Tailoring the Specificity of a Plant Cystatin toward Herbivorous Insect Digestive Cysteine Proteases by Single Mutations at Positively Selected Amino Acid Sites\textsuperscript{1}[OA]

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Plant cystatins, similar to other defense proteins, include hypervariable, positively selected amino acid sites presumably impacting their biological activity. Using 29 single mutants of the eighth domain of tomato (\textit{Solanum lycopersicum}) multicystatin, SlCYS8, we assessed here the potential of site-directed mutagenesis at positively selected amino acid sites to generate cystatin variants with improved inhibitory potency and specificity toward herbivorous insect digestive cysteine (Cys) proteases. Compared to SlCYS8, several mutants (22 out of 29) exhibited either improved or lowered potency against different model Cys proteases, strongly suggesting the potential of positively selected amino acids as target sites to modulate the inhibitory specificity of the cystatin toward Cys proteases of agronomic significance. Accordingly, mutations at positively selected sites strongly influenced the inhibitory potency of SlCYS8 against digestive Cys proteases of the insect herbivore Colorado potato beetle (\textit{Leptinotarsa decemlineata}). In particular, several variants improved inhibitory potency against both cystatin-sensitive and cystatin-insensitive digestive Cys proteases of this insect. Of these, some variants also showed weaker activity against leaf Cys proteases of the host plant (potato [\textit{Solanum tuberosum}]) and against a major digestive Cys protease of the two-spotted stink bug \textit{Perillus bioculatus}, an insect predator of Colorado potato beetle showing potential for biological control. Overall, these observations suggest the usefulness of site-directed mutagenesis at positively selected amino acid sites for the engineering of recombinant cystatins with both improved inhibitory potency toward the digestive proteases of target herbivores and weaker potency against nontarget Cys proteases in the host plant or the environment.

The expression of recombinant cystatins in transgenic crops has been proposed by several authors to prevent herbivory or pathogenic infection (Arai and Abe, 2000; Atkinson et al., 2003; Haq et al., 2004). Cystatins, similar to other competitive protease inhibitors, form a tight complex with the active site of target proteases to cause inhibition and interfere with dietary protein digestive functions in herbivorous organisms (Arai et al., 2002). Cystatins expressed in transgenic plant lines were shown to alter the development of herbivorous arthropods and root parasitic nematodes relying on Cys proteases for extracellular protein digestion (e.g. Leplé et al., 1995; Atkinson et al., 2003; Ouchkourof et al., 2004). These proteins also show potential for microbial control, as suggested by their deleterious effects against a number of fungal and viral pathogens of agronomic significance (Gutierrez-Campos et al., 1999; Martinez et al., 2005; Yang and Yeh, 2005; Christova et al., 2006).

Despite promising developments, the successful use of cystatins in plant protection still remains confined to specific examples. Insect herbivores have developed over time effective strategies to elude the inhibitory effects of plant protease inhibitors (Broadway, 2000), including: (1) the use of complex digestive protease systems with proteases from different mechanistic classes acting in a complementary, coordinated manner (Terra and Ferreira, 1994; Brunelle et al., 1999, 2004); (2) the production of alternative, insensitive protease forms following ingestion of protease inhibitors (Jongsma et al., 1995; Bown et al., 1997; Cloutier et al., 1999, 2000; Mazumdar-Leighton and Broadway, 2001a, 2001b; Zhu-Salzman et al., 2003; Brunelle et al., 2004); and (3) the degradation of defensive protease inhibitors using nontarget, insensitive digestive proteases (Michaud et al., 1995a; Michaud, 1997; Girard et al., 1998a; Giri et al., 1998; Gruden et al., 2003; Zhu-Salzman et al., 2003). It is now generally recognized

\textsuperscript{1} This work was supported by the Natural Science and Engineering Research Council of Canada (Discovery grant to D.M., and graduate scholarships to M.-C.G., C.G., and M.-O.D.), by the Government of Egypt (Ph.D. fellowship to M.K.), and by the Government of Tunisia (Ph.D. fellowship to A.M.B.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.115741
that protease/inhibitor interactions in plant-insect systems are the result of a long, coevolutionary process triggering the continuous diversification of proteolytic and protease inhibitory functions in the competing organisms (Lopes et al., 2004; Valueva and Mosolov, 2004; Christeller, 2005; Kiggundu et al., 2006; Girard et al., 2007).

In this context, the development of recombinant protease inhibitors with strong inhibitory effects specific to the organism targeted represents a worthwhile but challenging task. Several approaches based on structure/function models have been used over the years to improve the inhibitory potency of protease inhibitors against specific proteases, including site-directed mutagenesis of specific amino acids or amino acid strings (e.g. Qasim et al., 1997; Mason et al., 1998; Ogawa et al., 2002; Pavlova and Björk, 2003) and molecular phage display procedures involving random mutagenesis in specific regions of the inhibitor sequence (e.g. Laboissiere et al., 2002; Ceci et al., 2003; Melo et al., 2003; Stoop and Craik, 2003). A structural model for the human stefin B:papain complex (Stubbs et al., 1990), in particular, has been instrumental in the identification of relevant target sites for the molecular improvement of cystatins against Cys proteases. Koiva et al. (2001), for instance, used this model to confirm the importance of the first and second inhibitory loops of soyacystatin N in the inhibition of papain, and isolated improved variants of this plant cystatin from combinatorial phage display libraries after inducing random mutations within these two regions. In an early study, Urwin et al. (1995) engineered a variant of oryzacystatin I with improved nematicidal activity by deleting the residue Asp-86 from the original sequence, after deducing the possible interfering effect of this amino acid during the inhibitory process. More recently, Melo et al. (2003) produced a phage display library derived from a nematode cystatin randomly mutagenized in the first (central) inhibitory loop to select mutated cystatins with high inhibitory activity against digestive proteases of the major bean weevil Acanthoscelides obtectus.

From a biotechnology viewpoint, one challenge now is to devise cystatin variants tailored for the inhibition of specific proteases, in such a way that the activity of target proteases is efficiently hindered, but the activity of nontarget (Cys) proteases in the host or the environment is not compromised (Malone and Burgess, 2000; Visal-Shah et al., 2000; Bouchard et al., 2003a). Several studies used protein engineering approaches to improve the effectiveness of protease inhibitors toward herbivorous insect or nematode proteases (Urwin et al., 1995; Inanaga et al., 2001; Koiva et al., 2001; Ceci et al., 2003; Martinez et al., 2003; Melo et al., 2003), but little attention has been paid to the impact of such modifications in a multitrrophic context. The ability of recombinant plant cystatins to alter digestive protease activities in insect predatory arthropods via their herbivorous prey fed the modified plant has been documented in recent years (Bouchard et al., 2003a, 2003b; Ferry et al., 2003; Alvarez-Alfageme et al., 2007), as well as some unintended pleiotropic effects of these cystatins in planta significantly altering developmental processes and stress responses in the modified plant (Gutierrez-Campos et al., 2001; Belenghi et al., 2003; Van der Vyver et al., 2003; Vaillancourt, 2005). Using a collection of 29 cystatin variants derived from the eighth inhibitory unit of tomato (Solanum lycopersicum) multicystatin, SICYS8 (formerly LeCY8; Girard et al., 2007), we assessed here the usefulness of site-directed mutagenesis at hypervariable (positively selected) amino acid sites for the improvement of both the inhibitory potency and specificity of plant cystatins toward herbivorous insect digestive Cys proteases. We observed recently that single mutations at positively selected amino acid sites on SICYS8 were strongly impacting the inhibitory efficiency of this cystatin against different model Cys proteases (Kiggundu et al., 2006). Here, we discuss, using a well characterized plant-insect tritrophic model (Bouchard et al., 2003a, 2003b), the potential of this approach to generate insect-specific cystatin variants with improved activity against digestive proteases of a target herbivore but decreased activity against nontarget Cys proteases in the surrounding environment, including the host plant itself.

RESULTS

Twenty-nine variants of SICYS8 were engineered by single mutations at three amino acid positions recently identified as positively selected (Fig. 1A; see Kiggundu et al. [2006] for codon numbering). The second amino acid of the cystatin, a Pro residue (Pro-2, or P2) adjacent to the conserved GG motif in the N-terminal trunk, was substituted by the 19 other amino acids to estimate the overall functional variability possible at this position. Mutations were also induced at the sixth and 31st amino acid sites, substituting the original Thr-6 (T6) or Glu-31 (E31) residues by five residues representative of the major amino acid families. The wild-type and 29 SICYS8 mutants were expressed in and purified from Escherichia coli using the glutathione S-transferase (GST) gene fusion system as described recently for SICYS8 (Girard et al., 2007) and then compared in their ability to inhibit Cys proteases. All variants were isolated under a soluble form and cleaved from the GST affinity tag by treatment with human factor Xa, giving proteins of the expected size, free of protein contaminants or degradation intermediates when resolved by SDS-PAGE (Fig. 1B).

Inhibitory Potency against Model Cys Proteases

Inhibitory assays were first carried out with four model Cys proteases of the papain family to assess the antiprotease functional variability among SICYS8 variants. Point mutations at positions 2 and 6 in SICYS8 were shown recently to significantly alter the inhibitory profile of the cystatin, with some mutations either favoring or interfering with the inhibitory process,
depending on the target protease tested (Kiggundu et al., 2006). In line with these observations, the variants mutated at position 2, 6, or 31 showed important differences in their inhibitory potency against the four Cys proteases papain, cathepsin B, cathepsin L, and cathepsin H, compared to the wild-type cystatin interacting with the same enzymes (Table I). As inferred from inhibition constants (or $K_i$ values) for the different cystatin:protease complexes, some mutations (i.e. mutations P2H, P2K, and T6P) had a general negative impact on the cystatin (increased $K_i$ values), whereas other mutations (i.e. mutations P2C, P2V, and E31F) had a general positive impact (lower $K_i$ values). Interestingly, most mutations (22 out of 29; e.g. P2F, P2Q, P2S, P2W, and T6E) had a differential impact on inhibitory activity depending on the protease tested. The P2F variant, for instance, showed improved activity against papain and cathepsin L but decreased activity against cathepsin B and cathepsin H. In a similar way, the T6I variant exhibited a slightly lowered activity against papain and cathepsin B but improved activity against cathepsin H and cathepsin L, which suggests the potential of single mutations at hypervariable (positively selected) amino acid sites to generate recombinant inhibitors optimized for the inhibition of specific target proteases in complex biological systems.

Inhibitory Potency against Colorado Potato Beetle Digestive Cys Proteases

To test this hypothesis, protease inhibitory assays were performed for the different SICYS8 variants with digestive (midgut) proteases of the insect herbivore Colorado potato beetle (Leptinotarsa decemlineata), which relies on both cystatin-sensitive and cystatin-insensitive Cys proteases for dietary protein digestion (Michaud et al., 1993, 1995b; Gruden et al., 2003). Fluorimetric protease assays were first conducted to identify synthetic peptide substrates allowing us to discriminate these two differentially sensitive Cys protease populations in insect extracts (Fig. 2). The substrate Z-Phe-Arg-methylcoumarin (Z-Phe-Arg-MCA), used routinely to monitor Cys protease activities in biological extracts (Barrett and Kirschke, 1981; Tchoupe et al., 1991), was cleaved mainly by proteases sensitive to both the low-molecular-weight diagnostic inhibitor of Cys proteases, trans-exopopyruvyl-L-leucylamido-4(guanidino)butane (E-64) and the wild-type form of SICYS8 (Fig. 2A), making it a useful tool to monitor SICYS8-sensitive Cys protease activities in the insect midgut. By contrast, the substrate Z-Arg-Arg-MCA, cleaved specifically by cathepsin B-like enzymes (Barrett and Kirschke, 1981), was hydrolyzed mainly by E-64-sensitive proteases poorly sensitive to SICYS8 (Fig. 2B), in line with an early study reporting the negligible inhibitory potency of plant (rice [Oryza sativa]) cystatins against Colorado potato beetle midgut Cys proteases with cathepsin B-like activity (Michaud et al., 1993).

Protease inhibitory assays were performed with these two substrates to compare the 30 SICYS8 variants in their inhibitory efficiency against the insect cystatin-sensitive (Z-Phe-Arg-MCA-hydrolyzing) and cystatin-insensitive (Z-Arg-Arg-MCA-hydrolyzing) proteases (Table II; Fig. 3). As observed with model papain-like proteases (Table I), mutations at positions 2, 6, and 31 strongly altered the inhibitory profile of SICYS8 against the insect cystatin-sensitive proteases (Table II). For instance, mutating the residue Pro-2 for a His, Asn, Gln, or Thr negatively altered the inhibitory potency of the cystatin for these proteases, giving $IC_{50}$ values (i.e. inhibitor concentrations required to reduce protease activity by 50%) increased by 2- to more than 10-fold using Z-Phe-Arg-MCA as a substrate. In contrast, mutating the same residue for a Phe, Leu, Met, or Val...
improved the inhibitory effect of the cystatin, giving $K_i$ values decreased by 2- to 3-fold. As for Pro-2, variations in inhibitory potency were observed for mutations exchanging residues Thr-6 or Glu-31 for alternative residues having a strong impact on cystatin:plant protease complexes, with most mutations either increasing or weakening the inhibitory interaction. The P2D and P2E variants, for instance, gave $IC_{50}$ values increased by approximately 15-fold, in contrast with decreases $IC_{50}$ values for the P2H, P2R, and T6S mutants indicating an increased inhibitory effect against the plant proteases. Interestingly, some variants that showed increased inhibitory activity against both cystatin-sensitive and cystatin-insensitive proteases of Colorado potato beetle (e.g. P2F, P2L, and P2R; Table II; Fig. 3) exhibited unaltered or even decreased potency against the potato proteases, unlike other mutants (e.g. P2V) showing increased activity against both the insect and plant proteases. As inferred above with papain and human cathepsins, these data illustrate the potential of mutations at positively selected amino acid sites for the generation of functional variability against Cys proteases, allowing notably for the engineering of cystatin variants with both improved activity toward the digestive Cys proteases of an insect herbivore and weakened activity against endogenous (nontarget) Cys proteases of its primary host plant.

### Inhibitory Potency against Potato Cys Proteases

Inhibitory assays were then performed with the different SICY8 variants to compare their potency to inhibit the leaf Cys proteases of potato (*Solanum tuberosum*), using Z-Phe-Arg-MCA as a general substrate for plant Cys, E-64-sensitive proteases (Michaud, 1998; Table II). As observed for the herbivore proteases, mutating the residue Pro-2, Thr-6, or Glu-31 for alternative residues had a strong impact on cystatin:plant protease complexes, with most mutations either increasing or weakening the inhibitory interaction. The P2D and P2E variants, for instance, gave $IC_{50}$ values increased by approximately 15-fold, in contrast with decreases $IC_{50}$ values for the P2H, P2R, and T6S mutants indicating an increased inhibitory effect against the plant proteases. Interestingly, some variants that showed increased inhibitory activity against both cystatin-sensitive and cystatin-insensitive proteases of Colorado potato beetle (e.g. P2F, P2L, and P2R; Table II; Fig. 3) exhibited unaltered or even decreased potency against the potato proteases, unlike other mutants (e.g. P2V) showing increased activity against both the insect and plant proteases. As inferred above with papain and human cathepsins, these data illustrate the potential of mutations at positively selected amino acid sites for the generation of functional variability against Cys proteases, allowing notably for the engineering of cystatin variants with both improved activity toward the digestive Cys proteases of an insect herbivore and weakened activity against endogenous (nontarget) Cys proteases of its primary host plant.

### Inhibitory Potency against Stinkbug Predator Cys Proteases

Complementary zymographic assays were carried out to measure the inhibitory potency of SICY8 variants against protease I, a major digestive Cys protease of the stinkbug predator *Perillus bioculatus* sensitive to plant cystatins (Ashouri et al., 1998; Overney et al., 1998; Fig. 4). Strong plant cystatin:Cys protease complexes (i.e. with $K_i$ values in the nanomolar or subnanomolar range) remain stable in the presence of SDS, unlike weaker complexes (with $K_i$ values in the micromolar range or higher) breaking down and restoring protease activity in gel zymograms (Michaud, 1998). Similar to rice cystatin I expressed in potato (Bouchard et al., 2003a, 2003b), SICY8 formed a strong, SDS-stable complex with the predator protease, giving a residual protease activity of about 25% following nonreducing SDS-PAGE, compared to the activity of the same protease with no cystatin added (0% inhibition, 100% residual activity; Fig. 4). In a similar way, the P2V and T6R variants formed SDS-stable complexes leading to residual activities of 5% to 10%, suggesting an even stronger inhibitory potency for these mutants, potentially causing increased inhibitory effects in the predator via its herbivorous prey fed the recombinant...
inhibitor (Bouchard et al., 2003a). In sharp contrast, the P2F, P2I, P2L, and P2Y variants formed SDS-labile complexes with the same protease, leading to complete restoration of protease activity after electrophoresis (Fig. 4) and suggesting an eventual negligible impact of these mutants in the predator.

DISCUSSION

Current strategies for the molecular improvement of plant protease inhibitors include rational mutagenesis based on protease:inhibitor structural models, and stochastic methods involving random mutagenesis in specific regions of the gene/protein sequence followed by selection of improved inhibitor variants by molecular phage display. As a complement, we document here the potential of adaptive evolutionary models as useful tools to rapidly identify candidate target sites for the generation of functional diversity by site-directed mutagenesis. We recently established a link between the occurrence of positively selected (hypervariable) amino acid sites in the primary sequence of plant cystatins and the inhibitory spectrum of these inhibitors against Cys proteases of the papain family (Kiggundu et al., 2006). Using a collection of cystatin variants derived from the tomato multicystatin unit SLCY8 and digestive Cys proteases from a well-characterized plant-herbivore-predator tritrophic model, we show here that a limited, finite number of single mutations at positively selected amino acid sites can generate enough functional diversity toward Cys proteases to allow for the rapid identification of new variants with improved activity and specificity against Cys proteases of a target herbivore. Out of 29 mutants tested, four variants, namely, the P2F, P2I, P2L, and P2Y mutants, exhibited improved inhibitory activity against both cystatin-sensitive and cystatin-insensitive digestive Cys proteases of Colorado potato beetle, while also exhibiting lowered (or unaltered) activity against Cys protease(s) of potato leaves and protease I, a major digestive Cys protease of the insect predator P. bioculatus.

Figure 2. Cystatin-sensitive and cystatin-insensitive digestive Cys proteases in Colorado potato beetle extracts. A, Z-Phe-Arg-MCA hydrolysis (at pH 5.5) by E-64-sensitive Cys proteases sensitive to SLCY8. B, Z-Arg-Arg-MCA hydrolysis (at pH 6.0) by E-64-sensitive Cys proteases insensitive to SLCY8.

Figure 3. Inhibitory potency of SLCY8 and SLCY8 variants against Colorado potato beetle Z-Arg-Arg-MCA-hydrolyzing, cystatin-insensitive Cys proteases. Total protease activity was assayed in the presence of 1 μM or 5 μM of either E-64 or SLCY8 variant, as described in “Materials and Methods.” The dashed line refers to the mean inhibitory potency of wild-type SLCY8. Each bar is the mean of three independent measurements ± si.
Table II. IC50 values for SICY8 variants interacting with SICY8-sensitive Colorado potato beetle digestive Cys proteases or with potato Cys, Z-Phe-Arg-hydrolyzing proteases

<table>
<thead>
<tr>
<th>Variant</th>
<th>IC50a,b nM</th>
<th>Potato Cys Proteases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>20.0</td>
<td>8.5</td>
</tr>
<tr>
<td>P2A</td>
<td>n.i.</td>
<td>13.5</td>
</tr>
<tr>
<td>P2C</td>
<td>43.5</td>
<td>10.9</td>
</tr>
<tr>
<td>P2D</td>
<td>n.i.</td>
<td>115</td>
</tr>
<tr>
<td>P2E</td>
<td>n.i.</td>
<td>116</td>
</tr>
<tr>
<td>P2F</td>
<td>8.5</td>
<td>10.0</td>
</tr>
<tr>
<td>P2G</td>
<td>n.i.</td>
<td>17.1</td>
</tr>
<tr>
<td>P2H</td>
<td>76.5</td>
<td>4.9</td>
</tr>
<tr>
<td>P2I</td>
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<td>12.2</td>
</tr>
<tr>
<td>P2K</td>
<td>n.i.</td>
<td>3.0</td>
</tr>
<tr>
<td>P2L</td>
<td>9.0</td>
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<td>P2S</td>
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<td>T6P</td>
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*Each value is the mean of three independent measurements. All ± values for these measures fell within a 10% range compared to the mean value reported. b n.i., No measurable inhibition.

Several studies considered the use of recombinant cystatins expressed in transgenic plants for herbivorous insect control (e.g. Benckey et al., 1995; Leple et al., 1995; Girard et al., 1998a, 1998b; Cloutier et al., 1999, 2000; Lecardonnel et al., 1999; Ouchkourov et al., 2004; Ribeiro et al., 2006; Alvarez-Alfageme et al., 2007), but nonconclusive results were obtained in several cases, correlated with the onset of effective adaptive processes allowing target pests to overcome the deleterious effects of digestive protease inhibition. In practice, the inhibitory potency of protease inhibitors is critical for an effective inhibition of target pest digestive proteases in vivo (Urwin et al., 1995), as it directly determines the amount of recombinant protein needed in the modified host plant to provide sufficient anti-digestive or pesticidal effects (Jongsma and Bolter, 1997). In an early study, De Leo et al. (1998) illustrated the dose-dependent impact of a protease inhibitor from mustard (Sinapis alba) expressed in tobacco (Nicotiana tabacum) or Arabidopsis (Arabidopsis thaliana) on growth and foliar consumption of the herbivorous insect Spodoptera littoralis. This study was notably reporting strong deleterious effects for transgenic lines expressing the recombinant inhibitor at high levels (e.g. approximately 1.5% of leaf total soluble proteins), but null or even positive effects associated with overcompensatory responses in the target insect for sister lines expressing the same inhibitor at levels 3 times lower (i.e. approximately 0.5%). In this perspective, SICY8 variants such as P2F and P2L, which exhibit IC50 values decreased by 2- to 3-fold for the Colorado potato beetle cystatin-sensitive proteases (Table II), could provide improved antidigestive effects at given expression rates in planta and thus help avoid effective compensatory processes in this target herbivore (Cloutier et al., 1999, 2000; Gruden et al., 2003, 2004; Brunelle et al., 2004). An inhibitory spectrum extended to the so-called cystatin-insensitive proteases for these same two variants also could make them more effective in vivo, as suggested by a recent study reporting increased deleterious effects for a hybrid corn cystatin II bearing an extra protease inhibitory function (Brunelle et al., 2005). SICY8 variants such as P2F and P2L, which combine an improved inhibitory potency against cystatin-sensitive proteases of Colorado potato beetle with a broader inhibitory spectrum against the overall digestive protease complement of this same insect, would thus likely represent promising candidates for the design of insect-resistant transgenic potato lines.

From a physiological viewpoint, these improved variants could not only help overcome compensatory processes to cystatins in the target insect but also help avoid possible metabolic interference effects of these proteins in planta. Several transgenic plant lines expressing exogenous cystatins were successfully produced over the last 15 years (e.g. Masoud et al., 1993; Benckey et al., 1995; Leple et al., 1995; Gutierrez-Campos et al., 1999; Van der Vyver et al., 2003; Ouchkourov et al., 2004), but recent studies reported pleiotropic effects of these proteins affecting major biological processes such as flowering, programmed cell death, tolerance to biotic stresses, and defense protein induction (Gutierrez-Campos et al., 2001; Belenghi et al., 2003; Van der Vyver et al., 2003; Rojo et al., 2004; Vaillancourt, 2005). Little is still known about the exact regulatory functions of Cys proteases in plant cells, but their obvious importance at the genome level (Beers et al., 2004) and their broad distribution in plant tissues and cells (Grudkowska and Zagdanska, 2004; Schaller, 2004) suggest a significant role for these enzymes in various cellular and developmental processes. In this perspective, cystatin variants with increased specificity toward herbivorous insect proteases might prove useful not only in a direct manner by strongly inhibiting the digestive proteases of herbivore pests, but also indirectly by allowing to elude eventual interfering effects in planta that could affect fitness of the host plant.
or compromise efficient accumulation of the recombinant cystatin and thus limit the repulsive, antidigestive potential of the modified plant.

Optimizing the inhibitory specificity of recombinant cystatins toward target pests also could prove attractive from an ecological viewpoint, considering the complex multitrophic interactions known to take place at the ecosystem level (Groot and Dicke, 2002). The ability of recombinant plant cystatins to inhibit the digestive Cys proteases of insect predatory arthropods via their herbivorous prey fed the modified plant has been documented recently (Bouchard et al., 2003a, 2003b; Ferry et al., 2003; Alvarez-Alfageme et al., 2007), thus stressing the importance of not only monitoring protease: inhibitor interactions expected to take place between the plant and its herbivorous enemy but also to assess the interactions likely to occur in the proximate environment. SICYS8 variants such as P2F and P2L, which exhibit improved activity against Colorado potato beetle digestive proteases but weaker activity against protease I of _P. bioculatus_ (see Fig. 4), could represent in this regard useful tools for the production of insect-resistant transgenic potato lines compatible with complementary protection approaches involving arthropod biocontrol agents. Comparative studies are now being conducted to assess the possible unintended effects of wild-type SICYS8 and its functional variants in planta and ex planta. Work is also under way to estimate the actual antidigestive effects of these same variants against the Colorado potato beetle, keeping in mind the well-documented ability of this insect to overcome the inhibitory effects of plant protease inhibitors by various compensatory and hydrolytic processes. Studies documented the deleterious effects of broad-spectrum low-molecular-weight, nonprotein protease inhibitors (Bolter and Latoszek-Green, 1997) or the significant antidigestive effects of a hybrid, dual cystatin with an extended inhibitory spectrum (Brunelle et al., 2005) against Colorado potato beetle larvae, but effective compensatory processes following cystatin ingestion cannot be ruled out at this stage.

### MATERIALS AND METHODS

#### Proteases

Papain (from papaya latex; E.C.3.4.22.2), cathepsin B (from human liver; E.C.3.4.22.1), cathepsin H (from human liver; E.C.3.4.22.16), and cathepsin L (from human liver; E.C.3.4.22.15) were from Sigma-Aldrich. Digestive proteases of the Colorado potato beetle _Leptinotarsa decemlineata_ were isolated as described earlier from fourth-instar larvae reared in greenhouse on _Norland_ potato ( _Solanum tuberosum_) plants (Brunelle et al., 2004). Digestive proteases of the stinkbug predator _Perillus bioculatus_ were isolated as described earlier from larvae provided with potato-fed Colorado potato beetle larvae (Overney et al., 1998). In brief, the insect midgut soluble proteins were extracted in 100 mM citrate-phosphate, pH 6.0, containing 10% (v/v) ethylene glycol. After 10 min on ice, the mixtures were centrifuged at 13,000 g for 10 min at 4°C and the supernatant used as a source of insect digestive proteases for subsequent analyses. Protein content in the extracts was adjusted to 5 mg mL⁻¹ by the addition of extraction buffer, after assaying the proteins according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as a protein standard.

Potato leaf proteases (from various stages) were extracted in 50 mM Tris-HCl, pH 7.0, containing 2% (v/v) polyvinylpyrrolidone (Sigma-Aldrich). After 15 min on ice, the mixtures were centrifuged at 10,000 g for 10 min at 4°C and the supernatant used for subsequent analyses. Protein concentration in the extracts was assayed as described for the insect extracts.

#### SICYS8 Variants

Variants of SICYS8 were generated by exchanging the residue P2 for the 19 other amino acids; the residue T6 for Glu (E), Ile (I), Pro (P), Arg (R), or Ser (S); or the residue E31 for Ala (A), Phe (F), Gly (G), Lys (K), or Thr (T). A cDNA encoding SICYS8 (Genbank accession no. AF198399; Girard et al., 2007) was first amplified from a tomato _Solanum lycopersicum_ leaf RNA population using the following primers, including BamHI and EcoRI cloning sites (underlined): 5′-A AAGGATCCCA AAT CCT GGG GCC ATT ACC AAT GTT TCC CAA CAA CAA CAA GCT GTC ACT C-3′ for the 5′-end; and 3′-TAGATACCCCA AAT XXX GGG GCC ATT ACC AAT GGT CCA T-3′ for the 3′-end. After digestion with EcoRI and BamHI, the PCR amplicon was inserted into pGEX-3X (Amersham Biosciences) in frame with the GST-encoding gene. This vector was introduced into _Escherichia coli_ strain BL21 (Strategene) by electroporation and used to produce the original, wild-type cystatin SICYS8 (see below). SICYS8 variants were amplified by PCR using the pGEX-3X/SICYS8 plasmid as a template by substituting the P2-, T6-, or E31-encoding codon by alternative codons for the amino acids identified above (see Fig. 1A for codon substitutions). The following 5′-end primers were used to introduce the mutations: 5′-AAGGATCCCA AAT XXX GGG GCC ATT ACC AAT GGT CCA T-3′ for the P2 mutants; 5′-AAGGATCCCA AAT CAA CGG GCC ATT ACC AAT GGT CCA T-3′ for the T6 mutants; and 3′-CAAGATAT AAC AAA XXX AAT GCT CAT TTG (G)(AG)-3′ for the E31 mutants, where XXX corresponds to the alternative codon. All constructs were subject to automatic sequencing (Service de Séquençage de l’Université Laval) to proof check the mutated sequences and were reintroduced into _E. coli_ strain BL21 for heterologous expression. Expression and purification of the SICYS8 variants were carried out using the GST gene fusion system (Amersham Biosciences) as described recently (Girard et al., 2007). The GST affinity tag was removed from the cystatin variants by cleavage with human factor X₉ (Novagen), following the supplier’s instructions.

### Kᵢ Values (Model Papain-Like Proteases)

Kᵢ for the interaction of SICYS8 variants with the model Cys proteases papain, human cathepsin B, human cathepsin H, and human cathepsin L were determined by the monitoring of substrate hydrolysis progress curves (Salvesen and Nagase, 1989), based on the linear equation of Henderson (1972). Papain activity was measured in 50 mM Tris-HCl, pH 7.0, using the synthetic fluorogenic substrate Z-Phε-Arg-MCA (Sigma-Aldrich). Cathepsin B activity

![Figure 4](image-url)
was measured in 50 mM MES buffer, pH 6.0, using the substrate Z-Arg-Arg-MCA (Sigma-Aldrich). Cathepsin H aminopeptidase activity was measured in 50 mM Tris-HCl, pH 6.8, using Z-Arg-MCA (Sigma-Aldrich) as a substrate. Cathepsin L activity was measured in 50 mM MES buffer, pH 5.5, using Z-Phe-Arg-MCA as a substrate. Hydrolysis was allowed to proceed at 25°C (papain, cathepsin L) or 37°C (cathepsins B and H) in reducing conditions (10 mM L-Cys) with the substrate in large excess after adding (or not) the mutant cystatins dissolved in a minimal volume. Activity levels were monitored using a Fluostar Galaxy fluorimeter (BMG), with excitation and emission filters of 360 nm and 450 nm, respectively. Kᵢ values were calculated after empirically estimating Kᵢ and Kₐ values, using the following equation: 

$$Kᵢ = Kᵢ + (1 + [S] / Kₐ) \cdot Kᵢ$$

where Kᵢ values for papain, cathepsin B, cathepsin H, and cathepsin L with their test substrate were estimated at 13.6 μM, 2.0 μM, 6.5 μM, and 1.0 μM, respectively.

IC₅₀ Values (Herbivore and Plant Cystatin-Sensitive Proteases)

Inhibitory potency of the SCSY8 variants against Colorado potato beetle digestive Cys proteases and potato leaf Cys proteases was assessed by the estimation of IC₅₀ values, defined here as the concentration of cystatin needed to reduce proteolytic activity by 50% under our assay conditions. Protease inhibitory activities were assayed by the monitoring of substrate hydrolysis progress curves, using the synthetic fluorogenic substrate Z-Phe-Arg-MCA. Substrate hydrolysis by insect digestive proteases (12.5 ng protein) was carried out allowing to proceed at 25°C in 50 mM MES, pH 5.5, containing 10 mM L-Cys. Hydrolysis by plant leaf proteases (17.7 ng protein mL⁻¹) was allowed to proceed at 25°C in 50 mM Tris-HCl, pH 6.8, containing 10 mM L-Cys. Activity levels were monitored using a Fluostar Galaxy fluorimeter (BMG), with excitation and emission filters of 360 nm and 450 nm, respectively.

Maximal Inhibitory Rates (Herbivore Cystatin-Insensitive Cys Proteases)

Inhibitory effects of SCSY8 variants against Colorado potato beetle cystatin-insensitive Cys proteases were monitored by the estimation of maximal inhibitory rates (%) for protease activities cleaving the synthetic fluorogenic substrate Z-Arg-Arg-MCA. Protease and protease inhibitory activities were measured by the monitoring of substrate hydrolysis progress curves, using 25 ng of insect midgut protein per microliter of assay mixture. The recombinant cystatins were used at 1 or 5 μM concentration, similar to the concentrations typically observed for recombinant protease inhibitors expressed in transgenic plant lines. Hydrolysis was allowed to proceed at 25°C in 50 mM MES, pH 6.0, containing 10 mM L-Cys. Activity levels were monitored using a Fluostar Galaxy fluorimeter (BMG), with excitation and emission filters of 360 nm and 450 nm, respectively. Maximal inhibitory controls were carried out using E-64 (Sigma-Aldrich) as a broad-spectrum inhibitor for Colorado potato beetle midgut Cys proteases (Michaud et al., 1993).

Nonreducing Gelatin/SDS-PAGE (Predator Cys Proteases)

Inhibitory potency of the SCSY8 variants against the major digestive Cys protease of P. bicalvatus (protease I; see Bouchard et al., 2003a) was monitored by mildly denaturing (nonreducing) gelatin/SDS-PAGE (Michaud et al., 1996). The test proteases were incubated with recombinant cystatins (5 pmol cystatin μg⁻¹ of insect protein) or with an equivalent volume of 50 mM Tris-HCl, pH 8.0 (noninhibited control) for 10 min at 37°C before electrophoresis. In-gel quantitation of gelatinase activity was done by densitometry of the clear (proteolytic) bands, using a Microtek Scanner II scanner (Microtek Lab) and the image analysis software NIH Image v.1.51 (National Institutes of Health).

Received January 1, 2008; accepted January 8, 2008; published January 11, 2008.

**LITERATURE CITED**


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