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

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ABSTRACT: Cystatins are cysteine protease inhibitors involved in defence mechanisms against pests and pathogens. Here, the cystatin *CaCPI* gene was isolated from a cDNA library of the Siam tulip (*Curcuma alismatifolia* cv. Chiang Mai Pink). The full length cDNA of 601 bp contains 372 bp of an open reading frame encoding 123 amino acids flanked by 5′ and 3′ untranslated regions of 32 and 197 bp, respectively. The deduced amino acid sequence consists of a putative N-terminal secretory signal peptide of 22 amino acids and an estimated molecular mass of 11.2 kDa of the mature protein. The CaCPI protein contains all of the highly conserved blocks included Gly^{31}-Gly^{32}, the reactive site motif QXVXG ($Q^{76}V^{77}V^{78}$ $A^{79}G^{80}$), the P^{106}-W^{107}, and the [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N ($L^{49}G^{50}R^{51}$ $F^{52}A^{53}V^{54}$ $D^{55}Q^{56}$ $H^{57}N^{58}$) block that is common among plant cystatins. The *CaCPI* gene was cloned into a pDEST17 expression vector and was then transformed into *Escherichia coli* strain BL21-Star to produce a recombinant CaCPI protein. After induction with 1 mM IPTG, the cell lysate of *E. coli*-carrying pDEST17-CaCPI generated a CaCPI protein about 12 kDa in size as measured using SDS-PAGE. Pre-incubation of the 5–30 μM CaCPI protein samples with 5 μM papain is known to decrease papain activity. Antifungal activities of the purified recombinant CaCPI protein against phytopathogenic fungi were tested. The CaCPI protein could suppress mycelium growth of *Fusarium oxysporum*, *Colletotrichum capsici*, and *Pyricularia grisea* phytopathogenic fungi.

KEYWORDS: cysteine protease, cysteine protease inhibitor, phytopathogenic fungus

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[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[4], protein turnover during seed maturation, and germination[5–7] and programmed cell death[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[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[14–17]. Furthermore, some plant cystatins have been shown to have detrimental effects against pathogenic fungi[13, 18–22].

Plant disease is the 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[23]. More than 70% of all major crop diseases are caused by fungi[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

water melons[25, 26]. *C. capsici* is known to cause the anthracnose disease for many economically important plants such as pepper (*Capsicum* spp.)[27–29], and papaya (*Carica papaya*)[30, 31]. 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[32, 33]. 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[34] incorporating an autosequencer (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 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 Protino 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 by CaCPI was determined using BANA (N_α-Benzoyl-DL-arginine β-naphthylamide hydrochloride) as a substrate according to the previously described method[35] with some modification. Briefly, different concentrations of CaCPI were pre-incubated with 5 μM papain (Sigma, USA) in an activation buffer (100 mM Na_3PO_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_{540}. 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 10^4 spores of *F. oxysporum* and *C. capsici* or 10^4 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
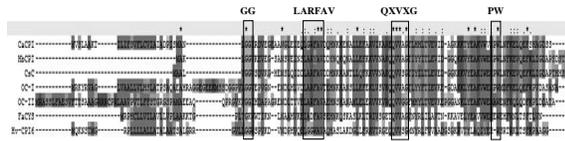
Fig. 1 Sequence alignment of cystatins from the Siam tulip (CaCPI), rubber latex (HbCPI), chestnut (CSC), rice (OC-I, OC-II), jelly fig (FaCYS) and barley (Hv-CPI6). The black blocks indicate 4 highly conserved sequences which are common among plant cystatins.

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 in, 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[36], 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*, *Amblyomma 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 (Gly^{31}-Gly^{32}) in the N-terminal region, the reactive site motif QXVXG ($Q^{76}V^{77}V^{78}$ $A^{79}G^{80}$) and the A/PW motif (P^{106}-W^{107}) in the C-
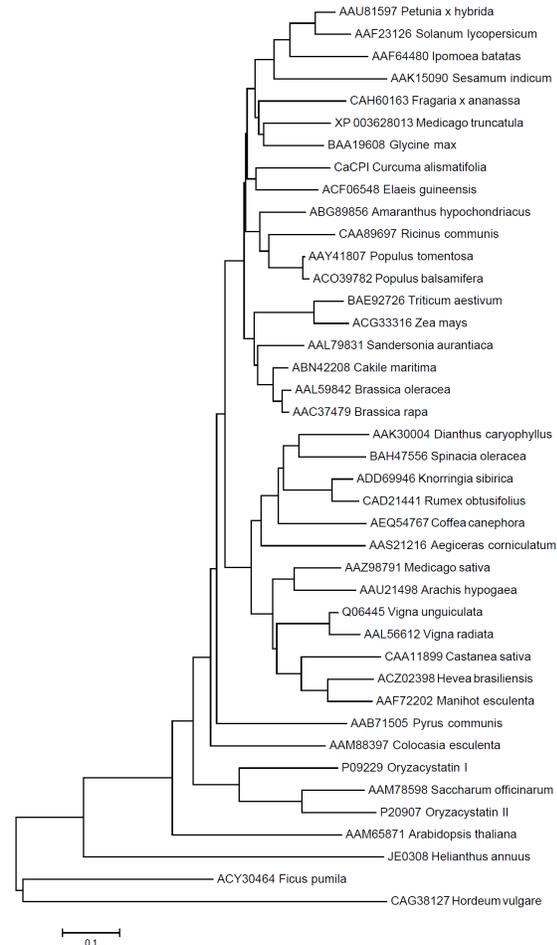

Fig. 2 Phylogenetic tree of the Siam tulip cystatin (CaCPI) and cystatins from 40 different plants.

terminal region. In addition, a unique consensus motif, [LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-X-[EDQV]-[HYFQ]-N ($L^{49}G^{50}R^{51}$ $F^{52}A^{53}V^{54}$ $D^{55}Q^{56}H^{57}$ N^{58}), was also found in these phytocystatins[37–39]. 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
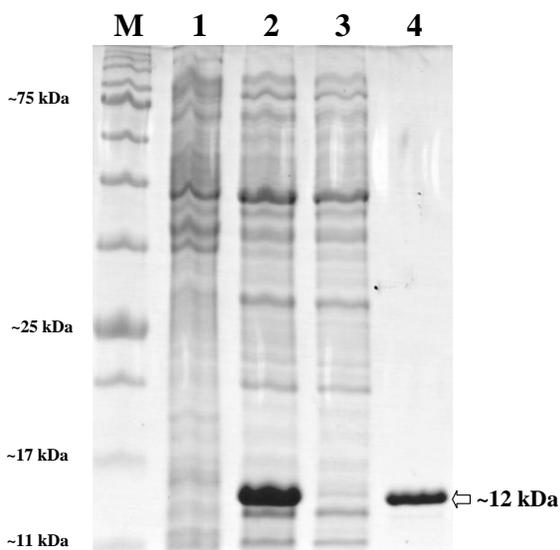
Fig. 3 Bacterial expression of the recombinant CaCPI protein with and without IPTG induction by SDS-PAGE (12%). Lane (1) total protein fraction before IPTG induction, lane (2) total protein fraction after IPTG induction, lane (3) soluble fraction and lane (4) purified 6× His-tagged CaCPI protein. Lane (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 w (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[21]. However, the inhibition type of papain activity in some plant species such as rubber latex, corn, rice,
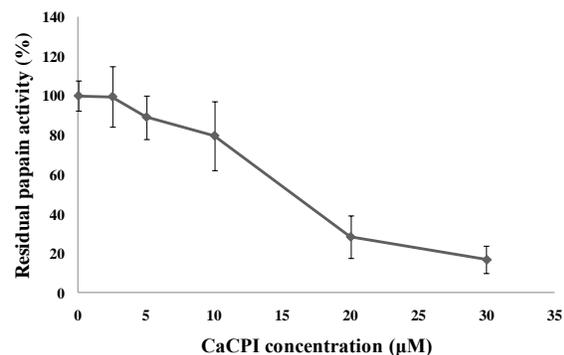

Fig. 4 Inhibition activity of purified CaCPI to papain. The inhibition was expressed as a residual enzyme activity in the presence of different inhibitor concentrations. Papain was incubated at 37 °C for 10 min and the residual proteolytic activity was measured using BANA as substrate. Vertical bars represented standard deviations.

soya bean, tomato, and strawberry were shown to be non-competitive inhibition[13, 14, 19, 20].

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[40]. 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
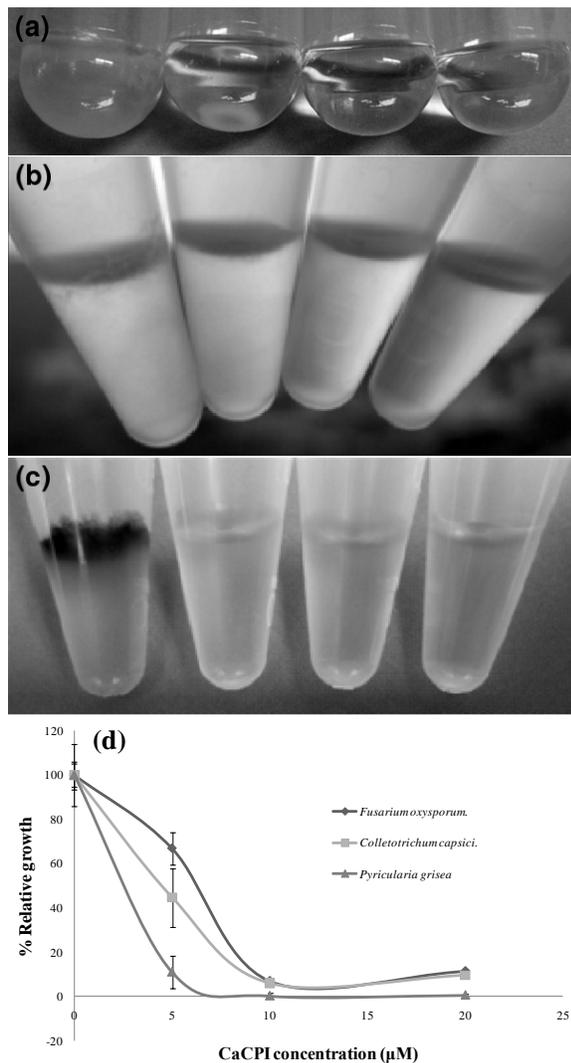
Fig. 5 Growth inhibition assay of three fungal pathogens (a) *F. oxysporum*, (b) *C. capsici*, (c) *P. grisea* by the recombinant CaPI protein. The protein concentrations were varied from 0–20 μM. (d) relative growth of three fungal species cultured in 1/3 PDB with and without various concentrations 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 μM[18]. 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 (EC_{50}) against *P. grisea* was 0.18–5.08 μM[40]. 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-
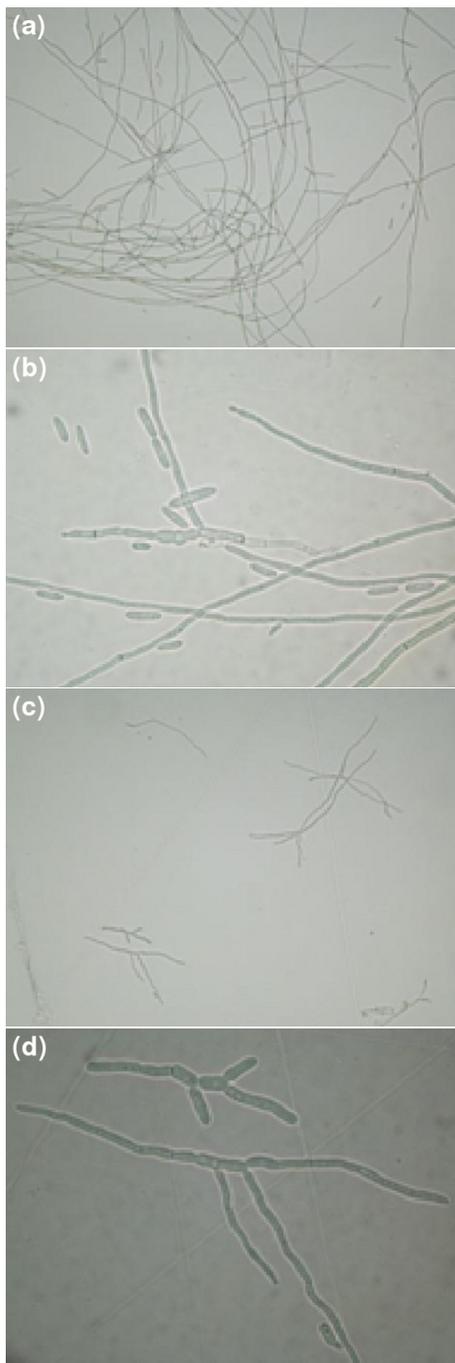
Fig. 6 Microscopic photographs of *F. oxysporum* growth suppression with the recombinant CaCPI under light microscopy at 40× (a, c) and 400× (b, d) magnifications. *F. oxysporum* growth without CaCPI (a, c), and with 10 μM CaCPI (b, d).
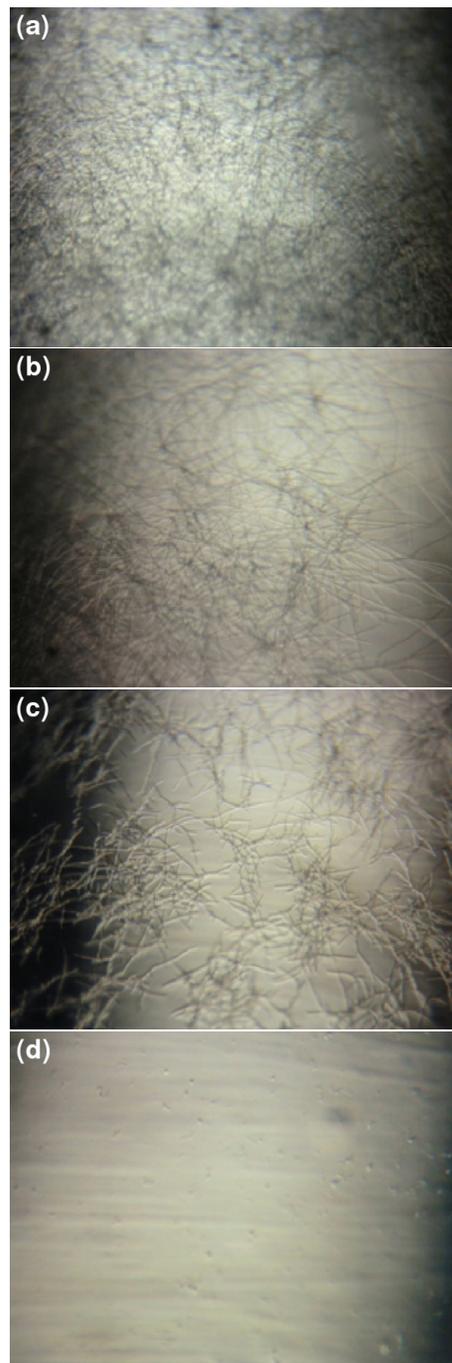

Fig. 7 Microscopic photographs of *C. capsici* growth suppression with the recombinant CaCPI under light microscopy at 40× magnification. (a) *C. capsici* growth without CaCPI, (b) with 5 μM CaCPI, (c) with 10 μM CaCPI, and (d) with 20 μM CaCPI.

tremely wide host range including cereals, legumes, ornamentals, vegetables, and fruit trees[25, 26, 42].

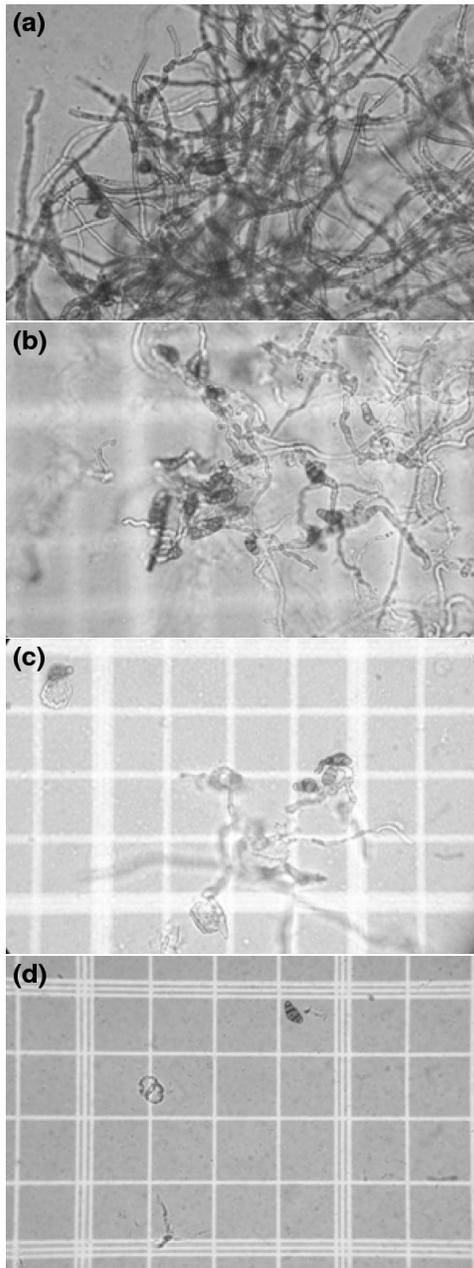*P. grisea* is the causal agent of the rice blast disease, the main fungal disease of rice. Our results show

Fig. 8 Microscopic photographs of *P. grisea* growth suppression by the recombinant CaCPI under light microscopy at 400× magnifications. (a) *P. grisea* growth without CaCPI, (b) with 5 μM CaCPI, (c) with 10 μM CaCPI, and (d) with 20 μM CaCPI.

that the Siam tulip cystatin could inhibit growth of these pathogenic fungi. Hence this protein may be useful for fungal disease control in these economically important crops in the future.

There are several reports on plant cystatins with antifungal property such as wheat[13], barley[43], strawberry[21], sugarcane[19], taro[22], chestnut[18], hevea rubber[44], and cacao[45]. 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[20, 46]. Furthermore, the investigation of cystatins from chestnut[18], Taro[22] and amaranth[41] 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 antifugal 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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