Plant Protection, 08 (04) 2024. 621-633 DOI:10.33804/pp.008.04.5335

Available Online at EScience Press
Plant Protection
ISSN: 2617-1287 (Online), 2617-1279 (Print)
http://esciencepress.net/journals/PP

Research Article

CLONING AND OVEREXPRESSION OF *ZEA MAYS* CYSTATIN 2 (CCII) GENE IN *BACILLUS SUBTILIS* TO REDUCE ROOT-KNOT NEMATODE INFECTION IN CUCUMBER PLANTS IN IRAQ

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ARTICLE INFO

Article history
Received: 12th August, 2024
Revised: 9th October, 2024
Accepted: 23rd October, 2024

Keywords
Cystatin 2 (CCII)
Bacillus subtilis
Root-knot nematode
Maize
Cucumber
Biocontrol

ABSTRACT

Root-knot nematodes significantly reduce cucumber yields, prompting the development of a genetic engineering strategy as an alternative to chemical nematicides. In this study, the maize *CCII* gene was cloned into the pHT1469 shuttle vector and transformed into the *Bacillus subtilis* expression system to produce a cystatin that interferes with nematode digestive proteins. The full-length *CCII* cDNA sequence was optimized for overexpression in *B. subtilis*, driven by the *Pgrac* promoter, resulting in a protein of 134 amino acids with a deduced molecular mass of 15.01 kDa. After optimization, the relative adaptiveness in the heterologous *B. subtilis* system improved by 6.72% compared to non-optimized codons. Purification results using immobilized metal affinity chromatography (IMAC) indicated that the third fraction exhibited the highest inhibition activity, and the purified cystatin was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to be approximately 15 kDa. Treatments with nematode juveniles showed the highest mortality percentages at 30% and 40% concentrations of purified cystatin isolated from both competent and wild *B. subtilis* cells, yielding mortality rates of 65%, 73.7%, 75.3%, and 89% respectively after 72 h. Furthermore, the recombinant cystatin isolated from the first *B. subtilis* (wild) strain had a greater impact on juvenile mortality than the second strain. This study aimed to clone and express corn cystatin in heterologous *B. subtilis* and evaluate its effectiveness in reducing nematode populations, exploring its potential as a biocontrol agent.

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INTRODUCTION

Some nematodes are obligate plant-parasitizing biotrophs that cause a range of above-ground symptoms, including leaf necrosis, chlorosis, wilting, stunted growth, and increased susceptibility to other diseases (Ali et al., 2017). Therefore, it is crucial to control these pests. However, the use of chemically derived nematicides poses environmental hazards, contributing to pollution and leading to the development of resistant nematode strains, which ultimately result in significant agricultural yield losses

(Aljuboori et al., 2022). This has made the search for safe and effective nematicides a priority, with considerable research focusing on biocontrol strategies as a potential solution (Xia et al., 2011; Afzal and Mukhtar, 2024).

Several organisms have been employed as biocontrol agents against root-knot nematodes (Azeem et al., 2021; Mukhtar et al., 2021; Haq et al., 2022; Saeed et al., 2021, 2023; Yaseen et al., 2024). Common fungal species such as *Actylellina*, *Arthrobotrys*, and *Aspergillus* manage nematodes by penetrating and breaking down their cuticles (Abd-Elgawad and Askary, 2018). Additionally, bacterial species including *Bacillus*, *Pseudomonas*, and *Serratia* exhibit nematicidal activity through the production of proteases that disrupt nematode physiology (Hallmann et al., 2009).

Genetic engineering has also been applied to introduce disease- and pest-resistance genes into crop plants, such as *Bt* toxins for insect resistance, herbicide resistance, R genes against nematodes (Fuller et al., 2008; Gulzar et al., 2020), and protease inhibitors targeting nematodes (Lilley et al., 1999). Recently, *Bacillus subtilis* has gained global attention for its potential as a biopesticide against plant-damaging nematodes due to its production of bioactive metabolites such as exotoxins, enzymes, and nematicidal compounds (Ramezani Moghaddam et al. 2014; Yu et al., 2015; Hadri et al., 2019).

As one of the most well-known non-pathogenic microorganisms, *B. subtilis* is considered eco-friendly and is widely used as a biocontrol agent (Okay and Alshehri 2020; Aziz et al., 2024). It is a gram-positive bacterium that is safe for humans, as it does not produce harmful exotoxins (Souza et al., 2021). Moreover, *B. subtilis* has been extensively studied and is frequently utilized for recombinant protein production (Rafique et al., 2021).

Cysteine protease inhibitors, commonly known as phytocystatins, are plant-derived proteins with crucial roles in seed development, germination, plant growth, and tolerance to both biotic and abiotic stresses (Yu et al., 2017). These low-molecular-weight proteins, secreted by several plant species, act as potent inhibitors of extracellular proteases, including the digestive enzymes of harmful microbes and pests (Papolu et al., 2016). Transgenic plants expressing cystatins, such as oryzacystatin I from corn and rice have shown enhanced resistance to insect gut proteinases and nematode parasites (Gutierrez-Campos et al., 1999). The efficacy of cysteine protease inhibitors in controlling parasitic nematodes on plants has been well-documented (Lilley et al., 2004).

To produce heterologous proteins, conventional expression systems generate recombinant *B. subtilis* strains by introducing high-copy-number plasmids (Tran et al., 2020). Recent advancements in vector design, such as the development of plasmids with pMTBs72 backbones such as pHCMC and pHT01, have improved recombination efficiency and structural stability through the theta replicating mechanism (Le et al., 2019). In this study, recombinant cystatin protease (CCII) was overexpressed in *B. subtilis* using the structurally stable pHT1469 plasmid under the control of the *Pgrac* promoter. The aim was to investigate the anti-nematodal effects of recombinant cystatin protease on root-knot nematodes infecting cucumber plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media

According to Saad and Melconian (2014), *Escherichia coli* DH5α (Cat: MB12001) was provided by NZYtech Life Science for plasmid propagation. *B. subtilis* WB800N and the pHT1469 vector from MoBiTech (Molecular Biotechnology, GmbH, Germany) were used to express genes involved in protease synthesis and secretion (Rafique et al., 2021). The wild-type *CCII* cystatin gene was codon-optimized without altering the amino acid sequence and synthesized by GenScript (USA).

The *E. coli* DH5α strain was cultured in Luria-Broth (LB) medium at 37°C, serving as the host for recombinant plasmid propagation to achieve a high plasmid copy number. *B. subtilis* WB800N was grown in 2xYT medium (16 g tryptone, 10 g yeast extract, and 5 g NaCl) at 37°C, functioning as the expression system for protease production. Wild-type *B. subtilis* was isolated from soil following the protocol outlined by Abdulhussein and Hussein (2022) for bacterial transformation.

Cloning and PCR program

The CCII-optimized gene was cloned downstream of the *Pgrac* promoter in the pHT1469 vector (MoBiTec GmbH, Germany), along with the *amyQ* signal peptide, using the ABP One Step Cloning Kit (Catalog No. D017-01, ABP Biosciences, USA). For gene insertion into the pHT1469 vector, *AatII* (Biolabs) and *BamHI* (TransGen) restriction enzymes were employed, along with the ABP One Step Cloning Kit. A His-tag peptide was added to the C-terminal region of the CCII gene to facilitate enzyme purification.

The primers used for cloning the CCII gene were: ccii-qF 5′-ACACCGCATCGTTTCACTGGT-3′ and ccii-qR 5′-

GCCGACAAGCGTCCCAGTATT-3′.

The PCR protocol included an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 40 sec, and extension at 72°C for 30 sec. A final extension step at 72°C for 7 min was also performed. The PCR product, with a size of 115 bp, was confirmed by 1.2% agarose gel electrophoresis.

CCII-cystatin transformation and expression

The transformation of *E. coli* NZY5α competent cells was performed following the protocol outlined by Couto et al. (2021). To quickly verify the transformed cells, colony PCR was conducted to identify the cloned gene, as described by Wang et al. (2019). The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 40 sec, and extension at 72°C for 30 sec, with a final extension step at 72°C for 7 min. The resulting PCR product was 115 bp. DNA fragments were separated by electrophoresis on a 1.2% agarose gel.

The transformation of *B. subtilis* (wild type and competent cells) was carried out using the protocol provided by MoBiTec GmbH (Göttingen), as described by Ng (2021). To screen for transformed colonies, bacterial cells were cultured on selective 2xYT medium containing 5 µg/ml chloramphenicol. Growth of colonies on this medium indicated successful transformation. Chloramphenicol-resistant colonies were then used for plasmid isolation.

For plasmid isolation from both wild-type and competent-transformed *B. subtilis*, the resistant colonies were grown overnight at 37°C in fresh 2xYT broth (16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter). After centrifugation at 12,500 rpm for 5 min, the bacterial pellets were resuspended in 100 µl phosphate-buffered saline (PBS 10%) with 20 µl lysozyme, followed by incubation at 56°C for 45 min in a thermomixer. Plasmid extraction was performed using the QIAprep® Spin Miniprep Kit (Cat. No: 27104), according to the manufacturer's instructions (Kodackattumannil et al., 2023). For PCR amplification, the OneTaq 2X Master Mix (Biolabs) was used, and the PCR products were analyzed by electrophoresis on a 1.2% agarose gel.

Isolation of root-knot nematodes from plant material

Root-knot nematodes were isolated from infected cucumber roots following Brooks (2004), with slight modifications. The infected roots were cut into approximately 1 cm pieces and thoroughly mixed. To improve the isolation process, the root tissue was treated with 0.5% NaOCl for 3 min. The macerated tissue was then placed on two layers of paper towels in a Baermann funnel, ensuring the water level just touched the tissue. After 48 h, the water was drained, replaced with fresh water, and nematodes were counted from two 5-ml samples of the mixture. Regular water changes during incubation enhanced extraction efficiency by increasing oxygen levels.

Protease inhibitor assay

The cysteine protease inhibitor (cystatin) activity produced by transformed *B. subtilis* cells, which impairs the proper intestinal digestion of dietary proteins in nematodes (as described by Roderick et al., 2016), was evaluated using the following procedure.

A colony of transformed *B. subtilis* was cultured in 2xYT broth at 37°C for 70 h. The culture supernatant was then centrifuged at 7,000 rpm for 15 min. The resulting pellet was discarded, and 5 column volumes of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole) were added to equilibrate the beads. According to Abd and Luti (2017), 500 ml of the culture supernatant was dialyzed using a dialysis membrane (MD341M, USA) to concentrate the extracellular cystatin protease. Ni-NTA 6FF resin (BioBasic, Catalog Number #SA005025) was used for the purification of cystatin protease. The column was washed with 10 volumes of washing buffer (50 mM NaH2PO4, 300 mM NaCl, and 20 mM imidazole), followed by elution of the cystatin protease using elution buffer (50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole). The eluate was collected and dialyzed again to remove NaCl and imidazole, thus purifying the cystatin protease.

The cysteine protease inhibitor activity was measured following the method of Bijina et al. (2011). A 1 ml solution of trypsin (Sigma, CAS: 9002-07-7), 0.5 mg/ml in 0.1 M phosphate buffer (pH 7), was incubated with 1 ml of appropriately diluted culture supernatant and purified protease at 37°C for 15 min. Following incubation, 2 ml of 1% casein solution, prepared in 0.1 M phosphate buffer, was added. The reaction was stopped by adding 2.5 ml of 0.44 M trichloroacetic acid (TCA), and the mixture was centrifuged at 10,000 rpm for 15 min. The absorbance of the clear supernatant was measured at 280 nm.

Tyrosine (0, 20, 40, 60, 80, and 100 µg/ml) was used to construct a standard curve. Bijina et al. (2011) defined a

"unit of inhibitor activity" as the reduction of one absorbance unit of the TCA-soluble casein hydrolysis product released by trypsin, measured at 280 nm per min under the assay conditions. Furthermore, one unit of trypsin activity was defined as the amount of enzyme that released 1 µg of tyrosine per milliliter of the reaction mixture per min.

SDS-PAGE electrophoresis of recombinant cystatin protease produced by transformed *Bacillus subtilis*

The SDS-PAGE protocol, as described by Al-Amery and Annon (2024), was used to analyze the recombinant cystatin protease. The components for the SDS-PAGE included 30% acrylamide-bisacrylamide solution, 10% ammonium persulfate (APS), 0.1% Bromophenol Blue (BMB), 0.1% Coomassie Brilliant Blue R-250, sample loading buffer, electrophoresis buffer (pH 8.3), stacking gel (5%, pH 6.8), and separating gel (10%, pH 8.8).

Purified samples were loaded into the gel wells and separated by electrophoresis at 20 mA for 1.5 h. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R-250 for 2 h with gentle shaking. The gel was then destained in a solution of 10% acetic acid for more than 2 h with shaking.

Determination of cysteine protease inhibitor effect on root-knot nematodes

The nematicidal activity of cystatin was assessed following the method described by Kepenekci et al. (2016). Various dilutions of purified bacterial cystatin (0%, 10%, 20%, 30%, 40%, and 100%) and culture supernatant were prepared. These dilutions were applied for different durations (24, 48, and 72 h) to competent and wild-type transformed *B. subtilis* cells, with each treatment performed in triplicate. The average results from the triplicates were recorded for each dilution.

For each treatment, 200 µl of the cystatin dilutions were incubated with samples containing approximately 100 juveniles (J2) of root-knot nematodes in 100 µl, at 30°C, for 24, 48, and 72 h. A control group, consisting of 100 µl containing only J2 nematodes in 200 µl of distilled water, was maintained in separate Eppendorf tubes. Nematode mortality was observed and calculated at 24, 48, and 72-h intervals using an optical microscope, following the specified equation for mortality calculation.

Statistical Analysis

Statistical analysis was performed using SAS software (2018 version, 9.6th ed., USA) to evaluate the mortality of juvenile stage 2 nematodes, as described by Alrikabi (2023). The impact of various purified cystatin dilutions on nematode mortality was assessed across different exposure times. The Least Significant Difference (LSD) method was employed to compare multiple means using ANOVA (Two-way), with significance determined at *P ≤ 0.05. The formula for LSD is as follows:

*LSD = $\sqrt{2}$mse/r*t *P= probability

RESULTS

Analysis of CCII optimized gene

The coding sequence of the *Zea mays* CCII gene was optimized for expression in *B. subtilis* using the GenSmart Codon Optimization tool (GenScript). The optimized sequence was then compared to the non-optimized sequence in the *B. subtilis* expression system using the Graphical Codon Usage Analyzer 2.0, as illustrated in Figure 1.

The goal of codon optimization was to facilitate the expression of the *CCII* coding sequence in *B. subtilis* by replacing potentially limiting codons with the most favored synonymous codons, as outlined by Haridhasapavalan et al. (2020). The analysis confirmed that the optimized codons do not include any that could impede enzyme expression. Furthermore, the relative adaptiveness of the optimized codons in the heterologous *B. subtilis* host increased by 6.72% compared to the non-optimized codons.

As noted by Yang et al. (2021), different organisms exhibit varying codon usage frequencies, each favoring distinct synonymous codons, a phenomenon known as codon usage bias. For instance, the use of the CGC synonymous codon increased adaptiveness by 10% compared to CGA, thereby enhancing arginine expression. Similarly, substituting the TCT synonymous codon for the TCG codon, which encodes the serine amino acid, also demonstrated improved efficiency, as shown in Figure 2.

CCII gene cloning and expression of cystatin

The synthetic and optimized *CCII* gene (GenBank ID: LOC100101525) was amplified via PCR. The 405 bp amplified gene was inserted into the pHT1469 shuttle vector at its cloning site using the *BamHI* and *AatII* restriction sites. The resulting plasmid, pHT1469-*ccii*, was propagated in the *E. coli* DH5α strain, and colony PCR confirmed the presence of the *CCII* gene, yielding a 115 bp product (Figure 3).

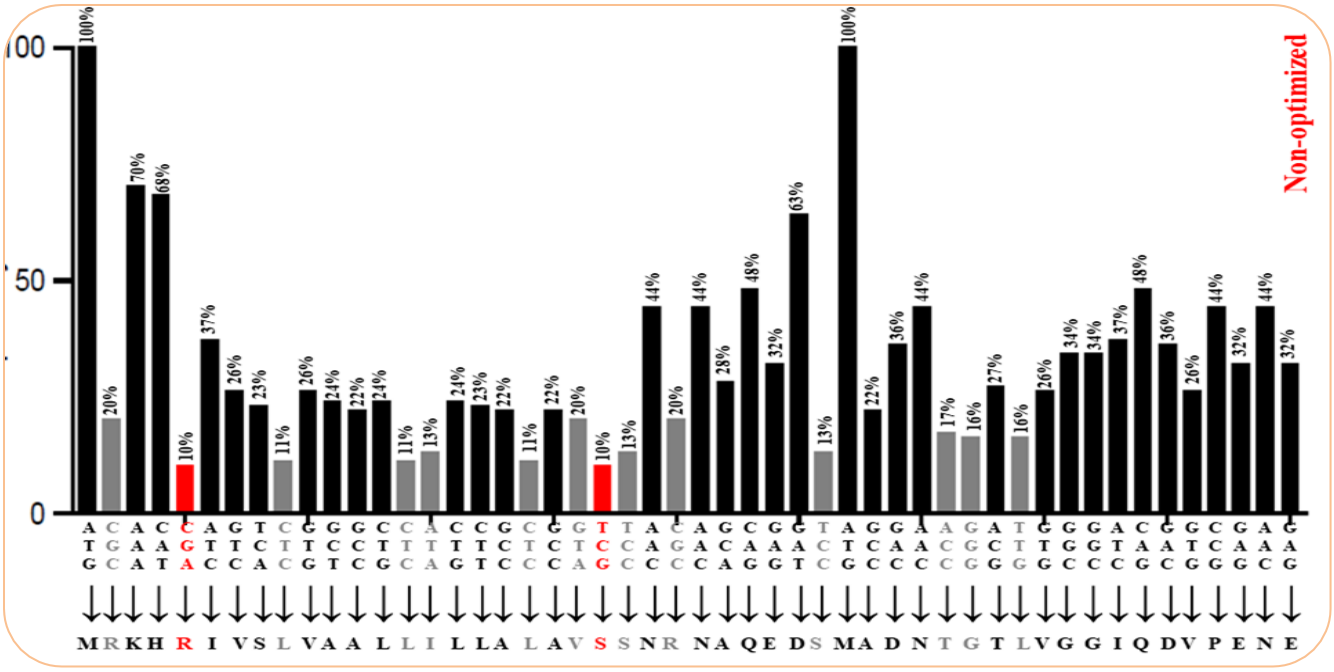

Figure 1. Analysis of non-optimized codon usage in the CCII gene using the Graphical Codon Usage Analyzer. The chart displays the first fifty codons of the non-optimized CCII gene, with red columns indicating codon mismatches in the heterologous *B. subtilis* expression system.

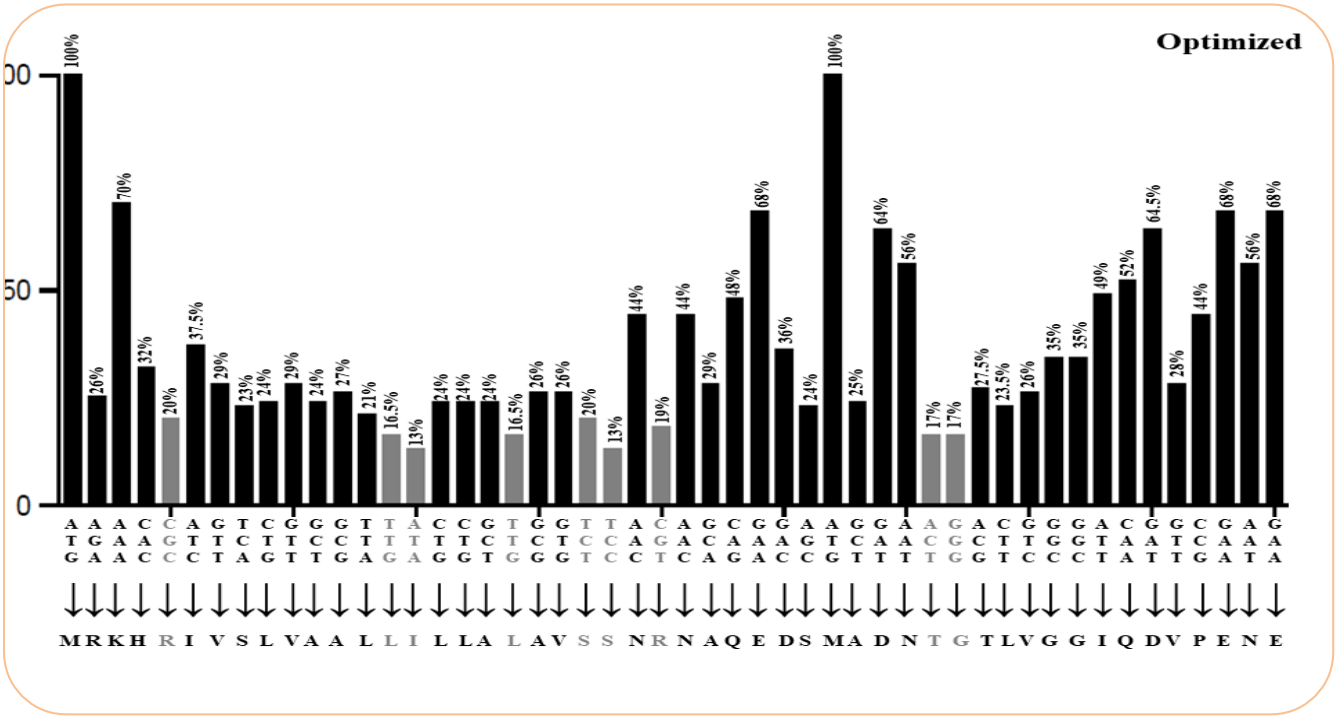

Figure 2. Analysis of codon optimization using the Graphical Codon Usage Analyzer. Columns 5 and 22 illustrate the increase in adaptiveness following the conversion of codons to preferred synonymous optimized codons for heterologous expression in the *B. subtilis* system.

CCII gene cloning and expression of cystatin

The synthetic and optimized *CCII* gene (GenBank ID: LOC100101525) was amplified via PCR. The 405 bp amplified gene was inserted into the pHT1469 shuttle vector at its cloning site using the *BamHI* and *AatII* restriction sites. The resulting plasmid, pHT1469-*ccii*, was propagated in the *E. coli* DH5α strain, and colony PCR confirmed the presence of the *CCII* gene, yielding a 115 bp product (Figure 3).

The transformant *B. subtilis* strains (both competent and wild-type), resistant to chloramphenicol, were successfully grown on 2xYT plates containing the antibiotic. Agarose gel electrophoresis confirmed the 115 bp PCR product of the cloned *CCII* gene (Figure 3), with two distinct bands indicating that the genomic DNA of *B. subtilis* (both wild and competent) had successfully incorporated the cloned *CCII* gene.

The optimized *CCII* gene was aligned with the original *Z. mays* cystatin 2 gene (GenBank Sequence ID: NM_001112543.1), showing a 76% match, with 24% of the codons being synonymous. The pHT1469-*ccii* plasmid was transformed into both competent and wild *B. subtilis* WB800N strains, where it successfully expressed cystatin protease in the host cells.

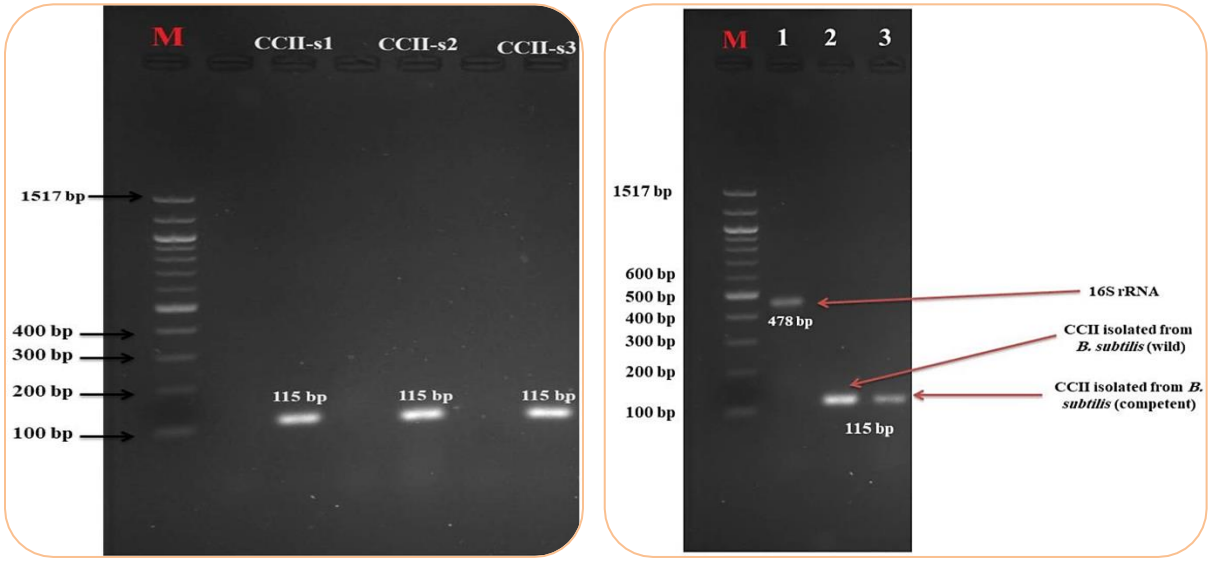

Figure 3. Electrophoresis analysis of cloned CCII gene. (A) Triplicate samples of amplified fragments from colony PCR of the *CCII* cloned gene in *E. coli* DH5α, displaying a 115 bp product alongside a DNA marker ladder ranging from 100 to 1517 bp. (B) PCR products of the optimized cystatin gene cloned into the pHT1469 plasmid and transformed into *B. subtilis*. Line M indicates the DNA marker (Catalog # N3231S). Line 1 shows the 478 bp PCR product of the *16S rRNA* gene from *B. subtilis*, Line 2 presents the 115 bp PCR product of the *CCII* cloned gene isolated from wild-type *B. subtilis* transformed cells, and Line 3 illustrates the 115 bp PCR product of the CCII cloned gene isolated from competent *B. subtilis* transformed cells.

Determination of cystatin activity and sds-page analysis

The expressed recombinant *CCII* was secreted into the liquid medium, and the culture supernatant was collected by centrifugation. The supernatant was then dialyzed and purified using immobilized metal affinity chromatography (IMAC). Trypsin was used as a substrate to evaluate the ability of recombinant CCII-cystatin to inhibit protease activity. All elution fractions from the cystatin purification exhibited inhibitory activity, with fraction 3 showing the highest inhibition, as illustrated in Figure 4.

To predict the molecular weight of cystatin produced by transformed *B. subtilis* (WB800N) with the optimized *CCII* sequence, we used an online protein molecular weight calculator (https://www.genecorner.ugent.be/protein_mw.html), which estimated a molecular weight of 15.01 kDa for the cystatin protease. A prominent band of approximately 15 kDa was identified in the purified bacterial sample containing the pHT1469-ccii construct. This is consistent with most plant cystatins, which range from 12 to 16 kDa (Martinez et al. 2005), confirming the expression of

cystatin in the transformed *B. subtilis* system based on SDS-PAGE analysis.

The electrophoresis of the purified sample revealed that cystatin protease was among the few proteases produced by competent *B. subtilis*. The recombinant cystatin protease was secreted extracellularly due to the presence of a signal peptide located at the C-terminus of the pHT1469-ccii construct. The SDS-PAGE results (Figure 4) further confirmed that the recombinant cystatin protease was optimally expressed from pHT1469-ccii under the control of the *Pgrac* promoter.

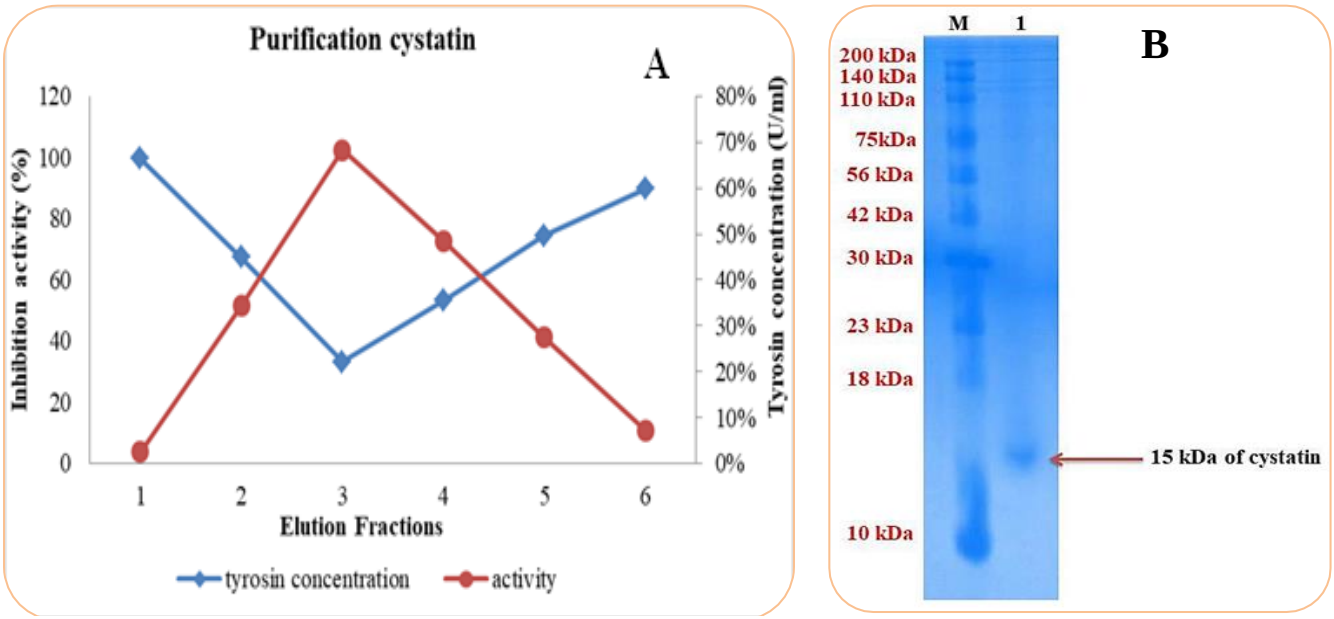

Figure 4. Analysis of cystatin curve inhibition and protease electrophoresis. (A) Inhibition activity of CCII-cystatin on the trypsin substrate, demonstrating its ability to liberate tyrosine. The enzyme activity is expressed as residual enzyme activity in the presence of increasing concentrations of the inhibitor. (B) SDS-PAGE (10%) analysis of purified cystatin protease extracted from transformed competent *B. subtilis* cells. Line M represents the protein ladder (Catalog # P7717S), while Line 1 corresponds to the 15 kDa band of recombinant cystatin produced extracellularly.

Cystatin nematicidal activity

According to the ANOVA results, there were significant differences in the percentage of nematode mortality ($P \leq 0.05$) among the tested *B. subtilis* strains (both wild-type and competent). Nematodes were considered dead if they failed to respond to gentle tapping with a needle or showed no movement when observed under a dissecting microscope (Figure 5).

After 72 h, the 30% and 40% purified cystatin concentrations resulted in the highest mortality rates, with 65.00 ± 1.00 and 73.67 ± 1.76, respectively. These results were significantly higher compared with other treatments.

Similarly, the cystatin purified from transformed *B. subtilis* (wild strain) recorded the highest juvenile mortality rates during the 24-h exposure, with no significant difference between the 30% and 40% concentrations. However, after 72 h, the 30% purified cystatin concentration showed a higher mortality rate than the 40% concentration after 48 h. Ultimately, the 40% concentration exhibited the highest mortality rate compared with the other treatments, as shown in Table 2.

Overall, based on the data analysis of nematode mortality, the cystatin protease purified from transformed *B. subtilis* (wild strain) had a greater impact on mortality than the cystatin purified from the competent strain. This is likely due to the ability of *Bacillus spp.* to produce various chitinases and proteases with nematicidal activity, as mentioned by Jimenez-Aguirre et al. (2023), in addition to the effect of recombinant cystatin protease. Thus, this study confirms that recombinant cystatin produced by transformed *B. subtilis* plays a critical role in affecting the nematode life cycle and can be considered a potential nematicidal agent.

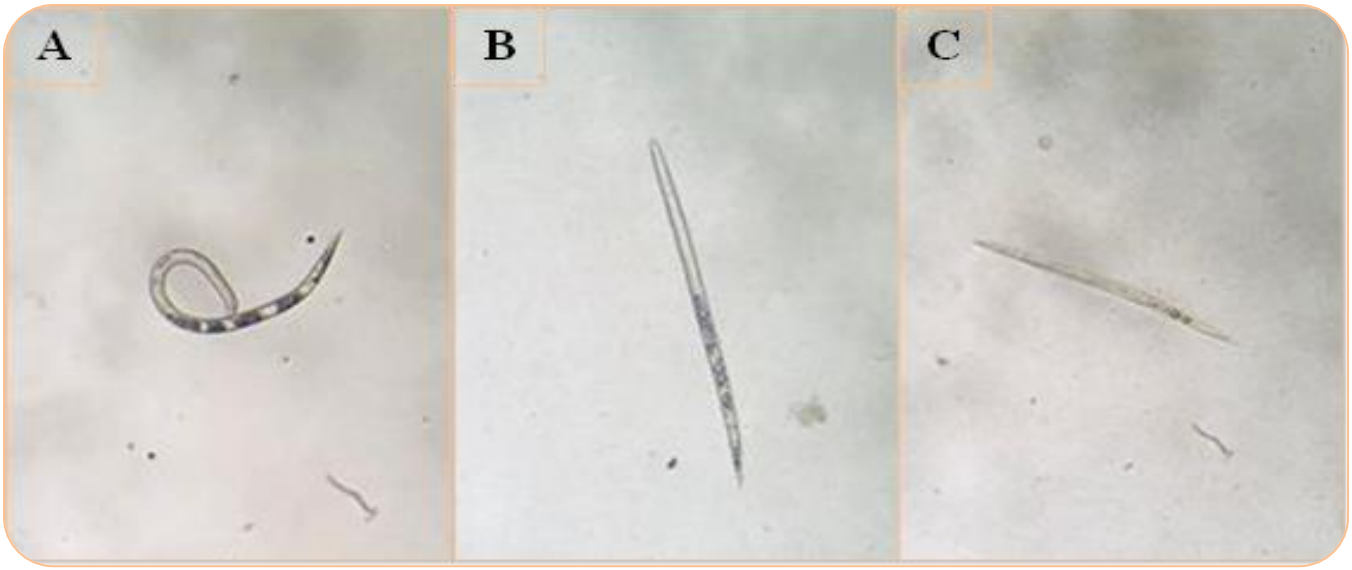

Figure 5. Effects of purified cystatin on nematode behavior and morphology over time. (A) Control sample exhibiting normal nematode rotational motion. (B) Nematodes displaying a pin-shaped morphology after 24 h of treatment with 40% purified cystatin. (C) Initial degradation of nematodes observed after 72 h of treatment with 40% purified cystatin.

Table 1. The effect of different concentrations of purified cystatin from transformed *B. subtilis* (competent strain) on nematode mortality at various exposure times (P ≤ 0.05).

Concentration (%)	Exposure time (h)			LSD value
	24 h	48 h	72 h	
0%	0.00 ± 0.00 B a	0.33 ± 0.17 E a	0.33 ± 0.17 E a	0.33 NS
10%	0.667 ± 0.33 B c	23.00 ± 1.15 D b	35.00 ± 1.15 D a	3.92 *
20%	3.67 ± 0.33 B c	43.67 ± 0.88 C b	52.67 ± 2.18 C a	4.08 *
30%	3.67 ± 0.33 B c	55.33 ± 1.76 B b	65.00 ± 1.00 B a	6.71 *
40%	6.67 ± 0.33 B c	59.67 ± 0.88 B b	73.67 ± 1.76 B a	4.95 *
100%	88.67 ± 0.67 A b	97.67 ± 1.20 A a	100 ± 0.00 A a	5.02 *
culture supernatant	90.33 ± 0.88 A b	100 ± 0.00 A a	100 ± 0.00 A a	5.17 *
LSD value	8.93 *	8.11 *	9.62 *	---

Means followed by different uppercase letters within the same column and different lowercase letters within the same row differ significantly. * (P≤0.05).

DISCUSSION

The infestation of root-knot nematodes presents a significant challenge to the growth and yield of many economically important crops (Mukhtar and Kayani, 2020; Yaseen et al., 2023; Saeed and Mukhtar, 2024). Understanding management strategies for controlling root-knot nematode infections is crucial for improving crop quality and ensuring sustainable long-term production. Genetic engineering techniques have been used to develop biological processes that bacteria utilize to combat nematode infections. Cystatin, a protease inhibitor, plays an essential role in controlling nematode infections by interfering with the digestive enzymes of nematodes. Cloning cystatin and transforming it into the *B. subtilis* expression system is one method that has proven effective in reducing nematode infections in cucumber plants.

Several studies have demonstrated that cystatin protease is involved in the biological control of nematodes. Cystatins are protease inhibitors that bind

tightly and reversibly to their target enzymes. The fundamental interactions between cystatins and their target proteases have been largely elucidated through the examination of crystal structures of various cathepsins, steins, cystatins, and their complexes as enzyme inhibitors (Turk et al., 2008).

In the current study, we demonstrated the effectiveness of recombinant cystatin CCII-maize produced by a transformed *B. subtilis* expression system in reducing nematode populations *in vitro*. One of the advantages of using genetically engineered *B. subtilis* is that it is environmentally friendly, plant-symbiotic, soil-enhancing, continuously produces the target recombinant protease as a form of plant protection, and is particularly useful in limited agricultural settings such as cucumber greenhouses. However, scaling up this approach poses challenges, including the difficulty and cost of purifying the target protease in large quantities. To address this, direct injection of genetically engineered *B. subtilis* into the infected cucumber rhizosphere may be a more practical approach.

Table 2. The impact of different concentrations of purified cystatin from transformed *B. subtilis* (wild strain) on nematode mortality at various exposure times (P ≤ 0.05).

Concentration (%)	Exposure time (h)			LSD value
	24 h	48 h	72 h	
0%	0.00 ± 0.00 D a	0.33 ± 0.17 E a	0.00 ± 0.00 F a	0.33 NS
10%	2.67 ± 0.66 D c	27.33 ± 1.76 D b	34.00 ± 0.57 E a	5.02 *
20%	10.67 ± 1.20 C c	53.33 ± 0.88 C b	60.33 ± 1.20 D a	4.96 *
30%	18.33 ± 1.33 B c	60.67 ± 1.45 C b	75.33 ± 0.88 C a	7.31 *
40%	23.00 ± 1.15 B c	70.00 ± 2.31 B b	89.00 ± 1.52 B a	7.57 *
100%	99.33 ± 0.33 A a	100 ± 0.00 A a	100 ± 0.00 A a	3.02 NS
culture supernatant	99.00 ± 0.58 A a	100 ± 0.00 A a	100 ± 0.00 A a	2.79 NS
LSD value	7.94 *	9.35 *	9.16 *	---

Means followed by different uppercase letters within the same column and different lowercase letters within the same row differ significantly. * (P≤0.05).

Our study employed the pHT 1469 vector for successful cystatin expression. Previous work by Kang et al. (2004) reported successful cloning of cystatin from soybeans into *B. subtilis* using the pWB980 vector. In this study, electrophoresis results showed that the purified cystatin produced by transformed *B. subtilis* had a molecular weight of approximately 15 kDa. This is consistent with findings by Lima et al. (2020), who isolated the *PnCPI* gene from the roots of black pepper (*Piper nigrum L.*), encoding cystatin, and cloned it into the pET29 vector for expression in *E. coli* (BL21), resulting in a molecular mass of 12.3 kDa. Similarly, Wang et al. (2012) successfully expressed recombinant BvM14-cystatin in *E. coli*, also reporting a molecular weight of 15 kDa.

When J2 nematodes were treated with various concentrations of purified cystatin extracted from transformed *B. subtilis*, the results indicated that the 40% concentration showed the highest level of nematode mortality, significantly outperforming other concentrations. This finding aligns with the study by Andrade et al. (2010), which found that the proteinaceous papain inhibitor (CpPRI), purified from *Crotalaria pallida* roots, affected the mobility of J2 nematodes at doses of 25 and 50 µg of purified enzyme after 24 and 36 h of incubation.

Moreover, our results indicated that wild-type *B. subtilis* was more effective in reducing nematode populations than the competent strain. Moreover, the present study confirms that recombinant cystatin isolated from genetically modified *B. subtilis* has a significant impact on the nematode life cycle, making it a promising candidate for use as a nematicidal agent. This conclusion is consistent with findings by Cervantes-Juan et al. (2023), who observed nematicidal activity in cystatin extracted from *Amaranthus hypochondriacus* (AhCPI).

In previous research, cysteine protease inhibitors (cystatins) have also been shown to possess antifungal (Yu et al., 2017), antiviral (Gutierrez-Campos et al., 1999), and insect-resistant (Kim et al., 2016) properties.

CONCLUSION

Briefly, *CCII*, a gene encoding a cystatin in *Zea mays*, was cloned using the pHT1469 shuttle vector and successfully transformed into a *B. subtilis* expression system. The full-length eukaryotic *CCII* gene was efficiently expressed in the prokaryotic *B. subtilis*, producing a protein consisting of 132 amino acids with a deduced molecular mass of 15.01 kDa. The recombinant cystatin protein exhibited strong trypsin protease inhibition activity and had a significant impact on the nematode life cycle by binding to nematode digestive proteins. These findings enhance our understanding of the molecular function of cystatin as a biocontrol agent against nematodes.

ACKNOWLEDGEMENT

We extend our sincere gratitude to the staff of the Nematodes Laboratory, Plant Protection Department, Office of Agricultural Research, and the Ministry of Agriculture for their invaluable assistance and cooperation in the isolation of root-knot nematodes for this study. We would also like to express our heartfelt thanks to Dr. Karam Dawood from the Nabu Scientific Foundation for his significant contribution and support in the molecular aspects of this research. Additionally, we offer special acknowledgment to Dr. Mohammed Fadhil Kadhim of AL Karkh General Hospital, Iraqi Ministry of Health, for his expert assistance in the statistical analysis.

AUTHORS' CONTRIBUTIONS

AAS designed, planned, and organized the work; MRA conducted field and laboratory experiments; Both MRA and SMS wrote the manuscript and analyzed the data.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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