

# DELETERIOUS EFFECTS OF PLANT CYSTATINS AGAINST THE BANANA WEEVIL *Cosmopolites sordidus*

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*The general potential of plant cystatins for the development of insect-resistant transgenic plants still remains to be established given the natural ability of several insects to compensate for the loss of digestive cysteine protease activities. Here we assessed the potential of cystatins for the development of banana lines resistant to the banana weevil *Cosmopolites sordidus*, a major pest of banana and plantain in Africa. Protease inhibitory assays were conducted with protein and methylcoumarin (MCA) peptide substrates to measure the inhibitory efficiency of different cystatins in vitro, followed by a diet assay with cystatin-infiltrated banana stem disks to monitor the impact of two plant cystatins, oryzacystatin I (OC-I, or OsCYS1) and papaya cystatin (CpCYS1), on the overall growth rate of weevil larvae. As observed earlier for other Coleoptera, banana weevils produce a variety of proteases for dietary protein digestion, including in particular Z-Phe-Arg-MCA-hydrolyzing (cathepsin L-like) and Z-Arg-Arg-MCA-hydrolyzing (cathepsin B-like)*

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proteases active in mildly acidic conditions. Both enzyme populations were sensitive to the cysteine protease inhibitor E-64 and to different plant cystatins including OsCYS1. In line with the broad inhibitory effects of cystatins, OsCYS1 and CpCYS1 caused an important growth delay in young larvae developing for 10 days in cystatin-infiltrated banana stem disks. These promising results, which illustrate the susceptibility of *C. sordidus* to plant cystatins, are discussed in the light of recent hypotheses suggesting a key role for cathepsin B-like enzymes as a determinant for resistance or susceptibility to plant cystatins in Coleoptera. © 2009 Wiley Periodicals, Inc.

**Keywords:** banana weevil (*Cosmopolites sordidus*); banana (*Musa* spp.); cathepsin B-like proteases; cathepsin L-like proteases; plant cystatins

## INTRODUCTION

The banana weevil *Cosmopolites sordidus* (Germar) (Coleoptera: Curculionidae) is an insect pest of considerable importance in Africa, associated with the rapid decline of banana and plantain plantations (Gold et al., 1999a, 2005; Gold, 2000; Swennen and Vuylsteke, 2001). At the adult stage, banana weevil females deposit their eggs inside host plant tissues, at the base of the pseudo-stem or on an exposed corm. On hatching, the larvae tunnel through the corm to feed and develop, where they damage tissues, compromise water and mineral uptake, and weaken the colonized organs. Banana weevil infestations cause important losses in the field by a direct negative impact on harvestable bunch weight and a weakening effect on infested organs causing plant toppling during windstorms (Sengooba, 1986; Rukazambuga et al., 1998).

Banana weevil control in Africa relies essentially on cultural and sanitation practices, such as the use of clean planting material, the systematic trapping of adult weevils to prevent population build-up, and the removal of plant residues serving as potential breeding grounds (Gold, 2000; Gold et al., 2002). High labour input and material requirements, however, often represent limiting factors for the effective implementation of sanitation practices in banana plantations, which makes it essential to develop complementary protection strategies (Gold, 1998; Gold et al., 2001). As for other staple crops, commercial insecticides have been considered to control insect populations, but the general adoption of these compounds in small-scale subsistence farming systems such as most banana plantations in Africa is hardly feasible from a socioeconomic standpoint, and potentially inefficient owing to the rapid development of genetic resistance in target weevil populations (Collins et al., 1991; Gold et al., 1999b). Biological control agents have also been considered for banana weevil control, but the actual effectiveness of biocontrol tools in field situations remains to be established (Paparou et al., 2008).

At this point, the most straightforward way to prevent banana weevil infestations likely resides in the introduction of insect resistance in the banana genome (Kiggundu et al., 2003a). The sterile status of most banana varieties makes it unpractical to establish conventional crossbreeding programmes with elite cultivars, despite the wide diversity of potentially useful traits in the banana germplasm worldwide (Kiggundu et al., 2003b; Heslop-Harrison and Schwarzacher, 2007). By contrast, significant progress has been made in recent years towards the genetic transformation and in

in vitro regeneration of commercial banana and plantain varieties (Sagi et al., 1995; Pérez Hernandez et al., 2006; Pommerrenig et al., 2006; Khanna et al., 2007), paving the way to the development of insect-resistant transgenic banana lines. A number of recombinant proteins with pesticidal, antidiigestive, or antifeedant properties have been identified or devised over the years for the development of herbivorous pest-resistant crops by genetic transformation (Carlini and Grossi-de-Sá, 2002; Christou et al., 2006; Gatehouse, 2008). An engineered version of the rice cysteine protease inhibitor oryzacystatin I (OC-I) (Urwin et al., 1995) was shown, notably, to confer nematode resistance in transgenic banana lines genetically transformed with the corresponding transgene sequence (Atkinson et al., 2004a).

Recombinant cystatins appear to be of particular interest for the design of pest-resistant transgenic crops intended for human use, given the absence of target cysteine proteases in the human gut and the negligible negative effects expected for these proteins in food products (Arai and Abe, 2000; Atkinson et al., 2004b). The potential of midgut cysteine proteases as relevant targets for the control of coleopteran plant pests has also been underlined recently (*Tribolium* Genome Sequencing Consortium, 2008), taking into account the number and diversity of gene-coding sequences for cysteine cathepsins in the genome of the model coleopteran herbivore *Tribolium castaneum* (Wang et al., 2007), the widespread occurrence of these enzymes among herbivorous Coleoptera (Murdock et al., 1987), and the detrimental effects of the broad-spectrum inhibitor of papain-like cysteine proteases, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), on the growth and development of several coleopteran herbivores (e.g., Murdock et al., 1988; Bolter and Latoszek-Green, 1997; Fabrick et al., 2002). In this study, we assessed the potential of OC-I and other plant cystatins as possible tools for the production of transgenic banana lines resistant to the banana weevil. Several reports have been published assessing the effectiveness of plant cystatins against herbivorous Coleoptera, with conclusive results in some cases (Leplé et al., 1995; Kuroda et al., 1996; Lecardonnell et al., 1999; Koiwa et al., 2000; Alvarez-Alfageme et al., 2007; Ninkovic et al., 2007) but disappointing results in other cases owing to the ability of the target insects to readily elude or even counteract the antidiigestive effects of natural or recombinant inhibitors (e.g., Girard et al., 1998a,b; Cloutier et al., 1999, 2000; Zhu-Salzman et al., 2003; Gruden et al., 2003, 2004; Ahn et al., 2004, 2007; Oppert et al., 2005; Koo et al., 2008). Here, we document the broad inhibitory effects of plant cystatins against midgut cysteine cathepsins of the banana weevil, and the detrimental effects of these proteins on larval growth using a novel bioassay setup involving cystatin-infiltrated banana tissues.

## MATERIALS AND METHODS

### *Banana Weevils*

Adult banana weevils were collected from commercial banana (*Musa* sp.) plantations in the South African province of Kwazulu Natal. The weevils were maintained in a greenhouse at room temperature in 10-L plastic containers kept moist with water, and provided with fresh banana stems for oviposition. The weevils were removed after two days to allow for the development of freshly laid eggs. Third and fourth instars for protease assays were collected by dissection of infected stems, and weighed individually

before use or storage at  $-20^{\circ}\text{C}$ . Newly hatched larvae for bioassays (see below) were produced with fresh eggs extracted from infected stems, after gently removing thin slices of outer tissue with a sharp paring knife. The eggs were washed quickly in 70% (v/v) ethanol, rinsed twice in distilled water, incubated at  $25^{\circ}\text{C}$  in the dark on sterile moistened filter paper, and placed in a sterile Petri dish for hatching.

### Midgut Proteases

Frozen larvae were thawed on ice and dissected in cold distilled water under a stereomicroscope to extract whole midguts. Midgut tissues were ground to a fine powder in liquid nitrogen, and homogenized in 150 mM  $\text{CaCl}_2$  in water containing 0.1% (v/v) Triton X-100, with 200 mg of tissue per mL of extraction solution. The mixtures were incubated on ice for 30 min, and then centrifuged at  $12,000g$  for 10 min to remove cellular debris and insoluble material. The supernatants were collected and protein contents adjusted to 1 mg/ml with extraction solution. Insect material for gelatinase zymography (see below) was homogenized directly in 100  $\mu\text{L}$  of gelatin-PAGE sample loading buffer (62.5 mM Tris-HCl, pH 8.0, 2% [w/v] sucrose, and 0.001% [w/v] bromophenol blue) (Michaud et al., 1996a). Protein concentration in the extracts was determined using the Bio-Rad Protein Assay Kit<sup>TM</sup> (Bio-Rad, Mississauga ON, Canada), with bovine serum albumin as a protein standard. Protein samples were used immediately for in vitro assays, or stored at  $-20^{\circ}\text{C}$  until use.

### Protease Substrates and Inhibitors

The protein and synthetic protease substrates azocasein, gelatin (porcine type A), Z-Arg-Arg-7-amido-4-methylcoumarin-hydrochloride (Z-RR-MCA), Z-Phe-Arg- (Z-FR-) MCA, Arg- (R-) MCA, Z-Arg- (Z-R-) MCA, and succinyl-Ala-Ala-Pro-Phe- (suc-AAPF-) MCA were purchased from Sigma (Oakville ON, Canada). The protease inhibitors bovine aprotinin, E-64, ethylenediamine tetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin-chymotrypsin inhibitor (SBBI), and L-3-trans-(propylcarbonyl)oxirane-2-carbonyl-L-isoleucyl-L-proline (CA-074) were also from Sigma. The cathepsin L inhibitor Z-Phe-Tyr(*t*Bu)-diazomethylketone (Z-FY-DMK) was from Calbiochem (San Diego, CA). Recombinant forms of OC-I (referred to as *O*sCYS1: Girard et al., 2007), oryzacystatin II (OC-II, or *O*sCYS2), corn cystatin II (CC-II, or *Zm*CYS2), and human stefin A were produced in *Escherichia coli* as glutathione S-transferase fusion proteins (Smith and Johnson, 1988), as described earlier (Michaud et al., 1994, 1996b; Brunelle et al., 2005). *O*sCYS1 was also expressed as a histidine (His)-tagged protein using the 6  $\times$  Histidine Tagging System<sup>TM</sup> (Qiagen, Mississauga ON, Canada), with the vector pAOCI-3 (Leplé et al., 1995) as a DNA template. An *O*sCYS1-encoding DNA fragment excised from pAOCI-3 by *Eco*RI/*Pst*I treatment was first cloned between the *Eco*RI and *Pst*I cloning sites of pBlueScript (Stratagene, La Jolla, CA), re-excised as a *Bam*HI/*Kpn*I fragment, and finally introduced in the expression vector pQE31 (Qiagen, Valencia, CA), in frame with the poly-His tag. The resulting plasmid, pQOC-I, was introduced into *E. coli* strain M15 for heterologous expression and purification of the (His)<sub>6</sub>-*O*sCYS1 fusion by nickel ( $\text{Ni}^{2+}$ ) affinity chelation, according to the instructions of the provider (Qiagen). Papaya cystatin (*Cp*CYS1) was cloned, expressed as a (His)<sub>6</sub>-tagged protein, and purified by  $\text{Ni}^{2+}$  affinity chelation as described for *O*sCYS1, using the vector pBlCYS1 as starting material and giving the vector pQPC for (His)<sub>6</sub>-*Cp*CYS1 heterologous expression.

### **Protease and Protease Inhibitory Assays**

Protease activities were assayed *in vitro* with the colorimetric protein substrate azocasein and a number of MCA fluorogenic substrates specific to different protease families. Overall protease activity in the extracts was monitored with azocasein at different pH values as described earlier (Michaud et al., 1995a). The assay buffers were as follows: 100 mM citrate phosphate for pH 4.0–7.0; 100 mM Tris-HCl for pH 7.5–8.5; and 100 mM glycine for pH 9.0–10.0. In brief, 50  $\mu$ L of midgut extract (50  $\mu$ g protein) was mixed with 450  $\mu$ L of assay buffer containing 5 mM L-cysteine. After 10 min at 37°C, 500  $\mu$ L of 2% (w/v) azocasein dissolved in assay buffer was added, and the whole mixture was incubated for 180 min at 37°C. Proteolysis was stopped by adding 100  $\mu$ L of 10% (w/v) cold trichloroacetic acid and incubating the whole mixture for 30 min at 4°C. Residual azocasein was removed by centrifugation at 12,000g for 5 min at 4°C. 1 N NaOH (1 mL) was finally added to 1 mL of supernatant, and the absorbance was read at 440 nm using a Spectronic 1000 Plus spectrophotometer (Milton Roy, Rochester, NY). The  $A_{440}$  of blanks, which consisted of complete mixtures incubated for 0 min, was subtracted from each value. One unit of activity was defined as the amount of insect extract needed to produce an absorbance change of  $1.0 \text{ h}^{-1}$  in a 1-cm cuvette. Each measure was repeated three times.

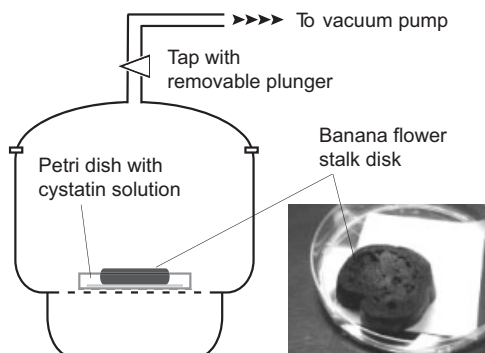
Substrate-specific protease activities were assayed with synthetic fluorogenic MCA substrates by the monitoring of substrate hydrolysis progress curves (Salvesen and Nagase, 1989). Cathepsin L-like, cathepsin B-like, and aminopeptidase (cathepsin H-like) activities were detected in 100 mM citrate phosphate buffer, pH 6.0, using the substrates Z-FR-MCA, Z-RR-MCA, and R-MCA, respectively. Trypsin-like and chymotrypsin-like activities were detected in 50 mM Tris-HCl buffer, pH 8.0, using the substrates Z-R-MCA and *suc*-AAPF-MCA, respectively. Proteolysis was allowed to proceed at 22°C with the substrate in large excess, after adding (or not) protease inhibitors in a minimal volume (Kiggundu et al., 2006). Activity levels were monitored using a Fluostar Galaxy fluorimeter (BMG, Offenburg, Germany), with excitation and emission filters of 360 and 450 nm, respectively. Protease activity rates in the presence or absence of inhibitor were inferred from the slope of the progress curves. All measures were repeated three times.

### **Gelatin SDS-PAGE**

The inhibitory potency of *Os*CYS1, *Cp*CYS1, SBBI, and bovine aprotinin against banana weevil midgut proteinases was also monitored by submitting the protease: inhibitor complexes to mildly-denaturing (non-reducing) gelatin/SDS-PAGE (Michaud, 1998). The insect extracts (3  $\mu$ L, for 3  $\mu$ g of protein) were incubated with excess amounts of protein inhibitor (25  $\mu$ g of inhibitor in 5  $\mu$ L), or with 5  $\mu$ L of 50 mM Tris-HCl, pH 8.0 (non-inhibited control) for 10 min at 37°C before electrophoresis. Protease (gelatinase) forms were visualized as clear bands against a Coomassie blue-stained background following electrophoresis (Michaud et al., 1996a).

### **Diet Bioassay**

A diet bioassay was devised for the monitoring of banana weevil larval growth in *Os*CYS1- and *Cp*CYS1-infiltrated banana stem disks (Fig. 1). Inner stems, which form part of the fruit (bunch) stalk but run in the centre of the pseudo-stem from the bunch to the underground stem, were collected fresh in the greenhouse, cut into 1-cm-thick



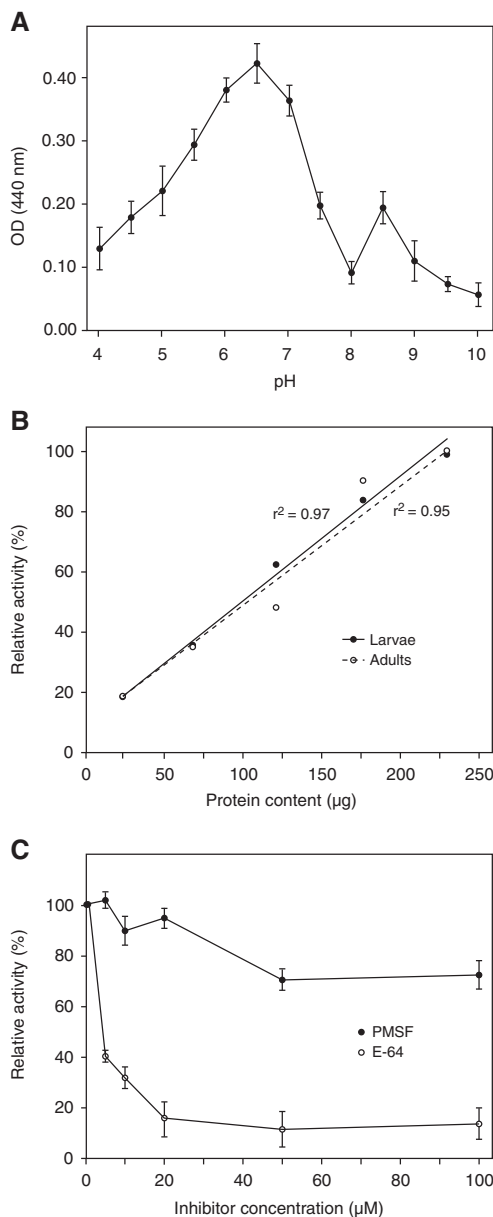
**Figure 1.** Experimental setup for the monitoring of banana weevil larval growth in banana stem disks infiltrated with plant cystatins. The stem disks were first infiltrated with a cystatin solution using a vacuum pump, and then transferred in a Petri dish for larva inoculation and growth monitoring (see text for details).

disks (ca. 15 g fresh weight) and dipped into a 1% (v/v) sorbic acid solution in water for 5 min at 60°C to prevent tissue oxidation and deterioration. The disks were used immediately for the bioassays or wrapped in polythene bags for storage at 4°C. For disk infiltration, 2 ml of *OsCYS1* or *CpCYS1* solution (corresponding to 10 mg of cystatin) was placed in a clean, 5-cm-diameter Petri dish. Three 4-cm-long/1-mm-thick plastic rods were placed in the Petri dish and one banana stem disk was placed on the rods. This allowed placing the disk just above the bottom of the dish and providing uniform contact with the solution. The complete setup was placed in a vacuum desiccator attached to an SC100 Thermo Savant SpeedVac™ vacuum pump (Thermo Fisher Scientific, Waltham, MA) equipped with a Savant RT100 refrigerated condensation trap (Thermo Fisher Scientific). A vacuum was applied until observing bubbling on both the surface of the tissue and the solution. The vacuum was then rapidly removed by unplugging using a conveniently placed tap plunger, causing rapid infiltration of the banana stem disk with the cystatin solution. The infiltrated stem disk was removed and placed on a filter paper in a clean Petri dish, and a newly hatched banana weevil larva was placed in a small hole made on the disk. Treated disks were stored in the dark at 25°C, and larvae dissected out and weighed after 10 days. Disks infiltrated with elution buffer were used as negative controls. Each treatment was repeated with 10 to 15 larvae and disks.

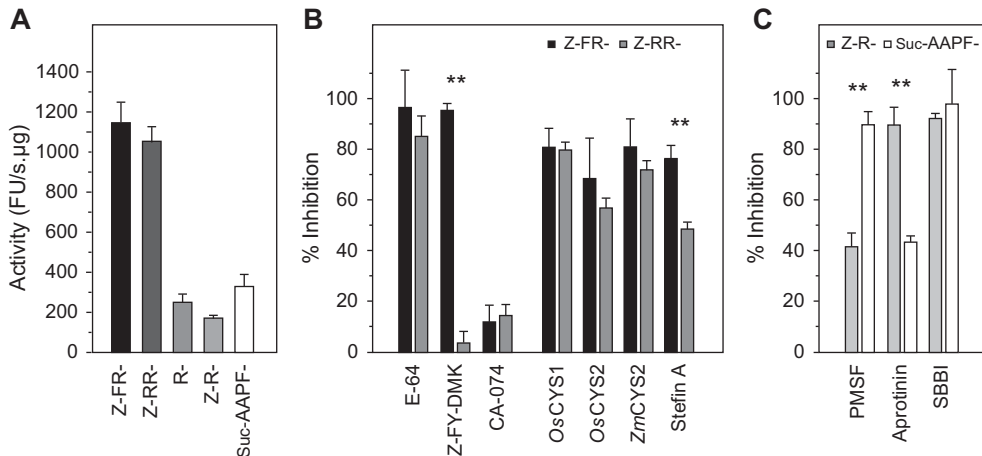
## RESULTS

### *Cysteine and Serine Proteases in the Banana Weevil Midgut*

Protein and fluorogenic peptide substrates were used to characterize banana weevil midgut proteases and their interactions with different synthetic and natural protease inhibitors. Azocasein was first used as a general, non-specific protein substrate to draw an overview of major protease activities in the midgut as a function of pH (Fig. 2A). The highest peak of activity in reducing conditions was found at mildly acidic pH values, with maximal protein hydrolysis at pH 6.5. A second, minor peak was detected at pH 8.5, in line with Montesdeoca et al. (2005) reporting the occurrence of trypsin- and chymotrypsin-like serine proteases in larval midgut extracts. Azocasein hydrolysis



**Figure 2.** Proteinase (azocaseinase) activity in midgut protein extracts of the banana weevil. **A:** Proteinase profile from 3rd-/4th-instars larval extracts, as a function of pH. Each datum is the mean of three independent values  $\pm$  SE. **B:** Proteinase activities in 3rd-/4th-instars and adult extracts. Data are expressed as relative activities at pH 6.5 compared to the activity measured with 225  $\mu$ g of larva protein. Straight lines were fitted to the data by the least-squares method. **C:** Dose-response effects of the diagnostic protease inhibitors *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64) and phenylmethylsulfonyl fluoride (PMSF) on midgut proteinases of 3rd-/4th-instars larvae active at pH 6.5. Data are expressed as relative activities compared to a non-inhibited control (100%). Each datum is the mean of three independent values  $\pm$  SE. Protease activities measured for PMSF- and E-64-treated extracts were significantly different at all inhibition concentrations tested (Student's *t*-test;  $P < 0.01$ ).



**Figure 3.** Cysteine cathepsin and serine protease activities in midgut protein extracts of banana weevil 3rd/4th-instar larvae. **A:** Z-FR-MCA- (cathepsin L-like), Z-RR-MCA- (cathepsin B-like), R-MCA- (amino-peptidase), Z-R-MCA- (trypsin-like), and *suc*-AAPF-MCA- (chymotrypsin-like) hydrolyzing specific activities. **B:** Inhibitory effects of *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), Z-Phe-Tyr(*t*Bu)-diazomethylketone (Z-FY-DMK), L-3-*trans*-(propyl-carbamyl)oxirane-2-carbonyl-L-isoleucyl-L-proline (CA-074), and different cystatins on cysteine cathepsin enzymes. **C:** Inhibitory effects of phenylmethylsulfonyl fluoride (PMSF), bovine aprotinin, and soybean trypsin-chymotrypsin inhibitor (SBBI) on trypsin-like and chymotrypsin-like enzymes. Assays were performed at pH 6.5 for cysteine cathepsins and at pH 8.0 for trypsin and chymotrypsin. Each bar is the mean of three independent measurements  $\pm$  SE. On B and C, asterisks (\*\*) indicate highly significant differences for inhibitory rates measured with the two substrates assessed (Student's *t*-test;  $P < 0.01$ ).

at pH 6.5, detected at similar specific protein levels in larval and adult extracts (Fig. 2B), was weakly sensitive to the serine protease inhibitor PMSF in reducing conditions (Fig. 2C). By contrast, the cysteine protease inhibitor E-64 strongly inhibited most protease activity in the same conditions (Fig. 2C), suggesting the predominance of papain-like cysteine (endo)proteases in the digestive tract of banana weevil larvae.

MCA fluorogenic substrates were used to characterize protease activities in a more specific way, and to assess their sensitivity to different proteinaceous cysteine- and serine-type inhibitors (Fig. 3). Proteases active at pH 6.5 readily hydrolyzed the substrates Z-FR-MCA, Z-RR-MCA, and, to a lesser extent, R-MCA (Fig. 3A), suggesting the presence of cathepsin L-like, cathepsin B-like, and aminopeptidase (cathepsin H-like) activities in larval extracts, as observed earlier for a number of coleopteran insects (e.g., Thie and Houseman, 1990; Michaud et al., 1993; Bown et al., 2004; Montesdeoca et al., 2005; Vinokurov et al., 2006a,b; Prabhakar et al., 2007). Trypsin- and chymotrypsin-like serine proteases hydrolyzing, respectively, Z-R-MCA and *succinyl*-AAPF-MCA, were detected at pH 8.0 (Fig. 3A) but barely detectable at pH 6.5 (not shown), which points again to the presence of serine proteinases in the midgut extracts, as also reported for other Coleoptera predominantly relying on cysteine proteases for dietary protein digestion (e.g., Novillo et al., 1997; Vinokurov et al., 2006a,b, 2009; Prabhakar et al., 2007).

### Cystatin-Sensitive Cathepsins in the Banana Weevil Midgut

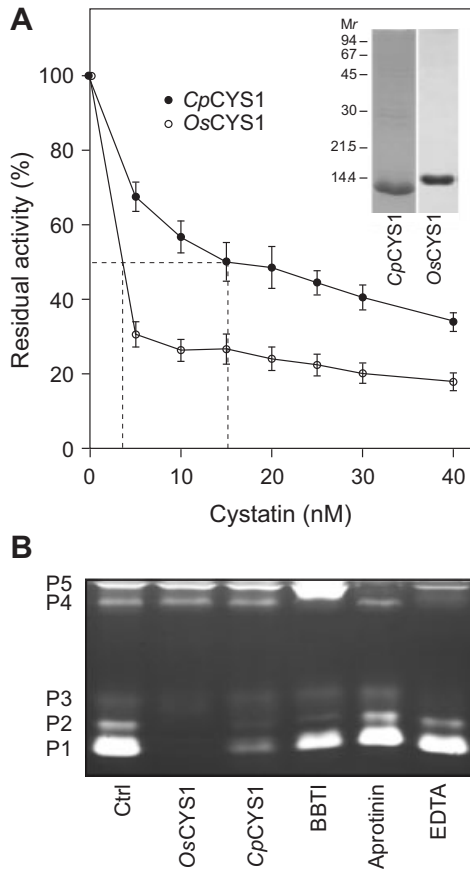
As expected, the broad-spectrum inhibitor of cysteine cathepsins E-64 inhibited most Z-FR-MCA- and Z-RR-MCA-hydrolyzing proteases (Fig. 3B), compared to PMSF

inhibiting most *suc*-AAPF-MCA-hydrolyzing chymotrypsin-like proteinases and a significant fraction of Z-R-MCA-hydrolyzing, trypsin-like proteinases (Fig. 3C). Confirming the occurrence of cathepsin L-like activity, the irreversible inhibitor Z-FY-DMK, specific to cathepsin L-like enzymes (Shaw et al., 1993), inhibited Z-FR-MCA-hydrolyzing enzymes while inhibiting only a small fraction of the Z-RR-MCA-hydrolyzing enzymes (Fig. 3B). By contrast, the E-64 derivative CA-074, specific to cathepsin B-like enzymes (Murata et al., 1991), showed no inhibitory activity against most Z-RR-MCA- and Z-FR-MCA-hydrolyzing enzymes (Fig. 3B), as also reported earlier for midgut cysteine cathepsins of the Western corn rootworm *Diabrotica virgifera* (Bown et al., 2004). Unlike cysteine-type inhibitors (not shown), the potent trypsin inhibitor bovine aprotinin inhibited most trypsin-like and some chymotrypsin-like activities in the extracts, compared to the soybean Bowman-Birk (trypsin-chymotrypsin) inhibitor completely inactivating both Z-R-MCA- and *suc*-AAPF-MCA-hydrolyzing proteinases (Fig. 3C). Similar to E-64, human stefin A and different plant cystatins including *Os*CYS1 inhibited an important fraction of the activity detected with the Z-FR-MCA and Z-RR-MCA substrates (Fig. 3B). Overall, the well-documented preference of coleopteran cathepsin L-like enzymes for Z-FR-MCA despite the Z-RR-MCA-hydrolyzing activity of some isoforms (Bown et al., 2004; Cristofolletti et al., 2005), the specificity of cathepsin B-like enzymes for Z-RR-MCA (Mort, 1998), and the differential inhibitory effects of Z-FY-DMK and human stefin A against banana weevil Z-FR- and Z-RR- hydrolyzing proteinases (Fig. 3B) suggest the occurrence of cathepsin L-like and cathepsin B-like proteinase populations in the banana weevil midgut, both sensitive to E-64 and cystatins but exhibiting distinct preferences for the Z-FR-MCA and Z-RR-MCA substrates.

#### ***Deleterious Effects of Plant Cystatins Against Banana Weevil Larvae***

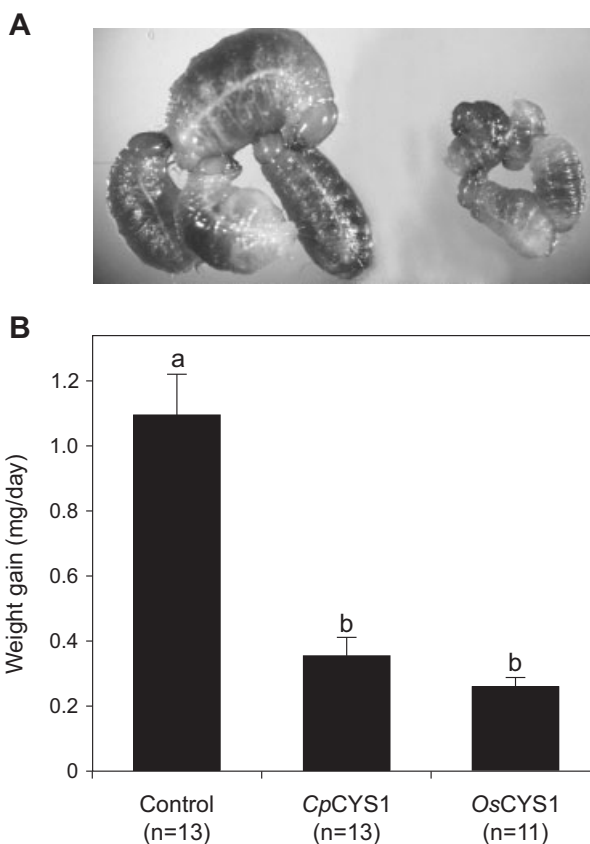
Dose-response protease inhibitory assays and gelatin/SDS-PAGE zymography were carried out with *Os*CYS1 and *Cp*CYS1 to estimate the inhibitory potency of different plant cystatins against banana weevil Z-FR-MCA- and gelatin-hydrolyzing cysteine cathepsins (Fig. 4). The two cystatins were expressed in *E. coli* as His-tagged proteins and purified under a stable form by immobilized metal ( $\text{Ni}^{2+}$ ) affinity chromatography (Fig. 4A, inset gel).  $IC_{50}$  values, i.e., the amount of inhibitor needed for 50% inhibition, of 3.7 and 15.2 nM were calculated, respectively, for the inhibition of cathepsin L-like proteinases by *Os*CYS1 and *Cp*CYS1 under our assay conditions (Fig. 4A), which suggests a stronger inhibitory effect of the first inhibitor at low concentrations. The higher potency of *Os*CYS1 was also inferred by submitting the *Os*CYS1: and *Cp*CYS1:weevil proteinase complexes to gelatin/SDS-PAGE in non-reducing conditions (Fig. 4B). Out of five gelatinases detected on the zymograms (referred to as proteinases P1 to P5), two (P1 and P2) were completely inhibited by *Os*CYS1 following electrophoresis in the presence of SDS, indicating a high stability for the *Os*CYS1:P1 and *Os*CYS1:P2 complexes, with  $K_i$  values presumably in the nanomolar or subnanomolar range (Michaud et al., 1996a). *Cp*CYS1 also caused an important loss of P1 and P2 activity, but part of the inhibition was restored following electrophoresis, which suggests a weaker inhibitory effect against the two enzymes and  $K_i$  values in the [10, 100] nanomolar range for the *Cp*CYS1:P1 and *Cp*CYS1:P2 complexes (Michaud et al., 1996a).

A diet bioassay was conducted with banana weevil larvae and cystatin-infiltrated banana stem disks (see Fig. 1) to measure the impact of *Os*CYS1 and *Cp*CYS1 on



**Figure 4.** Inhibition of banana weevil larval midgut cysteine cathepsins by recombinant (His)<sub>6</sub>-tagged variants of oryzacystatin I (*OsCYS1*) and papaya cystatin (*CpCYS1*). **A:** Dose-response of Z-FR-MCA-hydrolyzing enzymes to *OsCYS1* and *CpCYS1* at pH 6.5. Data are expressed as residual activity following inhibition, compared to the activity of a non-inhibited control (100%). The dashed lines indicate *IC*<sub>50</sub> values, defined as inhibitor concentrations required for a residual activity of 50%. Each datum is the mean of three values ± SE. Residual activities at cystatin concentrations of 5 and 15 nM were significantly different at *P* = 0.01 (Student's *t*-test). The inset gel is a Coomassie blue-stained image of the purified cystatins following 15% (w/v) SDS-PAGE. Mr, commercial molecular weight markers (kDa). **B:** Stability of *OsCYS1*: and *CpCYS1*:banana weevil proteinase complexes submitted to non-reducing gelatin/SDS-PAGE. Proteinase (gelatinase) activities were visualized as clear lysis areas against a dark Coomassie blue-stained gelatin substrate background after protease digestion (see Materials and Methods). A low residual activity (i.e., no or little activity detected on gel) indicates a strong interaction (*K*<sub>i</sub> value in the nanomolar range or lower), and a high residual activity (i.e., with complete or partial restoration of activity) indicates a weaker or negligible interaction (*K*<sub>i</sub> value in the micromolar range of higher) (Michaud et al., 1996a). Numbers on the left (P1, P2, P3, P4, and P5) refer to the insect gelatinases. Ctrl, non-inhibited control; BBTI, soybean Bowman-Birk trypsin/chymotrypsin inhibitor; EDTA, ethylenediamine tetraacetic acid.

banana weevil larval growth (Fig. 5). As shown in Figure 5A, larvae provided with cystatin-infiltrated stems were dramatically affected after 10 days compared to larvae provided with control, cystatin-free material. No mortality due to cystatin ingestion was observed over the period assessed, but early larval growth expressed as body weight gain per day was significantly reduced by either cystatin (Fisher's LSD test; *P* < 0.01), with measured growth rates of 0.25 to 0.35 mg/day compared to 1.1 mg/day



**Figure 5.** Impact of dietary cystatins on growth of newly hatched banana weevil larvae. **A:** Larvae collected after 10 days from cystatin-free (control) (left) and *OsCYS1*-infiltrated (right) stem disks. **B:** Daily weight gain of larvae developing in *OsCYS1*, *CpCYS1*, or cystatin-free (control) stem disks. Final cystatin concentrations after infiltration were estimated at  $\sim 0.6$  mg per g fresh weight, as measured following protein re-extraction. Each datum is the mean of 11 or 13 values  $\pm$  SE. Means with the same letter are not significantly different (protected Fisher's LSD test;  $P < 0.01$ ).

for larvae fed the control diet (Fig. 5B). Comparable growth decreases of about 70% relative to the controls were observed for the two cystatins under our experimental conditions ( $P > 0.05$ ), despite the stronger inhibitory potency of *OsCYS1* against Z-FR-MCA-hydrolyzing enzymes (see Fig. 4).

## DISCUSSION

Despite some promising developments (Leplé et al., 1995; Kuroda et al., 1996; Koiwa et al., 2000; Liu et al., 2004; Alvarez-Alfageme et al., 2007; Ninkovic et al., 2007), the general usefulness of plant cystatins for the control of coleopteran herbivores still remains to be established. These insects have developed over time effective strategies to elude the inhibitory effects of plant protease inhibitors, involving the use of complex digestive protease systems with proteases from different mechanistic classes acting in a complementary, coordinated manner (Brunelle et al., 1999; Hernandez et al., 2003; Gruden et al., 2003; Vinokurov et al., 2006a,b; Prabhakar et al., 2007); the

over-expression of target proteases following cystatin ingestion to outnumber the inhibitory proteins (Cloutier et al., 2000; Ahn et al., 2004); the constitutive or diet-induced expression of cysteine cathepsins weakly sensitive to the ingested cystatin (Michaud et al., 1993, 1995a,b; Girard et al., 1998a; Cloutier et al., 1999, 2000; Zhu-Salzman et al., 2003; Brunelle et al., 2004; Gruden et al., 2004; Liu et al., 2004; Koo et al., 2008); the over-expression of proteases from alternative mechanistic classes (Zhu-Salzman et al., 2003; Brunelle et al., 2004; Rivard et al., 2004; Oppert et al., 2005); and the degradation of defensive protease inhibitors using non-target, insensitive proteases (Michaud et al., 1995b; Girard et al., 1998b; Giri et al., 1998; Gruden et al., 2003; Zhu-Salzman et al., 2003). Taking this into account, the aim of the present study was to assess the possible detrimental effects of plant cystatins against the banana weevil *C. sordidus*, and to interpret these effects in the light of their inhibitory spectrum against cysteine cathepsin activities eventually detected in the midgut. Protease inhibitory assays were first conducted to measure the inhibitory efficiency of different cystatins in vitro, followed by a diet bioassay with cystatin-infiltrated banana stem disks to visualize the impact of two plant cystatins, *OsCYS1* and *CpCYS1*, on the overall growth rate of young larvae.

Similar to other successful examples (e.g., Zhao et al., 1996; Koiwa et al., 2000; Liu et al., 2004; Alvarez-Alfageme et al., 2007; Ninkovic et al., 2007), our data indicate a dramatic negative impact of plant cystatins on larval growth, associated with the presence of cystatin-sensitive cysteine cathepsins in midgut extracts. As suggested from studies reporting detrimental effects for the broad-spectrum inhibitor of papain-like proteases E-64 against Coleoptera the negative effects of *OsCYS1* and *CpCYS1* were likely the result of a broad sensitivity of the insect digestive cathepsins to the ingested inhibitors. The negative effects of E-64 on growth and fecundity of several coleopteran species, including cystatin-resistant insects such as the Colorado potato beetle *Leptinotarsa decemlineata* (Bolter and Latoszek-Green, 1997) or the cowpea weevil *Callosobruchus maculatus* (Murdock et al., 1988), were systematically associated with a broad inhibition of cysteine proteases in midgut extracts (Michaud et al., 1993; Fabrick et al., 2002; Kim and Mullin, 2003). By contrast, the reported inefficiency of plant cystatins against a number of Coleoptera was typically associated with the presence of cystatin-insensitive cysteine proteases, thought to help the target insects compensating for the loss of sensitive protease activities following cystatin intake (Cloutier et al., 1999, 2000; Zhu-Salzman et al., 2003; Gruden et al., 2003, 2004). In line with previous studies reporting detrimental effects for cystatins or other plant cysteine-type inhibitors against some coleopteran species (Zhao et al., 1996; Koiwa et al., 2000; Fabrick et al., 2002), the negative effects of *OsCYS1* and *CpCYS1* against the banana weevil were correlated with a broad inhibitory spectrum of the two cystatins against Z-FR-MCA-hydrolyzing (cathepsin L-like) and Z-RR-MCA-hydrolyzing (cathepsin B-like) cysteine proteases in midgut extracts, comparable to the overall inhibitory effect of E-64 against the same enzymes. Our data support, in sum, the hypothesis of a close relationship between the inhibitory spectrum of plant cystatins against E-64-sensitive cysteine proteases in midgut extracts and the potential of these inhibitors as effective candidates for the development of transgenic plant lines resistant to coleopteran pests.

The detection of cathepsin L-like and cathepsin B-like activities in banana weevil midgut extracts is not surprising considering the prevalence and numerical importance of cathepsin L and cathepsin B coding sequences in the recently described genome of *T. castaneum* (*Tribolium* Genome Sequencing Consortium, 2008), a

coleopteran herbivore of the same evolutionary clade (Hunt et al., 2007). On the other hand, the occurrence of cathepsin B variants sensitive to cystatins in the midgut of the banana weevil (Fig. 3B) (and other cystatin-susceptible insects; Bown et al., 2004) is somewhat intriguing since these enzymes were identified as possible key determinants in the resistance of Coleoptera to dietary cystatins (Michaud et al., 1993; Cloutier et al., 2000; Goulet et al., 2008; Koo et al., 2008). Unlike other proteases of the papain family, cathepsin B and cathepsin B-like homologues bear an extra structural element, the “occluding loop,” which physically hinders the substrate binding cleft and prevents free access to the inhibitory loops of large competitive inhibitors such as cystatins (Musil et al., 1991; Illy et al., 1997). In agreement with these structural inferences, a bacterially expressed variant of CmCatB1, a midgut cathepsin B-like enzyme synthesized *de novo* in the cowpea weevil *C. maculatus* challenged with dietary cystatins (Liu et al., 2004; Moon et al., 2004), was shown to bear an occluding loop-like structure, slightly shorter than the occluding loop of human cathepsin B but long enough to cause steric hindrance in the active site cleft and prevent inhibition by cystatins (Koo et al., 2008).

A possible explanation for the broad-spectrum inhibition of cysteine cathepsins by plant cystatins in some Coleoptera might be the predominance of cathepsin L-like enzymes in the midgut, including some forms able to cleave both the Z-RR-MCA and Z-FR-MCA substrates (see Bown et al., 2004 and Cristofolletti et al., 2005). An alternative explanation would be the predominance of cystatin-sensitive cathepsin B-like enzymes in some Coleoptera. Additional empirical data are now needed at the physiological and structural levels to document further the role of cathepsin B variants as possible determinants for the resistance or susceptibility of herbivorous Coleoptera to plant cystatins. Additional work is also required to compare the antidigestive potential of different plant cystatins against the banana weevil. Our bioassays with *OsCYS1* and *CpCYS1* were conducted using excess concentrations of inhibitor, estimated at ~0.6 mg/g fresh weight of infiltrated stem disk after re-extraction (not shown). Such concentrations would represent accumulation rates reaching ~2% of leaf soluble proteins in tissues of transgenic banana lines, higher than the rates of 0.1–1.0% typically observed in cystatin-expressing transgenic plants (Cloutier et al., 2000; Van der Vyver et al., 2003). In practice, a strong inhibitory potency of the protease inhibitor selected is critical for an effective inhibition effect, as it directly determines the minimal amount of recombinant inhibitor required in the host plant to provide sufficient pesticidal effects (De Leo et al., 1998; Goulet et al., 2008). On this basis, the strong binding activity of *OsCYS1* against cysteine cathepsins of banana weevil larvae compared to *CpCYS1* (see Fig. 4) would likely make this cystatin an interesting starting point for the development of banana weevil-resistant transgenic banana lines.

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