	Percentage (%)	Name	Quantity
Hydrophobic amino acids	43.10	Ala	21
		Ile	19
		Leu	41
		Met	4
		Phe	17
		Pro	21
		Try	3
Hydrophilic amino acids	29.90	Val	15
		Asp	10
		Cys	9
		Glu	15
		Gly	31
		Ser	26
		Thr	15
Basic amino acids	13.90	Tyr	15
		Arg	12
		His	6
Acidic amino acids	13.10	Lys	12
		Asp	21
		Glu	19

that *ZmSCP* has closer kinship with *Oryza sativa* and sorghum, which belong to the same evolutionary branch (Fig. 2).

3.2 Construction of Recombinant Plasmid pET32a(+)-*ZmSCP*

The pMD18-*ZmSCP* and the pET32a(+) were

double digestion with *EcoR* I and *Not* I. Competent cells DH5 α in *E. coli* were transformed by ligation products. Forty-eight monoclonals were picked for PCR detection. Results indicate that all the 48 monoclonals were positive. The sizes of fragments after single and double enzyme digested were correct (Figs. 3a and 3b). The cDNA sequence of inserted reading frame was confirmed by sequencing. No frameshift mutation occurred indicated that the recombinant expression plasmid pET32a(+)-*ZmSCP* was built successfully.

3.3 SDS-PAGE and Western Blot Analysis

E. coli BL21 cells were transformed and expression conditions were optimized at 37 °C and 28 °C, after induction with 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mM IPTG for various time periods, 0, 2, 4, 6, 8, and 10 h, respectively. The mRNA encoding *ZmSCP* was fused to His-Tag coding sequence at the 3' end, for comparison to cells containing recombinant plasmid without induction. Results showed a distinguishable extra band around 58 kD, the size of the *ZmSCP*-thioredoxin fusion protein in *E. coli*. The optimal induction occurred at 28 °C with 0.4 mM IPTG for 4 h (Fig. 4).

The recombinant plasmid was transformed into the

Fig. 2 Phylogenetic analysis of *ZmSCP* and other SCP from higher plants. Sequences were downloaded from GenBank and have the following accession numbers: *P. trichocarpa* (XM_002298750); *S. berthaultii* (AF006078); *S. bicolor* (XM_002446512); *A. Strigosa* (XM_002265806); *A. Thaliana* (NM_123009); *O. sativa* (NM_001074392); *R. communis* (XM_002525247); *V. Vinifera* (XM_002265806).

Fig. 3 Identification of recombinant plasmid pET32a(+)-*ZmSCP*. (a) Results of PCR with the template of plasmid; (b) Detection of double restriction enzyme digestion of plasmid pET32a(+)-*ZmSCP*. D stands for double restriction enzyme digestion, S stands for double restriction enzyme digestion.

Fig.4 SDS-PAGE of optimization of prokaryotic expression system.

host strain BL21 (DE3) and induced at 28 °C with 0.4 mM IPTG at 4 h. The expressed proteins were analyzed by SDS-PAGE and western blot analysis. Western blot analysis revealed the presence of one protein in the induced cells with apparent molecular weight of approximately 58 kD, which is bound to the anti-His-Tag antibody (Fig. 5). For analysis of antimicrobial activity of the fusion protein *in vitro*, the protein was purified by SDS-PAGE, which revealed highly pure protein (Fig. 6).

3.4 Purification and Antimicrobial Activity Assays of *ZmSCP* *in Vitro*

The recombinant protein concentration was measured with the Bradford method. The possible antifungal activity of *ZmSCP* against *Rhizoctonia solani* was investigated *in vitro* with three replications. Result showed that the *ZmSCP* protein exhibited an inhibitory effect *in vitro* on hyphal growth of *Rhizoctonia solani*. *Rhizoctonia solani* treated with *ZmSCP* at 60 µg/mL showed significantly slower

hyphal growth compared with the PBS control (Fig. 7a-7c).

4. Discussion

SCPs have been identified in a wide array of organisms and comprise a large family of hydrolyzing enzymes, which are believed to play roles in processing and degradation of proteins/peptides. Compared to the large number of members in the SCP family, only a few of them have been studied in detail for their biochemical activity and biological roles in

Fig.5 Identification of recombinant protein by Western Blot.

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Fig. 6 SDS-PAGE analysis of purified protein.

Fig. 7 Inhibitory effect of recombinant protein to *Rhizoctonia solani*. (a) antifungal activity of *ZmSCP* protein in culture dish; (b) statistical analysis of inhibitor zone (different letters indicate significant difference ($p < 0.01$) between treatments); (c) antifungal activity of *ZmSCP* protein under a microscope; Bars = 100 µm.

Many studies verified gene function using prokaryotic expressive system, for example, Lee et al. obtained a large amount of high purity expressive proteins using prokaryotic expressive system, and demonstrated the disease resistance using antimicrobial test *in vitro* [20]. Similar antimicrobial analyses have been conducted in studies involved rice, plantains, tobacco, and cotton [20-25]. Results of previous studies indicate part of pathogenesis-related proteins (e.g. PR2, PR3 and PR4) with antibacterial properties in different degrees [26]. The possible reason is that these pathogenesis-related proteins are extracellular proteins, and when the invasion of the pathogen occurred, pathogenesis-related proteins can quickly assembled to resistance to pathogens [26]. Although the present studies have demonstrated that

plants. OsBISCPL1 which encodes a protein containing typical conserved structural features plays roles in regulating defense responses against pathogen infection and oxidative stress. In the previous study, *ZmSCP* isolated from maize was up-regulated under different stress conditions [19]. In the present study, recombinant protein inhibited hyphal growth of *Rhizoctonia solani*, suggesting that *ZmSCP* plays an important role in the disease resistance response.

ZmSCP plays important roles in defense responses against biotic and abiotic stress, the mechanism by which *ZmSCP* regulates plant defense responses against biotic and abiotic stresses remains to be further explored.

5. Conclusion

The authors cloned and identified a new maize serine carboxypeptidase gene named *ZmSCP*. *ZmSCP* encodes a 332 amino acid protein with a predicted molecular mass of 36.5 kDa and pI of 4.75. Phylogenetic analysis revealed that *ZmSCP* showed closer kinship with *Oryza sativa* and sorghum, which belong to the same evolutionary branch. The authors subsequently purified the recombinant protein which expressed in *Escherichia coli* BL21 and analyzed its

antimicrobial activities *in vitro*. Results showed that the recombinant protein inhibited hyphal growth of *Rhizoctonia solani*. The study suggests that the expression of *ZmSCP* is closely related to maize sheath blight resistance caused by *Rhizoctonia solani*.

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