Expression of a barley cystatin gene in maize enhances resistance against phytophagous mites by altering their cysteine-proteases

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Abstract Phytocystatins are inhibitors of cysteine-proteases from plants putatively involved in plant defence based on their capability of inhibit heterologous enzymes. We have previously characterised the whole cystatin gene family members from barley (HvCPI-1 to HvCPI-13). The aim of this study was to assess the effects of barley cystatins on two phytophagous spider mites, *Tetranychus urticae* and *Brevipalpus chilensis*. The determination of proteolytic activity profile in both mite species showed the presence of the cysteine-proteases, putative targets of cystatins, among other enzymatic activities. All barley cystatins, except HvCPI-1 and HvCPI-7, inhibited in vitro mite cathepsin L- and/or cathepsin B-like activities, HvCPI-6 being the strongest inhibitor for both mite species. Transgenic maize plants expressing HvCPI-6 protein were generated and the functional integrity of the cystatin transgene was confirmed by in vitro inhibitory effect observed against *T. urticae* and *B. chilensis* protein extracts. Feeding experiments impaired on transgenic lines performed with *T. urticae* impaired mite development and

reproductive performance. Besides, a significant reduction of cathepsin L-like and/or cathepsin B-like activities was observed when the spider mite fed on maize plants expressing HvCPI-6 cystatin. These findings reveal the potential of barley cystatins as acaricide proteins to protect plants against two important mite pests.

Introduction

Crop losses due to herbivorous pest, mainly insects and mites, are estimated to be about 8–15% of the total yield for major crops worldwide, despite pesticide use (Oerke 2006). Phytophagous mites are members of the Acari subclass that feed by puncturing the plant parenchyma cells and sucking out their content. Particularly, Tetranychidae species have highly increased in Europe in the past years producing severe plant damages (Carbonelle et al. 2007). One of the major mite pests on agricultural crops is the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae), a polyphagous species that has been recorded from over 150 plants of economic interest. It represents a key pest for greenhouse crops and ornamentals, annual field crops and perennial cultures all over the world (Jeppson et al. 1975). Other mites with an increasingly important economic impact are specific to certain latitude localizations such as the grape false spider mite *Brevipalpus chilensis* Baker (Acari: Tenuipalpidae), considered a Chilean native species that feed over 100 plants. This mite species is particularly important in red wine grapes where they can reduce yields over 30% (Fuentealba

2003). Because of their short generation time and high population growth rates, mites have developed resistance to the major pesticide groups (Croft and Van de Baan 1988). In addition, no resistant plant cultivars are currently available, and phytophagous mites are not sensitive to toxins encoded by Bt genes (Rovenska et al. 2005; Obrist et al. 2006; Alvarez-Alfageme et al. 2008; Li and Romeis 2010).

Generate plants expressing transgenes targeted to silence or inhibit essential mite-specific genes could be one of these alternatives to combat them and to be included in conventional plant-assessment programmes. For this purpose, it is crucial to identify mite targets and define the processes or pathways that can be altered or inhibited. Genetically engineered plants expressing putative anti-mite genes have been generated as plant protection strategy. McCafferty et al. (2006) reported a significant reduction in the multiplication of carmine spider mite (*Tetranychus cinnabarinus*) reared on papayas expressing a chitinase gene. The transgenic papayas showed an increased tolerance both under laboratory conditions and field trials where natural mite infestation occurred. Similarly, an improved resistance to *T. cinnabarinus* was observed in papaya leaves transformed with the snowdrop GNA lectin gene (McCafferty et al. 2008). In contrast, tomato plants expressing the *Gox* (glucose oxidase) or *KTI3* (soybean Kunitz inhibitor) genes enhanced the *T. urticae* population growth under greenhouse conditions (Castagnoli et al. 2003).

Another putative target process is mite digestive physiology. Mites use both extracellular and intracellular digestion, with the latter occurring in free-floating gut wall-derived epithelial cells that ingest and digest food particles. The midgut is the site for synthesis and secretion of digestive enzymes and absorption of nutrients. Processed food and cells pass into the posterior midgut, subsequently compacted in the hindgut and excreted as faecal pellets (Hamilton et al. 2003). Mite species that feed on plants rely mostly on cysteine peptidase activities for the digestion of dietary proteins (Nisbet and Billingsley 2000). Particularly, protease activity in extracts from *T. urticae* was almost totally explained by cysteine type protease activity in vitro assays and in zymograms (Michaud et al. 1996). Therefore, a control strategy based on the use of phytocystatins (PhyCys) would provide new tools to protect crops against spider mites.

PhyCys are plant proteinaceous inhibitors of cysteine-proteases of the papain C1A family integrated into 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) conforming 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 is involved in the inhibition of a second family of cysteine-proteases, 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 proteases from herbivorous arthropods in vitro, as well as by bioassays in artificial diets and on transgenic plants over-expressing PhyCys genes (Pernas et al. 1998; Alvarez-Alfageme et al. 2007; Carrillo et al. 2010). Deleterious effects against phytopathogenic fungi and viruses have also been described for these inhibitors (Gutierrez-Campos et al. 1999; Martinez et al. 2003; Abraham et al. 2006). To the best of our knowledge, there is only a reference showing the acaricide properties of a phytocystatin, the CsC cystatin from chestnut (Pernas et al. 2000). The CsC inhibited the crude digestive protease activity of the house dust mite *Dermatophagoides farinae* and drastically increased the mite larval mortality when was added to a semisynthetic diet.

The aim of this study was to determine the potential acaricide properties of barley cystatins against phytophagous mites. We analysed the in vitro inhibitory effects and specificity of whole gene family members of barley cystatins (HvCPI-1 to HvCPI-13, Martinez et al. 2009) on two spider mites, *T. urticae* and *B. chilensis*, that rely on cysteine-proteases for digestion. Transgenic maize plants expressing the cystatin HvCPI-6 were prepared and their effects on *T. urticae* were analysed. Moreover, we have characterized further the protease activity profiles of protein extracts from these two mite species to determine the proteolytic alterations after feeding on transgenic plants.

Materials and methods

Purification of recombinant cystatins and inhibitory activity against mite 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 *Escherichia coli* as described in Martinez et al. (2009). The fusion proteins with a His6 tag were purified using a His-Bind Resin (Novagen) following the manufacturer's instructions, and purification checked by SDS-PAGE.

A *T. urticae* colony was reared on maize plants in environmental chambers at 25°C, 70% relative humidity and a 16h/8h day/night photoperiod. Mites were collected and stored frozen (−20°C) until needed. Lyophilized samples of *B. chilensis* were provided by Dr. Marina Gambardella (Pontificia Universidad Católica de Chile, Chile). Mites from both species were homogenized in 0.15 M NaCl (600 mg/ml), centrifuged at 10,000g for 5 min and the supernatants pooled to obtain soluble protein extracts for enzymatic activity assays.

For inhibitory enzymatic activity assays, hydrolysis of substrates containing the AMC (7-amino-4-methyl coumarin) fluorophore was carried out in microlitre plate format. The standard assay volume was 100 µl, 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 0.5 µg and 1.0 µg of soluble protein extracted from *T. urticae* and *B. chilensis*, respectively. The cystatins, at a final concentration of 0.4 µg were incubated at 30°C with the protein extracts for 10 min, prior to adding the substrate at a final concentration of 0.2 mM. Cathepsin B- and L-like activities were assayed using Z-RR-AMC (*N*-carbobenzoxy-loxy-Arg-Arg-7-amido-4-methylcoumarin) and Z-FR-AMC (*N*-carbobenzoxyloxy-Phe-Arg-7-amido-4-methylcoumarin) substrates, respectively. 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. Results were expressed as a percentage of protease 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.

Gelatin-PAGE gels

Electrophoretic detection of proteolytic forms of mite 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 mites (3 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 proteases. 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 distained in 25% (v/v) methanol and 10% (v/v) acetic acid.

Enzyme activity assays

A series of overlapping buffers were used to generate a pH range from 2.0 to 11.0. Reaction buffers for the characterization of mite proteases 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 7–9.5) 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 fluorophore was carried out as described earlier. The standard assay contained 0.5–2.0 µg of protein extract prepared from the two mites and the corresponding substrate added to a final concentration of 0.2 mM. Cathepsin B-like and L-like activities were assayed as described earlier. Trypsin-like activity was assayed using ZLA-AMC (z-L-arginine-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 activity using LL-AMC (L-leucine 7-amido-4-methyl coumarin). The reaction was incubated at 30°C and the fluorescence emission was measured as indicated earlier.

Cathepsin D-like activity was assayed in 0.5 ml of reaction buffer containing 10 µg of mite extract, using 0.2% haemoglobin as described by Novillo et al. (1997). Carboxypeptidases A and B-like activities were assayed in 0.5 ml of reaction buffer containing 20 µg of mite extracts, using 1 mM HPA (hippuryl-phenylalanine) for carboxypeptidase A-like activities and 1 mM HA (hippuryl-L-arginine) for carboxypeptidase B-like activities as described by Ortego et al. (1996).

The proteolytic activities of *T. urticae* and *B. chilensis* extracts were assayed for characterization purposes in the presence of the following class-specific diagnostic inhibitors: the cysteine protease inhibitor, E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butano); the serine protease inhibitor, STI (soybean trypsin inhibitor); the aspartyl protease inhibitor, Pep-A (pepstatin-A); the carboxypeptidase inhibitor, PCPI (potato tuber carboxypeptidase inhibitor); and the heavy metal CdCl$_2$ as inhibitor of aminopeptidases and some other metalloproteases (Salvesen

and Nagase, 1989). Protease 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 that was 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.

Plasmid constructs and plant transformation

The pUC-Icy6 plasmid used in the maize transformation is a vector derived from the pUC19 containing entire coding region of the barley cystatin *Icy6* gene under the control of the maize ubiquitine promoter plus its first intron, followed at the 3′ end by the nopaline synthase gene (NOS) 3′-terminator (Fig. 2a). As selectable marker was used the *bar* gene included in the pAHC25 plasmid, which also contained the reporter *uidA* gene encoding the β-glucuronidase (Christensen and Quail 1996).

Immature zygotic embryos of the South African elite white maize M37W inbred were transformed by particle bombardment according to Ramessar et al. (2008). Bombarded embryos were incubated on N6-based medium under dark for 15 days and then transferred every 2 weeks to fresh N6-based medium containing 3 mg/l phosphinothricin (PPT). Four to six weeks after bombardment, pieces of PPT-resistant embryogenic type I callus were cultured on regeneration medium plus 3 mg/l PPT for 2–4 weeks with a 16 h/8 h day/night photoperiod. Development plantlets with well-formed shoots and roots were hardened off in soil. Independent events were pollinated with nontransformed white maize M37W pollen to produce T_1 seeds and plants containing the barley *Icy6* gene were used for further analysis.

Nucleic acid analysis

Total DNA was isolated from transformed and control maize leaves essentially as described by Taylor and Powell (1982) and tested for the presence of the cystatin gene by PCR. Specific primer pair derived from the *Icy6* gene was used: forward: 5′-CGA**GGTACC**ATGGCCACCTCGGCC CTCGGC-3′, from the ATG translation initiation codon (in bold) to +21 nt, and reverse: 5′-CGA**GGTACC**TTA GCCGCCGGCAGCCGG-3′, positions +294 to +312 corresponding to the stop codon (in bold). Both primers included the Kpn 1 restriction site (underlined). The reaction products were separated on 0.8% agarose electrophoretic gels.

For real-time quantitative RT-PCR studies, T_1 transformed and control leaves of maize 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 7300 Real-Time PCR System (Applied Biosystems) using a SYBR Green detection system. Quantification was standardized to maize immunophilin mRNA levels using the primers described by Hueros et al. (1998). The primers used for PCR amplification were

- ZmFKBP-66 s -forward: 5′-GGGTGCTGTTGTTGAA GTCA-3′;
- ZmFKBP-66as -reverse: 5′-GCAATAACTTCCTCAT CG-3′.
- MqrtIcy6 -forward: 5′-GCGGACGGCTCCGGCAAG AG-3′;
- MqrtIcy6 -reverse: 5′-AAGGACGTGAGCTTGCGG GT-3′;

Inhibitory activity of protein extracts from maize plants expressing the barley cystatin

Total protein extracts from leaves were obtained from T_1 transformed and control maize 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 in Alvarez-Alfageme et al. (2007). Inhibitory activity of plant protein extracts (20 μg) 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 *T. urticae* and *B. chilensis* were also performed using the substrates Z-RR-AMC and Z-FR-AMC, following procedures described earlier.

Mite bioassays on maize plants expressing the barley cystatin

Mite bioassays were conducted on maize leaf fragments derived from T_1 plants of two transgenic lines (L1 and L2) and from the control M37W. Leaf fragments of 3.0 cm long were placed onto wet cotton, surrounded by wet filter paper to avoid mite escapes and confined in petridishes. Dishes were maintained in controlled conditions at 25 ± 2°C, 70% relative humidity and a 16 h/8 h day/night photoperiod.

To study mite development, newborn larvae of *T. urticae* were placed on each leaf fragment and observed daily until mites became adults. Everyday the emerged adults were separated from the leaf fragments. Ten mites were placed on each leaf fragment and ten replicates (independent plants) of every line were done. To study the reproductive performance, two mite females and four mite males were placed on each test leaf fragment and the progeny produced scored after 7 days. The period was limited to 7 days to avoid the first generation progeny to reach the adult stage and start producing second-generation eggs. Twenty replicates (independent plants) of every line were used.

Results

Inhibitory properties of the barley cystatins against proteolytic activities of mites

The inhibitory capability of the whole cystatin members from barley was tested against the protease activity of two phytophagous mites. The 13 cystatins, expressed in *E. coli* cultures were purified to homogeneity by affinity chromatography to a Ni^{2+} column and finally eluted from it. Examples of some of them are shown by the stained bands detected after SDS-PAGE. In addition, the barley cysteine-protease cathepsin L-like EPB-2, target of cystatins is included in the sample as positive control (Fig. 1). Crude protein extracts from *T. urticae* and *B. chilensis* were prepared to perform in vitro inhibition assays with all recombinant barley cystatins. Specific substrates susceptible to be degraded by cathepsin L- and B-like were used.
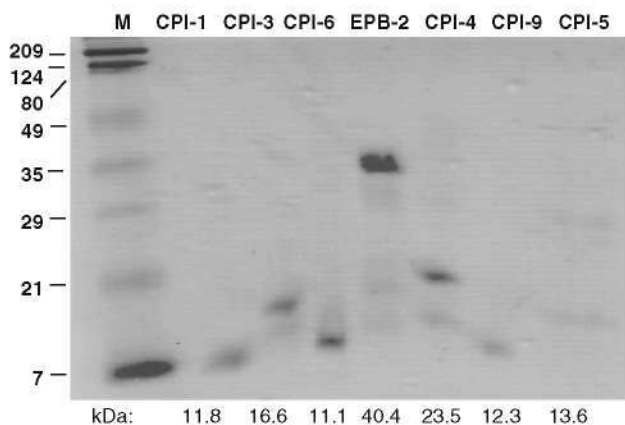

Fig. 1 Purification of the recombinant barley cystatins HvCPI-1, -3, -4, -5, -6 and -9 proteins (CPI-1, CPI-3,CPI-4, CPI-5, CPI-6, CPI-9) and cathepsin L-like- EPB-2 (EPB-2) from *E. coli* cultures by SDS-PAGE. 2 µg of each protein was loaded. The gel was stained with *Comassie Brillant Blue G*. Molecular markers (*M*). Molecular size (kDa) of each cystatin is indicated under its corresponding gel lane

The inhibition profiles showed that *T. urticae* protease activities were more susceptible to be inhibited by barley cystatins than the *B. chilensis* enzymes (Table 1). Thus, cathepsin L-like activity detected in the crude extracts from *T. urticae* was inhibited by 11 barley cystatins, seven of these cystatins being also good inhibitors of cathepsin B-like activity. In contrast, in vitro assays performed with the crude protein extracts from *B. chilensis* showed that cathepsin L- and B-like activities were only inhibited by two and four different barley cystatins, respectively. However, among the 13 cystatin tested, the HvCPI-6 recombinant protein was the strongest inhibitor against proteolytic activities of both mites. It reduced about 90% their cathepsin L-like activity and diminished the cathepsin B-like activity by 90 and 47% in *T. urticae* and *B. chilensis* extracts, respectively. In contrast, no inhibition was detected when HvCPI-7 and HvCPI-12 proteins were used.

The inhibitory properties of the HvCPI-6 were further analysed using mildly denaturing gelatine-PAGE. After electrophoretic mite protein separation and subsequent renaturation, active proteases hydrolysed gelatine showing clear lysis bands against the dark Coomassie blue stained-gelatin in the gel. As shown in Fig. 2, band profiles resulting from the two mites were very different. While only two mainly bands were resolved from *T. urticae* extracts, a multi-band pattern of protease activity appeared in *B. chilensis* sample. However, when mite extracts were pre-incubated with the HvCPI-6 recombinant cystatin purified form *E. coli* cultures, before loading the gel, most of the hydrolytic bands from the Chilean mite disappeared. In contrast, an almost undetectable inhibition of *T. urticae* bands was observed. Similar inhibitory effect was found after pre-treatment with the synthetic cysteine protease inhibitor E-64, although it showed a weaker inhibitory capability.

Characterization of *T. urticae* and *B. chilensis* protease activities

The proteolytic activity profile present in the protein extracts prepared from the two phytophagous mites was characterized. We analysed the optimum pH, specific activity and effect of different protease inhibitors on the hydrolysis of specific substrates (Table 2). The two mites presented marked proteolytic activities of different mechanistic classes, having in common cathepsin B-like, cathepsin L-like and leucine aminopeptidase-like activities. These three types of proteolytic activities were detected throughout the range from pH 5.5 to 7.5. Cathepsin B-like and L-like activities showed a maximum peak of activity at pH 5.5 in *T. urticae* extracts while the optimum pH detected in *B. chilensis* for these two proteases was 7.0. In these two mite extracts both cathepsin activities were

Table 1 In vitro inhibitory activity of the recombinant barley cystatins HvCPI-1 to HvCPI-13 against the two mite species, using the substrates Z-FR-AMC and Z-RR-AMC susceptible to be degraded by cathepsin L- and B-like, respectively

Cystatins	Inhibition[a] (%)			
	T. urticae		*B. chilensis*	
	Z-FR-AMC substrate	Z-RR-AMC substrate	Z-FR-AMC substrate	Z-RR-AMC substrate
HvCPI-1	61.6 ± 4.3	51.4 ± 3.8	ne	ne
HvCPI-2	51.3 ± 4.1	40.6 ± 4.1	ne	ne
HvCPI-3	55.8 ± 4.5	47.0 ± 2.2	ne	ne
HvCPI-4	55.2 ± 2.6	40.8 ± 2.2	ne	ne
HvCPI-5	90.6 ± 1.0	52.6 ± 5.3	ne	39.5 ± 0.6
HvCPI-6	90.3 ± 1.0	92.0 ± 4.4	86.3 ± 0.7	46.8 ± 0.6
HvCPI-7	ne	ne	ne	ne
HvCPI-8	64.5 ± 0.3	24.5 ± 5.2	ne	40.9 ± 3.2
HvCPI-9	61.8 ± 3.1	ne	ne	ne
HvCPI-10	24.2 ± 3.2	ne	ne	54.9 ± 0.8
HvCPI-11	33.0 ± 0.8	ne	ne	ne
HvCPI-12	ne	ne	ne	ne
HvCPI-13	24.9 ± 2.0	ne	29.2 ± 5.3	ne

No effect (ne) was considered for percentages of inhibition inferior to 20%

[a] Values expressed in percentage are a mean $\pm$ SE of triplicate measurements from a pool of extracts treated with the inhibitor (0.4 μg of cystatin) versus its corresponding control without it
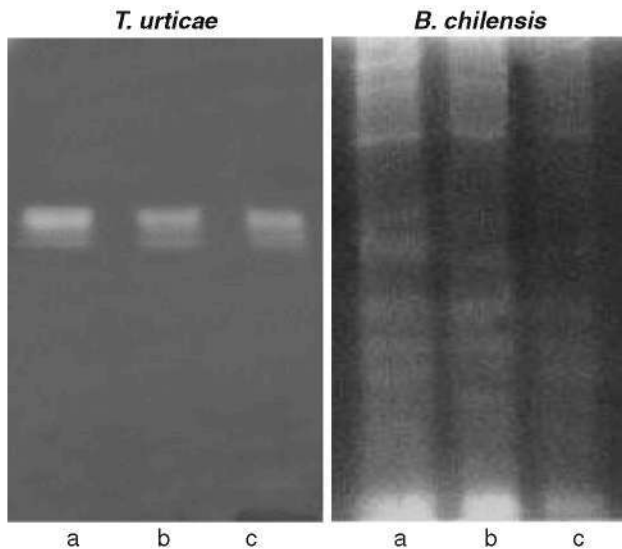

Fig. 2 Gelatin-containing SDS-PAGE gels of extracts from *T. urticae* and *B. chilensis*. Before electrophoresis, samples were treated with buffer alone (**a**), 100 μM E-64 (**b**) or 70 μM HvCPI-6 (**c**), for 15 min at 30°C. Gels were incubated with 0.1 M phosphate, pH 6.0 and 10 mM cysteine for 20 h at 30°C

strongly inhibited by E-64, a cysteine-specific protease inhibitor. CdCl$_2$ and soybean trypsin inhibitor (STI) presented a minor inhibition of these cysteine-protease activities. Besides, the leucine aminopeptidase-like activity showed the maximum proteolytic capability at pH 7.5 and

was inhibited by CdCl$_2$ and pepstatin A in both mite species. In contrast, aspartic protease cathepsin D-like activity was specifically found in *T. urticae* protein extracts, while carboxypeptidases A- and B-like activities were only detected in extracts from *B. chilensis*. 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 8.0 and 7.0, respectively, and were highly inhibited by CdCl$_2$ and potato tuber carboxypeptidase inhibitor (PCPI). No hydrolysis of trypsin-, chymotrypsin- and elastase-like substrates was detected in the two mite extracts, even after 24 h of incubation.

Characterization of transgenic maize plants containing the barley cystatin

Transgenic maize lines were recovered in a phosphinotricine medium after culturing bombarded embryos with microparticles coated with the pUC-Icy6 (Fig. 3a) and pAHC25 constructs. Plants were screened by genomic PCR to identify primary cystatin transformants. In all ten lines analysed, the UbiIcy6 transgene presence was confirmed as is shown by amplifying a 321-bp diagnostic fragment (Fig. 3b). Lines L1 and L2 were selected for further experiments and the plants were crossed with wild-type M37W pollen to produce T$_1$ seeds. These two lines were representative of the Icy6-cystatin lines and were

Table 2 Proteolytic activities from *T. urticae* and *B. chilensis* against specific substrates and effect of protease inhibitors at their optimum pH

Protease	Optimum pH[a]	Specific activity[b]	Inhibition[c] (%)				
			Pep-A	E-64	STI	PCPI	CdCl$_2$
Tetranychus urticae							
CTB	5.5	19.8 ± 1.1	ne	99 ± 0.1	ne	ne	23 ± 3
CTL	5.5	22.2 ± 2.1	ne	88 ± 2	ne	ne	ne
CTD	3.0	36.4 ± 0.0	68 ± 8	ne	ne	ne	ne
TRY		nd	–	–	–	–	–
CHY		nd	–	–	–	–	–
ELA		nd	–	–	–	–	–
LAP	7.5	0.65 ± 0.2	47 ± 2	ne	25 ± 4	ne	87 ± 2
CPA		nd	–	–	–	–	–
CPB		nd	–	–	–	–	–
Brevipalpus chilensis							
CTB	7.0	4.8 ± 0.2	ne	91 ± 1	26 ± 1	ne	ne
CTL	7.0	15.3 ± 0.5	ne	89 ± 0.2	ne	ne	ne
CTD		nd	–	–	–	–	–
TRY		nd	–	–	–	–	–
CHY		nd	–	–	–	–	–
ELA		nd	–	–	–	–	–
LAP	7.5	3.5 ± 0.1	39 ± 2	ne	ne	ne	59 ± 4.7
CPA	8.0	36.2 ± 0.1	ne	ne	ne	82 ± 1.4	83 ± 1.3
CPB	7.0	22.7 ± 0.1	ne	ne	ne	78 ± 0.7	75 ± 1.0

CTD cathepsin D, *CTB* cathepsin B, *CTL* cathepsin L, *TRY* trypsin, *CHY* chymoptrypsin, *ELA* elastase, *LAP* leucine aminopeptidase, *CPA* carboxypeptidase A and *CPB* carboxypeptidase B, *Pep-A* pestatin A, *E-64* L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane), *STI* soybean trypsin inhibitor and *PCPI* potato tuber carboxypeptidase inhibitor

[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 haemoglobin as mU Δ Abs 280 nm/min/mg protein. Data are the mean ± SE of triplicate measurements. *nd* No activity detected

[c] 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%

characterised fully molecularly and biochemically through T$_1$ generation.

The expression of the cystatin gene in leaves of ten T$_1$ plants from each line (L1.1–L1.10 and L2.1–L2.10) and non-transformed control leaves (M37W) was analysed by real-time quantitative RT-PCR (qRT-PCR) using specific primers. The content of cystatin mRNA was normalised to maize immunophilin transcript levels. Differences in the cystatin expression among the different transgenic plants within L1 and L2 lines were observed (Fig. 4a). No transcripts were found in the RNA isolated from non-transformed maize. Maize leaves were also used to prepare protein extracts and to analyse their in vitro inhibitory activity against the commercial papain. Results, quantified by the decrease amount of Z-FR-AMC-hydrolysed by the papain, were expressed of percentage of inhibitory enzyme activity (Fig. 4b). All transgenic plants showed inhibitory activity over the values obtained with protein extracts from the non-transformed control. Again, differences in the

inhibitory capability among plants were observed. While plants 1.8, 1.9, 2.1, 2.5 and 2.7 inhibited almost completely the papain proteolytic activity, plants 1.3, 1.4, 1.6 and 2.3 had very low or even no antipapain effect. The five plants within each line showing the stronger inhibitory properties against papain were selected to perform in vitro inhibitory assays against mite extracts.

To assess the mite sensitivity to transgenic maize extracts, Z-FR-AMC and Z-RR-AMC substrates susceptible to be hydrolysed by cathepsin L-and B-like activities, respectively, were used. As is shown in Fig. 4c, both protease activities were inhibited in the two mites. In general, *T. urticae* extracts were more susceptible to be inhibited by this barley cystatin than B. *chilensis* proteins. Interestingly, all transgenic plants derived from line L2 inhibited preferentially cathepsin B-like activity in *T. urticae* extracts (in the 65–100% range), whereas they reduced mainly cathepsin L-like activity in *B. chilensis* samples (about 50–80%). In contrast, plants from line L1 did not show any
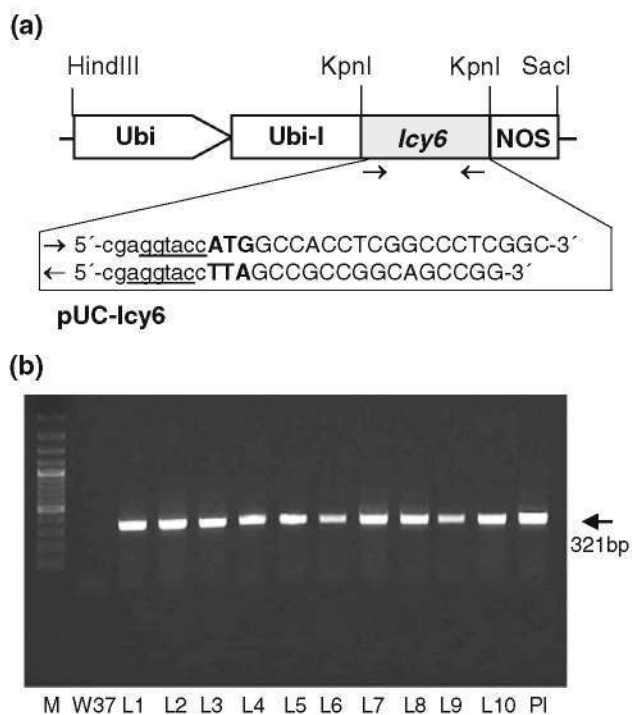
(a)

HindIII   KpnI   KpnI SacI

Ubi   Ubi-I   *Icy6*   NOS

→ 5′-cga<u>ggtacc</u>**ATG**GCCACCTCGGCCCTCGGC-3′
← 5′-cga<u>ggtacc</u>**TTA**GCCGCCGGCAGCCGG-3′

pUC-Icy6

(b)

M W37 L1 L2 L3 L4 L5 L6 L7 L8 L9 L10 Pl

← 321bp

Fig. 3 Schematic representation of the pUC-Icy6 plasmid used for maize transformation and PCR analysis of the maize transgenic lines. **a** The PUC-Icy6 plasmid contains the barley cystatin gene (*Icy6*) flanked by the ubiquitine promoter (Ubi) plus its first intron (Ubi-I) and the nopaline synthase gene (NOS) 3′ terminator. Sites of restriction enzymes are included and specific primer pair (sense → and antisense ←) derived from the *Icy6* gene used for PCR assays are indicated. The ATG translation initiation site and stop codon are in *bold*. Restriction Kpn I sites are *underlined* and added nucleotides to enhance restriction enzyme action are in *lower letters*. **b** PCR analysis of transgenic maize lines (L1–L10) and non-transformed control plant (M37W) using sense and antisense primers indicated in (**a**). Molecular size marker (*M*) and the plasmid pUC-Icy6 (*Pl*) used as positive control are marked

preference for a specific cathepsin activity, with the exception of the plant 1.2 which seems to be a stronger inhibitor of catehpsin L-like in the two mite samples. No inhibitory effects were detected when protein extracts from MW37 control leaves were used.

Effects of maize transgenic plants on *T. urticae*

T_1 plants of the transgenic lines L1 and L2 were used for the evaluation of *T. urticae* performance in maize leaves expressing the HvCPI-6 cystatin. Spider mite 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 immature stages fed on transgenic L1 and L2 plants to become adults suffered retardation of almost 1 day in comparison with those fed on non-transformed leaves. In addition, mite reproductive performance was significantly reduced after feeding on

transgenic L2 plants, while it was not altered in those mites fed on L1 plants (Table 3).

Effects of maize transgenic plants on *T. urticae* proteolytic activity

To investigate the effect of the cystatin ingestion on the proteolytic pattern of *T. urticae*, protein extracts were prepared from the red spider mite reared on transgenic maize plants for 7 days. Both cathepsin L-like and cathepsin B-like activities were significantly reduced in mites fed on L2 maize plant expressing the barley cystatin, whereas only cathepsin L-like activity was inhibited in mites fed on the transgenic line L1, as is shown in Table 4.

Discussion

The general usefulness of PhyCys for pest control have been shown, particularly against phytophagous coleopteran species which rely mostly on cysteine protease activities for the digestion of dietary proteins (Fabrick et al. 2002; Lalitha et al. 2005; Alvarez-Alfageme et al. 2007). Moreover, many other studies have shown that PhyCys also affect the growth or survival of other insects, nematodes and slugs on artificial diets or delivered through transgenic plants (Walker et al. 1999; Lilley et al. 2004; Ribeiro et al. 2006; Konrad et al. 2009; Carrillo et al. 2010). In contrast, the anti-mite properties of cystatins have only been proven using the chestnut cystatin against house dust mite species (Pernas et al. 2000). In this context, the aim of the present study was to assess the putative acaricide effects of the whole barley gene cystatin family against two phytophagous mites, *T. urticae* and *B. chilensis*, with impact on agricultural crops and to select the best cystatin as trangene to study its in vivo impact.

We have previously characterized the in vitro inhibitory properties of the 13 members of the barley cystatin family purified as recombinant proteins against commercial and endogenous barley proteases (Abraham et al. 2006; Martinez et al. 2009). Here, these recombinant proteins were tested towards extracts prepared from *T. urticae* and *B. chilensis*. Our data showed that all barley cystatins, except HvCPI-7 and HvCPI-12, were able to in vitro inhibit cysteine-like proteases in at least one of two mite species tested. These results were in accordance with the specific inhibitory capability reported for these cystatins against different cathepsin-like proteases which also showed the lack of the inhibitory activity of HvCPI-7 and HvCPI-12 cystatins (Abraham et al. 2006; Martinez et al. 2009). Interestingly, cysteine-protease activities, particularly cathepsin L-like, from *T. urticae* were inhibited by most of the barley cystatins while only some cystatins inhibited

Fig. 4 Molecular analysis of maize plants transformed with the *Icy6* barley gene encoding the HvCPI-6 cystatin.
a Analysis of the expression of the barley cystatin in 10 T_1 transgenic maize plants derived from two transgenic lines (L1 and L2) and non-transformed control (M37W) by real-time quantitative PCR. Values expressed as the relative mRNA contents of barley cystatin gene were standardized to the maize inmunophilin gene expression.
b Inhibitory activity of protein extracts from 10 T_1 transgenic maize plants derived form two transgenic lines (L1 and L2) and non-transformed control (M37W) against papain using Z-FR-AMC as substrate. Data are mean ± SE of triplicate measurements of each sample.
c Inhibitory activity of protein extracts from five T_1 transgenic maize plants derived from two transgenic lines (L1 and L2) and non-transformed control (W37M) against protein extracts from *T. urticae* and *B. chilensis* using Z-FR-AMC and Z-RR-AMC as substrates for cathepsin L- (CTL) and cathepsin B-like proteases (CTB), respectively. Data are mean ± SE of triplicate measurements of each sample
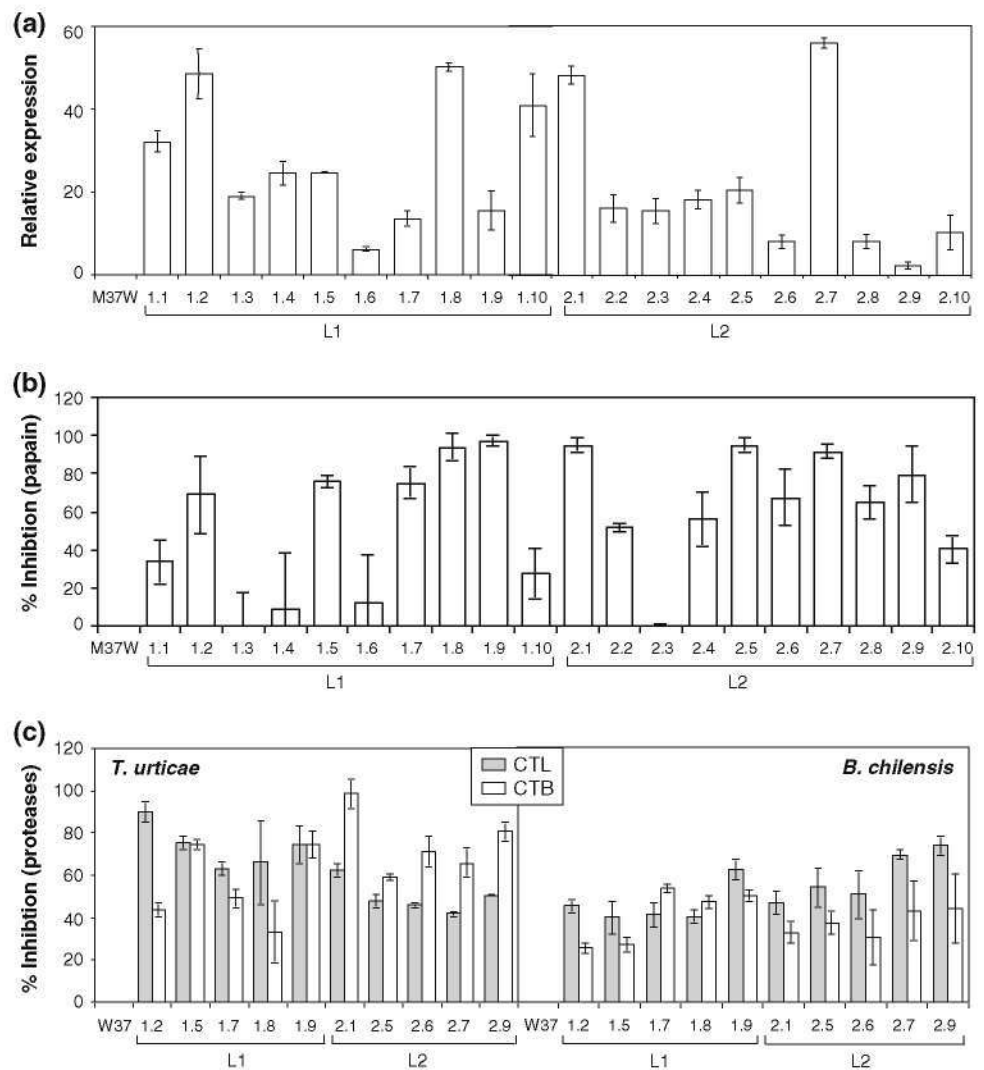

Table 3 Effects of the barley cystatin HvCPI-6 expressed on maize T_1 plants on development and reproductive performance of *T. urticae* after feeding assay

Maize	M37W	L1	M37M	L2
Development[a] (days)	7.4 ± 0.1	8.2 ± 0.1*	6.5 ± 0.1	7.5 ± 0.1*
Reproductive performance[b]	30.3 ± 2.0	25.8 ± 2.1	50.0 ± 3.2	41.7 ± 3.0*

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

[a] Number of days needed for the neonate larvae to become adult

Results are expressed as mean ± SE using ten mites per maize leaf fragment and ten replicates of each transgenic line (L1 and L2) or its respective controls (M37W)

[b] Total progeny produced by two mite females and four mite males after 7 days feeding assay. Results are mean ± SE of 20 replicates (leaves from independent plants) of each transgenic line (L1 and L2) or control plant (M37W). Two sets of experiments were conducted (10 replica per line) and the statistical analysis used a complete block design

efficiently *B. chilensis* protein extracts. Our findings indicated HvCPI-6 protein was the strongest inhibitor of cathepsin L- and B-like activities of both mites. However, the efficient inhibitory effects observed in vitro against *T. urticae* proteolytic activity differed from the lack of inhibition on the gelatinolytic bands detected on the zymogram. In contrast, an almost complete inhibition of the gelatinase bands from the *B. chilensis* extracts were detected after being preincubated with the HvCPI-6 cystatin. These differences could be determined by the stability

Table 4 Proteolytic activity profile *T. urticae* fed on transgenic maize T_1 plants (L1 and L2) or on control plants (M37W)

Proteolytic activity[a]	Specific activity[b]			
	M37W	L1	M37W	L2
CTB	12.4 ± 0.4	11.7 ± 0.3	15.2 ± 0.3	12.1 ± 0.2*
CTL	13.1 ± 0.7	9.5 ± 0.2*	16.7 ± 0.7	14.8 ± 0.4*
CTD	36.6 ± 1.6	35.8 ± 1.9	34.4 ± 2.0	34.0 ± 1.5
LAP	0.8 ± 0.0	0.7 ± 0.0	0.6 ± 0.2	0.6 ± 0.1

Data are mean ± SE of triplicate measurements from three biological samples

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

[a] Protease type abbreviations: as in Table 2

[b] Specific activities as in Table 2

of each cystatin/cysteine–protease complex (Michaud et al. 1996; Kiggundu et al. 2010), which in the case of *T. urticae* may favour dissociation during the electrophoretic process. According to Michaud et al. (1996), it is considered a weak complex stability when Ki values are higher than 10^{-8} M. In our hands the HvCPI-6 Kis were in the 10^{-6}–10^{-7} M range against commercial cathepsin B- and H-like proteases, respectively, but its Ki values against most cathepsin L-like tested was 10^{-9} M (Abraham et al. 2006; Martinez et al. 2009).

The characterization of protease activities of the two mites clarified the impact of cystatins and corroborated the presence of target enzymes in the spider mites. Michaud et al. (1996) had previously shown that protease activity in extracts from *T. urticae* was almost totally explained by cysteine-protease type in both in vitro assays and in zymograms. Likewise, proteolytic activities from *T. urticae* extracts were inhibited by E-64 and pepstatin A indicating cysteine- and aspartic-protease activities, respectively, in this mite (Nisbet and Billingsley 2000). We have detected both cathepsin B- and L-like activities in *T. urticae* and *B. chilensis* whole extracts using specific substrates. These results are not surprising considering the prevalence and numerical importance of cysteine-protease coding sequences annotated in the genome of *T. urticae* (our unpublished results). In addition, *T. urticae* extracts presented cathepsin D-like activity whereas this activity was absent in the Chilean mite. Besides, leucine aminopeptidase-like activity was also present in both mites while carboxypeptidase A- and B- activities were only found in *B. chilensis*. Band proteolytic profiles of the two mites were also very different, with only two main bands in *T. urticae*, whereas *B. chilensis* yielded multiple-diffuse bands of protease activity. It has been suggested that the spectrum of digestive enzymes detected in mites reflect trophic specialization (Bowman 1984).

The expression of the HvCPI-6 cystatin in transgenic maize allowed us to perform bioassays with *T. urticae*. We studied the potential interest of these transgenes to control spider mites as an alternative to Bt genes, which resulted unsuccessful against mite pests (Rovenska et al. 2005; Alvarez-Alfageme et al. 2008; Li and Romeis 2010). The transgenic maize lines generated (L1 and L2) presented different transgene expression levels. This maybe due to the insertion of multiple copies of transgene in different chromosomal loci. Moreover, in many cases transgene expression levels are not always correlated to inhibition activity. This maybe due to the differences in protein levels. This is quite general characteristic described in transgenic plants. For these reasons, we selected the transgenic lines for performing bioassays based on their inhibitory capabilities. Interestingly, a significant development delay of *T. urticae* larvae to reach the adult stage *in planta* (transgenic lines L1 and L2) evidenced the anti-mite effects of the barley cystatin. However, the mite reproductive rate was only significantly affected after feeding on transgenic line L2. Probably, the higher inhibitory activity shown by maize L2 line against *T. urticae* cathepsin B-like, and the preference of L1 line to inhibit cathepsin L-like proteases could support the mite differential responses. The observed differences between control plants (M37W) were probably due to differences on the mite and/or plant physiology between the two sets of experiments conducted. As expected, the effect of the cystatin HvCPI-6 retarding development time and reducing reproductive performance of *T. urticae* were correlated with a decrease of cathepsin L- and B-like protease activities after rearing on transgenic maize. It is important to have in mind that crude protein extracts were prepared from whole mites, and their cysteine-proteases can be involved in several physiological processes developed in different mite compartments. However, cathepsin D- and leucine aminopeptidase-like activities were not affected, suggesting that the spider mite did not develop a compensatory response to the inhibitory effect mediated by the barley cystatin. The overproduction of non-target proteases is usually found in other phytophagous arthropods as a response to host plants expressing distinct sets of defence proteins (Lara et al. 2000; Alvarez-Alfageme et al. 2007).

Taken together, all these results indicate that PhyCys could be a reliable source of plant resistance genes to be used as transgenes for conferring enhanced levels of resistant to phytophagous mites. As a proof of concept, transformation of maize with the barley cystatin HvCPI-6 impaired development and reproductive performance of *T. urticae*, highlighting the potential of this type of transgenic-derived plants for mite control.

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