Barley cystatin variants against phytopathogenic fungi, pests and their impact on natural enemies

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Abstract: The goal of this study has been to know the effects of the barley cystatin Hv-CPI and seven derived variants generated by direct-mutagenesis, on the growth and digestive physiology of the Colorado potato beetle (CPB), Leptinotarsa decemlineata and to assess the potential impact of these proteins on the spiner soldier bug (SSB), Podisus maculiventris, a generalist hemipteran predator. Among the different cystatins tested, the variant C⁶⁸G, in which the only cysteine residue was changed to glycine, showed the highest inhibitory activity when tested in vitro against commercial cysteine proteases and CPB digestive enzymes. Feeding trials conducted with CPB larvae reared on transgenic potato plants expressing this variant, resulted in significant lower weight gains compared to those fed on non-transformed plants. No effects on survival, development, and weight, were observed when SSB nymphs fed on prey CPB reared with transgenic potato plants. To investigate the physiological background, biochemical analysis were carried out on guts of insects dissected at the end of the feeding assays. The effects of the barley inhibitor Hv-CPI and its variants on the growth of the the necrotrophic fungus Fusarium oxysporum have been also analysed. The cystatin Hv-CPI inhibited fungal spore germination by 25%, the five point mutations inhibited spore germination by 18 to 40%, while the two truncated forms had no antifungal effect.

Key words: cystatin, insect pest, natural enemy, protein defence, transgenic potatos

Introduction

Plant cystatins are a group of specific inhibitors of cysteine proteases that have been involved in the regulation of protein turnover and in plant defence. The protective role is supported by in vitro inhibition data and bioassays against different pests and by the enhanced resistance towards insects, nematodes, slugs and potyviruses in transgenic plants expressing plant cystatins (for a review see Haq et al., 2004). As far as we know, only few cystatin genes from rice, maize and Arabidopsis have been used as transgenes in crop protection assays. Fungicidal and antimicrobial activities have been also described for several cystatins (Pernas et al., 2000; Martinez et al., 2003, 2005). However, the mechanism responsible of their antifungal properties is still unknown, although we have shown that is not associated with its role as cysteine protease inhibitor (Martinez et al., 2003).

We have reported previously the characterization of a barley cystatin Hv-CPI (Gaddour et al., 2001) and several variants derived from it with different Ki (inhibition constant) against cysteine proteases and antifungal properties against Botryis cinerea (Martinez et al., 2003). Our aim is to search for improved cystatin genes to be used as transgenes and to investigate their direct and indirect impacts on insect pests, their natural predators and on fungal pathogens.
The necrotrophic fungus *Fusarium oxysporum* causes vascular wilt diseases in many plant species worldwide. Their clamydospores persist in dead tissues or are released into the soil where they remain dormant until suitable conditions allow them to germinate and enter the vascular tissues producing the death of the plants (Jimenez-Casco et al., 2004).

The Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) is one of the most serious pest of potato worldwide. Protein digestion in CPB larvae and adults is carried out by a complex proteolytic system. Purification and characterization of their digestive protease activities have shown that they are mainly of the cysteine protease type (Novillo et al., 1997). The spined soldier bug (SSB), *Podisus maculiventris* (Hemiptera: Pentatomidae) is a generalist predator used as a biological control agent of lepidopteran and coleopteran pests. Protease activity in SSB midguts is mainly based on cysteine proteases (Bell et al., 2005). The presence of digestive cysteine-proteases both in prey (CPB) and in the predator (SSB) suggested their potential for studying a possible interference mediated by cystatins.

This study has been focused on two main goals, i) to determine the in vitro inhibitory activity of the Hv-CPI cystatin and their derived variants against commercial cathepsin B and the phytopathogenic fungus *F. oxysporum* and ii) to analyse the effects of transgenic potato plants expressing a selected variant of the barley cystatin on the *L. decemlineata* and on its natural enemy, *P. maculiventris*.

**Materials and methods**

**Inhibitory activity of recombinant barley cystatins in vitro**

The wild type Hv-CPI, five mutant variants and two truncated forms derived from it were purified from recombinant *E. coli* cultures as described previously (Martinez et al., 2003). Their inhibitory properties in vitro against commercial cathepsin B and digestive cathepsin B-like activity from guts extracts of CPB larvae and SSB nympha were assayed, using ZAA2MNA (N-carbobenzyoxy-alanine-arginine-arginine-4-methoxy-β-naphtylamide) as substrate and the conditions described by Novillo et al. (1997). Fungal growth inhibitory assays were performed as described Martinez et al. (2005), by incubating $10^4$ spores of *F. oxysporum* in potato dextrose broth (PDB) liquid medium in the absence and presence of 6 µM of each cystatin variant. Inhibition constant ($K_i$) values against commercial cathepsin B were determined from Dixon plots (1/V versus [I]). The effect on insects and fungi was monitored by measuring the percentage of enzyme inhibition and of spore germination, respectively.

**Insect bioassays with potato transgenic plants**

Different transgenic potato lines expressing the C68→G barley cystatin variant were produced (Alvarez-Alfageme et al., submitted) and used to feed second instar *L. decemlineata* for 4 days. In addition, third instar nymphs of *P. maculiventris* were reared with CPB fed on potato lines until they reach the fifth instar. To determine the effect of transgenic cystatin-potato plants on both insects, CPB larvae and SSB nymphs were weighed and dissected for digestive protease assays (Ortego et al., 1999).
Results and discussion

Inhibition of fungal growth and determination of Ki values against cathepsin B

In vitro bioassays of the necrotrophic fungus *F. oxysporum* were carried out adding at the PDB medium the seven recombinant cystatin variants and the wild type Hv-CPI purified from *E. coli* cultures at the 6 µM concentration. After incubation of 48 h, the wild-type Hv-CPI produced an inhibition of fungal spore germination of 35% and the five point variants inhibited between 18 (R58→G) to 40% (Q63→L), as compared to the 100% of germination control obtained in the medium without cystatin (Table 1). The two truncated forms did not affect spore germination, even at concentrations of 12 µM (data not shown), as well as the protein purified from *E. coli* transformed with the expression vector alone.

The biochemical characterization of these cystatin variants against commercial cathepsin B showed that the variant Q63→P was unable to inhibit cathepsin B, as well as other cysteine proteases (Martinez et al., 2003), but maintained the antifungal properties against *F. oxysporum*, indicating that the cystatin role as fungicide is not associated with its function as inhibitor of cysteine proteases. We have previously reported this effect mediated by Hv-CPI cystatin and their variants after studying the *in vitro* inhibition growth of another important phytopathogenic fungus such is *B. cinerea* (Martinez et al., 2003).

Table 1. Inhibition of spore germination of *F. oxysporum* and of digestive cathepsin B-like activity (CTB) from *L. decemlineata* midgut extracts and determination of Ki values against commercial cathepsin B. *a* 6 µM of each cystatin was used for fungal assays, and *b* 1 µM was used for insect digestive extracts. ni: no inhibitory activity detected.

<table>
<thead>
<tr>
<th>Cystatin variants</th>
<th>a Spore germination of <em>F. oxysporum</em></th>
<th>b Inhibition of CTB digestive activity of CPB (%)</th>
<th>Ki (M) against cathepsin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No protein</td>
<td>100 ± 2</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>HvCPI</td>
<td>75 ± 6</td>
<td>22 ± 1</td>
<td>2.5 x 10⁻⁶</td>
</tr>
<tr>
<td>Q63→L</td>
<td>60 ± 4</td>
<td>26 ± 3</td>
<td>4.5 x 10⁻⁵</td>
</tr>
<tr>
<td>Q63→P</td>
<td>75 ± 6</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>R58→G</td>
<td>82 ± 8</td>
<td>10 ± 1</td>
<td>5.9 x 10⁻⁵</td>
</tr>
<tr>
<td>C68→G</td>
<td>67 ± 7</td>
<td>43 ± 2</td>
<td>2.7 x 10⁻⁸</td>
</tr>
<tr>
<td>K92→P</td>
<td>88 ± 6</td>
<td>8 ± 1</td>
<td>3.1 x 10⁻⁴</td>
</tr>
<tr>
<td>N-termΔQ62</td>
<td>100 ± 3</td>
<td>ni</td>
<td>ni</td>
</tr>
<tr>
<td>ΔQ63C-term</td>
<td>100 ± 3</td>
<td>ni</td>
<td>ni</td>
</tr>
</tbody>
</table>

Inhibition of CPB and SSB digestive proteases

Barley cystatin variants were tested *in vitro* against the main midgut protease activity belonging to the cathepsin B-like from CPB larvae. The wild-type protein produced an inhibition of 22% of enzyme activity. However, the variant C68→G was a much better inhibitor than the wild-type because this substitution reduced the digestive activity to almost 45%. This data indicated that the G68 substitution was an important amino acid for the inhibitory properties of the Hv-CPI cystatin. The other point variants seemed to be less effective as inhibitors, being the Q63→P protein, in which the target Q of the reactive site was
substituted by the cyclic amino acid proline, devoid of cysteine protease inhibitory capacity (Table 1). No effect was observed when the two truncated forms were used. These results are according with the $K_i$ values determined against cathepsin B.

Similar in vitro assays were performed with the wild-type and the $C^{68}\rightarrow G$ proteins against extracts from midgut of SSB nymphs and no inhibitory activity of cathepsin B-like was observed (data not shown). However, it is important to mention that the digestive activity in SSB midguts is mainly based on cysteine proteases (Bell et al., 2005).

Taking in mind all these data, we decided to use the $C^{68}\rightarrow G$ cystatin variant as transgene to fight against the main potato insect pest, the Colorado potato beetle and to study their impact on its natural enemy, the spined soldier bug.

**Effect of transgenic potatoes expressing the $C^{68}\rightarrow G$ cystatin on CPB larvae and SSB nymphs**

Larvae of CPB were reared on the transgenic potato lines expressing the $C^{68}\rightarrow G$ variant as well as on non-transgenic plants (NT), to assess the cystatin effect on larval growth (Alvarez-Alfageme et al., submitted). While insect survival was not affected, larvae that had fed on some transgenic potato lines showed presented significantly lower weight gains, particularly those fed on line G16 (Table 2). Protein extracts from this line showed the highest inhibitory activity against cysteine proteases (Alvarez-Alfageme et al., submitted). Moreover, biochemical analysis carried out on guts of CPB insects dissected at the end of the feeding assay, showed higher cathepsin B-like activity levels than those fed on non transformed isogenic plants, probably produced as an adaptation response to the presence of the cystatin in the transgenic lines (Alvarez-Alfageme et al., submitted).

**Table 2. Growth of *L. decemlineata* larvae feeding on different lines of transgenic potato plants expressing the $C^{68}\rightarrow G$ cystatin (G) or on its corresponding non-transformed isogenic plants (NT). a Feeding assays were performed for 4 days from second to fourth instar. Larval growth is expressed in mg of fresh weight. Data are the mean ± SE (n = 48-64). *Significantly different from NT (Dunnet two-tailed test P < 0.05).**

<table>
<thead>
<tr>
<th>Potato plants</th>
<th>NT</th>
<th>G2</th>
<th>G5</th>
<th>G10</th>
<th>G11</th>
<th>G14</th>
<th>G16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval growtha (mg fw)</td>
<td>59 ± 3</td>
<td>57 ± 5</td>
<td>62 ± 5</td>
<td>58 ± 3</td>
<td>56 ± 4</td>
<td>52 ± 3</td>
<td>46 ± 2*</td>
</tr>
</tbody>
</table>

To study the prey-mediated effects at the third trophic level, SSB nymphs were fed on CPB larvae reared with G16 and NT potato lines. No differences in development or weight of this natural enemy were observed, whereas a slight reduction on midgut cathepsin B-like activity of SSB nymphs fed on CPB larvae reared on G16 plants was detected (Alvarez-Alfageme et al., submitted). These results indicate that the cystatin interactions that take place in the prey produce an insecticide effect and trigger an over-expression of the target digestive proteases of the Colorado potato beetle, while the possible impact on the spined soldier bug seems to be no harmful. The absence of detrimental effects on other insect predators via their herbivorous preys feeding on different species of transgenic plants such are potato and oilseed rape expressing the oryzacystatin-I from rice or the chicken egg white cystatin, has been also reported (Bouchard et al., 2003; Ferry et al., 2003; Cowgill et al., 2004). More work has to be done for clarifying the importance of direct and indirect effects of transgenic plants in multitrophic interactions.
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References


