A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic *Arabidopsis* plants

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Abstract Cystatins from plants have been implicated in plant defense towards insects, based on their role as inhibitors of heterologous cysteine-proteinases. We have previously characterized thirteen genes encoding cystatins (HvCPI-1 to HvCPI-13) from barley (*Hordeum vulgare*), but only HvCPI-1 C68 → G, a variant generated by direct-mutagenesis, has been tested against insects. The aim of this study was to analyze the effects of the whole gene family members of barley cystatins against two aphids, *Myzus persicae* and *Acyrthosiphon pisum*. All the cystatins, except HvCPI-7, HvCPI-10 and HvCPI-12, inhibited in vitro the activity of cathepsin L- and/or B-like proteinases, with HvCPI-6 being the most effective inhibitor for both aphid species. When administered in artificial diets, HvCPI-6 was toxic to *A. pisum* nymphs (LC_{50} = 150 μg/ml), whereas no significant mortality was observed on *M. persicae* nymphs up to 1000 μg/ml. The effects of HvCPI-6 ingestion on *A. pisum* were correlated with a decrease of cathepsin B- and L-like proteinase activities. In the case of *M. persicae*, there was an increase of these proteolytic activities, but also of the aminopeptidase-like activity, suggesting that this species is regulating both target and insensitive enzymes to overcome the effects of the cystatin. To further analyze the potential of barley cystatins as insecticidal proteins against aphids, *Arabidopsis* plants expressing HvCPI-6 were tested against *M. persicae*. For *A. pisum*, which does not feed on *Arabidopsis*, a combined diet-*Vicia faba* plant bioassay was performed. A significant delay in the development time to reach the adult stage was observed in both species. The present study demonstrates the potential of barley cystatins to interfere with the performance of two aphid species.

Keywords Aphid · Artificial diet · Cathepsin · Cystatin · Cysteine-proteinase · Transgenic plant

Introduction

Aphids are important agricultural pests that cause damage by sucking the plant juices and act as vectors of over 100 plant viruses. Currently, the most widely available method to control aphid populations is the use of chemicals, but alternative methodologies such as plant genetic engineering offer new possibilities to enhance plant resistance to aphids. Among transgenes tested, lectins and proteinase inhibitors have been reported to promote deleterious effects on aphids (Gatehouse et al. 2003; Rahbé et al. 2003a,b; Dutta et al. 2005; Shahidi-Noghabi et al. 2009). In contrast, *Bacillus thuringiensis* δ-endotoxins exhibit low to moderate toxicity in terms both of mortality and growth rate (Lawo et al. 2009; Porcar et al. 2009). Among the over 250 species of aphids considered as serious pest for agriculture, the green peach aphid *Myzus persicae* (Sulzer) and the pea aphid *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) are probably two of the most intensively studied and adopted for laboratory and genetic studies. The rapid behavior response of these aphids on diets containing toxic compounds and the easy handling to perform bioassays in plants expressing insecticidal transgenes makes them useful models to study defense metabolites for phloem-feeding insects.

Aphids are strict phloem-feeding insects that feed exclusively on photoassimilates translocated in phloem sieve elements where the sap is particularly rich in carbohydrates and free/bound amino acids. Nonetheless, phloem sap may contain relatively high concentrations of proteins depending on plant species (Kehr 2006). To hydrolyze these proteins aphids require proteolytic enzymes and cysteine-like proteinases have been identified in several species of aphids, including *M. persicae* (Rahbé et al. 2003a) and *A. pisum* (Cristofoletti et al. 2003). Therefore, a strategy based on the use of cysteine proteinase inhibitors could protect crops against sap-sucking aphids.

Phytocystatins (PhyCys) are plant proteinaceous inhibitors of cysteine-proteinases of the papain C1A family integrated in an independent subfamily on the cystatin phylogenetic tree (Margis et al. 1998; Martinez and Diaz 2008). The cystatin inhibitory mechanism is produced by a tight and reversible interaction with their target enzymes. It involves a tripartite wedge formed by the partially flexible *N*-terminus containing a glycine residue and two hairpin loops carrying a conserved QxVxG motif and a tryptophan residue, respectively (Stubbs et al. 1990). In addition to these three motifs common to all cystatins, PhyCys contain a particular consensus sequence ([LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N) that conforms a predicted secondary α-helix structure (Margis et al. 1998). Most PhyCys are small proteins with a molecular mass in the 12–16 kDa range, but there are some with a molecular weight of 23 kDa. These PhyCys have a carboxy-terminal extension which has been involved in the inhibition of a second family of cysteine-proteinases, the C13 legumain peptidases (Martinez et al. 2007; Martinez and Diaz, 2008). Several 85–87 kDa multicystatins, with eight cystatin domains, have also been described (Girard et al. 2007; Nissen et al. 2009). From a functional viewpoint, PhyCys have been implicated in regulation of the protein turn-over and as defense proteins. The defense role has been inferred from the ability of PhyCys to inhibit digestive proteinases from herbivorous arthropods in vitro, in artificial diets as well as by bioassays on transgenic plants over-expressing PhyCys genes (Pernas et al. 1998; Álvarez-Alfageme et al. 2007). Anti-mite activities and deleterious effects against phytopathogenic fungi and viruses have also been described for these inhibitors (Gutierrez-Campos et al. 1999; Pernas et al. 2000; Martinez et al. 2003; Abraham et al. 2006).

The insecticidal properties of PhyCys against aphids have been previously reported. Several studies have shown that the oryzacystatin I (OC-I) from rice or a derived variant added into artificial diets affect growth and significantly alter demographic parameters of several aphids (Cowgill et al. 2002; Rahbé et al. 2003a; Azzouz et al. 2005). Moreover, the stable expression of the oryzacystatin gene in oilseed rape and eggplant produced negative impact on aphid population growth (Rahbé et al. 2003a; Ribeiro et al. 2006). In contrast, no effects were observed in aphids fed with potato plants expressing chicken egg white cystatin (CEWc) (Cowgill et al. 2002).

The aim of this study was to analyze the effects of the whole gene family members of barley (*Hordeum vulgare* L.) cystatins against two aphids, *M. persicae* and *A. pisum*, which rely on cysteine-like proteinases for digestion. We have characterized further the proteinase activities in protein extracts from these two species and determined the in vitro inhibitory

effects and specificity of the barley cystatins (HvCPI-1 to HvCPI-13). In vivo assays by feeding *M. persicae* and *A. pisum* aphids on artificial diets containing the most effective inhibitor for both aphid species (HvCPI-6) have been performed to study toxicity patterns. In addition, transgenic *Arabidopsis* plants expressing the cystatin HvCPI-6 have been prepared to determine their effects on the viability, development and reproduction of *M. persicae* after feeding transgenic lines. A combined diet-plant bioassay has also been designed to study the HvCPI-6 cystatin effects on *A. pisum*, an aphid species that does not feed on *Arabidopsis* plants.

Materials and methods

Insects

Laboratory colonies of the aphids *M. persicae* and *A. pisum* were reared on potato (*Solanum tuberosum* L.) and bean (*Phasoleus vulgaris* L.) plants, respectively, in environmental chambers at 25°C, 70% relative humidity and 16/8 day/night photoperiod. Aphids were homogenized in 0.15 M NaCl (60 mg/ml), centrifuged at 10,000 *g* for 5 min and the supernatants pooled and stored frozen (−20°C) to obtain soluble protein extracts for enzymatic activity assays.

Enzyme activity assays

A series of overlapping buffers were used to generate a pH range from 2 to 11. Reaction buffers for the characterization of aphid proteinases were 0.1 M citric acid NaOH (pH 2.0–3.0), 0.1 M citrate (pH 3.0–6.0), 0.1 M phosphate (pH 6.0–7.0), 0.1 M Tris–HCl (pH 7.0–9.5) and 0.1 M glycine-NaOH (pH 9.5–11.0). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$.

Hydrolysis of substrates containing the AMC (7-amino-4-methyl coumarin) fluorophore was carried out in microtiter plate format. The standard assay volume was 100 µl, using 5 µg of protein extract prepared from *M. persicae* and *A. pisum* adults and the corresponding substrate added to a final concentration of 0.2 mM. Cathepsin B-like and L-like activities were assayed using Z-RR-AMC (*N*-carbobenzoxy-Arg-Arg-7-amido-4-methylcoumarin) and Z-FR-AMC (*N*-carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin) substrates, respectively, and a buffer containing 8 mM DTT. Trypsin-like activity was assayed using Z-R-AMC (*N*-carbobenzoxy-Arg-7-amido-4-methyl coumarin), chymotrypsin-like activity using SAAPP-AMC (Suc-Ala-Ala-Pro-Phe-7-amido-4-methyl coumarin), elastase-like activity using MeOSAAPV-AMC (MeOSuc-Ala-Ala-Pro-Val-7-amido-4-methyl coumarin) and leucine aminopeptidase-like using L-AMC (Leu-7-amido-4-methyl coumarin). The reaction was incubated at 30°C and emitted fluorescence measured with a 365 nm excitation wavelength filter and a 465 nm emission wavelength filter. The system was calibrated with known amounts of AMC hydrolysis product in a standard reaction mixture.

Cathepsin D-like activity was assayed in 0.5 ml of reaction buffer containing 30 µg of protein extract, using 0.2% hemoglobin as described by Novillo et al. (1997). Carboxypeptidases A and B-like activities were assayed in 0.5 ml of reaction buffer containing 30 µg of protein extracts, using 1 mM HPA (hippuryl-phenylalanine) for carboxypeptidase A-like and 1 mM HA (hippuryl-L-arginine) for carboxypeptidase B-like as described by Ortego et al. (1996).

The proteolytic activities of *M. persicae* and *A. pisum* extracts were assayed for characterization purposes in the presence of the following specific inhibitors: the cysteine-proteinase inhibitor E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butano), the two serine proteinase inhibitors STI (soybean trypsin inhibitor) and SBBI (soybean Bowman-Birk inhibitor), the aspartyl proteinase inhibitor Pep-A (pepstatin-A), the carboxypeptidase inhibitor PCPI (potato tuber carboxypeptidase inhibitor), and the two heavy metals $CdCl_2$ and $CuCl_2$ as inhibitors of aminopeptidases and other metalloproteinases. Proteinase inhibitors were incubated at 30°C for 15 min with the extracts prior to adding the substrate. All compounds were added to a final concentration of 0.2 µM, except PCPI, $CuCl_2$ and $CdCl_2$ that were added to a final concentration of 0.02 µM.

Total protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as a standard. All assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of substrates.

Zymogram analysis

Electrophoretic detection of proteolytic forms of aphid extracts was performed using 0.1% (w/v)

gelatin-containing 0.1% (w/v) SDS–12% (w/v) polyacrylamide slabs gels according to Lantz and Ciborowski (1994). The acrylamide to bis-acrylamide ratio was 37.5:1. Protein extracts from *M. persicae* and *A. pisum* (10–15 μg of total protein) were diluted into two-fold in electrophoresis sample buffer containing 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 0.05% (w/v) bromophenol blue, and subjected to electrophoresis using a Bio-Rad Mini-Protein II Electrophoresis Cell System. After migration at 4°C, gels were transferred into a 2.5% (v/v) aqueous solution of Triton X-100 for 30 min at room temperature, to allow restoration of the proteinases. Gels were then placed in an activation buffer (0.1 M phosphate pH 6.0 with 10 mM cysteine) for 20 h at 30°C. Proteolysis was stopped by transferring the gels into a staining solution 0.3% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid. The gels were destained in 25% (v/v) methanol and 10% (v/v) acetic acid.

Purification of recombinant cystatins and inhibitory activity against aphid extracts

The cDNA fragments spanning the whole cystatin ORFs, aside from their signal peptide sequences, were inserted in frame into the expression vector pRSETB (Invitrogen). The recombinant plasmids for barley HvCPI-1 to HvCPI-13 cystatins were produced and introduced in *E. coli* as described in Martinez et al. (2009). The fusion proteins with a histidine tail were purified using a His-Bind Resin (Novagen) following the manufacturer's instructions, and purification checked by SDS–PAGE.

For inhibitory enzymatic activity assays, cathepsin B-like and L-like activities were assayed using substrates containing the AMC fluorophore as previously described, using a buffer containing 100 mM sodium phosphate pH 6.0, 10 mM L-cysteine, 10 mM EDTA and 0.01% (v/v) Brij35, and adding18 μg and 30 μg of soluble protein extracted from *M. persicae* and *A. pisum*, respectively. The cystatins, at a final concentration of 0.4 μg were incubated at 30°C with the protein extracts for 10 min, prior adding the substrate at a final concentration of 0.2 mM. Results were expressed as a percentage of proteinase activity relative to that in the absence of the inhibitor. All assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of substrates.

Artificial diet bioassay

Reproductive adults of *M. persicae* and *A. pisum* from the laboratory colonies were transferred to fresh potato and bean leaves placed in Petri dishes and allowed to produce nymphs for 12 h. Experimental arenas consisted in sachets containing 200 μl of artificial diet as described by Shahnaz et al. (2008). Newborn nymphs were then brushed carefully onto sachets containing 0–1000 μg of HvCPI-6 per ml of artificial diet. Fifteen nymphs were confined in each sachet and nymphal survival was scored after 3 days. Abbott's correction for natural mortality was applied (Abbott 1925). Three to six sachets were used per treatment and held in a growth chamber at 25°C, 70% relative humidity and a 16/8 day/night photoperiod for the entire duration of the experiment.

Plasmid constructs and plant transformation

The ORF of the barley cystatin *Icy6* gene encoding the mature HvCPI-6 protein (from Ala23 to Gly124) devoid of its signal peptide sequence was amplified using exact match primers containing *Bam*HI restriction sites to facilitate the cloning of the sequence between the CaMV35S promoter and the NOS terminator of the binary pROKII vector (Baulcome et al. 1986). The resultant plasmid was used to transform *Arabidopsis thaliana* plants from ecotype Columbia (Col-0) by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). T_1 seeds from the transformed plants were harvested and plated on MS-medium, containing 50 μg/ml kanamycin to select 35S-*Icy6*-plants. Kanamycin-resistant seedlings were transplanted to soil and allowed to set seed. T_2 seeds were harvested from them and subjected to a second round of kanamycin selection and again transplanted to soil. T_3 seeds were harvested and subjected to kanamycin selection to determine if T_2 plants were homozygous.

Nucleic acid analysis

Total DNA was isolated from transformed and control *Arabidopsis* leaves essentially as described by Taylor and Powell (1982) and tested for the

presence of the *Icy6* cystatin gene by PCR using the primer pairs, araIcy6-forward: 5′-CACTATCCTTC GCAAGACC-3′ and araIcy6-reverse: 5′-CGAGGT ACCTTAGCCGCCGGCAGCCGG-3′. The reaction products were separated on 1% agarose electrophoretic gels.

For real-time quantitative RT–PCR (qRT–PCR) studies, transformed and control leaves of *Arabidopsis* were collected and frozen into liquid N_2 and stored at -80°C until used for RNA extraction. Total RNA was extracted by the phenol/chloroform method, followed by precipitation with 8 M LiCl (Oñate-Sanchez and Vicente-Carbajosa 2008). cDNAs were synthesized from 2 μg of RNA using the High-capacity cDNA reverse transcription kit (Applied Biosystems) following manufacturer's instructions. qRT–PCR analyses were performed for duplicated samples by means of a 7,300 Real-Time PCR System (Applied Biosystems) using a SYBR Green detection system Quantification was standardized to *Arabidopsis* ubiquitin mRNA levels (At5g25760). The primers used for PCR amplification were:

- qrtIcy6-forward: 5′-ATCTACGAGCACTGGAG CAGGA-3′;
- qrtIcy6-reverse: 5′-CAGATGAATTTAACCGG CAGC-3′;
- AtUbi-forward: 5′-GCTCTTATCAAAGGACCT TCGG-3′;
- AtUbi-reverse: 5′-CGAACTTGAGGAGGTTGC AAAG-3′.

Inhibitory activity of protein extracts of *Arabidopsis* plants expressing the barley cystatin

Total protein extracts from leaves were obtained from transformed and control *Arabidopsis* plants. Leaves were ground and resuspended in 0.15 M NaCl sodium phosphate pH 6.0, 2 mM EDTA for 1 h at 4°C and treated as described Álvarez-Alfageme et al. (2007). Inhibitory activity of plant protein extracts was in vitro tested against 10 ng of commercial papain (EC 3.4.22.2) from Sigma using Z-FR-AMC as substrate. Similarly, inhibitory assays against 10 μg of protein extracts prepared from *M. persicae* were also performed using the substrates Z-RR-AMC and Z-FR-AMC, following procedures described above.

Bioassays with aphids feeding on plants expressing the barley cystatin

Prior to the assays, *M. persicae* colony was adapted to live on *Arabidopsis* for several generations. To study aphid development, reproductive *M. persicae* adults from the laboratory rearing were transferred to fresh 4-week old *Arabidopsis* plants and allowed to produce nymphs for 12 h. Newborn nymphs were placed on test plants (ten aphids per plant and ten plants of each *Arabidopsis* line: transgenic lines L5.4 and L9.8, and the non transformed line Col-0) and observed daily until they reached adulthood, being these adults removed daily from the plant. To study aphid reproductive rate, last instar nymphs from the laboratory colony were transferred to fresh *Arabidopsis* plants. Newly emerged adults (<24 h old) were placed on test plants (seven aphids on each plant and twenty replicates of each *Arabidopsis* line) and the number of nymphs produced scored after 7 days. The period was limited to 7 days to avoid the first generation nymphs to reach the adult stage and start producing second generation nymphs. In both assays, plants were confined and maintained in controlled conditions of 25°C, 70% relative humidity and a 16/8 day/night photoperiod.

Combined diet-plant feeding bioassays

Due to the fact that *A. pisum* aphids do not feed on *Arabidopsis* plants, a combined diet-plant bioassay was performed. Aphids were fed for 1 day on artificial diet incorporating the cystatin and subsequently transferred to *Vicia faba* plants until the end of the assay.

To study effects on development, newborn nymphs from the laboratory colony were brushed carefully onto sachets containing 400 μg of HvCPI-6 or HvCPI-12 per ml of artificial diet for 1 day (ten aphids per sachet and ten replicates per treatment) and subsequently placed on bean plants until they reached adulthood. Emerging adults were counted daily and separated from the plant. To study effects on reproductive rate, newly emerged adults from the laboratory colony were fed on artificial diet for 1 day and subsequently placed on bean plants, where the produced nymphs were scored after 7 days. For this experiment, seven aphids were placed on each diet-plant combination and ten replicates of every

treatment were done. Plants were confined and maintained in controlled conditions of 25°C, 70% relative humidity and a 16/8 day/night photoperiod.

Molecular modeling of cystatin-cathepsin interaction

Homology-based three-dimensional models were obtained by the automated SWISS-MODEL program (Bordoli et al. 2009). The molecular models of HvCPI-6 and HvCPI-12 were built using the NMR structure of oryzacystatin-I as a template (Protein Data Bank identifier 1EQK). The three-dimensional structures of the cathepsin B and L proteins from *A. pisum* (accession numbers NP_001119608 and NP_001156569) were modeled using as templates the structures of the human cathepsin B (3PBH) and the human cathepsin L (1CS8), respectively. The template structures were selected on the basis of highest sequence similarities. The complexes formed by aphid cathepsins and barley cystatins were modeled by superimposing the structures of the inhibitors and cathepsins onto the three-dimensional structures of the stefin A–cathepsin B complex (1K9 M) for aphid cathepsin B, and stefin B-papain complex (1STF) for aphid cathepsin L, using the fit routines of the SWISS-PDB viewer program (Guex and Peitsch 1997).

Statistical analysis

Development, reproductive rate and proteinase activities of aphids were analyzed by means of Dunnett two-tailed-test. Differences between treatments were considered significant at the $P < 0.05$ level. Mortality data were used to determine the lethal concentration for 50% mortality (LC_{50}) by probit analysis using the computer program POLO-PC (LeOra Software, Berkeley, CA).

Results

Characterization of *M. persicae* and *A. pisum* proteinase activities

The proteolytic activities of *M. persicae* and *A. pisum* were characterized using whole aphid crude protein extracts from both aphid species. We analyzed the optimum pH, specific activity and effect of different proteinase inhibitors on the hydrolysis of specific substrates (Table 1). The two aphids presented proteolytic activities of different mechanistic classes, having in common cathepsin B- and L-like and leucine aminopeptidase-like activities. These three types of proteolytic activities were detected throughout the range from pH 3 to 7. Cathepsin B-like activity showed a maximum peak of activity at pH 6.5 for both species and was highly inhibited by E-64, a cysteine specific proteinase inhibitor. The hydrolysis of cathepsin L-like substrate gave two peaks of optimum activity at 3.0 and 5.5 for *M. persicae* and at pH 3.5 and 6.0 for *A. pisum*, which were also completely inhibited by E-64. Pepstatin A, STI, $CdCl_2$ and $CuCl_2$ presented minor inhibition of these cysteine-proteinase activities. On the contrary, leucine aminopeptidase-like activity presented the maximum proteolytic capability at pH 7.0 and was only inhibited by $CdCl_2$ or $CuCl_2$.

In addition, aspartic proteinase cathepsin D-like activity was specifically found in *M. persicae* protein extracts, while carboxypeptidases A- and B-like activities were only detected in extracts from *A. pisum*. Cathepsin D-like optimal activity occurred at pH 3.0 and it was specifically inhibited by pepstatin-A. The carboxypeptidases A- and B-like showed the highest activity at pH 7.0 and 8.0, respectively, and were almost completely inhibited by $CuCl_2$ and PCPI. No hydrolysis of trypsin-, chymotrypsin-, and elastase-like substrates was detected in the two aphid extracts, even after 24 h of incubation (data not shown).

Inhibitory properties of the barley cystatins against proteolytic activities of aphid pests

Crude protein extracts from the aphids were prepared to perform in vitro inhibition assays with the 13 recombinant barley cystatins, using the Z-RR-AMC and Z-FR-AMC substrates susceptible to be degraded by cathepsin B- and L-like, respectively. As is shown in Table 2, proteolytic activities in the two aphids were differentially inhibited by the barley cystatins. In general, the in vitro inhibition profile indicated a higher susceptibility of cathepsin L-like proteinases than the B-like proteinases to be inhibited by barley cystatins. The recombinant cystatin HvCPI-6 resulted particularly efficient and reduced about 70% and 50% cathepsin L- and B-like activities, respectively, in both aphids. Similarly, the HvCPI-5 and HvCPI-9 barley proteins also inhibited cathepsin L-and B-like

Table 1 Proteolytic activities of *M. persicae* and *A. pisum* against specific substrates and effect of proteinase inhibitors at their optimum pH

Proteinase	Optimum pH[a]	Specific activity[b]	% Inhibition[c]				
			Pep-A (0.2μM)	E-64 (0.2μM)	STI (0.2μM)	PCPI (0.02μM)	$CdCl_2$ (0.02μM)
(a) *M. persicae*							
CTB	6.5	1.3 ± 0.1	21 ± 2	99 ± 1	23 ± 7	ne	ne
CTL	5.5	1.3 ± 0.2	20 ± 4	98 ± 1	ne	ne	ne
	3.0	0.5 ± 0.1	ne	99 ± 1	ne	ne	26 ± 9
CTD	3.0	20.5 ± 0.1	48 ± 4	ne	ne	ne	ne
TRY		nd	–	–	–	–	–
CHY		nd	–	–	–	–	–
ELA		nd	–	–	–	–	–
LAP	7.0	2.5 ± 0.1	ne	ne	ne	ne	59 ± 3
CPA		nd	–	–	–	–	–
CPB		nd	–	–	–	–	–
Proteinase	Optimum pH[a]	Specific activity[b]	% Inhibition[c]				
			Pep-A (0.2μM)	E–64 (0.2μM)	SBBI (0.2μM)	PCPI (0.02μM)	$CuCl_2$ (0.02μM)
(b) *A. pisum*							
CTB	6.5	4.5 ± 0.4	ne	84 ± 1	ne	ne	ne
CTL	6.0	15.9 ± 0.6	ne	92 ± 1	ne	ne	ne
	3.5	20.5 ± 0.1	ne	86 ± 3	ne	ne	21 ± 2
CTD		nd	–	–	–	–	–
TRY		nd	–	–	–	–	–
CHY		nd	–	–	–	–	–
ELA		nd	–	–	–	–	–
LAP	7.0	13.9 ± 2.0	ne	ne	ne	ne	81 ± 2
CPA	7.0	14.2 ± 0.8	ne	ne	ne	94 ± 2	99 ± 1
CPB	8.0	26.6 ± 0.1	ne	ne	ne	93 ± 2	98 ± 1

Proteinase type abbreviations: *CTD* cathepsin D, *CTB* cathepsin B, *CTL* cathepsin L, *TRY* trypsin *CHY* chymotrypsin, *ELA* Elastase, *LAP* leucine aminopeptidase, *CPA* Carboxypeptidase A and *CPB* Carboxypeptidase B

[a] pH measurements were taken in the range 2.0–11.0, with increments of 0.5 pH units

[b] Specific activities as nmoles of substrate hydrolyzed/min/mg protein, except for proteolytic activity against hemoglobin as mU Δ Abs 280 nm/min/mg protein. Data are the mean ± SE of triplicate measurements. No activity detected (nd)

[c] Inhibitor abbreviations: *Pep-A* pepstatin A, *E-64* L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, *STI* soybean trypsin inhibitor, *SBBI* soybean Bowman-Birk inhibitor, and *PCPI* potato tuber carboxypeptidase inhibitor. Values are mean ± SE of triplicate measurements treated with the inhibitor versus its corresponding controls without it. No effect (ne) was considered for inhibition under 20%. No analyzed (–)

activities in the aphids, although in a weaker way. On the contrary, no inhibition was detected when HvCPI-7, Hv-CPI-10 and HvCPI-12 proteins were used.

Feeding results using artificial diets

The strong effect of the inhibitor HvCPI-6 on cysteine-proteinase activities from both aphids observed in vitro was further investigated in vivo by feeding assays of *M. persicae* and *A. pisum* aphids on artificial diets (Fig. 1a, b). *A. pisum* nymphs reared for 3 days on diets containing 10–300 μg/ml of the protein HvCPI-6 exhibited significantly higher mortality compared to nymphs reared on the control diet. The percentage mortality reached up to 80% when *A. pisum* were fed with 300 μg/ml cystatin. As is shown in the insert of Fig. 1a, the effective HvCPI-6

Table 2 In vitro inhibitory activity of the recombinant barley cystatins (HvCPI-1 to HvCPI-13) against cathepsin L (CTL) and cathepsin B (CTB) like activities in *M. persicae* and *A. pisum*

Cystatin	% Inhibition[a]			
	M. persicae		*A. pisum*	
	CTL	CTB	CTL	CTB
HvCPI-1	49.8 ± 4.2	ne	34.9 ± 1.1	31.2 ± 6.0
HvCPI-2	38.4 ± 3.0	ne	41.9 ± 2.4	ne
HvCPI-3	29.2 ± 3.6	ne	33.7 ± 1.9	ne
HvCPI-4	27.0 ± 7.0	ne	ne	ne
HvCPI-5	40.2 ± 3.1	26.5 ± 11.3	56.1 ± 3.6	51.8 ± 5.5
HvCPI-6	70.3 ± 3.3	47.2 ± 10.9	74.1 ± 1.0	52.2 ± 3.2
HvCPI-7	ne	ne	ne	ne
HvCPI-8	ne	ne	ne	41.8 ± 5.5
HvCPI-9	21.3 ± 0.5	21.6 ± 2.0	39.0 ± 4.8	40.6 ± 5.2
HvCPI-10	ne	ne	ne	ne
HvCPI-11	ne	22.4 ± 3.2	30.1 ± 4.1	ne
HvCPI-12	ne	ne	ne	ne
HvCPI-13	32.3 ± 2.0	30.9 ± 2.7	30.4 ± 5.9	ne

[a] Values expressed in percentage, are a mean ± SE of triplicate measurements from extracts treated with the inhibitor (0.4 µg cystatin) versus its corresponding controls without it. No effect (ne) was considered for percentages of inhibition inferior to 20%

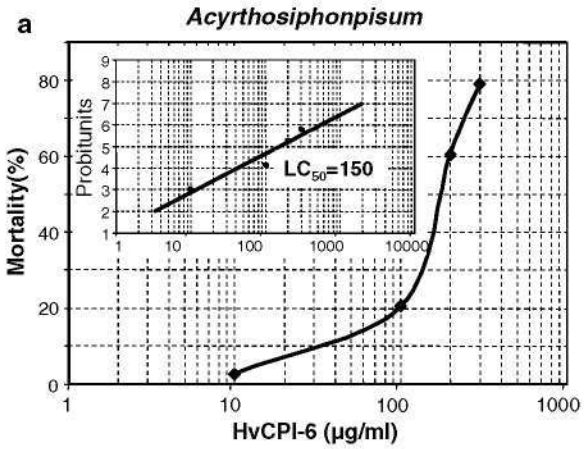
cystatin concentration for 50% mortality (LC_{50}) was also determined at the third day of feeding by Probit analysis. The LC_{50} value was 150 µg/ml (95% Confidence Limits = 105.2–213.9). In contrast, HvCPI-6 did not cause any mortality during the 3-days feeding trial on *M. persicae* aphids, even at cystatin concentrations of 1000 µg/ml (Fig. 1b).

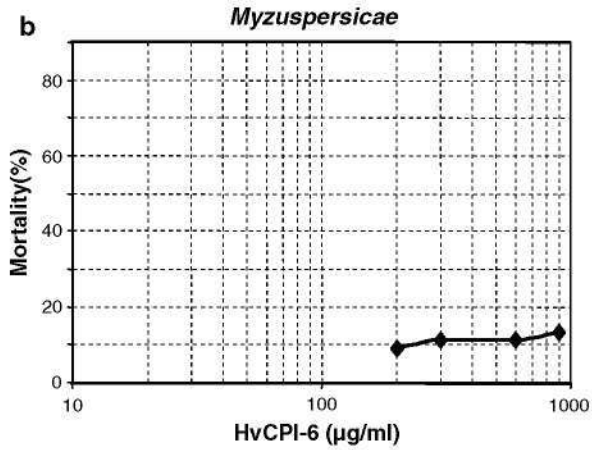
To investigate the aphid responses to the cystatin ingestion, possible alterations in their proteolytic activities were analyzed. Protein extracts were prepared from the two aphid species reared on a diet containing 400 µg/ml cystatin for 1 day. At this dose, mortality rate of *A. pisum* after 3 days of feeding was extremely severe and no survivors were found, whereas after 1 day of feeding the nymphal survival was similar to the control diet. As expected, the two cysteine-proteinase activities analyzed in *A. pisum* extracts, cathepsin B- and L-like, were significantly reduced (between 30% and 55%) in aphids fed on cystatin containing diet in comparison to the activity values obtained after fed on control diet (Fig. 1c). However, there were no differences for the other proteolytic activities (Fig. 1c), and no differences in the band pattern of the gelatin substrate-gel electrophoresis analysis of *A. pisum* extracts fed with cystatin- or control-diet could be observed (Fig. 1e). In contrast, three of the four classes of proteolytic activities detected in *M. persicae* extracts were induced in response to cystatin-diet ingestion for 24 h (Fig. 1d). Regarding their cysteine-proteinases, both cathepsin B- and L-like activities showed a respective increase of about 4- and 8-fold compared to extracts from aphids fed on the control diet. An increase of leucine aminopeptidase-like activity of about 2.6-fold was also detected. In contrast, the aspartic proteinase cathepsin D-like activity was not modified by cystatin-feeding (Fig. 1d). These alterations in the proteolytic activity were corroborated by a higher intensity of the gelatinolytic activity bands visualized in gel samples derived from cystatin-fed aphids (Fig. 1f).

Molecular characterization of *Arabidopsis* expressing the HvcPI-6 barley cystatin

To further analyze the cystatin effect on *M. persicae*, the *Icy6* barley gene encoding the HVCPI-6 cystatin was stably expressed in *Arabidopsis* plants. In the transformation process mediated by *Agrobacterium*, nine independent *Arabidopsis* lines were selected after germinating T1 seeds on kanamycin MS-medium. The presence of the *Icy6* gene in the transgenic lines was determined by PCR amplification. Eight of the nine T_1 transgenic lines exhibited

Fig. 1 Effects of the recombinant barley cystatin HvCPI-6 on ▶ the survival and the proteolytic activity of the aphids *A. pisum* and *M. persicae* fed on artificial diets. **a**, **b** Percentage of mortality of aphid newborn nymphs reared for 3 days on artificial diets supplemented with different concentrations of the recombinant barley cystatin HvCPI-6. Three to six replicates with 15 nymphs each were used per concentration. The effective HvCPI-6 cystatin concentration for 50% mortality (LC_{50}) of *A. pisum* expressed in probit units is indicated in the insert of *panel a*. **c**, **d** Proteolytic activities of aphids after 1 day feeding on a diet containing 400 µg/ml cystatin. Data are mean ± SE of triplicate measurements from three replicates. *Significantly different from control (Dunnett two-tailed test, $P < 0.05$). **e**, **f** Zymograms of the gelatinolytic activity of aphid extracts (10–15 µg total protein), after feeding for 1 day on artificial diet containing 400 µg/ml cystatin or on control diet. *Lanes a, b* and *c* represent three independent biological samples



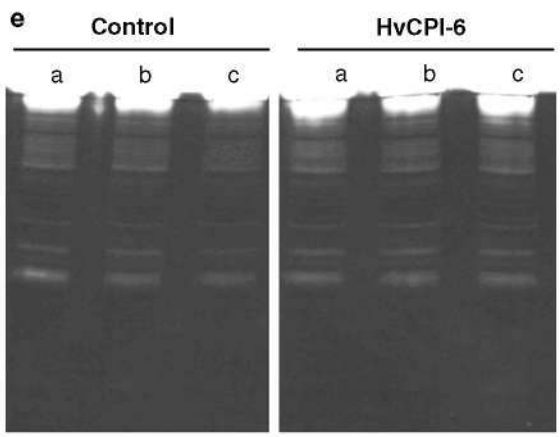


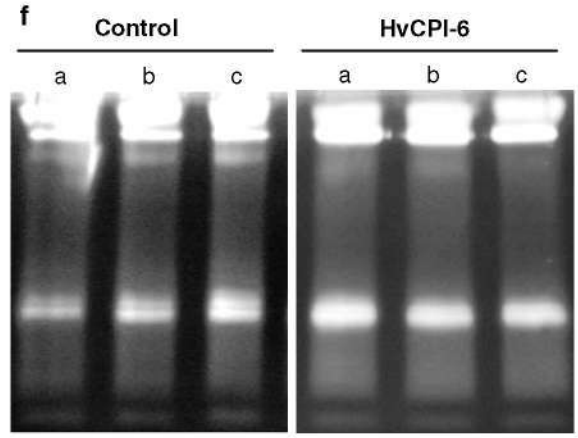
c

Proteolytic activity	pH	Specificactivity	
		Control	HvCPI-6
CTB	6.5	4.00 ±0.36	1.77 ±0.25*
CTL	5.5	13.88 ±1.43	9.40 ±0.81*
	3	18.35 ±0.87	9.78 ±0.54*
LAP	7	8.67 ±0.93	12.29 ±1.89
CPA	7	9.20 ±0.50	9.12 ±2.18
CPB	8	12.65 ±0.44	11.6 ±0.09

d

Proteolytic activity	pH	Specificactivity	
		Control	HvCPI-6
CTB	6.5	1.20 ±0.01	5.03 ±0.68*
CTL	5.5	1.32 ±0.06	10.19 ±0.80*
	3	0.76 ±0.03	6.06 ±0.40*
LAP	7	2.80 ±0.07	7.35 ±0.81*
CTD	3	20.90 ±1.00	21.70 ±0.80





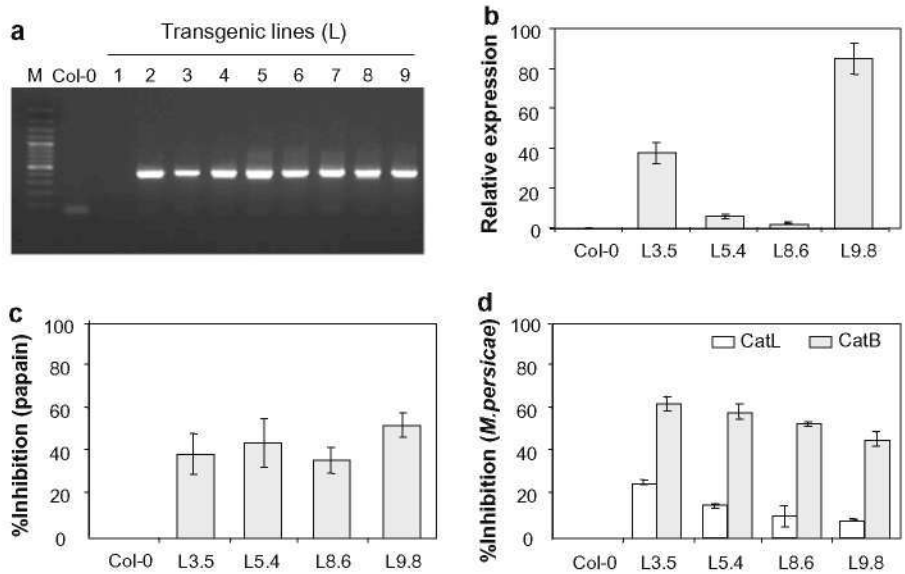


Fig. 2 Molecular analysis of *Arabidopsis* plants transformed with the *Icy6* barley gene encoding the HvCPI-6 cystatin. **a** PCR analysis of T_1 transgenic *Arabidopsis* lines (L1 to L9) and non transformed control (Col-0) using the sense and antisense primers derived from the CaMV35S promoter and the 3′region of the *Icy6* gene. M: molecular size marker. **b** Analysis of the expression of the barley cystatin in the T_2 transgenic *Arabidopsis* lines (L3.5, L5.4, L8.6 and L9.8) and non transformed control (Col-0) by real-time quantitative PCR. Values expressed as the relative mRNA contents of barley cystatin gene were standardized to the *Arabidopsis* ubiquitin gene expression. **c** Inhibitory activity of protein extracts from T_2 transgenic *Arabidopsis* lines (20 µg) against papain (10 ng) using Z-FR-AMC as substrate. **d** Inhibitory activity of protein extracts from T_2 transgenic *Arabidopsis* lines (20 µg) against protein extracts from *M. persicae* adults (10 µg) using Z-RR-AMC and Z-FR-AMC as substrates for cathepsin B- and cathepsin L-like proteinases, respectively

the expected 469 bp band after electrophoresis of the amplified products, which was absent in the non-transformed plant (Fig. 2a). Their seeds were germinated on selective medium to study segregation rate and to identify homozygous lines in subsequent generations.

The expression of the cystatin gene in leaves of transgenic *Arabidopsis* T_2 lines (L3.5, L5.4, L8.6 and L9.8) and control leaves (Col-0) was analyzed by real-time quantitative RT-PCR using specific primers, and the content of cystatin mRNA was normalized to *Arabidopsis* ubiquitin transcripts levels. Strong differences in the cystatin expression among the different transgenic lines were observed (Fig. 2b). No transcripts were found in the RNA isolated from non-transformed Col-0 leaves. The cystatin expression in the transgenic lines was also studied by assaying its in vitro inhibitory activity against the commercial papain. Results were quantified by the decrease amount of Z-FR-AMC-hydrolyzed by the papain and expressed of percentage of inhibitory enzyme activity. All transgenic lines showed inhibitory activity over the values obtained with protein extracts from the non-transformed control (Fig. 2c).

In a second approach, the inhibitory capability of protein extracts from the selected transgenic lines on the proteolytic activity of *M. persicae* extracts was analyzed using Z-FR-AMC and Z-RR-AMC substrates for cathepsin L- and B-like activities, respectively. As is shown in Fig. 2d, both activities were differentially inhibited; particularly cathepsin L-like proteinases were sensitive. Lines L5.4 and L9.8 were selected for further studies based on their protease inhibitory capability and their high number of seed production. These lines were assayed to be homozygous for the transgene.

Effects of *Arabidopsis* transgenic plants on *M. persicae*

T_2 plants of the transgenic lines L5.4 and L9.8 were used for the evaluation of *M. persicae* performance in plants expressing the HvCPI-6 cystatin (Table 3).

Table 3 Performance of *M. persicae* feeding on transgenic *Arabidopsis* lines expressing the HvCPI-6 cystatin (L5.4 and L9.8) or on control plants (Col-0)

	Col-0	L5.4	L9.8
Development[a] (days)	6.8 ± 0.1	7.3 ± 0.1*	7.4 ± 0.1*
Reproductive rate[b]	2.07 ± 0.10	1.95 ± 0.07	1.94 ± 0.09

* Significantly different from control (Dunnett two-tailed test, $P < 0.05$)

[a] Number of days needed by newborn nymphs to reach the adult stage. Results are expressed as mean ± SE using ten aphids per plant and 10 plants of each *Arabidopsis* line ($n = 100$)

[b] Reproductive rate (nymphs/adult/day) was estimated based on the nymphs produced by seven adult aphids feeding on a plant over a period of 7 days. Results are mean ± SE of 20 replicates of each transgenic line ($n = 20$)

Aphid survival on the plants was not affected by the expression of the cystatin in any of the transgenic lines over the assayed period. In contrast, the development of nymphs fed on transgenic L5.4 and L9.8 plants suffered retardation of about 12 and 14 h, respectively, in comparison with those nymphs fed on non-transformed plants. Differences among treatments were evident after the sixth feeding-day in which about 32% of individuals reached the adult stage in control plants, whereas only 9% in the L5.4 transgenic plants and no adults appeared in the L9.8 transgenic plants. The reproductive rate of aphids feeding on both transgenic lines was slightly lower than those feeding on control plants, but the differences were not significant.

Effects of the combination diet-plant feeding on *A. pisum*

The combined diet-plant bioassay showed that nymphs of *A. pisum*, exposed to the barley HvCPI-6 cystatin by feeding on artificial diet for 1 day and completing their development on *V. faba* plants, experienced a significant retardation in reaching the adult stage (Table 4). These nymphs required 19 h more to become adults, in comparison with those nymphs fed 1 day on artificial diets with the HvCPI-12 cystatin or without cystatin. The reproductive rate of aphids feeding for 24 h on a HvCPI-6 cystatin treated diet was slightly lower than those feeding on an artificial diet with the HvCPI-12 cystatin or on a control diet, but the differences were not significantly different (Table 4).

Table 4 Effects of barley cystatins (HvCPI-6 and HvCPI-12) on development and reproductive rate of A. *pisum* after a combined treated diet-plant feeding assay[c]

	Control	HvCPI-6	HvCPI-12
Development[a] (days)	7.6 ± 0.1	8.4 ± 0.1*	7.7 ± 0.1
Reproductive rate[b]	2.46 ± 0.12	2.19 ± 0.11	2.41 ± 0.12

* Significantly different from control (Dunnett two-tailed test, $P < 0.05$)

[a] Number of days needed by the newborn nymphs to reach the adult stage. Results are expressed as mean ± SE using ten aphids per diet-plant and ten replicates of every diet-plant combination ($n = 100$)

[b] Reproductive rate (nymphs/adult/day) was estimated based on the nymphs produced by seven adult aphids over a period of 7 days. Results are mean ± SE of 10 replicates of each diet-plant combination ($n = 10$)

[c] Aphids were fed for 24 h on artificial diet incorporating the cystatin and subsequently transferred to bean plants

Discussion

The importance of proteolytic digestion in aphids has only been recently recognized and cysteine-like proteinases have been identified in the gut of several species of aphids (Cristofoletti et al. 2003; Rispe et al. 2008). Rahbé et al. (2003a) detected cathepsin L/H- like cysteine-proteinases in whole protein extracts from *M. persicae*. Likewise, a cathepsin L-like proteinase has been purified from the gut of *A. pisum* (Cristofoletti et al. 2003) and five cathepsin B-like genes have been identified in the genome of *A. pisum* that were preferentially expressed in the gut (Rispe et al. 2008; International Aphids Genomics Consortium, 2010). Using specific substrates we detected both cathepsin B- and L-like proteolytic activities in *M. persicae* and *A. pisum* whole aphid extracts. The specific activities of these enzymes were low compared with those obtained in other insects using the same substrates (data not shown). This may be related to the type of feeding, since aphids are strict phloem feeders and, because of that, their diet is generally poor in proteins. Besides, some of the proteolytic activities measured may include proteases active in non-gut tissues, or in the haemolymph. No significant levels of trypsin-, chymotrypsin-, or elastase-like proteinase activities were detected in any of the two species, as already reported for *A. pisum* (Rahbé et al. 1995, 2003b). However, we have found that *M. persicae* extracts possess cathepsin

D-like activity, whereas this activity was not present in *A. pisum*, but it will be necessary to confirm its expression in gut tissues to prove that aspartyl-like proteinases are implicated in food digestion. A membrane-bound aminopeptidase has been purified from the midgut cells of *A. pisum* (Rahbé et al. 1995; Cristofoletti et al. 2003, 2006). We have also shown that leucine aminopeptidase-like activity was present in both *M. persicae* and *A. pisum*, indicative of its importance in aphids for the final steps of digestion, whereas carboxypeptidase A- or B-like activities were only detected in *A. pisum*.

Up to date, more than 80 PhyCys s from different plant species have been identified, and some of them are implicated in plant defense against pests and pathogens (Haq et al. 2004). In barley, thirteen cystatin genes have been characterized and the in vitro inhibitory properties of the corresponding recombinant proteins (HvCPI-1 to HvCPI-13) against purified and endogenous cysteine-proteinases determined (Abraham et al. 2006; Martinez et al. 2009). Our data indicated that all barley cystatins, except HvCPI-7, HvCPI-10 and HvCPI-12, were able to inhibit cysteine-like proteinases in at least one of the two species of aphids tested. Concerning their inhibitory capacity towards the different substrates assayed, we have shown that these cystatins were, in general, better inhibitors of the hydrolysis of the cathepsin L substrate Z-FR-AMC than of the cathepsin B substrate Z-RR-AMC. These results are in good agreement with the specificities reported for these cystatins against commercial enzymes: all cystatins, except HvCPI-7 and HvCPI-12, were able to inhibit papain (a cathepsin L-like enzyme) (Martinez et al. 2009); only HvCPI-1, HvCPI-5, HvCPI-6 and HvCPI-8 inhibited cathepsin B (Abraham et al. 2006 and data not published). Structural and functional particularities of mammalian and arthropod proteinases may account for the differences in the susceptibility to inhibitors. We have shown that HvCPI-6 was the most active inhibitor against both cathepsin L- and cathepsin B-like proteinases in both aphid species, while HvCPI-12 and -7 were not active. Molecular modeling of cystatins—*A. pisum* cathepsins interaction shows that HvCPI-6 closely fits the wedge of the cathepsins B and L where the catalytic triad is located (Fig. 3). In contrast, the most energetically favorable interactions between HvCPI-12 and the cathepsins imply that HvCPI-12 does not

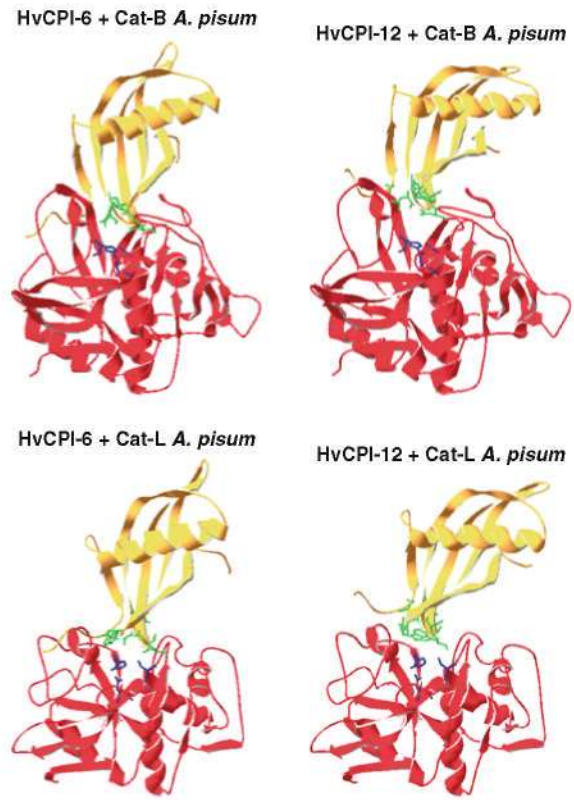


Fig. 3 Molecular modeling of cystatin-cathepsin interaction. The complexes formed by cathepsins B and L from *A. pisum* (*red*) and HvCPI-6 and HvCPI-12 barley cystatins (*yellow*) were modeled by superimposing their homology-based three-dimensional models onto the three-dimensional structures of the stefin A–cathepsin B complex for aphid cathepsin B, and stefin B-papain complex for aphid cathepsin L. The catalytic triad of cathepsins is depicted in *blue* and the residues involved in the proteinase inhibitory activity of cystatins are colored in *green*

interact with the catalytic sites of the aphid cathepsins B and L. The mature protein sequences of both HvCPI-6 and -12 have about 30% amino acid identity and 55% similarity with the mature protein sequence of Oryzacystatin-I used as template, which supports the accuracy of the results obtained. Likewise, the no inhibition of cysteine-like proteinase activity by HvCPI-7 could be related to the predicted changes in its tridimensional structure that disrupts the interaction with the cysteine-proteinases (Abraham et al. 2006).

Considering its wider range of target enzymes and the fact that it is specifically expressed in seeds, Abraham et al. (2006) suggested that HvCPI-6 may act as a defense protein against pest and pathogens.

We have found that, when administered in artificial diets, HvCPI-6 was toxic to *A. pisum* nymphs (LC_{50} = 150 μg/ml), whereas no significant mortality was observed on *M. persicae* nymphs up to 1,000 μg/ml. Previous studies have shown that cystatins affect the growth or survival of aphids on artificial diets. The cysteine-proteinase inhibitor OC-I induced moderate but significant growth inhibition on *A. pisum*, *M. persicae* and *Aphis gossypii* (Rahbé et al. 2003a). The survival and growth of *M. persicae* nymphs were adversely affected when a modified version of OC-I or CEWc was added to artificial diets (Cowgill et al. 2002). OC-I supplemented diets strongly reduced nymphal survival of the aphid *Macrosiphum euphorbiae* and prevented aphids from reproducing (Azzouz et al., 2005). The effects of HvCPI-6 ingestion on *A. pisum* were correlated with a decrease of cathepsin B- and cathepsin L-like proteinase activities, detected in whole insect extracts. However, when the proteinase forms from this species were resolved in gelatin-containing gels, no qualitative or quantitative differences were observed between *A. pisum* fed on control diets or diets containing the HvCPI-6 cystatin. The reason for this discrepancy is probably related to the ability of cystatin/cysteine-proteinase complexes to remain stable during electrophoresis. According to Michaud et al. (1996) complexes with Ki $\geq 10^{-8}$ M are partly or completely dissociated when using mildly-denaturing gelatin-polyacrylamide gel electrophoresis, leading to the complete restoration of gelatinase activity. Interestingly, the Ki value of HvCPI-6 against papain was 1.7×10^{-9} M, but those for commercial cathepsin B and H were 1.0×10^{-6} M and 6.2×10^{-7} M, respectively (Abraham et al. 2006). In the case of *M. persicae*, there was an increase in cathepsin B- and cathepsin L-like activities, but also in the aminopeptidase-like activity, for those insects fed on HvCPI-6 containing diets. This was associated with an increase in the activity of the two main proteolytic bands identified in the gelatin-gels, suggesting that *M. persicae* is regulating the proteolytic activities of both target and insensitive enzymes to overcome the effects of the cystatin.

The possibility of extra-digestive targets in aphids for proteinase inhibitors is a matter of discussion (Rahbé et al. 2003b). Rahbé et al. (2003a) showed that OC-I affects *M. persicae* through digestive tract targets, but also by reaching the hemolymph and internal organs, thereby inhibiting extra-digestive proteolytic activities and interacting with functions related to aphid reproduction. Our results using artificial diets support the possibility that the toxicity of the cystatins was not linked to food protein digestion, since the artificial diets were protein free. Moreover, aphids are also sensitive to some serine proteinase inhibitors (Rahbé et al. 1995, 2003b; Azzouz et al. 2005), despite the absence of serine peptidase activity in these species, suggesting that these deleterious effects could be the consequence of the disruption of non-digestive proteinases involved in other physiological processes (Azzouz et al. 2005).

To further analyze the potential of barley cystatins as insecticidal proteins against aphids, *Arabidopsis* plants expressing HvCPI-6 were tested against *M. persicae*. For *A. pisum*, that does not feed on *Arabidopsis*, a combined diet-plant bioassay was performed in which aphids were exposed to the cystatin by feeding on artificial diets for 1 day, and then placing the aphids on *V. faba* plants. A significant delay in the developmental time to reach the adult stage was observed in both aphid species when exposed to the cystatin HvCPI-6. The reproductive rate was also lower for *M. persicae* fed for 7 days on two different lines of *Arabidopsis* plants expressing HvCPI-6 and for *A. pisum* fed for 24 h on HvCPI-6 treated diet, but the differences were not significant. Nevertheless, these slight differences did not occur when *A. pisum* was fed for 24 h on a diet that contained HvCPI-12, a cystatin that lacks inhibitory activity against cysteine-like proteinases in this species. Enhanced resistance to aphids was also obtained when *M. persicae* was fed transgenic oilseed rape plants expressing OC-I (Rahbé et al. 2003a). Likewise, the expression of OC-I in eggplant has a negative impact on population growth and mortality rates of *M. persicae* and *M. euphorbiae* (Ribeiro et al. 2006). On the other hand, the performance of *M. persicae* was not affected when reared on potato plants expressing the cystatin CEWc (Cowgill et al. 2002). Interestingly, no significant mortality was observed when *A. pisum* was exposed to HvCPI-6 for 1 day in the combined diet-plant bioassay, which contrasts with the high level of mortality obtained when *A. pisum* was fed on HvCPI-6 containing diets for 3 days. This may be related to the different time of exposure to the cystatin, but also to the different nutrient composition of the artificial diet and the plant.

The present study demonstrates the potential of barley cystatins to interfere with the performance of two aphid species. Nevertheless, the expression of the barley cystatin HvCPI-6 in *Arabidopsis* has only a limited effect on aphid growth and reproduction; being necessary to improve is efficacy to make a meaningful contribution to plant resistance. Moreover, if transgenic plants expressing proteinase inhibitors are to be deployed for protection against insect pests, the range of potential target pests and the possible adverse effects on natural enemies should be considered. Previous studies indicated that larvae of the Colorado potato beetle, *Leptinotarsa decemlineata,* reared on transgenic potato plants expressing HvCPI-1 C68 → G, a variant generated by direct-mutagenesis, resulted in significantly lower weight gains compared to those fed on non-transformed plants, whereas prey-mediated effects were not observed on the predatory spined soldier bug, *Podisus maculiventris* (Álvarez-Alfageme et al. 2007). Furthermore, Martinez et al. (2003) described that barley cystatins inhibit the development of phytopathogenic fungi. Therefore, these properties make barley cystatins suitable candidates to be used as transgenes to develop transgenic plants against agronomically important insects and fungal pathogens.

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