Characterization of a cDNA encoding cystatin with antifungal activity from Siam tulip Curcuma alismatifolia

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INTRODUCTION

The cysteine protease inhibitors or cystatin superfamily regulates the proteolytic process of papain-like cysteine protease by binding to the active site of the target enzyme. In this superfamily, there are three commonly conserved motifs that are important for the protease inhibition activity. The first motif is the reactive site motif QXVXG, which physically interacts with the active site. The second motif contains the PW residues located in the C-terminal region of the protein. The last active site is a glycine residue near the N-terminal region of the inhibitor\textsuperscript{1–3}.

Cystatins exist widely in many organisms from microbes to plants and animals. In plants, several roles have been attributed to these proteins including the control of endogenous cysteine protease in physiological and developmental processes such as the development of stem and leaves\textsuperscript{4}, protein turnover during seed maturation, and germination\textsuperscript{5–7} and programmed cell death\textsuperscript{8}. Several lines of evidence suggest that plant cystatins are also responsive to abiotic stresses such as drought, salt, ABA and cold treatment and this helps plants to improve their ability to tolerate environmental stress\textsuperscript{9–13}. In addition, plant cystatins have a significant role in the plant defence mechanisms where they inhibit the activities of digestive cysteine proteases of herbivorous arthropods, field slugs, and parasitic nematodes\textsuperscript{14–17}. Furthermore, some plant cystatins have been shown to have detrimental effects against pathogenic fungi\textsuperscript{13,18–22}.

Plant disease is one of major limiting factors for world-wide food production. Since plants do not have an immune system to protect them from pathogens, plant infections can result in significant crop loss globally\textsuperscript{23}. More than 70% of all major crop diseases are caused by fungi\textsuperscript{24}. The fungi Fusarium oxysporum, Colletotrichum capsici, and Pyricularia grisea are the important and common fungal species which can cause significant economic damage to crops worldwide particularly in tropical, subtropical, and temperate regions. The fungus F. oxysporum can cause vascular wilt on more than 100 cultivated plants such as cereals, tomatoes, potatoes, bananas, and...
C. capsici is known to cause the anthracnose disease for many economically important plants such as pepper (Capsicum spp.) and papaya (Carica papaya). The fungus P. grisea (teleomorph = Magnaporthe grisea) is known to be the causal agent of the rice blast disease. This disease is considered to be the most prevalent fungal disease of rice because of its wide distribution and destructiveness and high degree of pathogenicity. Hence cystatin from Siam tulip was tested for antifungal activities against these three pathogenic fungi.

In this study, the Curcuma alismatifolia CPI homologue gene (CaCPI) was cloned from the Siam tulip cDNA library. The sequence analysis was performed and protein expression in bacterial system and protein purification were carried out. The protein activities and antifungal assays in growth inhibition of some plant fungi were also examined.

**Materials and Methods**

**Cloning of the CaCPI gene from the Siam tulip cDNA library**

Bract samples of the Siam tulip were prepared for mRNA isolation using the standard TRIzol reagent (Invitrogen, USA). The Creator SMART cDNA Library Construction Kit (Clontech, USA) was then used to generate the cDNA library according to the manufacturer’s instructions. DNA sequence analysis of the double-strand recombinant plasmids was performed using the dideoxynucleotide chain termination method incorporating an autoscaler (ABI). The CaCPI cDNA was identified directly from the sequenced EST clones using BLASTX against the data in the current non-redundant protein database of GenBank (www.ncbi.nlm.nih.gov). The signal peptide was analysed using the SignalP 4.0 server (www.cbs.dtu.dk/services/SignalP).

**Sequence alignment and phylogenetic analysis**

The CaCPI protein sequence was aligned to other known plant cystatins retrieved from GenBank using ClustalW2 from the EMBL-EBI database (www.ebi.ac.uk/Tools/msa/clustalw2/). The other plant cystatins which had been characterized previously were chosen to compare the Siam tulip cystatin sequence as a measure of similarity and to ensure the required active sites were presented in the newly discovered Siam tulip cystatin.

**Expression of a recombinant Siam tulip cystatin in E. coli**

A CaCPI fragment without a secretory signal sequence was amplified from the pDNR-CaCPI vector and cloned into the entry vector pENTR-3C (Invitrogen, USA). Then the CaCPI gene was transferred to the Gateway E. coli expression vector pDEST17 with the 6×His tag using the clonase reaction resulting in pDEST17-CaCPI. The pDEST17-CaCPI product was then transformed into E. coli BL21 (DE3) cells using the electroporation technique. Cells containing the pDEST17-CaCPI construct were grown at 37 °C in LB liquid medium until OD_600 of 0.5–1 was reached. The recombinant CaCPI expression was then induced by the addition of 1 mm isopropyl-β-D-thiogalactopyranoside and cultured at 21 °C overnight.

The recombinant proteins were finally extracted using sonication method and purified by Proteo Ni-IDA 1000 packed His-tag purified columns (5 mg binding capacity per column; Macherey-Nagel, Germany) according to the manufacturing procedure. The purified protein was quantitated with Bradford reagent, ready-to-use (Fermentas, USA) using BSA as a standard protein.

**Papain inhibitory activity measurement**

The proteinase inhibition assay using CaCPI was determined using BANA (Nα-Benzoyl-DL-arginine β-naphthylamide hydrochloride) as a substrate according to the previously described method with some modification. Briefly, different concentrations of CaCPI were pre-incubated with 5 μM papain (Sigma, USA) in an activation buffer (100 mM Na_2PO_4, pH 6.5, 10 mM EDTA, 10 mM 2-mercaptoethanol) at 37 °C for 10 min. The reaction was initiated by the addition of 100 μl of 200 μM BANA as a substrate. The reaction mixture was incubated at room temperature for 20 min then 300 μl of 2% HCl in ethanol (w/v) was added at the end to stop the reaction. The chromophore was developed by addition of 300 μl of 0.06% p-dimethylaminocinnamaldehyde in ethanol followed by incubation at room temperature for 15 min and the final measurement of the A_440. There were 3 replica of each treatment.

**Antifungal activity assay of CaCPI**

The phytopathogenic fungi F. oxysporum, C. capsici and P. grisea were cultivated on potato dextrose agar media at 28 °C. Relative fungal growth was estimated by cultivating 10⁴ spores of F. oxysporum and C. capsici or 10⁴ conidia of P. grisea in a microcentrifuge tube with 1/3 strength of potato dextrose broth (PDB) media and varying the concentration of CaCPI (0, 5, 10, and 20 μM) with 100 μl total volume. Ten samples were used in each treatment. The mycelial growth was then monitored by measuring the absorbance.
at 492 nm (BioRad, USA) after 48 h of culture in 28 °C. Once the growth conditions were satisfied, the actual amount of growth for each fungus was calculated based on ten replicates of the experiment as a percentage of the fungus growth in the absence of CaCPI.

RESULTS AND DISCUSSION

Sequence analysis of CaCPI

The cystatin gene was cloned from a cDNA library of Siam tulip. This cDNA fragment (GenBank Accession Number KC176357) comprised 601 nucleotides, including 32 nucleotides of the 5′ untranslated region (UTR) and 197 nucleotides at the 3′ UTR. This sequence also had an internal open reading frame of 372 bp, encoding a deduced amino acid sequence of 123 residues. Signal peptide analysis using the SignalP software showed that the encoded CaCPI protein had a putative N-terminal secretory signal of 22 amino acids. The predicted molecular masses of the precursor and the mature protein were 13.5 and 11.2 kDa, respectively, (expasy.org/tools/peptide-mass.html). The CDD search, with the deduced CaCPI protein sequence showing identity with the conserved cystatin-like domain (CY domain; cd00042).

A BLASTP analysis showed that the deduced amino acid sequence of the CaCPI shared a high level of identity with cystatin sequences from Amaranthus hypochondriacus, Sandersonia aurantiaca, Amblyommia maculatum, Vitis vinifera and Petunia × hybrid with 86%, 78%, 78%, 77%, and 79% similarity, respectively. Sequence alignment of the Siam tulip cystatin with other phytocystatins is shown in Fig. 1. The CaCPI sequence contained all of the highly conserved blocks that are essential for cysteine proteinase activity. These conserved regions included the GG doublet (Gly31-Gly32) in the N-terminal region, the reactive site motif QXVXG (Q76V77V78 A79G80) and the A/PW motif (P106-W107) in the C-terminal region. In addition, a unique consensus motif, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N (L49G50R51 F52A53V54 D55Q56H57 N58), was also found in these phytocystatins. The phylogenetic tree (Fig. 2) is based on a 100 sample bootstrapping analysis of the amino acid sequences for CaCPI and other 40 plant cystatins from different species and indicates that the CaCPI sequence for the Siam tulip is most closely related to E. guineensis. The internal node values show the percent conservation from the bootstrapping analysis where values over 50% are included and values over 80% indicates that there is a strong consensus for the generated phylogram.

Purification of recombinant proteins from E. coli

The CaCPI gene was cloned into a pDEST17 expression vector and then transformed into the E. coli strain.
Fig. 3 Bacterial expression of the recombinant CaCPI protein with and without IPTG induction by SDS-PAGE (12%). Lane (1) total protein fraction before IPTG induction, lane (2) total protein fraction after IPTG induction, lane (3) soluble fraction and lane (4) purified 6× His-tagged CaCPI protein. Lane (M) molecular weight peptide standards.

BL21-Star for recombinant CaCPI protein production. The recombinant CaCPI expressed in E. coli as a fusion protein with a histidine tag which was in the soluble form and therefore could be purified by affinity chromatography using a Ni-IDA column. The predicted molecular weight of the mature CaCPI protein without the secretory signal peptide was 11.2 kDa. But after automated tagging with 6× His in pDEST17, its predicted molecular weight increased to 12.06 kDa. The single band of the purified His-CaCPI with an apparent molecular mass of approximately 12 kDa in coomassie blue stained SDS-PAGE was observed in an eluted solution from the Ni-IDA column (Fig. 3).

Papain inhibitory activity measurement

To determine the papain inhibition assay, 5 µM of papain was preincubated with various concentrations of purified CaCPI protein at concentrations ranging from 0 µM (control) to 20 µM. Percentage of residual papain activity (Fig. 4). The residual papain activity was decreased when the CaCPI protein concentration was increased (from 0–30 µM) in the mixture. The previous report demonstrated that, in general, cystatins were a competitive inhibitor of papain. However, the inhibition type of papain activity in some plant species such as rubber latex, corn, rice, soya bean, tomato, and strawberry were shown to be non-competitive inhibition

Antifungal activity assay

The effect of purified CaCPI on the growth of fungal plant pathogens was determined using an in vitro bioassay. Purified recombinant CaCPI inhibited the growth of all three phytopathogenic fungi (F. oxysporum, C. capsici, and P. grisea), but the inhibitory effect varied depending on the fungal species. Ten thousand (10^4) fungal spores were cultured in 1/3 PDB with various concentration of CaCPI protein from 0 (control) to 20 µM. Then spores or conidia cultures were incubated at 28 °C for 48 h without the use of a shaker. Hyphae growths were then measured for their optical density at 492 nm and used for calculation for relative growth (Fig. 5d).

Hyphae growths of all fungal strains were suppressed after adding CaCPI protein into the medium (Fig. 5). Interestingly, comparison of the sensitivity of CaCPI protein against three pathogenic fungi in this experiment, P. grisea was the most sensitive to CaCPI. The growth of P. grisea was inhibited up to 90% in the presence at 5 µM CaCPI in the culture medium but others needed more CaCPI concentration for inhibit at the same level. Our results agreed with a previous report on barley cystatins. In vitro, most barley cystatins provided a greater growth inhibition in P. grisea than F. oxysporum or Plectosphaerella cucumerina. They explained this result as being due to the different actions of the pathogenic mechanism in different fungal groups. P. grisea was a fungus in the hemibiotrophic group but F. oxysporum and
Fig. 5 Growth inhibition assay of three fungal pathogens (a) *F. oxysporum*, (b) *C. capsici*, (c) *P. grisea* by the recombinant CaCPI protein. The protein concentrations were varied from 0–20 μM. (d) relative growth of three fungal species cultured in 1/3 PDB with and without various concentrations of CaCPI proteins. The mycelial growth was monitored by measuring absorbance at 492 nm after 48 h of incubation and was presented as a percentage of the fungus growth in the absence of CaCPI.

*P. cucumerina* belong to the necrotrophic group. The hemibiotroph *P. grisea* which lacks the C1A cysteine proteases (cystatin’s target enzyme) showed stronger inhibition by cystatins than the necrotrophic *F. oxysporum* or *P. cucumerina* which contain C1A cysteine proteases. It is possible that cystatin was targeted to another type of proteolytic enzymes or that growth inhibition was not mediated by inhibition of cystein proteinases. However, the mechanisms responsible for these differences are still unknown.40

When a sensitivity of fungi to CaCPI was compared between *C. capsici* and *F. oxysporum*. At 5 μM of CaCPI concentrations, the growth of *C. capsici* was lesser than that of *F. oxysporum*. While at 10 and 20 μM CaCPI, both fungi showed the same level of growth inhibition (Fig. 5). This indicated that *C. capsici* was more sensitive to the CaCPI protein than *F. oxysporum* at the lower concentrations of the CaCPI protein.

The maximum concentration of the purified His-tagged recombinant Siam tulip cystatin for the inhibition of the growth of the three phytopathogenic fungi (*F. oxysporum*, *C. capsici* and *P. grisea*) was 10 μM. The cystatin concentrations used to inhibit *F. oxysporum* growth in this study were found to be different from that previously used in other inhibitory assays such as amaranth cystatin AhCPI (17 μM)41 or strawberry cystatin (6 μM)21. Although we did not find any reports mentioned an effect of other plant cystatins against *C. capsici*, but there has been a report on chestnut cystatin against *C. graminicola*. The maximum inhibition level was found for a concentration of 9 μM18. This concentration was very similar to our result. In the case of *P. grisea*, there was no report on the maximum cystatin concentration for growth inhibition but there was some reports on barley cystatins (HvCPI-1 to HvCPI-12). The 50% growth inhibition (EC50) against *P. grisea* was 0.18–5.08 μM40. This concentration was also similar to our result (Fig. 5). Our results suggested that Siam tulip cystatin carried antifungal activities at the same level as other plant cystatins.

The inhibitory effects that the recombinant CaCPI exerted on the growth of these three phytopathogenic fungi were also monitored by microscopic observations (Figs. 6, 7, and 8). In the control (without CaCPI application), we observed the spores or conidia germination in all three fungal species with the development of hyphae (Figs. 6, 7, and 8). The mycelial growths were diminished compared to the control when CaCPI was present in the medium. Greater inhibition of hypha growth was observed at 10 μM CaCPI (Figs. 6, 7, and 8). Interestingly, at 20 μM CaCPI concentration, spore germination of *C. capsici* and conidia germination of *P. grisea* were fully suppressed (Figs. 7d and 8d). These results clearly indicated that the CaCPI contained antifungal activity against these pathogens, *F. oxysporum*, *C. capsici*, and *P. grisea*.

*Fusarium* and *Colletotrichum* are serious and common fungal genus which cause significant economic damage to crops worldwide, and have an ex-
Fig. 6 Microscopic photographs of *F. oxysporum* growth suppression with the recombinant CaCPI under light microscopy at 40× (a, c) and 400× (b, d) magnifications. *F. oxysporum* growth without CaCPI (a, c), and with 10 µM CaCPI (b, d).

Extremely wide host range including cereals, legumes, ornamentals, vegetables, and fruit trees\textsuperscript{25, 26, 42}. *P. grisea* is the causal agent of the rice blast disease, the main fungal disease of rice. Our results show
antifungal property such as wheat, barley, strawberry, sugarcane, taro, chestnut, hevea rubber, and cacao. But, the mechanisms of antifungal activity demonstrated by cystatins remain unclear. The antifungal properties are most likely related to their inhibition against fungal proteases. However, some reports on barley cystatin (HvCPI) and taro cystatins (CaCPI) demonstrated that the antifungal properties were not associated with activities as proteinase inhibitors. Furthermore, the investigation of cystatins from chestnut, taro and amaranth suggested that the antifungal activity of plant cystatins is probably not mediated by fungal proteinase inhibition because the cystatin concentration required to inhibit the mycelial proteolytic activity was very high compared to the concentration that inhibits fungal growth.

CONCLUSIONS

The cystatin CaCPI gene was cloned and isolated from cDNA library of Siam tulip (Curcuma alismatifolia cv. Chiang Mai Pink), a popular Thai ornamental plant for export. The recombinant CaCPI protein was successfully produced in bacterial expression system. The purified protein exhibited the antifungal activities against growth of phytopathogenic fungi; F. oxysporum, C. capsici, and P. grisea. This study of CaCPI protein function could serve as a knowledge base or could be useful for further applications for genetic improvement of economy crop in the future.

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REFERENCES


