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Research article

Recombinant amaranth cystatin (AhCPI) inhibits the growth of phytopathogenic fungi

Silvia Valdes-Rodriguez*, Alberto Cedro-Tanda, Victor Aguilar-Hernandez, Erick Cortes-Onofre, Alejandro Blanco-Labra, Armando Guerrero-Rangel

Departamento de Biotecnología y Bioquímica, Cinvestav, Km. 9.6 Libramiento Norte, A.P. 629, 36821 Irapuato, Gto., Mexico

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ABSTRACT

Phytocystatins are cysteine proteinase inhibitors from plants implicated in defense mechanisms against insects and plant pathogens. We have previously characterized an amaranth cystatin cDNA and analyzed its response to different kinds of abiotic stress [37]. In order to characterize amaranth cystatin, the coding sequence was expressed in *Escherichia coli* using the pQE-2 vector. Recombinant cystatin was predominantly found in the soluble fraction of the cell extract. Large amounts (266 mgL^{-1}) of pure recombinant protein were obtained by affinity chromatography in a single step of purification. The amaranth cystatin with a pI 6.8 and an apparent 28 kDa molecular mass inhibited papain (E.C.3.4.22.2) (Ki 115 nM), ficin (E.C.3.4.22.3) (Ki 325 nM) and cathepsin L (E.C.3.4.22.15) (Ki 12.7 nM) but not stem bromelain (E.C.3.4.22.32), and cathepsin B (E.C.3.4.22.1) activities, in colorimetric assays. Furthermore, it was able to arrest the fungal growth of *Fusarium oxysporum*, *Sclerotium cepivorum* and *Rhyzoctonia solani*. It was further demonstrated that recombinant AhCPI is a weak inhibitor of the endogenous cysteine proteinase activities in the fungal mycelium. These findings contribute to a better understanding of the amaranth cystatin activity and encourage further studies of this protein.

1. Introduction

Cystatins are proteinaceous inhibitors of cysteine proteinases that have been identified in animals and plants. Those from plants, known as Phytocystatins (PhyCys), cluster in a major evolutionary tree branch of the cystatin superfamily of proteins [16]. Most PhyCys are small proteins ranging from 12 to 16 kDa. However, a group of them with a molecular mass of 23 kDa containing a terminal extension has been described [18,31,37]. To date, the physiological significance of the C-terminal extension is unknown. However, recent evidence has demonstrated that the carboxy-terminal extensions are able to inhibit legumain peptidases [20]. Furthermore, an evolutionary comparative analysis of cysteine proteinases and their putative inhibitors suggested a gene duplication event associated with the increasing structural and functional complexities acquired in land plants [19]. Several 85 kDa multicystatins with eight cystatin domains, have been reported in tomato and potato [38,40]. As multiphytocystatin domains clustered with members of PhyCys from group I and are present in a restricted number of species, it has been proposed that they resulted from sequential duplication events of independent members of PhyCys from group I [17]. In addition to a glycine residue near the N-terminal part of the protein, the reactive site QXVXG and the PW residues located in the second half of the molecule, common to all cystatins, the PhyCys have a consensus LARFAVDEHN sequence in the N-terminal portion of the protein [16].

Several PhyCys have been isolated from different species such as soybean [24], maize [1], and barley [9]. They show variable expression patterns during plant development and different defense responses to biotic and abiotic stresses [9,22,37]. They exhibit a wide range of inhibitory activities against different cysteine proteinases. It has been proposed that PhyCys participate in the regulation of activity of endogenous cysteine proteinases during seed development and germination [30], in programmed cell death [34], and in plant cell growth and proliferation [36]. Furthermore, they also participate in protecting plants against insects and pathogens. In vitro studies have demonstrated phytocystatin inhibition of insect gut proteinases [13]. In vivo studies have demonstrated enhanced resistance obtained against insects

Abbreviations: AhCPI, *Amaranthus hypochondriacus* cysteine proteinase inhibitor; BANA, N-benzoyl-DL-arginine-2-naphthylamide; DTT, DL-dithiothreitol; DTE, DL-dithioerythrol; E-64, *trans*-epoxysuccinyl-L-leucylamino (4-guanidino)-butane; EDTA, ethylenediaminetetraacetate; IPTG, Isopropyl-β-D-Thiogalactoside; MALDI-ToF, Matrix-assisted laser desorption ionization-time of flight; PMF, peptide mass fingerprinting; PhyCys, phytocystatins.

* Corresponding author. Tel.: +52 462 6239638; fax: +52 462 62459 96.
E-mail address: svaldes@ira.cinvestav.mx (S. Valdes-Rodriguez).

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[3], nematodes [4], and potyvirus [12] in transgenic plants over-expressing phytocystatin genes. The induction of some PhyCys by wounding and methyl jasmonate further supports their role in defense [40]. The antifungal activity of PhyCys against phytopathogenic fungi has already been reported in sugarcane [33], barley [21], taro [41], strawberry [18], and wheat [7]. However, the mechanism by which phytocystatin inhibits mycelium growth is not yet understood. It has been suggested to be related to the direct inhibition of a fungal cysteine proteinase [41]. However, there are some reports in which the inhibition of plant-pathogenic fungi was not associated with cystatin inhibition of cysteine proteinases [21].

Amaranth (*Amaranthus hypochondriacus*; *Ah*) is an ancient crop native to the American continent. Its seed protein is characterized by high nutritional quality. It is cultivated as an alternative to cereal crops due to its capacity to produce reasonable yields in infertile soils and semi-arid conditions [14]. Considering that amaranth cultivation has been restricted to a few specific areas, it represents a potential source of genes for pest control. In this respect, leaves and seeds of amaranth accumulate cystatins and trypsin and α-amylase inhibitors which are considered to be defensive factors against insect pests. Moreover, their induction in plants following different types of (a) biotic stress and related elicitors further suggests that these inhibitors might contribute to the relatively high tolerance to unfavorable ambient conditions observed for this species in the field [32,37].

Recently we reported the cloning and molecular characterization of a cDNA from developing amaranth seed encoding the cystatin AhCPI. AhCPI is 24 kDa, and highly related in sequence to cystatins with the C-terminal extension. Its expression is induced by drought, salinity, and low temperatures [37]. In this paper, the coding sequence of the amaranth cystatin was efficiently over-expressed in *Escherichia coli*, demonstrating that the purified recombinant AhCPI was an efficient inhibitor of cystein proteinases and also has antifungal properties on several pathogenic fungi.

2. Results and discussion

2.1. Heterologous expression and purification of recombinant AhCPI

The AhCPI coding sequence obtained by PCR was subcloned in frame in the expression vector pQE-2. This construct was introduced into *E. coli* strain M15 (pREP4). The expression of AhCPI protein was induced by adding 100 μM IPTG to Super broth culture medium. SDS-PAGE analysis of recombinant protein clearly showed a highly expressed protein of approximately 28 kDa in the supernatant of bacterial lysate (Fig. 1A, lane 2), compared with the non-induced control (Fig. 1A, lane 1). The size of the expressed protein was larger than the expected size of 24 kDa. This difference in size could be caused by structural features of AhCPI fusion to a histidine tail. The identity of the recombinant AhCPI was confirmed by Peptide Mass Fingerprint in a mass spectrometry MALDI-ToF. The molecular mass of the peptides obtained by trypsin digestion agrees with information deduced from the open reading frame (data not shown).

To determine whether AhCPI was expressed in its soluble form, cells that underwent IPTG-induction for 2–5 h were sonicated, centrifuged and analyzed in SDS-PAGE (Fig. 1B). After different induction times, most of the cystatin was recovered in its soluble form and could be purified directly by affinity chromatography, using a Ni^{2+} column. The recombinant protein was eluted in lysis buffer containing 250 mM imidazole. This system was quite efficient, since SDS-PAGE analysis revealed the presence of a single band of AhCPI in the fraction collected (Fig. 1A, lane 4). The homogeneity of the purified cystatin was confirmed by 2D electrophoresis analysis that revealed a single 28 kDa spot with pI = 6.8

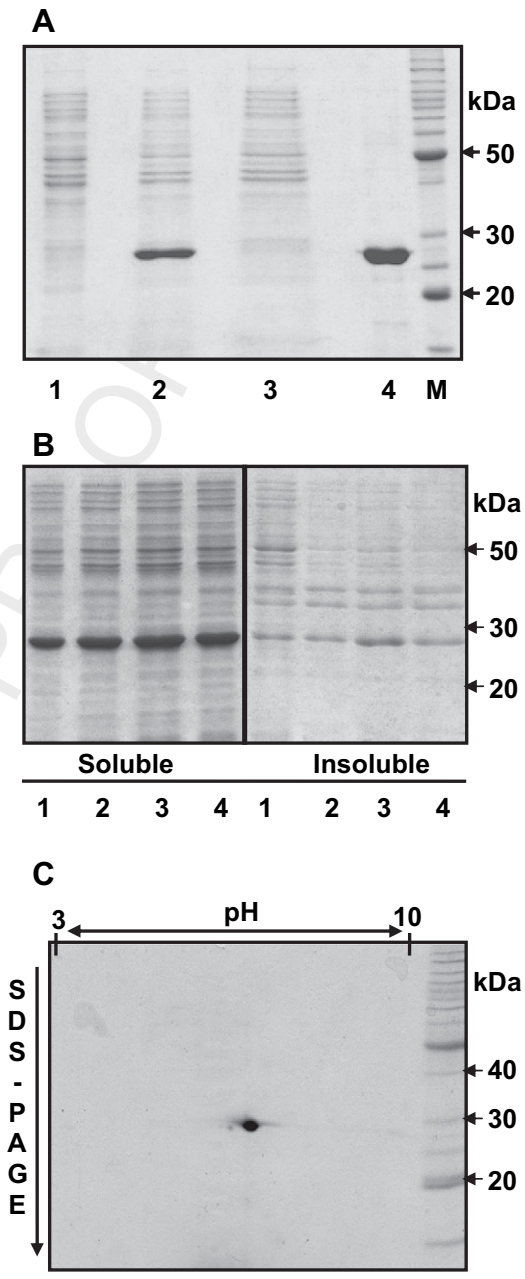

Fig. 1. Analysis of the expression, solubility test and purification of the recombinant AhCPI. (A) SDS-PAGE relating to the purification process of the AhCPI from *E. coli* cells: (1) Total soluble protein extract from bacterial lysates. (2) Total soluble protein extract from IPTG-induced bacterial lysates. (3) supernatant protein not retained in the Ni^{2+} columm. (4) purified AhCPI after elusion with 250 mM imidazol. (B) SDS-PAGE showing induction kinetics at 2 h (lane 1), 3 h (lane 2), 4 h (lane 3), and 5 h (line 4) and solubility test of AhCPI. (C) 2D electrophorethic profile of the purified AhCPI. The purified AhCPI was subjected to IEF in a 7 cm gel pH range 3–10, and the second dimension in SDS-PAGE 12%. All samples contain 10 μg of protein, except purified AhCPI that contain 5 μg. Molecular Markers (kDa) are indicated. The gels were stained with Coomassie Brillant Blue G.

(Fig. 1C). The pI value of this protein was close to the theoretical value (6.29).

The yield of purified amaranth cystatin was 266 mg per liter of cell culture. This yield is very high, as compared with the values reported for other PhyCys in other expression vectors. The expressed cane cyststatins CPI-2 and CPI-3 were fusioned to a histidine tail in pET 28; these constructs yielded 20 and 22 mg per liter of cell culture, respectively [10]. The sugarcane and the

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sunflower cystatins expressed in the same vector yielded around 10 mg pure protein per liter of culture [33]. The high levels of expression of recombinant AhCPI reported here suggest that the pQE-2 system is an excellent choice for the production of amaranth cystatin.

2.2. Inhibitory activity of the recombinant protein

The purified AhCPI was assayed against various cysteine proteinases by measuring the residual proteolytic activity after pre-incubation with different inhibitor concentrations. As shown in (Fig. 2), proteolytic activity of papain, ficin and cathepsin L gradually decreased as the AhCPI concentration was increased. AhCPI at 1.0 μM reduced papain activity by 50%, whereas 2.4 μM of cystatin produced a 47% decrease in ficin activity and 35 nM of cystatin reduces 52% of cathepsin L activity. In contrast, no inhibition was found against stem bromelain at the highest level tested (up to 20 μM of AhCPI). This lack of inhibition of bromelain is common to several plant cystatins [25].

The above results indicate that the recombinant cystatin was effective in inhibiting papain, ficin, and cathepsin L. In these cases, non-competitive inhibition kinetics were observed (data not shown). A similar inhibition model was reported in taro, strawberry and soybean, members of the group of cystatins with a C-extension [18,24,39]. Ki values of 115 nM for papain, 325 nM for ficin and 12.7 nM for carhepsin L. were determined from Dixon plot. Ki values for papain and ficin are one order of magnitude higher than those reported for most of the PhyCys [1,2,9]. It has been suggested that the C-extension affects the affinity of these inhibitors for cysteine proteinases. However, when comparing the Ki values of AhCPI with the structurally related cystatins, the results were very

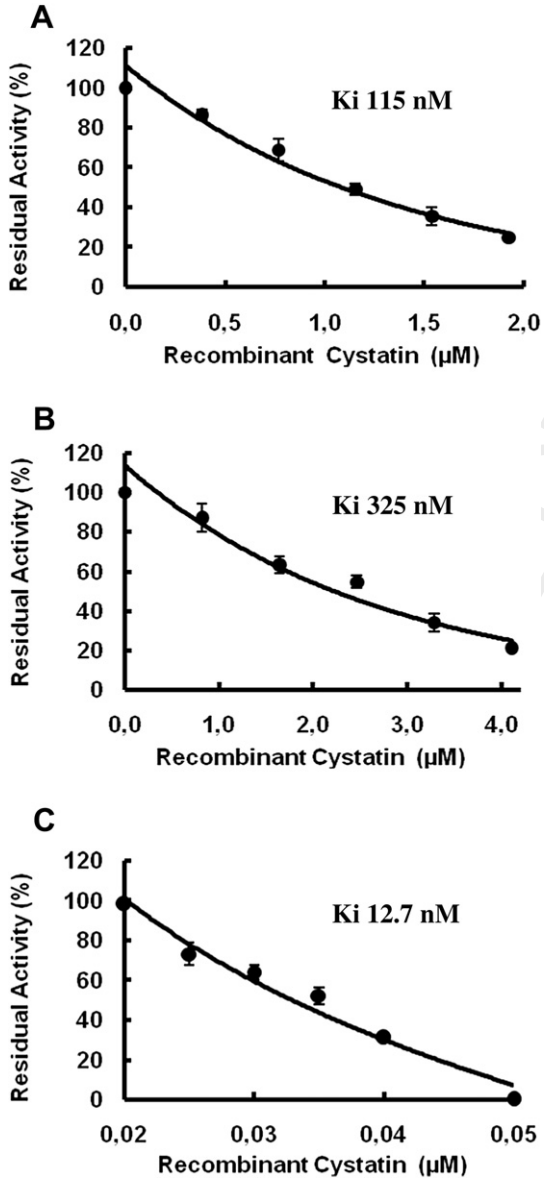

Fig. 2. Inhibitory activity of purified recombinant AhCPI. The inhibitory activity was assayed by measuring the remaining hydrolytic activity after pre-incubation (10 min) of active enzymes with different inhibitor concentrations. BANA was used as substrate for papain (A) and ficin (B) and Z-Phe-Arg-p-nitroanilide for cathepsin L (C). The Ki of the enzyme–inhibitor complex were calculated by Dixon plot. Experiments were repeated three times and the standard deviations are shown by vertical bars.

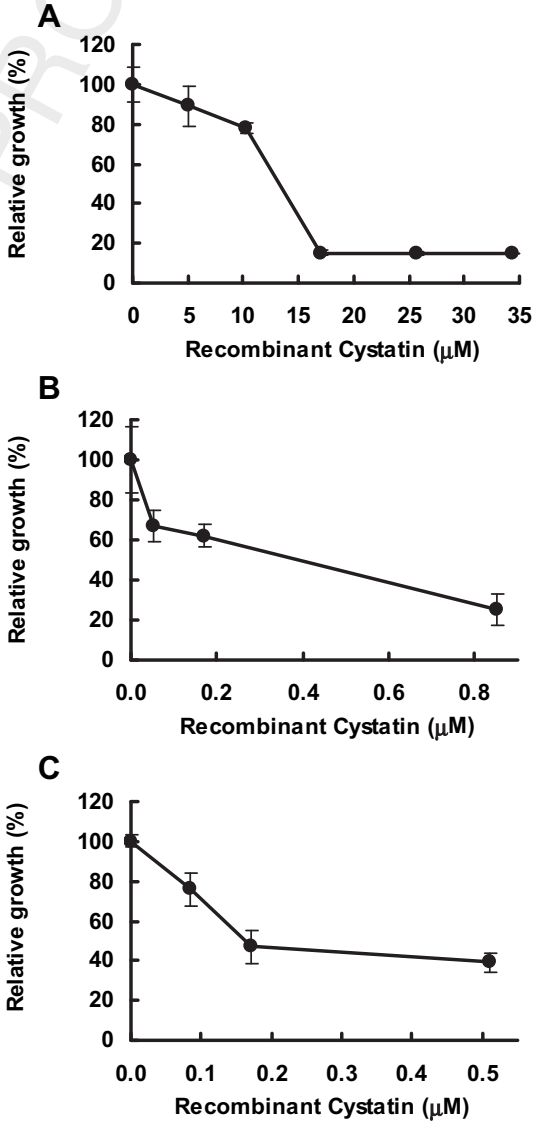

Fig. 3. Effects of AhCPI on the growth of *F. oxysporum* (A), *S. cepivorum* (B), and *R. solani* (C). Approximatelly one thousand spores from *F. oxysporum* and six pieces of sclerotinia from *R. solani* and *S. cepivorum* were inoculated in the absence and presence of different concentrations of the recombinant AhCPI. Fungal growth was monitored measuring absorbance at 492 nm and expressed as a percentage of growth in the absence of the cystatin. Data are mean values of three independent replicates and the standard errors are shown by vertical bars.

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variable. The highly homologous soybean (Ki 190 nM) and winter wheat (Ki 580 nM) cystatins had similar Ki values against papain, but the apple (Ki 1.2 nM) and strawberry (Ki 1.9 nM) cystatins showed Ki values two orders of magnitude lower [7,18,24,31]. Apple cystatin was a stronger inhibitor of ficin (Ki 3.2 nM) and bromelain (Ki 3.8 nM), while AhCPI was a weak ficin inhibitor (Ki 325 nM) and showed no activity against stem bromelain. In the inhibition of cathepsin L, the Ki for AhCPI was almost the same as corn cystatin I (17 nM), oryzacystatin I (51 nM) and oryzacystatin II (Ki 39 nM), whereas Ki values for *Phaseolus vulgaris* and kiwifruit cystatins were three (10 pM) and four orders (100 pM) of magnitude lower, respectively [1,6,28]. There is no information about the Ki values for cathepsin L with cystatins structurally related to AhCPI. However, data could not be strictly compared because the experimental conditions were different.

Here we demonstrated the ability of AhCPI to inhibit enzymatic activity, however, a more detailed analysis would require the excision of the polyhistidine tail, as it might interfere with the protein's function

2.3. Inhibition of fungal growth by recombinant AhCPI

The effect of purified AhCPI on the growth of three phytopathogenic fungi (*Fusarium oxysporum*, *Sclerotium cepivorum*, *and Rhyzoctonia solani*) was determined by in vitro bioassays. AhCPI inhibited the growth of the three fungi, but the inhibitory effect varied depending on fungal species. As shown in (Fig. 3A), the effective concentration for 50% growth inhibition was about 13 μM for *F. oxysporum*. This value is not in agreement with the activity reported by the barley and strawberry cystatins. In both species, EC50 values were always lower than 3 μM [2,18]. However, in the case of *S. cepivorum*, and *R. solani*, 380 nM and 160 nM concentrations were enough to obtain 50% growth inhibition, respectively. In those cases AhCP showed stronger antifungal activity, as demonstrated by the activity showed at lower concentrations than the levels reported for other PhyCys on different fungal species [2,7,18,21,35].

The toxic effects that the recombinant AhCPI exerted on the growth of fungi were also monitored by microscopic observations. In the control (without AhCPI), we observed the germination of spores and the development of hyphae (Fig. 4). The mycelial growth of *F. oxysporum* was weakly diminished at 10 μM of AhCPI, while 2.5 and 5.0 μM of AhCPI produced a strong mycelial growth inhibition in *S. cepivorum* and *R. solani*, respectively. Stronger spore germination inhibition was also observed in *F. oxysporum*, *S. cepivorum* and *R. solani* at 17.0 μM, 5.0 μM and 10.0 μM of AhCPI, respectively. These results clearly indicate that the AhCPI has antifungal activity. To further understand the effect of AhCPI on the inhibition of mycelium growth, crude protein samples were extracted from mycelium of fungal cultures. These extracts were assayed for proteolytic activity on 0.1% gelatin SDS-PAGE showing a single band of hydrolytic activity for *F. oxysporum*, whereas three bands were detected for *R. solani* and two bands for *S. cepivorum* (Fig. 5 lane 0). The high apparent molecular masses found agree with previous

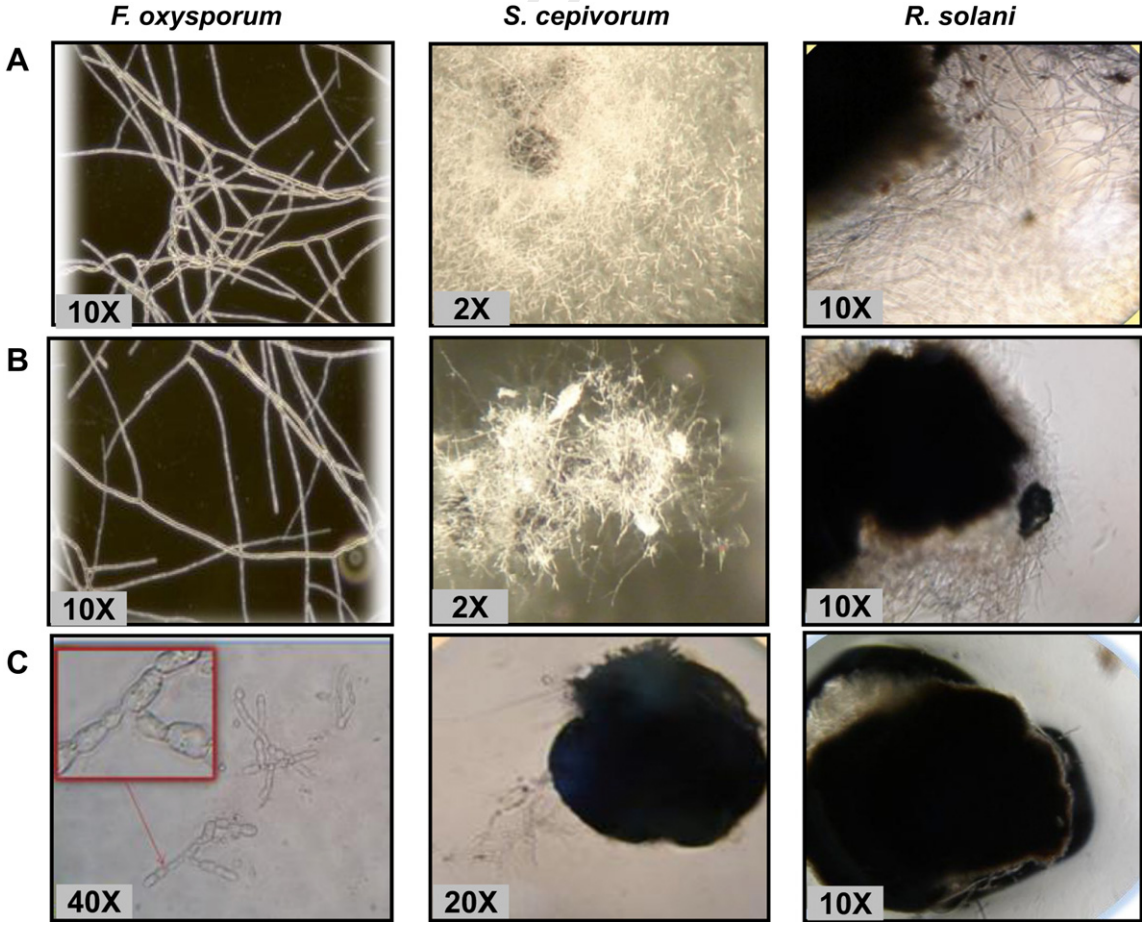

Fig. 4. Microscopic photographs under light microscopy. (A) Fungal growth control without AhCPI, (B) Mycelial growth inhibition: *F. oxysporum* (10 μM), *S. cepivorum* (2.5 μM), and *R. solani* (5 μM). (C) Spore germination inhibition of *F. oxysporum* (17.0 μM), *S. cepivorum* (5.0 μM) and *R. solani* (10.0 μM).

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reports of this kind of enzymes in other fungi [26]. Mycelial proteolytic activity of *S. cepivorum* was very low. Larger amount (23 μg) of the protein crude extract was required to detect proteolytic activity at pH 4.5. The results showed that crude protein samples from *F. oxysporum* and *R. solani* mycelium contain cysteine proteinases which were inhibited by E-64. On the contrary, the proteolytic activity detected for *S. cepivorum* was not inhibited by E-64 (data not shown). On the other hand, AhCPI was a weak inhibitor of the endogenous proteolytic activity of fungi. The addition of

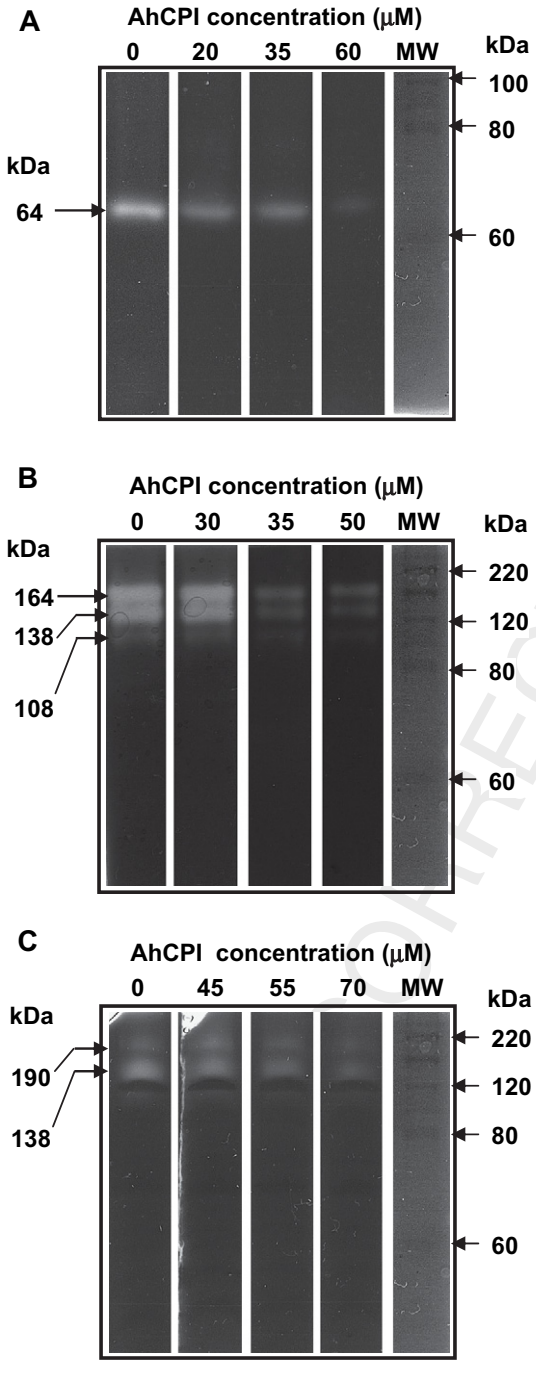

Fig. 5. Inhibition of recombinant AhCPI on fungal proteolytic activity. Crude protein extracts obtained from mycelium of (A) *F. oxysporum* (1 μg), (B) *R. solani* (4 μg) and (C) *S. cepivorum* (23.0 μg) were used to react with different concentrations of recombinant AhCPI for 10 min at 37 °C. Then, proteolytic activity was analyzed on 0.1% gelatin SDS-PAGE. The concentrations of AhCPI used are indicated above each lane. Molecular Markers (kDa) are indicated. The gels were stained with Coomassie Brillant Blue G.

20 μM, 30 μM and 45 μM of recombinant AhCPI produced a slight decrease in the proteolytic activity detected in *F. oxysporum*, *R. solani* and *S. cepivorum*, respectively (Fig. 5). The cystatin concentration required to arrest the mycelial proteolytic activity was very high compared to the concentration that inhibit fungal growth, suggesting that the antifungal activity of AhCPI is probably not mediated by the inhibition of fungal proteinases, as it has been demonstrated to occur for the chestnut [27] and taro cystatins [41]. One possibility would be that AhCPI is directed to extracelullar proteolytic enzymes. However, more detailed studies are required to explain the high antifungal activity shown by AhCPI. In a recent report it was shown that the antifungal effect of the barley cystatin (Hv-CPI) and its derived mutants does not correlate with their activities as proteinase inhibitors [21]. This is supported by evidence presented by Wang et al. with a cystatin from taro [39].

Tarocystatin from *Colocasia esculenta*, a group-2 phytocystatin is composed of a highly conserved N-terminal region, which is homologous to group-1 cystatin, and a repetitive peptide at the C-terminus. The kinetic analysis of the N-terminal region, the C-terminal and the full length peptide on papain activity allowed them to propose an inhibitory mechanism of group-2 PhyCyst. The full length peptide was a stronger papain inhibitor than the N-terminal peptide while the C-terminal lacks inhibitory activity. By contrast, the antifungal activity of the N-terminal peptide appeared to be greater than that of the full length peptide, and the C-terminal peptide showed no antifungal activity, indicating that the antifungal effect is not related to proteinase inhibitory activity.

It has been suggested that alterations in fungal membrane permeability could be the origin of the antifungal properties of the proteinase inhibitors [11]. Therefore, a future study, which provides more detailed analysis, will be necessary to reveal the mechanisms of antifungal activity demonstrated by cystatins. In previous work we showed that AhCPI mRNAs increase in response to heat, cold, water deficit and salinity treatments [37]. It is possible that AhCPI expression could be positively correlated with the known tolerance to arid and/or soil salinity conditions reported for field grown *A. hypochondriacus*. The overall results reported here indicate that AhCPI may be part of a mechanism designed to increase resistance to both biotic and abiotic stress in amaranth.

3. Conclusion

This work describes the cloning, expression, purification and characterization of the recombinant amaranth cystatin. This cystatin was expressed in very high levels in *E. coli*. The purified recombinant protein was an effective cysteine proteinase inhibitor and a strong fungal growth inhibitor. It was able to reduce the fungal growth of *F. oxysporum, S. cepivorum* and *R. solani*. However, AhCPI produced a very slight decrease in the proteolytic activity detected in mycelial fungi. It was suggested that antifungal activiy is not mediated by fungal proteinase inhibition. The overall results reported here indicate that AhCPI may be part of a mechanism designed to increase resistance to biotic stress in amaranth. Considering that amaranth cultivation has been restricted to a few specific areas, it represents a potential source of genes for pest control.

4. Methods

The cDNA encoding the amaranth cystatin (Gen-Bank, accession number DQ792503) was previously isolated by our group [37]. The pQE-2 vector and the M15 (pREP 4) strain of *E. coli* were provided by Qiagen. Immobilized nickel resin was supplied by Invitrogen. Papain, ficin, bromelain, cathepsin B, cathepsin L, DTT, DTE and the substrate BANA were purchased from Sigma. The substrate Z-Phe-

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Arg-p-nitroanilide for cathepsin L was provided by Alexis Biochemicals, Life Sciences, Inc. Fungal strains of *F. oxysporum, S. cepivorum* and *R. solani* were provided by the laboratory of Biochemical Ecology from Cinvestav.

4.1. Construction of the expression vector

The open reading frame coding for the AhCPI inhibitor, without putative signal peptide, was amplified by PCR. The oligonucleotide used as forward primer was: 5′-AATTCCATATG**CAG**GCTACTCTTGG TGGGTTACGT-3′ which incorporated an NdeI restriction site (underlined) and the substitution of the ATG start codon by a glutamine codon (CAG bolded). This last change was performed in order to introduce a stop dipeptidyl aminopeptidase I enzymatic activity to be used in combination with glutamine cyclotransferase in the future to remove the histidine tail, added to the recombinant protein in the pQE-2 vector. The reverse primer was: 5′-AA**CTGCAG** GCATTACTGGTTCTCAATCTC-3′ which added a PstI restriction site (underlined) at the 3′end of the amplified fragment. Briefly, 10 ng of template DNA, 200 mM each dNTP, 1× PCR buffer (20 mM Tris−HCl, 1.5 mM $MgCl_2$, and 50 mM KCl (pH 8.4)), 20 pmol of each primer, and 1 U of Platinum DNA polymerase (Roche Mannheim, Germany) were used in a 50 μl reaction. The temperature protocol began with a heating temperature of 95 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 60 °C and 1 min at 72 °C. The amplification product was cloned in frame in the restriction sites NdeI and PstI of pQE-2. Chemically competent *E. coli* M15 (pREP 4) cells were transformed using the described construct. The recombinant clones were sequenced on an ABI Prism 377 DNA sequencer using the Big Dye Terminator v 3.1 Cycle Sequencing kit (Applied Biosystems).

4.2. Expression of recombinant cystatin

Transformed cells were grown at 37 °C in Super Broth medium, containing 100 μg mL^{-1} of carbenicillin and 25 μg mL^{-1} of kanamycin, under agitation until they reached an OD_{600} of 0.5. Expression was then induced by the addition of 0.1 mM IPTG. Aliquots of the cell culture taken at 1 h intervals after up to 5 h of induction were harvested by centrifugation. The pellets, suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, (pH 8.0)) were frozen and subjected to sonication for cell disruption. The lysates were centrifuged at 13,000 × g at 4 °C for 15 min, soluble and insoluble fractions were analyzed in SDS-PAGE 12% according to the method of Laemmli [15].

4.3. Mass spectrometry identification

The identity of the IPTG-induced recombinant cystatin was determined by PMF using Maldi-ToF Mass Spectrometry (Ettan, Amersham Biosciences). The induced protein band recovered from the gel was digested by trypsin (EC 3.4.21.19), and the peptide mixture was analyzed by mass spectrometry. For identification, we used the SWISS-PROT and TrEMBL databases with publicly availble software (Profound, MASCOT and PepIdent (http://www.expasy.ch/toolspeptide.html)).

4.4. Purification of recombinant cystatin by affinity chromatography

AhCPI was purified using an affinity nickel resin column, previously equilibrated with lysis buffer. The soluble fraction was added and the column was washed with the same buffer. Proteins were eluted with lysis buffer containing 250 mM imidazole. Different protein fractions and purified product was analyzed on SDS-PAGE.

Homogeneity of the purified cystatin was determined by 2D electrophoresis. A 7 cm IPG strip (pH 3−10) was rehydrated overnight at 22 °C with 125 μL of isoelectric focusing buffer (7 M urea, 2 M thiourea, 2.65% CHAPS, 0.31% w/v DTT, 0.5% w/v pharmalyte pH 3−10) in which 5 μg of AhCPI were previously dissolved. IEF was conducted at 22 °C with an IPGphor (Amersham BioScience), applying 10000 Vh. The focused strip was equilibrated twice for 15 min. The first equilibration was performed in a solution containing 6 M urea, 30% w/v glycerol, 2% w/v SDS, 1% w/v DTT, and 50 mM Tris−HCl buffer, (pH 8.8). The second equilibration solution was modified by the replacement of DTT with 2.5% w/v iodoacetamide. Separation in the second dimension was performed by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue. The molecular mass of cystatin on gel was determined by co-electrophoresis of standard proteins markers (Amersham BioScience), pI was determined by migration of the protein spots on 7 cm IPG (pH 3−10) linear strip.

4.5. Inhibitory activity of the recombinant AhCPI

Purified cystatin was tested against papain (E.C.3.4.22.2), ficin (E:C.3.4.22.3), bromelain (E.C.3.4.22.32), and cathepsin B (E.C.3.4.22.1) essentially as described by Gaddour et al. [9], using BANA as substrate. The inhibitory activity against cathepsin L (E.C.3.4.22.15) was assayed according to a modification of the method of Rawlings and Barret [29], using Z-Phe-Arg-p-nitroanilide as substrate. This assay was performed in a 96-well microtiter plate. The reaction mixture (180 μl per well) consisted of 15 μl of cathepsin L (10 μM), the volume of substrate stock solution required for a final concentration of 480 μM, and reaction buffer. The buffer used was 50 mM acetate buffer containing 10 mM EDTA and 2.5 mM DTT (pH 6.0). Before addition of the substrate to the reaction mixture, the buffer and the cathepsin L were incubated for 15 min at 37 °C to activate the enzyme. After addition of the substrate, the mixture was incubated at 37 °C for 15 min. The reaction was monitored at 410 nm using a 680 model Benchmark plus microplate reader provided with Microplate Manager software Versión 5.2.1 (Bio-Rad). Rates were calculated by linear regression of the data. As a control for possible spontaneous release of *p*-nitroaniline, assays were performed with substrate and buffer only; as a background activity control. For inhibition assays, we used the same assay procedures, except that before addition of the substrate, the enzymes were incubated with the corresponding inhibitor for 10 min at 37 °C. In all assays the enzyme concentrations were calculated by active-site titration with E-64 [5]. Protein concentration was quantified by using the BioRad kit with bovine albumin as standard. Inhibition was expressed as % of remaining protease activity compared to controls without inhibitor. Ki values were determined from plot (1/v versus [I]) [8].

4.6. Growth inhibition assay of phytopathogenic fungi

For a survey of the antimicrobial toxicity of AhCPI, three phytopathogenic fungi were chosen for the growth inhibition assay: *F. oxysporum*, *S. cepivorum* and *R. solani.* They were used as models because of their pathogenicity against diverse plant species. Approximately 10^3 spores of *F. oxysporum* and six pieces of sclerotinia from *R. solani* and *S. cepivorum* were inoculated in 250 μl of 1/3 potato dextrose broth in the absence and presence of different concentrations of the recombinant amaranth cystatin. The incubation was carried out in sterile microtiter plate. *F. oxysporum* and *R. solani* were grown at 28 °C for 48 h, and *S. cepivorum* was grown at 24 °C for 72 h. Fungal growth was monitored by measuring absorbance at 492 nm and by microscopic observations. Results were expressed as the percentage of relative growth in the absence

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of the AhCPI. Experiments were repeated at least three times with similar results.

4.7. Inhibition of fungal proteinases

An inhibition test of AhCPI on fungal cysteine proteinase activity was carried out as follows: fungi were grown in PDB medium for 48 h (*F. oxysporum* and *R. solani*) or 72 h (*S. cepivorum*) at the same conditions described before. Mycelium was recovered by centrifugation and ground to a fine power with liquid nitrogen. The soluble proteins were extracted by shaking for 30 min at 4 °C with 50 mM phosphate buffer, containing 10 mM EDTA and 2.5 mM DTT (pH 6.0) (1:3 wt/vol). The homogenate was centrifuged at 12 000 × g for 30 min at 4 °C and the supernatants were recovered. Protein samples of the mycelium extracts were used to react with different concentrations of recombinant AhCPI and E-64 for 15 min at 37 °C. Then, proteolytic activity was analyzed on 0.1% gelatin SDS-PAGE according to the procedure described by Michaud et al. [23].

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