*Journal of Experimental Botany*, Vol. 61, No. 15, pp. 4169–4183, 2010
doi:10.1093/jxb/erq166 Advance Access publication 25 June, 2010

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

# Recombinant protease inhibitors for herbivore pest control: a multitrophic perspective

**Urte Schlüter[1,2,†], Meriem Benchabane[2,†], Aurélie Munger[2], Andrew Kiggundu[3], Juan Vorster[2,4], Marie-Claire Goulet[2], Conrad Cloutier[5] and Dominique Michaud[2,*]**

[1] Forestry and Agricultural Biotechnology Institute, Plant Science Department, University of Pretoria, Pretoria, South Africa
[2] Département de phytologie, CRH/INAF, Pavillon des Services (INAF), Université Laval, 2440 boul. Hochelaga, Québec (QC), Canada
[3] National Agricultural Research Organization, Kawanda Agricultural Research Institute, Kampala, Uganda
[4] Department of Plant Production and Soil Science, University of Pretoria, Pretoria, South Africa
[5] Département de biologie, Faculté des Sciences et de Génie, Université Laval, Québec (QC), Canada

[†] The first two authors contributed equally to this manuscript.
[*] To whom correspondence should be addressed: E-mail: dominique.michaud@fsaa.ulaval.ca

Received 22 March 2010; Revised 18 May 2010; Accepted 19 May 2010



## 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 multitrophic 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.*, 2009*a*, *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 protease 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 finite and limited 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 multitrophic 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, 1995*a*, *b*; Jongsma *et al.*, 1995*a*; Bown *et al.*, 1997; Girard *et al.*, 1998*a*; Cloutier *et al.*, 1999, 2000; Mazumdar-Leighton and Broadway, 2001*a*, *b*; Zhu-Salzman *et al.*, 2003*a*; 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.*, 2003*a*; 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.*, 1995*b*; Michaud, 1997; Girard *et al.*, 1998*b*; Giri *et al.*, 1998; Gruden *et al.*, 2003; Zhu-Salzman *et al.*, 2003*a*; 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

| Plant | Recombinant inhibitor | Target proteases | Target herbivore(s) | Reference |
|---|---|---|---|---|
| | | | **Herbivorous insects** | |
| Alfalfa | Oryzacystatin II | Cysteine | *Phytodecta fornicata* | Ninkovic *et al.*, 2007 |
| Apple | *Nicotiana alata* proteinase inhibitor | Serine | *Epiphyas postvittana* | Maheswaran *et al.*, 2007 |
| Arabidopsis | Mustard trypsin inhibitor 2 | Serine | *Plutella xylostella* | De Leo *et al.*, 2001 |
| Oilseed rape | Mustard trypsin inhibitor 2 | Serine | *P. xylostella* | De Leo *et al.*, 2001 |
| Potato | Multidomain cystatin fusions | Cysteine+Aspartate | *Frankliniella occidentalis* | Outchkourov *et al.*, 2004*a* |
| | Various animal and plant cystatins | Cysteine (+Aspartate) | *F. occidentalis* | Outchkourov *et al.*, 2004*b* |
| | Barley cystatin HvCPI-1 $C^{68}$ | Cysteine | *Leptinotarsa decemlineata* | Alvarez-Alfageme *et al.*, 2007 |
| | Locust serine proteinase inhibitors | Serine | *L. decemlineata* | Kondrak *et al.*, 2005 |
| Rice | Barley trypsin inhibitor | Serine | *Sitophilus oryzae* | Alfonso-Rubi *et al.*, 2003 |
| | Soybean trypsin inhibitor (+lectin) | Serine | *Nilaparvata lugens* | Li *et al.*, 2005 |
| | | | *Cnaphalocrocis medinalis* | Li *et al.*, 2005 |
| | Maize proteinase inhibitor (mpi) | Serine | *Chilo suppresssalis* | Vila *et al.*, 2005 |
| | Cowpea trypsin inhibitor (+Bt toxin Cry1Ac) | Serine | *C. medinalis* | Han *et al.*, 2007 |
| Sugarcane | Bovine pancreatic trypsin inhibitor (aprotinin) | Serine | *Scirpophaga excerptalis* | Christy *et al.*, 2009 |
| Tobacco | Bovine spleen trypsin inhibitor | Serine | *Helicoverpa armigera* | Christeller *et al.*, 2002 |
| | *Brassica juncea* trypsin inhibitor | Serine | *Spodoptera litura* | Mandal *et al.*, 2002 |
| | Mustard trypsin inhibitor 2 | Serine | *Spodoptera littoralis* | De Leo and Gallerani, 2002 |
| | Tobacco trypsin protease inhibitor | Serine | *S. litura* | Srinivasan *et al.*, 2009 |
| | | | *H. armigera* | Srinivasan *et al.*, 2009 |
| | Sporamin+Taro cystatin | Serine+Cysteine | *H. armigera* | Senthilkumar *et al.*, 2010 |
| | Buckwheat serine proteinase inhibitor | Serine | *Trialeurodes vaporariorum* | Khadeeva *et al.*, 2009 |
| | | | **Root parasitic nematodes** | |
| Alfalfa | Oryzacystatin I | Cysteine | *Pratylenchus penetrans* | Samac and Smigocki, 2003 |
| Potato | Oryzacystatin I | Cysteine | *Meloidogyne incognita* | Lilley *et al.*, 2004 |
| | | | *Globodera pallida* | Lilley *et al.*, 2004 |
| Tomato | Taro cystatin | Cysteine | *M. incognita* | Chan *et al.*, 2010 |
| Wheat | Potato proteinase inhibitor 2 | Serine | *Heterodera avenae* | Vishnudasan *et al.*, 2005 |
| | | | **Pathogens** | |
| Potato | Buckwheat serine proteinase inhibitor | Serine | *Pseudomonas syringae* | Khadeeva *et al.*, 2009 |
| | | | *Clavibacter michiganensis* | Khadeeva *et al.*, 2009 |
| Rice | Potato carboxypeptidase inhibitor | Carboxypeptidases A | *Magnaporthe oryzae* | Quilis *et al.*, 2007 |
| | | | *Fusarium verticillioides* | Quilis *et al.*, 2007 |
| Tobacco | Buckwheat serine proteinase inhibitor | Serine | *P. syringae* | Khadeeva *et al.*, 2009 |
| | | | *C. michiganensis* | Khadeeva *et al.*, 2009 |
| | Sporamin+Taro cystatin | Serine+Cysteine | *Pythium aphanidermatum* | Senthilkumar *et al.*, 2010 |

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 phage display procedures involving random mutagenesis in functionally significant regions of the inhibitor sequence (Jongsma *et al.*, 1995*b*; Koiwa *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.*, 2003*b*; Outchkourov *et al.*, 2004*a*; 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

**Table 2.** Overall impact of transgenic plants expressing protease inhibitors on predatory, parasitoid and herbivorous non-target arthropods: a summary of recent studies

| Plant | Recombinant inhibitor | Target herbivore | Non-target organism(s) [a] | Overall impact[b] | Reference |
|---|---|---|---|---|---|
| | | | **Predators** | | |
| Canola | Oryzacystatin I | *Plutella xylostella* | *Harmonia axyridis* | None | Ferry *et al.*, 2003 |
| | | *P. xylostella* | *Pterostichus madidus* | Negative | Ferry *et al.*, 2005 |
| | | *Deroceras reticulatum* | *Pterostichus melanarius* | None | Mulligan *et al.*, 2006 |
| Strawberry | Cowpea trypsin inhibitor | *Otiorynchus sulcatus* | Carabids | None | Graham *et al.*, 2002 |
| Potato | Cowpea trypsin inhibitor | *Lacanobia oleracea* | *Podisus maculiventris* | None | Bell *et al.*, 2003 |
| | Oryzacystatin I | *Leptinotarsa decemlineata* | *Perillus bioculatus* | None | Bouchard *et al.*, 2003*a*, *b* |
| | Barley cystatin HvCPI-1 $C^{68}$ | *L. decemlineata* | *P. maculiventris* | None | Alvarez-Alfageme *et al.*, 2007 |
| Tobacco | Bovine spleen trypsin inhibitor | *Spodoptera litura* | *Ctenoghathus novaezelandiae* | None | Burgess *et al.*, 2008 |
| | | | **Parasitoids** | | |
| Canola | Oryzacystatin I | *Myzus persicae* | *Diaeretiella rapae* | None | Schuler *et al.*, 2001 |
| Potato | Cowpea trypsin inhibitor | *L. oleracea* | *Eulophus pennicornis* | Negative | Bell *et al.*, 2001 |
| | Oryzacystatin I | *L. decemlineata* | *Aphidius nigripes* | Positive | Ashouri *et al.* 2001*a* |
| | | *Globodera pallida* | *Aphidius ervi* | None | Cowgill *et al.*, 2004 |
| | | *G. pallida* | *Aspahes vulgaris* | None | Cowgill *et al.*, 2004 |
| | | | **Non-target herbivores** | | |
| Canola | Oryzacystatin I | – | *Osmia bicornis* | None | Konrad *et al.*, 2008, 2009 |
| Potato | Chicken egg white cystatin | *G. pallida* | *Myzus persicae* | None | Cowgill *et al.*, 2002 |
| | | *G. pallida* | *Eupteryx aurata* | None | Cowgill and Atkinson, 2003 |
| | Oryzacystatin I | *G. pallida* | *M. persicae* | None | Cowgill *et al.*, 2002 |
| | | *G. pallida* | *E. aurata* | None | Cowgill and Atkinson, 2003 |
| | | *L. decemlineata* | *Macrosiphum euphorbiae* | Positive | Ashouri *et al.*, 2001*b* |
| Sugarcane | Soybean Bowman-Birk inhibitor | *Diatraea saccharalis* | *Scheloribates praeincisus* | None | Simoes *et al.*, 2008 |
| | Soybean Kunitz inhibitor | *D. saccharalis* | *S. praeincisus* | None | Simoes *et al.*, 2008 |

[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.*, 2003*a*, *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.*, 2001*a*, *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 stinkbug *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.*, 2003*a*, *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

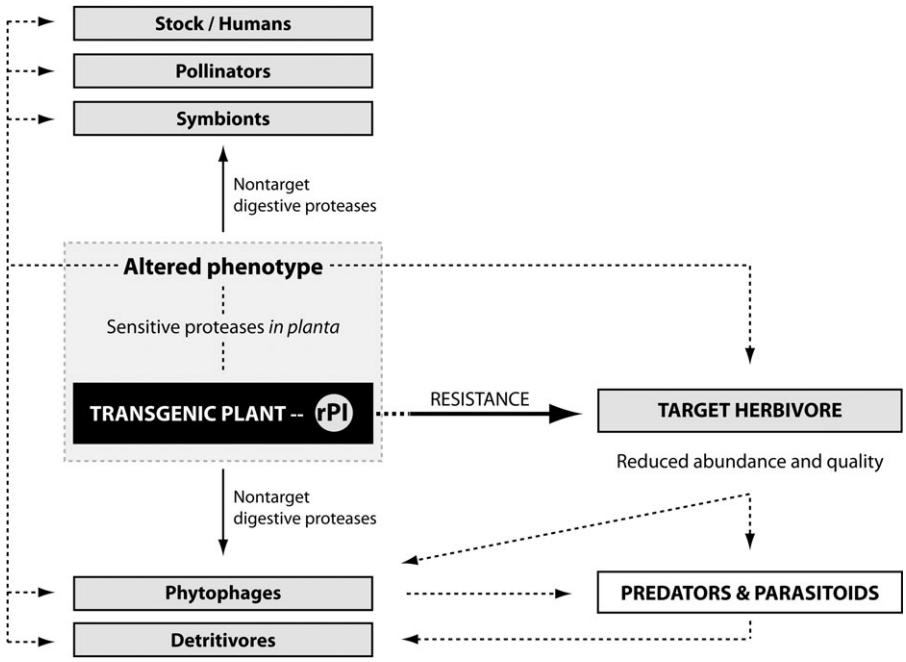


**Fig. 1.** Intended and unintended effects of protease inhibitor-expressing plants on non-target organisms. The main intended effect for a recombinant protease inhibitor usually is to provide resistance against an insect herbivore (horizontal plain arrow), but a number of unintended effects may be observed as a result of pleiotropic effects *in planta* changing the host plant's phenotype or composition (dashed arrows), or as a result of non-specific protease inhibitory effects due to a limited specificity of the recombinant inhibitor against extracellular (digestive) proteolytic enzymes in the ecosystem (vertical plain arrows). Direct unintended effects may be observed on different organisms interacting with or ingesting tissues from the host plant (grey boxes). Indirect unintended effects may also be observed at higher trophic levels, via target and non-target phytophages ingesting transgenic tissues (white box). rPI, recombinant protease inhibitor.

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.*, 2001*a*, *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.*, 2004*a*; Outchkourov *et al.*, 2004*a*, *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 physico-chemical 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 (Vellicce *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.*, 2003*a*, *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

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

## 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 $K_d$ 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, hypervariable 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 *Sl*CYS8 interacting with papain-like cysteine proteases (Kiggundu *et al.*, 2006). This latter strategy has recently proved useful to isolate *Sl*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 *Sl*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.*, 2004*b*), 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–insect 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.

## Acknowledgements

This article is a synthesis of two talks given at the Association of Applied Biologists conference, 'Agriculture: Africa's Engine for Growth–Plant Science and Biotechnology Hold the Key', Rothamsted Research, Harpenden (UK), 14 October 2009; and at the Africa College one-day symposium, 'Current Research in Plant Biology: Excellence with Application', University of Leeds, Leeds (UK), 16 October 2009. Research on protease inhibitors at Laval University is funded by the Natural Science and Engineering Research Council (NSERC) of Canada and the Fonds Québécois de recherche sur la nature et les technologies. Research on protease inhibitors at the University of Pretoria is funded by the National Research Foundation (NRF) of South Africa. M Benchabane was the recipient of an NSERC PhD scholarship. J Vorster was the recipient of an NRF post-doctoral fellowship.

## References

**Abdeen A, Virgos A, Olivella E, Villanueva J, Aviles X, Gabarra R, Prat S.** 2005. Multiple insect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. *Plant Molecular Biology* **57,** 189–202.

**Ahn J-E, Salzman RA, Braunagel SC, Koiwa H, Zhu-Salzman K.** 2004. Functional roles of specific bruchid protease isoforms in adaptation to a soybean protease inhibitor. *Insect Molecular Biology* **13,** 649–657.

**Alfonso-Rubi J, Ortego F, Castanera P, Carbonero P, Diaz I.** 2003. Transgenic expression of trypsin inhibitor CMe from barley in *indica* and *japonica* rice, confers resistance to the rice weevil *Sitophilus oryzae*. *Transgenic Research* **12,** 23–31.

**Alvarez-Alfageme F, Martinez M, Pascual-Ruiz S, Castanera P, Diaz I, Ortego F.** 2007. Effects of potato plants expressing a barley cystatin on the predatory bug *Podisus maculiventris* via herbivorous prey feeding on the plant. *Transgenic Research* **16,** 1–13.

**Andow DA, Lövei GL, Arpaia S.** 2009. Cry toxins and proteinase inhibitors in transgenic plants do have non-zero effects on natural enemies in the laboratory: rebuttal to Shelton et al., 2009. *Environmental Entomology* **38,** 1528–1532.

**Ashouri A, Michaud D, Cloutier C.** 2001*a*. Recombinant and classically selected factors of potato plant resistance to the Colorado potato beetle, *Leptinotarsa decemlineata*, variously affect the potato aphid parasitoid *Aphidius nigripes*. *Biocontrol* **46,** 401–418.

**Ashouri A, Michaud D, Cloutier C.** 2001*b*. Unexpected effects of different potato resistance factors to the Colorado potato beetle (Coleoptera: Chrysomelidae) on the potato aphid (Homoptera: Aphididae). *Environmental Entomology* **30,** 524–532.

**Atkinson HJ, Grimwood S, Johnston K, Green J.** 2004*a*. Prototype demonstration of transgenic resistance to the nematode *Radopholus similis* conferred in banana by a cystatin. *Transgenic Research* **13,** 135–142.

**Atkinson HJ, Johnston KA, Robbins M.** 2004*b*. Prima facie evidence that a phytocystatin for transgenic plant resistance to nematodes is not a toxic risk in the human diet. *Journal of Nutrition* **134,** 431–434.

**Badri MA, Rivard D, Coenen K, Michaud D.** 2009. Unintended molecular interactions in transgenic plants expressing clinically-useful proteins: the case of bovine aprotinin travelling the potato leaf cell secretory pathway. *Proteomics* **9,** 746–756.

**Belenghi B, Acconcia F, Trovato M, Perazzolli M, Bocedi A, Polticelli F, Ascenzi P, Delledonne M.** 2003. AtCYS1, a cystatin from *Arabidopsis thaliana*, suppresses hypersensitive cell death. *European Journal of Biochemistry* **270,** 2593–2604.

**Bell HA, Down RE, Fitches EC, Edwards JP, Gatehouse AMR.** 2003. Impact of genetically modified potato expressing plant-derived insect resistance genes on the predatory bug *Podisus maculiventris* (Heteroptera: Pentatomidae). *Biocontrol Science and Technology* **13,** 729–741.

**Bell HA, Fitches EC, Down RE, Ford L, Marris GC, Edwards JP, Gatehouse JA, Gatehouse AMR.** 2001. Effect of dietary cowpea trypsin inhibitor (CpTI) on the growth and development of the tomato moth *Lacanobia oleracea* (Lepidoptera: Noctuidae) and on the success of the gregarious ectoparasitoid *Eulophus pennicornis* (Hymenoptera: Eulophidae). *Pest Management Science* **57,** 57–65.

**Birch ANE, Geoghegan IE, Majerus MEN, McNicol JW, Hackett CA, Gatehouse AMR, Gatehouse JA.** 1999. Tri-trophic interactions involving pest aphids, predatory 2-spot ladybirds and transgenic potatoes expressing snowdrop lectin for aphid resistance. *Molecular Breeding* **5,** 75–83.

**Birk Y.** 2003. *Plant protease inhibitors*. New York: Springer.

**Benchabane M, Goulet M-C, Dallaire C, Côté P-L, Michaud D.** 2008. Hybrid protease inhibitors for pest and pathogen control–a functional cost for the fusion partners? *Plant Physiology and Biochemistry* **46,** 701–708.

**Bouchard É, Michaud D, Cloutier C.** 2003*a*. Molecular interactions between an insect predator and its herbivore prey on transgenic potato expressing a cysteine proteinase inhibitor from rice. *Molecular Ecology* **12,** 2429–2437.

**Bouchard É, Cloutier C, Michaud D.** 2003*b*. Oryzacystatin I expressed in transgenic potato induces digestive compensation in an insect natural predator via its herbivorous prey feeding on the plant. *Molecular Ecology* **12,** 2439–2446.

**Boulter D, Edwards GA, Gatehouse AMR, Gatehouse JA, Hilder VA.** 1990. Additive protective effects of different plant-derived insect resistance genes in transgenic tobacco plants. *Crop Protection* **9,** 351–354.

**Bown DP, Wilkinson HS, Gatehouse JA.** 1997. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. *Insect Biochemistry and Molecular Biology* **27,** 625–638.

**Broadway RM.** 2000. The adaptation of insects to protease inhibitors. In: Michaud D, ed. *Recombinant protease inhibitors in plants*. Georgetown TX: Landes Bioscience, 80–88.

**Brunelle F, Girard C, Cloutier C, Michaud D.** 2005. A hybrid, broad-spectrum inhibitor of Colorado potato beetle aspartate and

cysteine digestive proteinases. *Archives of Insect Physiology and Biochemistry* **60,** 20–31.

**Brunelle F, Cloutier C, Michaud D.** 2004. Colorado potato beetles compensate for tomato cathepsin D inhibitor expressed in transgenic potato. *Archives of Insect Physiology and Biochemistry* **55,** 103–113.

**Brunelle F, Nguyen-Quoc B, Cloutier C, Michaud D.** 1999. Protein hydrolysis by Colorado potato beetle, *Leptinotarsa decemlineata*, digestive proteases: the catalytic role of cathepsin D. *Archives of Insect Biochemistry and Physiology* **42,** 88–98.

**Burgess EPJ, Philip BA, Christeller JT, Page NEM, Marshall RK, Wohlers MW.** 2008. Tri-trophic effects of transgenic insect-resistant tobacco expressing a protease inhibitor or a biotin-binding protein on adults of the predatory carabid beetle *Ctenognathus novaezelandiae*. *Journal of Insect Physiology* **54,** 518–528.

**Callis J.** 1995. Regulation of protein degradation. *The Plant Cell* **7,** 845–857.

**Ceci LR, Volpicella M, Rahbé Y, Gallerani R, Beekwilder J, Jongsma MA.** 2003. Selection by phage display of a variant mustard trypsin inhibitor toxic against aphids. *The Plant Journal* **33,** 557–566.

**Chan YL, Yang A-H, Chen J-T, Yeh K-W, Chan M-T.** 2010. Heterologous expression of taro cystatin protects transgenic tomato against *Meloidogyne incognita* infection by means of interfering sex determination and suppressing gall formation. *Plant Cell Reports* **29,** 231–238.

**Chi YH, Salzman RA, Balfe S, *et al*.** 2009. Cowpea bruchid midgut transcriptome response to a soybean cystatin: costs and benefits of counter-defense. *Insect Molecular Biology* **18,** 97–110.

**Christeller JT.** 2005. Evolutionary mechanisms acting on proteinase inhibitor variability. *FEBS Journal* **272,** 5710–5722.

**Christeller JT, Burgess EPJ, Mett V, *et al*.** 2002. The expression of a mammalian proteinase inhibitor, bovine spleen trypsin inhibitor in tobacco and its effects on *Helicoverpa armigera* larvae. *Transgenic Research* **11,** 161–173.

**Christy LA, Arvinth S, Sarvanakumar M, Kanchana M, Mukunthan N, Srikanth J, Thomas G, Subramonian N.** 2009. Engineering sugarcane cultivars with bovine pancreatic trypsin inhibitor (aprotinin) gene for protection against top borer (*Scirpophaga excerptalis* Walker). *Plant Cell Reports* **28,** 175–184.

**Clark BW, Phillips TA, Coats JR.** 2005. Environmental fate and effects of *Bacillus thuringiensis* (Bt) proteins from transgenic crops: a review. *Journal of Agricultural and Food Chemistry* **53,** 4643–4653.

**Cloutier C, Boudreault S, Michaud D.** 2008. Impact de pommes de terre résistantes au doryphore sur les arthropodes non visés: une méta-analyse des facteurs possiblement en cause dans l'échec d'une plante transgénique Bt. *Cahiers Agriculture* **17,** 388–394.

**Cloutier C, Fournier M, Jean C, Yelle S, Michaud D.** 1999. Growth compensation and faster development of Colorado potato beetle (Coleoptera: Chrysomelidae) feeding on potato foliage expressing oryzacystatin I. *Archives of Insect Biochemistry and Physiology* **40,** 69–79.

**Cloutier C, Jean C, Fournier M, Yelle S, Michaud D.** 2000. Adult Colorado potato beetles, *Leptinotarsa decemlineata* compensate for nutritional stress on oryzacystatin I-transgenic potato plants by hypertrophic behaviour and over-production of insensitive proteinases. *Archives of Insect Physiology and Biochemistry* **44,** 69–81.

**Cowgill SE, Atkinson HJ.** 2003. A sequential approach to risk assessment of transgenic plants expressing protease inhibitors: effects on non-target herbivorous insects. *Transgenic Research* **12,** 439–449.

**Cowgill SE, Danks C, Atkinson HJ.** 2004. Multitrophic interactions involving genetically modified potatoes, non-target aphids, natural enemies and hyperparasitoids. *Molecular Ecology* **13,** 639–667.

**Cowgill SE, Wright C, Atkinson HJ.** 2002. Transgenic potatoes with enhanced levels of nematode resistance do not have altered susceptibility to non-target aphids. *Molecular Ecology* **11,** 821–827.

**De Leo F, Gallerani R.** 2002. The mustard trypsin inhibitor 2 affects the fertility of *Spodoptera littoralis* larvae fed on transgenic plants. *Insect Biochemistry and Molecular Biology* **32,** 489–496.

**De Leo F, Bonadé-Bottino M, Ceci LR, Gallerani R, Jouanin L.** 2001. Effects of a mustard trypsin inhibitor expressed in different plants on three lepidopteran pests. *Insect Biochemistry and Molecular Biology* **31,** 593–602.

**Deka S, Barthakur S.** 2010. Overview on current status of biotechnological interventions on yellow stem borer *Scirpophaga incertulas* (Lepidoptera: Crambidae) resistance in rice. *Biotechnology Advances* **28,** 70–81.

**Distefano G, La Malfa S, Vitale A, Lorito M, Deng Z, Gentile A.** 2008. Defence-related gene expression in transgenic lemon plants producing an antimicrobial *Trichoderma harzianum* endochitinase during fungal infection. *Transgenic Research* **17,** 873–879.

**Dively GP, Rose R, Sears MK, Hellmich RL, Stanley-Horn DE, Calvin DD, Russo JM, Anderson PL.** 2004. Effects on Monarch butterfly larvae (Lepidoptera: Danaidae) after continuous exposure to Cry1Ab-expressing corn during anthesis. *Environmental Entomology* **33,** 1116–1125.

**Down RE, Ford L, Woodhouse SD, Davison GM, Majerus MEN, Gatehouse JA, Gatehouse AMR.** 2003. Tritrophic interactions between transgenic potato expressing snowdrop lectin (GNA), an aphid pest (peach-potato aphid; *Myzus persicae* (Sulz.) and a beneficial predator (2-spot ladybird; *Adalia bipunctata* L.). *Transgenic Research* **12,** 229–241.

**Duan X, Li X, Xue Q, Abo-El-Saad M, Xu D, Wu R.** 1996. Transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene are insect resistant. *Nature Biotechnology* **14,** 494–498.

**Faye L, Boulaflous A, Benchabane M, Gomord V, Michaud D.** 2005. Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. *Vaccine* **23,** 1770–1778.

**Ferry N, Jouanin L, Ceci R, Mulligan EA, Emami K, Gatehouse JA, Gatehouse AM.** 2005. Impact of oilseed rape expressing the insecticidal serine protease inhibitor, mustard trypsin inhibitor-2 on the beneficial predator *Pterostichus madidus*. *Molecular Ecology* **14,** 337–349.

**Ferry N, Raemaekers RJ, Majerus ME, Jouanin L, Port G, Gatehouse JA, Gatehouse AM.** 2003. Impact of oilseed rape expressing the insecticidal cysteine protease inhibitor oryzacystatin on

the beneficial predator *Harmonia axyridis* (multicoloured Asian ladybeetle). *Molecular Ecology* **12,** 493–504.

**Firbank LG, Heard MS, Woiwod IP, *et al*.** 2003. An introduction to the farm-scale evaluations of genetically modified herbicide-tolerant crops. *Journal of Applied Ecology* **40,** 2–16.

**Gatehouse AMR, Ferry N, Raemaekers RJM.** 2002. The case of the monarch butterfly: a verdict is returned. *Trends in Genetics* **18,** 249–251.

**Gelvin SB.** 2003. *Agrobacterium*-mediated plant transformation: the biology behind the 'gene-jockeying' tool. *Microbiology and Molecular Biology Reviews* **67,** 16–37.

**Girard C, Rivard D, Kiggundu A, Kunert K, Gleddie SC, Cloutier C, Michaud D.** 2007. A multicomponent, elicitor-inducible cystatin complex in tomato, *Solanum lycopersicum*. *New Phytologist* **173,** 841–851.

**Girard C, Le Métayer M, Zaccomer B, Bartlet E, Williams I, Bonadé-Bottino M, Pham-Delegue MH, Jouanin L.** 1998*a*. Growth stimulation of beetle larvae reared on a transgenic oilseed rape expressing a cysteine protease inhibitor. *Journal of Insect Physiology* **44,** 263–270.

**Girard C, Le Métayer M, Bonadé-Bottino M, Pham-Delegue MH, Jouanin L.** 1998*b*. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. *Insect Biochemistry and Molecular Biology* **28,** 229–237.

**Giri AP, Harsulkar AM, Deshpande VV, Sainani MN, Gupta VS, Ranjekar PK.** 1998. Chickpea defensive proteinase inhibitors can be inactivated by podborer gut proteinases. *Plant Physiology* **116,** 393–401.

**Goulet C, Benchabane M, Anguenot R, Brunelle F, Khalf M, Michaud D.** 2010. A companion protease inhibitor for the protection of cytosol-targeted recombinant proteins in plants. *Plant Biotechnology Journal* **8,** 142–154.

**Goulet M-C, Dallaire C, Vaillancourt L-P, Khalf M, Badri MA, Preradov A, Duceppe M-O, Goulet C, Cloutier C, Michaud D.** 2008. Tailoring the specificity of a plant cystatin toward herbivorous insect digestive cysteine proteases by single mutations at positively selected amino acid sites. *Plant Physiology* **146,** 1010–1019.

**Graham J, Gordon SC, Smith K, McNicol RJ, McNicol JW.** 2002. The effect of the cowpea trypsin inhibitor in strawberry on damage by vine weevil under field conditions. *Journal of Horticultural Science and Biotechnology* **77,** 33–40.

**Groot AT, Dicke M.** 2002. Insect-resistant transgenic plants in a multi-trophic context. *The Plant Journal* **31,** 387–406.

**Gruden K, Kuipers AGJ, Guncar G, Slapar N, Strukelj B, Jongsma MA.** 2004. Molecular basis of Colorado potato beetle adaptation to potato plant defence at the level of digestive cysteine proteinases. *Insect Biochemistry and Molecular Biology* **34,** 365–375.

**Gruden K, Popovic T, Cimerman N, Krizaj I, Strukelj B.** 2003. Diverse enzymatic specificities of digestive proteases, 'intestains', enable Colorado potato beetle larvae to counteract the potato defence mechanism. *Biological Chemistry* **384,** 305–310.

**Gutierrez-Campos R, Torres-Acosta JA, Pérez-Martínez JDJ, Gómez-Lim MA.** 2001. Pleiotropic effects in transgenic tobacco plants expressing oryzacystatin I gene. *HortScience* **36,** 118–119.

**Han L, Wu K, Peng Y, Wang F, Guo Y.** 2007. Efficacy of transgenic rice expressing Cry1Ac and CpTI against the rice leaffolder, *Cnaphalocrocis medinalis* (Guenée). *Journal of Invertebrate Pathology* **96,** 71–79.

**Hansen Jesse LC, Obrycki JJ.** 2000. Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia* **125,** 241–248.

**Haq SK, Atif SM, Khan RH.** 2004. Protein proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. *Archives of Biochemistry and Biophysics* **431,** 145–159.

**Hellmich RL, Siegfried BD, Sears MK, Stanley-Horn DE, Daniels MJ, Mattila HR, Spencer T, Bidne KG, Lewis LC.** 2001. Monarch larvae sensitivity to *Bacillus thuringiensis*-purified proteins and pollen. *Proceedings of the National Academy of Sciences, USA* **98,** 11925–11930.

**Hernandez CA, Pujol M, Alfonso-Rubi J, Armas R, Coll Y, Pérez M, Gonzalez A, Ruiz M, Castanera P, Ortego F.** 2003. Proteolytic gut activities in the rice water weevil, *Lissorhoptrus brevirostris* Suffrian (Coleoptera: Curculionidae). *Archives of Insect Biochemistry and Physiology* **53,** 19–29.

**Hilder VA, Gatehouse AMR, Sheerman SE, Barker RF, Boulter D.** 1987. A novel mechanism of insect resistance engineered into tobacco. *Nature* **330,** 160–163.

**Huang Y, Xiao B, Xiong L.** 2007. Characterization of a stress responsive proteinase inhibitor gene with positive effect in improving drought resistance in rice. *Planta* **226,** 73–85.

**Huang J, Hu R, Rozelle S, Pray C.** 2005. Insect-resistant GM rice in farmers' fields: assessing productivity and health effects in China. *Science* **388,** 688–690.

**Imani J, Baltruschat H, Stein E, Jia G, Vogelsberg J, Kogel K-H, Hückelhoven R.** 2006. Expression of barley BAX Inhibitor-1 in carrots confers resistance to *Botrytis cinerea*. *Molecular Plant Pathology* **7,** 279–284.

**Inanaga H, Kobayasi D, Kouzuma Y, Aoki-Yasunaga C, Iiyama K, Kimura M.** 2001. Protein engineering of novel proteinase inhibitors and their effects on the growth of *Spodoptera exigua* larvae. *Bioscience, Biotechnology and Biochemistry* **65,** 2259–2264.

**James CA.** 2009. *ISAAA Brief No. 41. Global status of commercialized biotech/GM crops: 2009*. Ithaca, NY: ISAAA.

**Johnson R, Narvaez J, An G, Ryan CA.** 1989. Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. *Proceedings of the National Academy of Sciences, USA* **86,** 9871–9875.

**Jongsma MA, Bolter C.** 1997. The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* **43,** 885–895.

**Jongsma MA, Bakker PL, Peters J, Bosch D, Stiekema WJ.** 1995*a*. Adaptation of *Spodoptera exigua* larvae to plant proteinase inhibitors by induction of gut proteinase activity insensitive to inhibition. *Proceedings of the National Academy of Sciences, USA* **92,** 8041–8045.

**Jongsma MA, Bakker PL, Stiekema W, Bosch D.** 1995*b*. Phage display of a double-headed proteinase inhibitor: analysis of the binding

domains of potato proteinase inhibitor II. *Molecular Breeding* **1,** 181–191.

**Khadeeva NV, Kochieva EZ, Yu M, Tcherednitchenko MY, Yu E, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA.** 2009. Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress. *Biochemistry (Moscow)* **74,** 260–267.

**Khalf M, Goulet C, Vorster J, Brunelle F, Anguenot R, Fliss I, Michaud D.** 2010. Tubers from potato lines expressing a tomato Kunitz protease inhibitor are substantially equivalent to parental and transgenic controls. *Plant Biotechnology Journal* **8,** 155–169.

**Kiggundu A, Muchwezi J, Van der Vyver C, Viljoen A, Vorster J, Schlüter U, Kunert K, Michaud D.** 2010. Deleterious effects of plant cystatins against the banana weevil *Cosmopolites sordidus*. *Archives of Insect Biochemistry and Physiology* **73,** 87–105.

**Kiggundu A, Goulet M-C, Goulet C, Dubuc J-F, Rivard D, Benchabane M, Pépin G, van der Vyver C, Kunert K, Michaud D.** 2006. Modulating the proteinase inhibitory profile of a plant cystatin by single mutations at positively selected amino acid sites. *The Plant Journal* **48,** 403–413.

**Koiwa H, D'Urzo MP, Assfalg-Machleidt I, Zhu-Salzman K, Shade RE, An H, Murdock LL, Machleidt W, Bressan RA, Hasegawa PM.** 2001. Phage display selection of hairpin loop soyacystatin variants that mediate high affinity inhibition of a cysteine proteinase. *The Plant Journal* **27,** 383–391.

**Kondrak M, Kutas J, Szenthe B, Patthy A, Banfalvi Z, Nadasy M, Graf L, Asboth B.** 2005. Inhibition of Colorado potato beetle larvae by a locust proteinase inhibitor peptide expressed in potato. *Biotechnology Letters* **27,** 829–834.

**Konrad R, Connor M, Ferry N, Gatehouse AMR, Babendreier D.** 2009. Impact of transgenic oilseed rape expressing oryzacystatin-1 (OC-1) and of insecticidal proteins on longevity and digestive enzymes of the solitary bee *Osmia bicornis*. *Journal of Insect Physiology* **55,** 305–313.

**Konrad R, Ferry N, Gatehouse AMR, Babendreier D.** 2008. Potential effects of oilseed rape expressing oryzacystatin-1 (OC-1) and of purified insecticidal proteins on larvae of the solitary bee *Osmia bicornis*. *PLoS ONE* **3,** e2664.

**Koo YD, Ahn J-E, Salzman RA, Moon J, Chi YH, Yun D-J, Lee SY, Koiwa H, Zhu-Salzman K.** 2008. Functional expression of an insect cathepsin B-like counter-defence protein. *Insect Molecular Biology* **17,** 235–245.

**Laboissiere MC, Young MM, Pinho RG, Todd S, Fletterick RJ, Kuntz I, Craik CS.** 2002. Computer-assisted mutagenesis of ecotin to engineer its secondary binding site for urokinase inhibition. *Journal of Biological Chemistry* **277,** 26623–26631.

**Li G, Xu X, Xing H, Zhu H, Fan Q.** 2005. Insect resistance to *Nilaparvata lugens* and *Cnaphalocrocis medinalis* in transgenic indica rice and the inheritance of *gna+sbti* transgenes. *Pest Management Science* **61,** 390–396.

**Li X, Huang K, He X, Zhu B, Liang Z, Li H, Luo Y.** 2007. Comparison of nutritional quality between Chinese *Indica* rice with *sck* and *cry1Ac* genes and its nontransgenic counterpart. *Journal of Food Science* **72,** S420–S424.

**Lilley CJ, Urwin PE, Johnston KA, Atkinson HJ.** 2004. Preferential expression of a plant cystatin at nematode feeding sites confers resistance to *Meloidogyne incognita* and *Globodera pallida*. *Plant Biotechnology Journal* **2,** 3–12.

**Liu Y, Salzman RA, Pankiw T, Zhu-Salzman K.** 2004. Transcriptional regulation in southern corn rootworm larvae challenged by soyacystatin N. *Insect Biochemistry and Molecular Biology* **34,** 1069–1077.

**Lopes AR, Juliano MA, Juliano L, Terra WR.** 2004. Coevolution of insect trypsins and inhibitors. *Archives of Insect Biochemistry and Physiology* **55,** 140–152.

**Losey JE, Rayor LS, Carter ME.** 1999. Transgenic pollen harms monarch larvae. *Nature* **399,** 214.

**Lövei GL, Andow DA, Arpaia S.** 2009. Transgenic insecticidal crops and natural enemies: a detailed review of laboratory studies. *Environmental Entomology* **38,** 293–306.

**Maheswaran G, Pridmore L, Franz P, Anderson MA.** 2007. A proteinase inhibitor from *Nicotiana alata* inhibits the normal development of light-brown apple moth, *Epiphyas postvittana* in transgenic apple plants. *Plant Cell Reports* **26,** 773–782.

**Malone LA, Burgess EPJ.** 2000. Interference of protease inhibitors on non-target organisms. In: Michaud D, ed. *Recombinant protease inhibitors in plants*. Georgetown TX: Landes Bioscience, 89–106.

**Mandal S, Kundu P, Roy B, Mandal RK.** 2002. Precursor of the inactive 2S seed storage protein from the Indian mustard *Brassica juncea* is a novel trypsin inhibitor. Characterization, post-translational processing studies, and transgenic expression to develop insect-resistant plants. *Journal of Biological Chemistry* **277,** 37161–37168.

**Maqbool SB, Riazzudin S, Loc NT, Gatehouse AMR, Gatehouse JA, Christou P.** 2001. Expression of multiple insecticidal genes confers broad resistance against a range of different rice pests. *Molecular Breeding* **7,** 85–93.

**Marvier M, McCreedy C, Regetz J, Kareiva P.** 2007. A meta-analysis of effects of Bt cotton and maize on non-target invertebrates. *Science* **316,** 1475–1477.

**Mason RW, Sol-Church K, Abrahamson M.** 1998. Amino acid substitutions in the N-terminal segment of cystatin C create selective protein inhibitors of lysosomal cysteine proteinases. *Biochemical Journal* **330,** 833–838.

**Masoud SA, Johnson LB, White FF, Reeck GR.** 1993. Expression of a cysteine proteinase inhibitor (oryzacystatin-I) in transgenic tobacco plants. *Plant Molecular Biology* **21,** 655–663.

**Mazumdar-Leighton S, Broadway RM.** 2001*a*. Transcriptional induction of diverse midgut trypsins in larval *Agrotis ipsilon* and *Helicoverpa zea* feeding on the soybean trypsin inhibitor. *Insect Biochemistry and Molecular Biology* **31,** 645–657.

**Mazumdar-Leighton S, Broadway RM.** 2001*b*. Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. *Insect Biochemistry and Molecular Biology* **31,** 633–644.

**Melo FR, Mello MO, Franco OL, Rigden DJ, Mello LV, Genu AM, Silva-Filho MC, Gleddie S, Grossi-de-Sa MF.** 2003. Use of phage display to select novel cystatins specific for *Acanthoscelides obtectus* cysteine proteinases. *Biochimica et Biophysica Acta* **1651,** 146–152.

**Michaud D.** 2005. Impact environnemental des cultures transgéniques. II. L'impact des caractères recombinants. *Phytoprotection* **86,** 107–124.

**Michaud D.** 2000. *Recombinant protease inhibitors in plants*. Georgetown, TX: Landes Bioscience.

**Michaud D.** 1997. Avoiding protease-mediated resistance in herbivorous pests. *Trends in Biotechnology* **15,** 4–6.

**Michaud D, Bernier-Vadnais N, Overney S, Yelle S.** 1995*a*. Constitutive expression of digestive cysteine proteinase forms during development of the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae). *Insect Biochemistry and Molecular Biology* **25,** 1041–1048.

**Michaud D, Cantin L, Vrain TC.** 1995*b*. Carboxy-terminal truncation of oryzacystatin II by oryzacystatin-insensitive insect digestive proteinases. *Archives of Biochemistry and Biophysics* **322,** 469–474.

**Michaud D, Nguyen-Quoc B, Yelle S.** 1993. Selective inhibition of Colorado potato beetle cathepsin H by oryzacystatins I and II. *FEBS Letters* **331,** 173–176.

**Miki B, Abdeen A, Manabe Y, MacDonald P.** 2009. Selectable marker genes and unintended changes to the plant transcriptome. *Plant Biotechnology Journal* **7,** 211–218.

**Mulligan EA, Ferry N, Jouanin L, Walters KFA, Port GR, Gatehouse AMR.** 2006. Comparing the impact of conventional pesticide and use of a transgenic pest-resistant crop on the beneficial carabid beetle *Pterostichus melanarius*. *Pest Management Science* **62,** 999–1012.

**Munger A, Goulet C, Vaillancourt L-P, Schlüter U, Kiggundu A, Kunert K, Goulet M-C, Coenen K, Michaud D.** 2010. Constitutive expression of endogenous defense proteins in transgenic potato lines expressing the Cys protease inhibitor corn cystatin II. *Aspects of Applied Biology* **96** (in press).

**Naef A, Zesiger T, Défago G.** 2006. Impact of transgenic Bt maize residues on the mycotoxigenic plant pathogen *Fusarium graminearum* and the biocontrol agent *Trichoderma atroviride*. *Journal of Environmental Quality* **35,** 1001–1009.

**Naranjo SE.** 2009. Impacts of *Bt* crops on non-target invertebrates and insecticide use patterns. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **4,** 23.

**Ninkovic S, Miljus-Dukic J, Radovic S, Maksimovic V, Lazarevic J, Vinterhalter B, Neskovic M, Smigocki A.** 2007. *Phytodecta fornicata* Brüggemann resistance mediated by oryzacystatin II proteinase inhibitor transgene. *Plant Cell Tissue and Organ Culture* **91,** 289–294.

**O'Callaghan M, Glare TR, Burgess EPJ, Malone LA.** 2005. Effects of plants genetically modified for insect resistance on non-target organisms. *Annual Review of Entomology* **50,** 271–292.

**Ogawa M, Nakamura S, Scaman CH, Jing H, Kitts DD, Dou J, Nakai S.** 2002. Enhancement of proteinase inhibitory activity of recombinant human cystatin C using random-centroid optimization. *Biochimica et Biophysica Acta* **1599,** 115–124.

**Oppert B, Morgan TD, Hartzer K, Kramer KJ.** 2005. Compensatory proteolytic responses to dietary proteinase inhibitors in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology* **140,** 53–58.

**Outchkourov NS, de Kogel WJ, Wiegers GL, Abrahamson M, Jongsma MA.** 2004*a*. Engineered multidomain cysteine protease inhibitors yield resistance against western flower thrips (*Frankliniella occidentalis*) in greenhouse trials. *Plant Biotechnology Journal* **2,** 449–458.

**Outchkourov NS, de Kogel WJ, Schuurman-de Bruin A, Abrahamson M, Jongsma MA.** 2004*b*. Specific cysteine protease inhibitors act as deterrents of western flower thrips, *Frankliniella occidentalis* (Pergande), in transgenic potato. *Plant Biotechnology Journal* **2,** 439–448.

**Pavlova A, Björk I.** 2003. Grafting of features of cystatins C or B into the N-terminal region or second binding loop of cystatin A (stefin A) substantially enhances inhibition of cysteine proteinases. *Biochemistry* **42,** 11326–11333.

**Pigott CR, Ellar DJ.** 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiology and Molecular Biology Reviews* **71,** 255–281.

**Pimentel DS, Raven PH.** 2000. Bt corn pollen impacts on non-target Lepidoptera: assessment of effects in nature. *Proceedings of the National Academy of Sciences, USA* **97,** 8198–8199.

**Prabhakar S, Chen M-S, Elpidina EN, Vinokurov KS, Smith CS, Marshall J, Oppert B.** 2007. Sequence analysis and molecular characterization of larval midgut cDNA transcripts encoding peptidases from the yellow mealworm, *Tenebrio molitor* L. *Insect Molecular Biology* **16,** 455–468.

**Prihoda KR, Coats JR.** 2008. Fate of *Bacillus thuringiensis* (Bt) Cry3Bb1 protein in a soil microcosm. *Chemosphere* **73,** 1102–1107.

**Prins A, van Heerden PDR, Olmos E, Kunert KJ, Foyer CH.** 2008. Cysteine proteinases regulate chloroplast protein content and composition in tobacco leaves: a model for dynamic interactions with ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) vesicular bodies. *Journal of Experimental Botany* **59,** 1935–1950.

**Qasim MA, Ganz PJ, Saunders CW, Bateman KS, James MN, Laskowski Jr M.** 1997. Interscaffolding additivity. Association of P1 variants of eglin c and of turkey ovomucoid third domain with serine proteinases. *Biochemistry* **36,** 1598–1607.

**Qiu J.** 2008. Is China ready for GM rice? *Nature* **455,** 850–852.

**Quilis J, Meynard D, Vila L, Avilés FX, Guiderdoni E, San Segundo B.** 2007. A potato carboxypeptidase inhibitor gene provides pathogen resistance in transgenic rice. *Plant Biotechnology Journal* **5,** 537–553.

**Rivard D, Anguenot R, Brunelle F, Le VQ, Vézina L-P, Trépanier S, Michaud D.** 2006. An in-built proteinase inhibitor system for the protection of recombinant proteins recovered from transgenic plants. *Plant Biotechnology Journal* **4,** 359–368.

**Rivard D, Cloutier C, Michaud D.** 2004. Colorado potato beetles show differential digestive compensatory responses to host plants expressing distinct sets of defense proteins. *Archives of Insect Biochemistry and Physiology* **55,** 114–123.

**Romeis J, Bartsch D, Bigler F, *et al*.** 2008. Assessment of risk of insect-resistant transgenic crops to non-target arthropods. *Nature Biotechnology* **26,** 203–208.

**Romeis J, Meissle M, Bigler F.** 2006. Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nature Biotechnology* **24,** 63–71.

**Samac DA, Smigocki AC.** 2003. Expression of oryzacystatin I and II in alfalfa increases resistance to the root-lesion nematode. *Phytopathology* **93,** 799–804.

**Schaller A.** 2004. A cut above the rest: the regulatory function of plant proteases. *Planta* **220,** 183–197.

**Schuler TH, Denholm I, Jouanin L, Clark SJ, Clark AJ, Poppy GM.** 2001. Population-scale laboratory studies of the effect of transgenic plants on non-target insects. *Molecular Ecology* **10,** 1845–1853.

**Sears MK, Hellmich RL, Stanley-Horn DE, Oberhauser KS, Pleasants JM, Mattila HR, Sigfried BD, Dively GP.** 2001. Impact of Bt corn pollen on monarch butterfly populations: a risk assessment. *Proceedings of the National Academy of Sciences, USA* **98,** 11937–11942.

**Senthilkumar R, Cheng C-P, Yeh K-W.** 2010. Genetically pyramiding protease-inhibitor genes for dual broad-spectrum resistance against insect and phytopathogens in transgenic tobacco. *Plant Biotechnology Journal* **8,** 65–75.

**Shan L, Li C, Chan F, Zhao S, Xia G.** 2008. A Bowman–Birk protease inhibitor is involved in the tolerance to salt stress in wheat. *Plant, Cell and Environment* **31,** 1128–1137.

**Shelton AM, Sears MK.** 2001. The monarch butterfly controversy: scientific interpretations of a phenomenon. *The Plant Journal* **27,** 483–488.

**Shelton AM, Naranjo SE, Romeis J, *et al*.** 2009*a*. Appropriate analytical methods are necessary to assess non-target effects of insecticidal proteins in GM crops through meta-analysis (Response to Andow, et al., 2009). *Environmental Entomology* **38,** 1533–1538.

**Shelton AM, Naranjo SE, Romeis J, *et al*.** 2009*b*. Setting the record straight: a rebuttal to an erroneous analysis on transgenic insecticidal crops and natural enemies. *Transgenic Research* **18,** 317–322.

**Simoes RA, Silva-Filho MC, Moura DS, Delalibera Jr I.** 2008. Effects of soybean proteinase inhibitors on development of the soil mite *Scheloribates praeincisus* (Acari: Oribatida). *Experimental and Applied Acarology* **44,** 239–248.

**Sims SR.** 1995. *Bacillus thuringiensis* var. *kurstaki* [CrylA(c)] protein expressed in transgenic cotton: effects on beneficial and other non-target insects. *Southwestern Entomology* **20,** 493–500.

**Sims SR.** 1997. Host activity spectrum of the CrylIA *Bacillus thuringiensis* subsp. *kurstaki* protein: effect on Lepidoptera, Diptera, and non-target arthropods. *Southwestern Entomology* **22,** 395–404.

**Srinivasan T, Kumar KRR, Kirti PB.** 2009. Constitutive expression of a trypsin protease inhibitor confers multiple stress tolerance in transgenic tobacco. *Plant and Cell Physiology* **50,** 541–553.

**Stoop AA, Craik CS.** 2003. Engineering of a macromolecular scaffold to develop specific protease inhibitors. *Nature Biotechnology* **21,** 1063–1068.

**Streatfield SJ.** 2007. Approaches to achieve high-level heterologous protein production in plants. *Plant Biotechnolgy Journal* **5,** 2–15.

**Sullivan JA, Shirasu K, Deng XW.** 2003. The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nature Reviews Genetics* **4,** 948–958.

**Tang K, Tinjuangjun P, Xu Y, Sun X, Gatehouse JA, Ronald PC, Qi H, Lu X, Christou P, Kohli A.** 1999. Particle-bombardment-mediated co-transformation of elite Chinese rice cultivars with genes conferring resistance to bacterial blight and sap-sucking insect pests. *Planta* **208,** 552–563.

**Tomov BW, Bernal JS, Vinson SB.** 2003. Impacts of transgenic sugarcane expressing GNA lectin on parasitism of Mexican rice borer by *Parallorhogas pyralophagus* (Marsh) (Hymenoptera: Braconidae). *Environmental Entomology* **32,** 866–872.

**Urwin PE, McPherson MJ, Atkinson HJ.** 1998. Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs. *Planta* **204,** 472–479.

**Urwin PE, Atkinson HJ, Waller DA, McPherson MJ.** 1995. Engineered oryzacystatin-I expressed in transgenic hairy roots confers resistance to *Globodera pallida*. *The Plant Journal* **8,** 121–131.

**Vaillancourt L-P.** 2005. Effets pléiotropiques d'une forme recombinante de la cystatine II du maïs exprimée dans le cytosol des cellules de la pomme de terre. Québec QC, Canada: MSc thesis, Université Laval.

**van Baarlen P, Woltering EJ, Staats M, van Kan JAL.** 2007. Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three *Botrytis* species: an important role for cell death control. *Molecular Plant Pathology* **8,** 41–54.

**van den Hoorn RAL.** 2008. Plant proteases: from phenotypes to molecular mechanisms. *Annual Review of Plant Biology* **59,** 191–223.

**Van der Vyver C, Schneidereit J, Driscoll S, Turner J, Kunert K, Foyer CH.** 2003. Oryzacystatin I expression in transformed tobacco produces a conditional growth phenotype and enhances chilling tolerance. *Plant Biotechnology Journal* **1,** 101–112.

**van Frankenhuyzen K, Nystrom C.** 2002. The *Bacillus thuringiensis* toxin specificity database. Internet webpage: http://www.glfc.cfs.nrcan.gc.ca/bacillus (accessed Ferbruary 28, 2010).

**Vellicce GR, Diaz Ricci JC, Hernandez L, Castagnaro AP.** 2006. Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene *ch5B* in strawberry. *Transgenic Research* **15,** 57–68.

**Vila L, Quilis J, Meynard D, Breitler JC, Marfa V, Murillo I, Vassal JM, Messeguer J, Guiderdoni E, San Segundo B.** 2005. Expression of the maize proteinase inhibitor (*mpi*) gene in rice plants enhances resistance against the striped stem borer (*Chilo suppressalis*): effects on larval growth and insect gut proteinases. *Plant Biotechnology Journal* **3,** 187–202.

**Vinokurov KS, Elpidina EN, Zhuzhikov DP, Oppert B, Kodrik D, Sehnal F.** 2009. Digestive proteolysis organization in two closely related tenebrionid beetles: red flour beetle (*Tribolium castanuem*) and confused flour beetle (*Tribolium confusum*). *Archives of Insect Biochemistry and Physiology* **70,** 254–279.

**Vinokurov KS, Elpidina EN, Oppert B, Prabhakar S, Zhuzhikov DP, Dunaevsky YE, Belozersky MA.** 2006. Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionae) larvae. *Comparative Biochemistry and Physiology B* **145,** 126–137.

**Visal-Shah S, Brunelle F, Michaud D.** 2000. Multiple protease/inhibitor interactions in plant-pest systems. In: Michaud D, ed. *Recombinant protease inhibitors in plants*. Georgetown, TX: Landes Bioscience, 107–113.

**Vishnudasan D, Tripathi MN, Rao U, Khurana P.** 2005. Assessment of nematode resistance in wheat transgenic plants expressing potato proteinase inhibitor (*PIN2*) gene. *Transgenic Research* **14,** 665–675.

**Wolfenbarger LL, Naranjo SE, Lundgren JG, Bitzer RJ, Watrud LS.** 2008. Bt crops effects on functional guilds of non-target arthropods: a meta-analysis. *PLoS ONE* **3**, e2118.

**Wolt JD, Peterson RKD, Bystrak P, Meade T.** 2003. A screening level approach for non-target insect risk assessment: transgenic Bt corn pollen and the monarch butterfly (Lepidoptera: Danaidae). *Environmental Entomology* **32,** 237–246.

**Xu D, Xue Q, McElroy D, Mawal Y, Hilder VA, Wu R.** 1996. Constitutive expression of a cowpea trypsin inhibitor gene, *CpTi*, in transgenic rice plants confers resistance to two major rice insect pests. *Molecular Breeding* **2,** 167–173.

**Yang L, Fang Z, Dicke M, van Loon JJA, Jongsma MA.** 2009. The diamondback moth, *Plutella xylostella*, specifically inactivates Mustard Trypsin Inhibitor 2 (MTI2) to overcome host plant defence. *Insect Biochemistry and Molecular Biology* **39,** 55–61.

**Yuan L, Kurek I, English J, Keenan R.** 2005. Laboratory-directed protein evolution. *Microbiology and Molecular Biology Reviews* **69,** 373–392.

**Zhang X, Liu S, Takano T.** 2008. Two cysteine proteinase inhibitors from *Arabidopsis thaliana, AtCYSa* and *AtCYSb*, increasing the salt, drought, oxidation and cold tolerance. *Plant Molecular Biology* **68,** 131–143.

**Zhou J, Ma C, Xu H, Yuan K, Lu X, Zhu Z, Wu Y, Xu G.** 2009. Metabolic profiling of transgenic rice with *crylAc* and *sck* genes: an evaluation of unintended effects at metabolic level by using GC-FID and GC-MS. *Journal of Chromatogrphy B* **877,** 725–732.

**Zhu-Salzman K, Koiwa H, Salzman RA, Shade RE, Ahn J-E.** 2003*a*. Cowpea bruchid *Callosobruchus maculatus* uses a three-component strategy to overcome a plant defensive cysteine protease inhibitor. *Insect Molecular Biology* **12,** 135–145.

**Zhu-Salzman K, Ahn J-E, Salzman RA, Koiwa H, Shade H, Balfe S.** 2003*b*. Fusion of a soybean cysteine protease inhibitor and a legume lectin enhances anti-insect activity synergistically. *Agricultural and Forest Entomology* **5,** 317–323.

**Zwahlen C, Hilbeck A, Gugerli P, Nentwig W.** 2003. Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. *Molecular Ecology* **12,** 765–775.