REVIEW PAPER

Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective

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Abstract

Protease inhibitors are a promising complement to Bt toxins for the development of insect-resistant transgenic crops, but their limited specificity against proteolytic enzymes and the ubiquity of protease-dependent processes in living organisms raise questions about their eventual non-target effects in agroecosystems. After a brief overview of the main factors driving the impacts of insect-resistant transgenic crops on non-target organisms, the possible effects of protease inhibitors are discussed from a multitrophic perspective, taking into account not only the target herbivore proteases but also the proteases of other organisms found along the trophic chain, including the plant itself. Major progress has been achieved in recent years towards the design of highly potent broad-spectrum inhibitors and the field deployment of protease inhibitor-expressing transgenic plants resistant to major herbivore pests. A thorough assessment of the current literature suggests that, whereas the non-specific inhibitory effects of recombinant protease inhibitors in plant food webs could often be negligible and their "unintended" pleiotropic effects in planta of potential agronomic value, the innocuity of these proteins might always remain an issue to be assessed empirically, on a case-by-case basis.

Key words: Insect-resistant transgenic plants, non-target organisms, pleiotropic effects, protease–inhibitor interactions, protease inhibitors.

Introduction

The intense media coverage of a short scientific communication reporting the detrimental effects of a Bt toxin (Cry1Ab protein)-expressing corn (Zea mays L.) hybrid against the lepidopteran monarch butterfly, Danaus plexippus (L.) (Losey et al., 1999), has caused, ten years ago, an unprecedented controversy on the large-scale deployment of insect-resistant transgenic crops worldwide (Pimentel and Raven, 2000; Shelton and Sears, 2001). The conclusions of this laboratory study, then supported by feeding assays with corn pollen collected on milkweed (Asclepias syriaca L.) plants in a field experimental set-up (Hansen Jesse and Obrycki, 2000), could be explained by the documented toxicity of Cry1A proteins against different lepidopteran herbivores, the abundance of Cry1Ab toxins in the pollen tested, and the large quantities of pollen dusted on milkweed leaves for larval bioassays (Pimentel and Raven, 2000; Hellmich et al., 2001). Although the agronomic relevance of the two studies was seriously questioned and their conclusions tempered by follow-up studies suggesting a negligible impact of commercial Bt corn hybrids under field conditions (Sears et al., 2001; Gatehouse et al., 2002; Wolt et al., 2003; Dively et al., 2004), this new episode of
the ongoing debate over genetically modified organisms had the merit to put forward the ecologically relevant question of non-target organisms in transgenic crop fields, and to catalyze the funding of studies assessing the possible unintended effects of transgenic crops in the environment.

After a brief overview of the main factors driving the effects of insect-resistant transgenic crops on non-target organisms, the issue is addressed here from a multifaceted perspective using protease inhibitors as a model case. These ubiquitous regulators of proteolytic enzymes have readily been identified as potential candidates for the development of insect-resistant transgenic crops (Hilder et al., 1987; Johnson et al., 1989), and large-scale field trials are currently being conducted with transgenic rice (Oryza sativa L.) lines expressing serine protease inhibitors, before their eventual release for lepidopteran insect control (Qiu, 2008; Deka and Barthakur, 2010). This review discusses the possible impacts of recombinant protease inhibitors on target and non-target species including the host plant itself, keeping in mind the limited functional specificity of these proteins compared with the high specificity of currently used Bt toxins.

**The impact of insect-resistant transgenic plants on non-target organisms**

Taking into account the different modes of action and activity ranges of recombinant pesticidal proteins expressed in transgenic crops, the striking complexity of biotic interactions and food web relationships in agroecosystems, and the random insertion of transgene sequences in recipient plant genomes, three main factors generally determine the environmental impact of a given pest- (or pathogen-) resistant transgenic crop: (i) the overall efficiency of the introduced resistance trait against the target herbivore (or pathogen); (ii) the activity range—or functional specificity—of the recombinant trait; and (iii) the (bio)chemical composition and physiological status of the host plant following transgene insertion and expression:

(i) **Overall efficiency of the recombinant trait**

Currently, most transgenic crops grown in agricultural fields worldwide are used for weed or herbivorous insect control (James, 2009). By definition, any pest control measure adopted in the field, whether relying on transgenic crop lines or not, may exert direct and indirect effects on microbial, animal, and plant communities. Weed control strategies involving herbicide-tolerant transgenic crops, which also rely on broad-spectrum commercial pesticides, have a direct negative impact on the number and abundance of resident plant species, with an indirect negative impact on the abundance and diversity of refuges and food sources available to resident organisms (Firbank et al., 2003). By comparison, the direct effects of insect-resistant (e.g., Bt toxin-expressing) plants are limited essentially to target pest populations, but the high pesticidal efficiency of these plants may indirectly impact the fitness of non-target organisms and the overall organization of non-target populations in the field. A well-known example of this is the negative impact of Bt toxin (Cry protein)-expressing plants on arthropod parasitoids and predators provided with ‘poor quality’ herbivore hosts or preys suffering recombinant toxin ingestion (Naranjo et al., 2009). Another example is the adjustment of secondary herbivore, auxiliary carnivore, and soil detritivore arthropod populations in agricultural fields due to the efficient repression of primary herbivore pests with Bt toxin-expressing lines (Marvier et al., 2007; Cloutier et al., 2008). The abundance of non-target secondary pests such as aphids, for instance, can significantly increase as a result of released direct competition with the target herbivore, as notably observed with the Bt toxin-expressing potato (Solanum tuberosum L.) line Newleaf™, highly resistant to the Colorado potato beetle, Leptinotarsa decemlineata (Say) (Cloutier et al., 2008).

(ii) **Functional specificity of the recombinant trait**

Herbivore pest resistance traits introduced into crop plant genomes are usually selected for the control of a specific pest, but direct unintended effects on non-target organisms cannot be excluded de facto (Groot and Dicke, 2002). The monarch butterfly controversy (see above) and the reported toxicity of Cry1A and Cry2A toxins against different lepidopteran insects (Sims, 1995, 1997; van Frankenhuyzen and Nystrom, 2002) are examples of the preferential, but non-exclusive action of pesticidal proteins against specific target pests. Possible non-specific effects are also, if not even more, likely to occur for those newly developed pesticidal proteins, such as lectins and protease inhibitors, which exert their effects in a poorly specific manner on molecular targets found in most organisms in the environment (Malone and Burgess, 2000; O’Callaghan et al., 2005). Despite some controversy on methodological and interpretation issues (Andow et al., 2009; Lövei et al., 2009; Shelton et al., 2009a, b), it is usually considered that the direct unintended effects of Bt toxin-expressing plants on non-target organisms are negligible owing to the intrinsic functional specificity of Cry toxins, toxin concentrations in plant tissues below the thresholds required for significant side-effects, and a limited persistence of the recombinant toxins in natural ecosystems (Zwahlen et al., 2003; Clark et al., 2005; O’Callaghan et al., 2005; Romeis et al., 2006, 2008; Naef et al., 2006; Marvier et al., 2007; Prihoda and Coats, 2008; Wolfenbarger et al., 2008; Naranjo, 2009). By contrast, the question of direct unintended effects is of particular relevance for proteins such as lectins, which interact with the glycan moiety of many glycoproteins and glycolipids in eukaryotes; or protease inhibitors, which inhibit proteases from protease families widely distributed among plant, animal, and microbial taxa (Malone and Burgess, 2000; O’Callaghan et al., 2005).

(iii) **Alteration of the host plant’s characteristics**

The heterologous expression of metabolic regulators such as protease inhibitors in transgenic crops also raises
questions about their effects *in planta* and their resulting impact on the composition and physiology of the host plant, which represents a central element in the whole food chain (Visal-Shah *et al.*, 2000; Khalf *et al.*, 2010). Unlike Cry toxins acting on specific membrane protein receptors in the digestive tract of target herbivores (Pigott and Ellar, 2007), protease inhibitors may interact with plant endogenous proteases targets structurally and functionally related to herbivore digestive proteases (Goulet *et al.*, 2008). For recombinant Cry toxins, insertional mutagenesis events altering the host plant phenotype may occur in a plant line-specific manner as a result of transgene position effects following random integration of the toxin-encoding gene and/or transcription of regulatory sequences included in gene constructs (Gelvin, 2003; Miki *et al.*, 2009). Apart from a limited and fraction of the host cell tRNA and amino acid pools allocated to biosynthesis, Bt toxin expression is unlikely, however, to have a significant impact on the host plant in the absence of target receptors. In sharp contrast, non-specific ‘pleiotropic’ effects positively correlated with transgene expression remain possible, even plausible, with recombinant protease inhibitors such as those currently considered for pest control (Visal-Shah *et al.*, 2000), which usually show inhibitory activity towards widely distributed proteases like trypsin-, chymotrypsin-, and cathepsin L-like enzymes (Haq *et al.*, 2004).

Together, the three factors identified above determine, depending on their relative importance, the positive, negative or negligible (non-significant) net impact of a modified plant on the biotic component of its surrounding environment. Given the strong influence of cultural practices in agricultural fields and the number of parameters underlying biotic interactions in multitrrophic systems, the impact of transgenic crops on non-target organisms is typically determined on a comparative basis, using as comparators closely-related (e.g. isogenic or near-isogenic) plant varieties, or, on a larger scale, realistic production schemes with conventional plant lines and the usual cultural practices (Firbank *et al.*, 2003; Michaud, 2005). Several studies have been carried out over the last ten years assessing the impacts of protease inhibitor-expressing transgenic plants on the growth and development of non-target organisms. The next paragraphs summarize the conclusions of these studies, after a brief overview of recent data documenting the potential of these plants for herbivorous pest control.

**Recombinant protease inhibitors in plant protection**

Numerous papers have reported the potential of protease inhibitors as effective antidigestive compounds to protect crop plants from herbivory or pathogenic infection (Michaud, 2000; Haq *et al.*, 2004). Serine protease inhibitors, in particular, have been readily identified as potential candidates for the development of insect-resistant transgenic crops (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Duan *et al.*, 1996; Xu *et al.*, 1996), and their usefulness to reduce insecticide loads in the field has recently been documented (Huang *et al.*, 2005; Qiu, 2008). Most protease inhibitors in plants are proteinaceous, competitive inhibitors acting as pseudo-substrates to enter the active site of proteases (Birk, 2003). Following inhibition, the target proteases can no longer cleave peptide bonds, which causes a detrimental disruption of dietary protein assimilation in herbivorous pests leading to significant growth and development delays (Haq *et al.*, 2004).

Despite promising developments in recent years (Table 1), the pesticidal effects of protease inhibitors in plant protection are still to be improved in many cases. Insect herbivores have developed, over evolutionary time, effective strategies to cope with dietary protease inhibitors (Jongsma and Bolter, 1997; Broadway, 2000), such as the use of complex digestive protease complements with proteases from different functional classes acting on dietary proteins in a complementary manner (Brunelle *et al.*, 1999; Hernandez *et al.*, 2003; Gruden *et al.*, 2003; Vinokurov *et al.*, 2006, 2009; Prabhakar *et al.*, 2007; Kiggundu *et al.*, 2010); the over-expression of target proteases to outnumber the inhibitory proteins (Cloutier *et al.*, 2000; Ahn *et al.*, 2004); and the constitutive or diet-induced expression of proteases weakly sensitive to the ingested inhibitors (Michaud *et al.*, 1993, 1995a, b; Jongsma *et al.*, 1995a; Bown *et al.*, 1997; Girard *et al.*, 1998a; Cloutier *et al.*, 1999, 2000; Mazumdar-Leighton and Broadway, 2001a, b; Zhu-Salzman *et al.*, 2003a; Brunelle *et al.*, 2004; Gruden *et al.*, 2004; Liu *et al.*, 2004; Koo *et al.*, 2008). Other strategies involve the over-expression of proteases from alternative functional classes (Zhu-Salzman *et al.*, 2003a; Brunelle *et al.*, 2004; Rivard *et al.*, 2004; Oppert *et al.*, 2005; Vila *et al.*, 2005); the degradation of protease inhibitors with non-target, insensitive proteases (Michaud *et al.*, 1995b; Michaud, 1997; Girard *et al.*, 1998b; Giri *et al.*, 1998; Khalf *et al.*, 2003; Zhu-Salzman *et al.*, 2003a; Yang *et al.*, 2009); and a transcriptionally regulated reallocation of cellular resources towards protease inhibitor-induced compensatory processes (Liu *et al.*, 2004; Chi *et al.*, 2009). It is now well recognized that protease-inhibitor interactions in plant–insect systems are the result of a long, co-evolutionary process triggering the continuous diversification of (insect) proteolytic and (plant) protease inhibitory functions (Lopes *et al.*, 2004; Christeller, 2005; Kiggundu *et al.*, 2006; Girard *et al.*, 2007), with the practical implication that the ectopic expression of protease inhibitors *in planta* leads not only to the inhibition of constitutive target proteases in naive herbivores, but also to the induction of specific protease-encoding genes and a significant remodelling of the digestive proteome complement.

In this context, the development of recombinant protease inhibitors with strong inhibitory activity against specific herbivores is a worthwhile, but challenging task. Protein engineering approaches based on structure/function models have been used to improve the inhibitory potency of protease inhibitors against herbivore pest and parasitic
### Table 1. Protease inhibitor-expressing transgenic plants resistant to herbivore pests and pathogens: a summary of recent successful examples

<table>
<thead>
<tr>
<th>Plant</th>
<th>Recombinant inhibitor</th>
<th>Target proteases</th>
<th>Target herbivore(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfalfa</td>
<td>Oryzacystatin I</td>
<td>Cysteine</td>
<td>Phytophaga vaporaria</td>
<td>Schlueter et al., 2005</td>
</tr>
<tr>
<td>Apple</td>
<td>Nicotiana alata proteinase inhibitor</td>
<td>Serine</td>
<td>Phytomyza nunata</td>
<td>Maheswaran et al., 2005</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Mustard trypsin inhibitor 2</td>
<td>Serine</td>
<td>Plutella xylostella</td>
<td>De Leo et al., 2001</td>
</tr>
<tr>
<td>Oilseed rape</td>
<td>Mustard trypsin inhibitor 2</td>
<td>Serine</td>
<td>P. xylostella</td>
<td>De Leo et al., 2001</td>
</tr>
<tr>
<td>Potato</td>
<td>Multidomain cystatin fusions</td>
<td>Cysteine+Aspartate</td>
<td>F. occidentalis</td>
<td>Ouchkourou et al., 2004a</td>
</tr>
<tr>
<td></td>
<td>Various animal and plant cystatins</td>
<td>Cysteine+Aspartate</td>
<td>L. decemlineata</td>
<td>Ouchkourou et al., 2004b</td>
</tr>
<tr>
<td></td>
<td>barley cystatin HvCPI-1 C68</td>
<td>Cysteine</td>
<td>S. litura</td>
<td>Kondrak et al., 2005</td>
</tr>
<tr>
<td>Rice</td>
<td>barley trypsin inhibitor</td>
<td>Serine</td>
<td>Sitophilus oryzae</td>
<td>Alfonso-Rubi et al., 2003</td>
</tr>
<tr>
<td></td>
<td>soybean trypsin inhibitor (+lectin)</td>
<td>Serine</td>
<td>Nilaparvata lugens</td>
<td>Li et al., 2005</td>
</tr>
<tr>
<td></td>
<td>maize proteinase inhibitor (mpi)</td>
<td>Serine</td>
<td>Cnaphalocrosis medinalis</td>
<td>Li et al., 2005</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>cowpea trypsin inhibitor (+Bt toxin Cry1Ac)</td>
<td>Serine</td>
<td>Chilo suppressalis</td>
<td>Vila et al., 2005</td>
</tr>
<tr>
<td>Tobacco</td>
<td>bovine pancreatic trypsin inhibitor (aprotinin)</td>
<td>Serine</td>
<td>C. medinalis</td>
<td>Han et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Brassica juncea trypsin inhibitor</td>
<td>Serine</td>
<td>Heterodera avenae</td>
<td>Vishnudassan et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Mustard trypsin inhibitor 2</td>
<td>Serine</td>
<td>H. armigera</td>
<td>Senthilkumar et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Tobacco trypsin protease inhibitor</td>
<td>Serine</td>
<td>Trialeurodes vaporariorum</td>
<td>Khadeeva et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Sporamin+Taro cystatin</td>
<td>Serine+Cysteine</td>
<td>Pratylenchus penetrans</td>
<td>Sarnac and Smigocki, 2003</td>
</tr>
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<td></td>
<td>buckwheat serine protease inhibitor</td>
<td>Serine</td>
<td>Meloidogyne incognitae</td>
<td>Lilley et al., 2004</td>
</tr>
<tr>
<td></td>
<td>alfalfa Oryzacystatin I</td>
<td>Cysteine</td>
<td>Globodera pallida</td>
<td>Lilley et al., 2004</td>
</tr>
<tr>
<td></td>
<td>potato Oryzacystatin I</td>
<td>Cysteine</td>
<td>M. incognita</td>
<td>Chan et al., 2010</td>
</tr>
<tr>
<td></td>
<td>tomato Taro cystatin</td>
<td>Cysteine</td>
<td>Heterodera avenae</td>
<td>Vishnudassan et al., 2005</td>
</tr>
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<td></td>
<td>wheat Potato proteinase inhibitor 2</td>
<td>Serine</td>
<td>Pseudomonas syringae</td>
<td>Khadeeva et al., 2009</td>
</tr>
<tr>
<td></td>
<td>potato Buckwheat serine proteinase inhibitor</td>
<td>Serine</td>
<td>Clavibacter michiganensis</td>
<td>Khadeeva et al., 2009</td>
</tr>
<tr>
<td></td>
<td>rice Potato carboxypeptidase inhibitor</td>
<td>Carboxypeptidases A</td>
<td>Magnaporthe oryzae</td>
<td>Quilis et al., 2007</td>
</tr>
<tr>
<td>Tobacco</td>
<td>buckwheat serine protease inhibitor</td>
<td>Serine</td>
<td>Fusarium verticillioides</td>
<td>Quilis et al., 2007</td>
</tr>
<tr>
<td></td>
<td>Sporamin+Taro cystatin</td>
<td>Serine+Cysteine</td>
<td>P. syringae</td>
<td>Khadeeva et al., 2009</td>
</tr>
<tr>
<td></td>
<td>alfalfa Oryzacystatin I</td>
<td>Cysteine</td>
<td>C. michiganensis</td>
<td>Khadeeva et al., 2009</td>
</tr>
<tr>
<td></td>
<td>potato Oryzacystatin I</td>
<td>Cysteine</td>
<td>Pythium aphanidermatum</td>
<td>Senthilkumar et al., 2010</td>
</tr>
<tr>
<td></td>
<td>tomato Taro cystatin</td>
<td>Cysteine</td>
<td>H. armigera</td>
<td>Senthilkumar et al., 2010</td>
</tr>
</tbody>
</table>

Nematode digestive proteases, including site-directed mutagenesis of key amino acids (Urwin et al., 1995; Kiggundu et al., 2006; Goulet et al., 2008) and molecular phase display procedures involving random mutagenesis in functionally significant regions of the inhibitor sequence (Jongsma et al., 1995b; Koiva et al., 2001; Ceci et al., 2003; Melo et al., 2003). Fusion proteins integrating complete or partial inhibitor sequences have also been designed to broaden the activity range and improve the overall efficiency of protease inhibitors against target herbivores (Urwin et al., 1998; Inanaga et al., 2001; Zhu-Salzman et al., 2003b; Ouchkourou et al., 2004a; Brunelle et al., 2005; Benchabane et al., 2008). Such protein engineering strategies, together with ‘transgene stacking’ (or gene pyramiding) in planta involving protease inhibitor combinations (Abdeen et al., 2005; Senthilkumar et al., 2010) or protease inhibitors combined with other pesticidal proteins (Boulter et al., 1990; Tang et al., 1999; Maqbool et al., 2001; Han et al., 2007), have clearly confirmed the practical potential of these proteins in plant protection. From an ecological perspective, these advances underlined, on the other hand, the relevance of assessing their inhibitory effects against the proteases of non-target organisms, including the host plant’s own proteases.

#### Unintended protease–inhibitor interactions in non-target arthropods

Several laboratory studies have assessed the effects of recombinant protease inhibitors on non-target arthropods interacting with, or likely to interact with, the modified plant (Table 2). Protease inhibitors may impact non-target organisms either directly by the establishment of a formal trophic interaction, or indirectly through an intermediate herbivore ingesting the recombinant material (Malone and
Unintended effects of protease inhibitor-expressing crops

<table>
<thead>
<tr>
<th>Plant</th>
<th>Recombinant inhibitor</th>
<th>Target herbivore</th>
<th>Non-target organism(s)</th>
<th>Overall impact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>Oryzacystatin I</td>
<td>Plutella xylostella</td>
<td>Harmonia axyridis</td>
<td>None</td>
<td>Ferry et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P. xylostella</td>
<td>Pterostichus madidus</td>
<td>Negative</td>
<td>Ferry et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Deroceras reticulatum</td>
<td>Pterostichus melanarius</td>
<td>None</td>
<td>Mulligan et al., 2006</td>
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<td>Cowpea trypsin inhibitor</td>
<td>Otiorynchus scutatus</td>
<td>Carabids</td>
<td>None</td>
<td>Graham et al., 2002</td>
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<td>Potato</td>
<td>Cowpea trypsin inhibitor</td>
<td>Laccania oleracea</td>
<td>Podisus maculiventris</td>
<td>None</td>
<td>Bell et al., 2003</td>
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<td></td>
<td>Oryzacystatin I</td>
<td>Leptinotarsa decemlineata</td>
<td>Perillus bioculatus</td>
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<td>Bouchard et al., 2003a, b</td>
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<td>Barley cystatin H+CI-1</td>
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<td>Bovine spleen trypsin inhibitor</td>
<td>Spodoptera litura</td>
<td>Ctenogathus novaselundiae</td>
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<td>Burgess et al., 2008</td>
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Parasitoids

<table>
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<th>Recombinant inhibitor</th>
<th>Target herbivore</th>
<th>Non-target organism(s)</th>
<th>Overall impact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola</td>
<td>Oryzacystatin I</td>
<td>Myzus persicae</td>
<td>Diaeretiella rapae</td>
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<td>Schuler et al., 2001</td>
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<td>Negative</td>
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</tr>
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<td></td>
<td>Oryzacystatin I</td>
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<td>Aphidius avenae</td>
<td>Positive</td>
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<td></td>
<td></td>
<td>Globodera pallida</td>
<td>Aphidius ervi</td>
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<td></td>
<td></td>
<td>G. pallida</td>
<td>Aspalhes vulgaris</td>
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Non-target herbivores

<table>
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<tr>
<th>Plant</th>
<th>Recombinant inhibitor</th>
<th>Target herbivore</th>
<th>Non-target organism(s)</th>
<th>Overall impact</th>
<th>Reference</th>
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<td>Konrad et al., 2008, 2009</td>
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<td>Potato</td>
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<td>Myzus persicae</td>
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<td>Euphyrus avenae</td>
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<td>Cowgill and Atkinson, 2003</td>
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<td>M. persicae</td>
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<td>Cowgill et al., 2002</td>
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<td>E. avara</td>
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<td>L. decemlineata</td>
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<td>Diatraea saccharalis</td>
<td>Schelonthes praecincus</td>
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<td>Simoes et al., 2008</td>
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<td>S. praecincus</td>
<td>None</td>
<td>Simoes et al., 2008</td>
</tr>
</tbody>
</table>

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a Plant material ingested indirectly via herbivorous preys (predators) or hosts (parasitoids), or directly from host plant tissues (non-target herbivores).

b Significant positive (Positive), adverse (Negative) or null (None) impact on mortality, fecundity, weight gain and/or food consumption (usually at P=0.05).

Burgess, 2000) (Fig. 1). Along with their pesticidal effects against target herbivores, recombinant protease inhibitors might, in the field, influence digestive protease processes in pollinators, symbionts, detritivores, or non-target phytophages, and eventually alter the fitness or behaviour of these organisms establishing a physical relationship with the modified plant. They might also impact the proteases and compromise the ecological functions of insect natural predators, parasitoids, or pathogens by a two-step route, via their herbivore prey or host ingesting the modified plant tissues.

Similar to Bt toxin-expressing plants, negligible effects have been observed in several instances for protease inhibitor-expressing plants against diverse non-target arthropods including herbivore predators, parasitoids, secondary phytophages, and soil detritivores (Cowgill et al., 2002, 2004; Graham et al., 2002; Cowgill and Atkinson, 2003; Bell et al., 2003; Bouchard et al., 2003a, b; Ferry et al., 2003, 2005; Mulligan et al., 2006; Simoes et al., 2008; Burgess et al., 2008; Konrad et al., 2008, 2009). By contrast, negative (Bell et al., 2001; Ferry et al., 2005) or positive (Ashouri et al., 2001a, b) effects were recorded in other cases, as has also been observed for a number of lectin-expressing plants (Birch et al., 1999; Bell et al., 2003; Down et al., 2003; Tomov et al., 2003). Whereas the detection of positive effects in some instances remains surprising given the metabolic interference effects expected for protease inhibitors, the negative effects of these proteins could be explained, as noted for Bt toxins, by a poor quality of the herbivore preys or hosts ingesting transgenic plant tissues (Bell et al., 2001).

Protease inhibitory effects and midgut compensatory responses observed in some predators following ingestion of herbivore preys fed transgenic material also suggest a direct interfering effect at superior trophic levels. Articles describing the impact of L. decemlineata larvae fed oryzacystatin-expressing potato leaves on the hemipteran two-spotted stink bug Perillus bioculatus (F.), for instance, reported a strong inhibition of this predator’s midgut proteases, readily compensated by the secretion of proteases from alternative functional classes insensitive to the recombinant inhibitor (Bouchard et al., 2003a, b). In a similar way, oryzacystatin and MTI-2, a trypsin inhibitor from mustard, were shown to induce compensatory responses in the coleopteran predators Pterostichus madidus (F.) and P. melanarius (Illiger) via their herbivorous prey fed oryzacystatin- or MTI-2-expressing rapeseed lines (Ferry et al., 2005; Mulligan et al., 2006). Interestingly, transient negative effects observed for MTI-2 on growth rates of P. madidus were overcome gradually, along with the
secretion of inhibitor-insensitive proteases in the midgut (Ferry et al., 2005). These observations, while illustrating the remarkable adaptability of digestive protease complements at superior trophic levels of plant ecosystems, were also underlining the vectorial movement of protease inhibitors along food chains and the relevance of additional empirical studies assessing the spatio-temporal dynamics of protease–inhibitor interactions. The unexpected positive effects observed for oryzacystatin on secondary phytophages and associated parasitoids (Ashouri et al., 2001a, b) were also adding to the complexity of the whole picture, and perhaps pointing to a possible role for inhibitor-induced pleiotropic effects in planta altering some compositional or physiological characteristics of the transformed host plant.

**Unintended protease–inhibitor interactions in planta**

Proteolytic enzymes are ubiquitous biochemical effectors in plant cells, involved in the regulation of numerous metabolic processes ranging from housekeeping functions such as protein turnover and the elimination of misfolded proteins, to the processing of polypeptide pre- and pro-regions on maturing proteins (Sullivan et al., 2003; Schaller, 2004; Faye et al., 2005; van den Hoorn, 2008). At the cellular level, proteases are involved in virtually all biochemical processes, which raises questions about the possible impacts of ectopically expressed protease inhibitors on proteolytic processes and protease/inhibitor balances in the plant. Studies have reported negligible phenotypic effects for protease inhibitors in transgenic plants based on the assessment of macroscopic indicators such as growth rate, stem diameter or leaf number (Masoud et al., 1993; Brunelle et al., 2004; Rivard et al., 2006; Badri et al., 2009), but recent reports suggest significant effects at the metabolic level. Several papers documented, for instance, the pesticidal effects of recombinant cystatins in transgenic plants (Atkinson et al., 2004a; Ouchkourova et al., 2004a, b; Ninkovic et al., 2007; Chan et al., 2010), but the constitutive accumulation of these proteins in planta could also be associated with an alteration of flower development (Gutiérrez-Campos et al., 2001), an inhibition of the pathogen-inducible hypersensitive response (Belenghi et al., 2003), an improved stability of the photosynthetic apparatus under low temperature regimes (Van der Vyver et al., 2003), or an increased protein content in leaves (Prins et al., 2008). In a similar way, the potential of serine protease inhibitors for herbivorous insect control has been extensively discussed (see above), but significant metabolic interference effects impacting leaf protein levels were observed recently for the serine-type inhibitors bovine aprotinin and tomato Kunitz-type cathepsin D inhibitor expressed in potato (Badri et al., 2009; Goulet et al., 2010).
Subcellular targeting of the recombinant inhibitors using appropriate peptide targeting signals may represent an effective way to elude unintended inhibitory effects in planta. Organelles play specific, complementary roles in plant cells and harbour a specific protease complement, well adapted to their particular metabolic needs and physicochemical environment (Callis, 1995). Not surprisingly, a recent study assessing the possible effects of bovine aprotinin targeted to the secretory pathway of transgenic potato leaf cells using an N-terminal signal peptide confirmed the onset of organelle-dependent pleiotropy (Badri et al., 2009). Whereas retaining this inhibitor of serine proteases in the endoplasmic reticulum with a ‘KDEL’ retention signal negatively altered protein content in leaves via a proteome-wide negative feedback on protein biosynthesis, the same protein allowed to migrate further along the secretory pathway had a negligible impact on both the leaf proteome and total protein content. In agreement with the absence of aprotinin in the cytosol, no particular phenotype was observed at the proteome level in potato clones expressing aprotinin in this cell compartment (Badri et al., 2009), in sharp contrast with cytosol-targeted forms of corn cystatin II and tomato cathepsin D inhibitor both inducing important changes (Goulet et al., 2010; Munger et al., 2010). The latter inhibitor, a broad-spectrum inhibitor of serine and aspartate proteases, was shown to alter the overall protein biosynthesis/degradation balance in leaves positively, with a resulting positive impact on leaf protein content (Goulet et al., 2010). The corn cystatin had no impact on general protein content (Vaillancourt, 2005), but triggered the constitutive expression of several naturally inducible stress- and pathogenesis-related proteins (Munger et al., 2010), in line with earlier studies reporting modified responses to abiotic and biotic stress cues in plants expressing cystatins in the cytosol (Van der Vyver et al., 2003; Belenghi et al., 2003; Prins et al., 2008).

From a scientific viewpoint, the so-called pleiotropic effects of recombinant protease inhibitors in planta are typically considered as unintended metabolic interference, but they might simply illustrate a lack of knowledge on plant proteolytic processes and eventually represent useful traits for crop improvement (Table 3). Whereas pleiotropic effects such as a delayed development of floral organs (Gutierrez-Campos et al., 2001) or an inhibition of the hypersensitive response (Belenghi et al., 2003) may hardly be seen as positive or neutral phenotypes, the up-regulation of stress-related proteins in leaves (Munger et al., 2010) might, in contrast, represent an agronomically useful trait and account, at least in part, for the increased tolerance of protease inhibitor-expressing plants to abiotic stress conditions such as drought, salinity or chilling (Van der Vyver et al., 2003; Huang et al., 2007; Shan et al., 2008; Srinivasan et al., 2009). Likewise, the constitutive expression of pathogenesis-related proteins such as chitinases and β-glucanases in cystatin-expressing leaves (Munger et al., 2010), along with the repression of the hypersensitive response by recombinant cystatins (Belenghi et al., 2003), could explain the recently observed resistance of cystatin-expressing potato clones to the fungal necrotroph Botrytis cinerea (A Munger et al., unpublished data), which is both sensitive to chitinases (Vellicie et al., 2006; Distefano et al., 2008) and dependent on the hypersensitive response during infection (van Baarlen et al., 2007; Imani et al., 2006). Together, these findings suggesting eventual ‘beneficial pleiotropic effects’ for protease inhibitors in transgenic plants open new avenues for the use of these proteins in plant protection, but they also raise a number of new questions about the impact of such recombinant metabolic effectors in plant ecosystems. Could, for instance, the non-target effects of cystatin-expressing potato plants on predatory arthropods be related not only to a direct protease inhibitory interaction at the third trophic level (Bouchard et al., 2003a, b), but also to the constitutive expression of stress-related proteins in leaf tissues altering to some extent the chemical composition of the herbivore prey? Fourth instars of the coleopteran herbivore L. decemlineata are known to adjust the composition of their digestive protease complement to different sets of endogenous defence proteins induced in potato leaves with antagonistic defence elicitors (Rivard et al., 2004), which supports the idea of protease inhibitor-mediated pleiotropic effects having an impact on the (bio)chemical composition of insect herbivores.

**Table 3. Pleiotropic effects in transgenic plants expressing recombinant protease inhibitors**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Recombinant inhibitor</th>
<th>Target proteases</th>
<th>Induced phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>Wheat Bowman-Birk inhibitor (WRSI5)</td>
<td>Serine</td>
<td>Salt tolerance</td>
<td>Shan et al., 2008</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>AtCYSa and AtCYSb</td>
<td>Cysteine</td>
<td>Drought, salt, cold, and oxidation tolerance</td>
<td>Zhang et al., 2008</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>cystatin 1 (AtCYS1)</td>
<td>Cysteine</td>
<td>Inhibition of the hypersensitive response</td>
<td>Belenghi et al., 2003</td>
</tr>
<tr>
<td>Potato</td>
<td>Corn cystatin II (CC-II)</td>
<td>Cysteine</td>
<td>Up-regulation of PR-/stress-related proteins</td>
<td>Munger et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Tomato cathepsin D inhibitor</td>
<td>Serine/Aspartate</td>
<td>Increased leaf protein content</td>
<td>Goulet et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Bovine aprotinin</td>
<td>Serine</td>
<td>Decreased leaf protein content</td>
<td>Badri et al., 2009</td>
</tr>
<tr>
<td>Rice</td>
<td>Rice chymotrypsin inhibitor-like 1 (OCPII)</td>
<td>Serine</td>
<td>Drought tolerance</td>
<td>Huang et al., 2007</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Nicotiana tabacum trypsin inhibitor (NPII)</td>
<td>Serine</td>
<td>Salt tolerance</td>
<td>Srinivasan et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Oryzaeystatin I (OC-I)</td>
<td>Cysteine</td>
<td>Increased leaf protein content</td>
<td>Prins et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chilling tolerance</td>
<td>Van der Vyver et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Altered flowering</td>
<td>Gutierrez-Campos et al., 2001</td>
</tr>
</tbody>
</table>
Recombinant protease inhibitors in a multitrophic context

Overall, the recent literature on recombinant protease inhibitors clearly highlights the eventual impacts of these proteins in plant ecosystems, and the obvious relevance of additional studies on protease-inhibitor interactions in non-target species likely to interact, directly or not, with the modified host plant. Several conclusive papers have described protease inhibitory effects for native inhibitors such as cowpea trypsin inhibitor or oryzacystatin on non-target phytophages and auxiliary arthropods, but few studies addressed the effects of novel-generation inhibitors exhibiting improved potency against herbivorous pest proteases, or those of multifunctional (e.g. fusion) hybrid inhibitors active against proteases from different functional classes. In a similar way, most protein engineering attempts to improve the efficiency of pesticidal protease inhibitors—and to confirm their potential in plant protection—have put the focus on target herbivore proteases, but little attention has been paid to the activity of the improved inhibitors against non-target proteases. Although the net impacts of protease inhibitor-expressing plants might often be limited compared with pest control strategies relying on commercial chemical pesticides (Cowgill et al., 2002; Mulligan et al., 2006), significant impacts, either negative or positive, could be observed more frequently in future years, along with the design of highly potent inhibitors and the fine-tuning of strategies for the high-level expression of heterologous proteins in transgenic plants (Streatfield, 2007). The challenge, then, will be to approach the molecular improvement of protease inhibitors in a multitrophic perspective, looking for inhibitor variants with lower $K_d$ (or $K_i$) dissociation constants for (and increased activity against) the target herbivore proteases, but with higher $K_a$ values for (and weaker activity against) proteases of the same functional class in the host plant and non-target arthropods of the biological system considered.

A straightforward strategy to achieve this goal will be the adoption of a two-step approach first involving the generation and identification of inhibitor variants with increased potency against the herbivore target proteases, followed by the selection of candidate inhibitors among these variants also showing decreased potency against a number of selected non-target proteases. Rational design based on 3-D models for protease-inhibitor complexes has been instrumental over the years to decipher protease inhibitory mechanisms and to identify relevant target sites for site-directed mutagenesis (Urwin et al., 1995; Qasim et al., 1997; Mason et al., 1998; Ogawa et al., 2002; Pavlova and Björk, 2003), but this approach could be of limited value for engineering attempts requiring the analysis of multiple protease-inhibitor interactions. By comparison, in vitro molecular evolution schemes involving recombination or random mutagenesis in functionally relevant regions of the gene (protein) sequence, combined with high throughput screening approaches such as molecular phage display for the selection of improved inhibitor variants (Koiwa et al., 2001; Laboissiere et al., 2002; Ceci et al., 2003; Melo et al., 2003; Stoop and Craik, 2003; Yuan et al., 2005), probably represent an effective way to generate functionally diverse inhibitor populations. Site-directed mutagenesis of inhibitor-encoding DNA sequences at positively selected, hyper-variable codon (amino acid) sites may also be useful to induce significant functional diversity among a relatively small number of single mutants, as illustrated with the tomato cystatin S/CYS8 interacting with papain-like cysteine proteases (Kiggundu et al., 2006). This latter strategy has recently proved useful to isolate S/CYS8 variants with stronger inhibitory activity against digestive cysteine proteases of the potato pest L. decemlineata but with weaker activity against the digestive proteases of its natural predator, P. bioculatus (Goulet et al., 2008).

Some of the S/CYS8 variants in this study also exhibited positively or negatively altered inhibitory activity against potato leaf cysteine proteases, thereby providing a preliminary proof-of-concept for the feasibility to develop potent pesticidal inhibitors with the option of amplifying or minimizing protease inhibitory effects in planta (Goulet et al., 2008). Recombinant protease inhibitors may exhibit agronomically useful pleiotropic effects in the host plant (see Table 3), but a minimal monitoring of these effects should remain an important piece of the puzzle for any realistic account of protease inhibitor interfering effects in plant ecosystems. In practice, rationally controlling the specificity of recombinant protease inhibitors should allow useful traits to be amplified intentionally, or, on the contrary, metabolic interference to be avoided along the plant food chain. Successfully modulating the onset of in planta pleiotropic effects and the inhibitory specificity of recombinant inhibitors could also be of interest, finally, to preserve the nutritional quality of food products derived from transgenic crops expressing these proteins. Recent studies established ‘substantial equivalence’ between food products derived from protease inhibitor-expressing lines and conventional or transgenic comparator lines (Li et al., 2007; Khalf et al., 2010), but the increasing amount of data showing pleiotropic effects in vegetative organs raises questions about the occurrence of similar effects in storage organs (Zhou et al., 2009). In a similar way, the ectopic accumulation of cysteine protease inhibitors such as cystatins in food products may be of little concern given the absence of target proteases in the human gut (Atkinson et al., 2004b), but the expression of highly potent, broad-spectrum inhibitors of serine and aspartate proteases will raise a number of questions in future years. Despite serious doubts about their actual potential in plant protection, recombinant protease inhibitors have proved to be of particular value over the last 20 years as powerful models for studying the ecological impacts of insect-resistant transgenic plants, co-evolutionary processes in plant—pest systems, and recombinant protein-mediated pleiotropic effects in transgenic plants. Recent developments towards the successful implementation of inhibitor-expressing plant lines in agricultural fields, along with the numerous and complex questions raised by such promising developments,
should ensure the status of these metabolic effectors as useful working models for several more years.

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