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Specific cysteine protease inhibitors act as deterrents of western flower thrips, *Frankliniella occidentalis* (Pergande), in transgenic potato

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Summary

In this study, the effects of the accumulation of cysteine protease inhibitors on the food preferences of adult female western flower thrips, *Frankliniella occidentalis* (Pergande), were investigated. Representative members of the cystatin and thyropin gene families (stefin A, cystatin C, kininogen domain 3 and equistatin) were expressed in potato (*Solanum tuberosum*) cv. Impala, Kondor and Line V plants. In choice assays, a strong time- and concentration-dependent deterrence from plants expressing stefin A and equistatin was observed. Cystatin C and kininogen domain 3 were not found to be active. All tested inhibitors were equally or more active than stefin A at inhibiting the proteolytic activity of thrips, but, in contrast with stefin A, they were all expressed in potato as partially degraded proteins. The resistance of cysteine protease inhibitors against degradation *in planta* by endogenous plant proteases may therefore be relevant in explaining the observed differences in the deterrence of thrips. The results demonstrate that, when given a choice, western flower thrips will select plants with low levels of certain cysteine protease inhibitors. The novel implications of the defensive role of plant cysteine protease inhibitors as both deterrents and antimetabolic proteins are discussed.

Introduction

Western flower thrips (WFTs), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), are highly polyphagous insects (Jensen, 2000). They cause considerable economic losses on a large number of field-grown vegetable, fruit, ornamental and plantation crops (Lewis, 1997). In addition, WFTs are the most prevalent pest in greenhouses throughout the world (Parrella, 1995; Shipp *et al*., 1991). The small size of WFTs (1–2 mm), their sexual habit (arrhenotoky) and short generation time, combined with their polyphagous nature (Loomans *et al*., 1995), resistance to many pesticides (Jensen, 2000) and feeding habit in the inner whorls of flowers and buds, make this insect extremely difficult to control.

It has previously been established that the proteolytic activity of extracts of adult WFTs has an optimum at pH 3.5, and is nearly completely inhibited by protease inhibitors (PIs) specific for cysteine proteinases. The hypothesis that cysteine proteases are predominant in WFT digestive tracts was subsequently supported by the gradual reduction in WFT oviposition rate when purified potato cystatin and equistatin were fed to adult females in combination with a protein-rich pollen diet (Annadana *et al*., 2002). This demonstrated the potential protective role of cysteine PIs in plants, and the heterologous expression of cysteine PIs was proposed as a novel means to control WFTs.

PIs are proteins that form stoichiometric high-affinity complexes with proteases and inhibit their hydrolytic activity. Ryan (1978) first proposed that, in plants, PIs play an important biological role by inhibiting the digestive proteases of potential insect herbivores. Some insect herbivores adapt to PIs of host plants by the induction of proteases that are

insensitive to inhibition or by inactivation of the inhibitor by proteolysis (see Jongsma and Bolter, 1997 for a review). The effects of PIs on insect development, survival and fecundity have been reported in numerous studies. A limited number of studies have reported the effects of PIs on insect behaviour, but these studies used only artificial diets (Girard *et al*., 1998; Pham *et al*., 2000; Rahbe *et al*., 2003). Some studies have shown that jasmonate-induced responses in plants, which include the accumulation of PIs, are associated with changes in behavioural food preferences. However, these changes in food preference have so far not been associated with specific PIs (Thaler *et al*., 2001; van Dam *et al*., 2000).

The aim of this research was to investigate the effect of heterologous cysteine PIs, expressed in susceptible model plants, on the food preferences of thrips. For this purpose, we transformed thrip-susceptible potato varieties with five different cysteine PIs that are representative of cystatins and thyropins (kininogen domain 3, stefin A, cystatin C, potato cystatin, equistatin). We chose to localize all different cysteine PIs to the endoplasmic reticulum (ER), because tomato plants that expressed high levels of potato cystatin in the cytosol under the control of the *rbcS1* promoter showed an undesirable early senescent phenotype (N. S. Outchkourov *et al*., unpublished data, 2003). In contrast, the abundant accumulation of equistatin in the ER of potato leaf cells did not result in a deviating phenotype (Outchkourov *et al*., 2003a,b). Here, the food preferences of WFTs were assessed in choice assays. The results demonstrate that two of the five cysteine PIs tested are highly deterrent to thrips when expressed in transgenic potato plants.

Results

Inhibition of the total proteolytic activity of thrips

The inhibitory properties of the purified inhibitors, kininogen domain 3 (K), stefin A (A), cystatin C (C), potato cystatin (P) and equistatin (EI), were compared with those of the synthetic cysteine PI E-64. For this comparison, we used *in vitro* assays with total WFT homogenate as an enzyme source and fluorescein isothiocyanate (FITC)-labelled albumin as a substrate. Two concentrations of each inhibitor were tested (10 and 100 nM). At the high concentration (100 nM), most inhibitors efficiently inhibited 90–95% of proteolytic activity in total extracts of WFTs (Figure 1). Potato cystatin inhibited only 60% of the activity at this concentration, and therefore was classified as a weak inhibitor. In contrast, equistatin was a strong inhibitor, as > 90% of the total proteolytic activity of thrips was inhibited at the lower 10 nM concentration. The other three inhibitors, kininogen domain 3, stefin A and cystatin C, were intermediate, with around 40% inhibition at 10 nM inhibitor concentration. E-64 inhibited up to 90% of the total proteolytic activity, but was less efficient than equistatin. The strong activity of equistatin compared with the other inhibitors may be partly due to the fact that this inhibitor is active against both cysteine and aspartic proteases. However, based on the maximum inhibition values obtained for the cysteine PIs, the contribution of aspartic protease activity is no more than 10%.

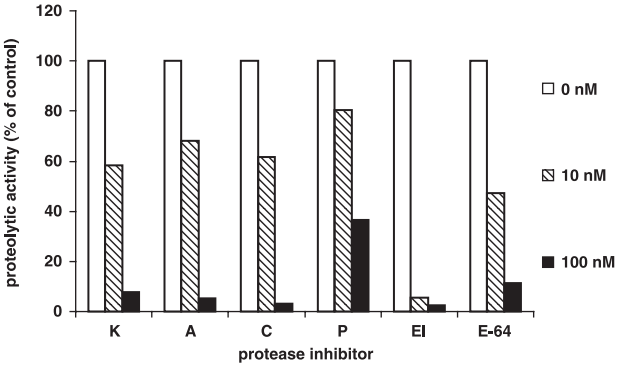

Figure 1 Effects of protease inhibitors on the hydrolysis of fluorescein isothiocyanate (FITC)-labelled albumin by proteases present in whole insect homogenates of *Frankliniella occidentalis* adults. The concentrations of the tested inhibitors are shown. K, kininogen domain 3; A, stefin A; C, cystatin C; P, potato cystatin; EI, equistatin; E-64, synthetic inhibitor of cysteine proteases (Scott and Whitton, 1988).

Expression of the cysteine protease inhibitors in transgenic potato plants and WFT choice assays on detached leaves

The cysteine PIs, kininogen domain 3 (K), stefin A (A), cystatin C (C) and potato cystatin (P), were expressed in potato cv. Kondor and Impala. Previously obtained transgenic plants of potato cv. Line V, expressing a modified version of the equistatin gene (*eim*) optimized for expression of the protein (EI), were also included in the bioassays (Outchkourov *et al*., 2003a). All inhibitors were expressed under the control of the rubisco promoter/terminator *rbcS1* from chrysanthemum (Outchkourov *et al*., 2003a), and targeted to the ER. This was achieved by fusing the cDNA fragments encoding the mature proteins at the N-terminus to the equistatin signal sequence, and at the C-terminus to the KDEL signal. The amounts of the expressed proteins were measured by dot blot immunological detection. It was not possible to quantify the amount of potato cystatin (P) by dot blot analysis, because of the presence of endogenous potato multicystatin in potato leaves that interfered with the analysis (see Figure 2; Western blot).

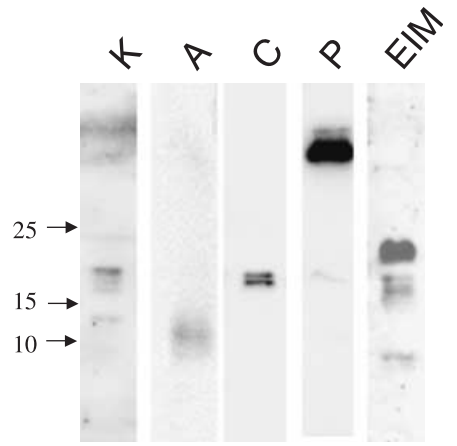

Figure 2 Combined results of different Western blot experiments on the transgenic potato plants that express: K, kininogen domain 3; A, stefin A; C, cystatin C; P, potato cystatin; EIM, equistatin in potato cv. Impala. Identical degradation patterns were obtained in potato cv. Kondor (not shown). EIM was expressed in potato cv. Line V. Arrows indicate the approximate positions of the molecular weight standards in kilodaltons. The additional lower bands are degradation products.

The expression levels were strongly dependent on the proteins involved, as shown in Table 1. The high expression levels of equistatin in the potato Line V could not be equalled in the Impala and Kondor cultivars by the other inhibitors kininogen domain 3, stefin A, cystatin C and potato cystatin.

The stability of the expressed proteins was assessed by Western blot analysis (Figure 2). Specific antibodies were used for each of the proteins. For potato cystatin, varying (sample-dependent) amounts of endogenous multicystatin inhibitor of 80 kDa were detected and were distinguishable from the transgenic inhibitor of 10 kDa. For kininogen domain 3 and cystatin C, a degradation pattern was observed which was identical in both potato cultivars Impala and Kondor. In contrast, stefin A was found as a single band in both Impala and Kondor. The expression of stefin A, however, was weaker compared with the other inhibitor proteins. Multiple degradation bands appeared for kininogen domain 3 and equistatin, indicating multiple cleavage sites in the polypeptide chains of these inhibitors. Cystatin C appeared as a double band and was probably cleaved *in planta* most efficiently at only one position.

The preferences of WFTs, when given a choice between plants expressing cysteine PIs at low and high levels, were monitored *in vitro* using detached leaves. During the time course of 24 h, the ingestion of high concentrations of stefin A (Figure 3) or equistatin (Figure 4) led to increasing deterrence of WFTs. The preference for the lower expresser tended to be most significant at the end of the 24–26 h experimental period. The effects on WFT behaviour correlated very well with the expression level differences. When 'high' expression level differences of stefin A were tested (Figure 3E: 0.34% TSP is the difference between inhibitor expressed at 0.50% and 0.16% of total soluble protein (TSP)), females responded more rapidly and significantly more strongly to the accumulated inhibitor for all the time points. Choice assays with smaller expression differences of 0.16% to 0.21% TSP stefin A (Figure 3B–D) resulted in significant thrip deterrence only after a prolonged period of feeding. No significant thrip food preferences were measured when the expression level differences of stefin A were 0.11% TSP or less (Figure 5).

Similar correlations, but at much higher expression level differences, were observed for equistatin (Figure 4). Equistatin appeared to require approximately 20-fold higher levels of accumulated inhibitor (7.3% TSP expression level difference)

Table 1 Quantitative expression levels achieved with the *rbcS1* promoter constructs from chrysanthemum in different potato cultivars

		Expression (% of total soluble protein)	
Gene	Potato cultivar	Mean	Maximum
Kininogen domain 3	Impala	0.81 (n = 9)*	1.7
	Kondor	0.31 (n = 8)*	0.63
Stefin A	Impala	0.18 (n = 9)*	0.32
	Kondor	0.28 (n = 5)*	0.50
Cystatin C	Impala	0.40 (n = 8)*	1.5
	Kondor	0.91 (n = 9)*	2.7
Potato cystatin	Impala	n.d.	n.d.
	Kondor	n.d.	n.d.
Equistatin	Line V	2.3 (n = 22†)*	7.5

n.d., not determined due to background signal.

*Plant numbers analysed given in parentheses.

†Transgenic plants obtained from the study of Outchkourov *et al*. (2003a). The levels of expression were quantified again in this study and differed slightly from those published in Outchkourov *et al*. (2003a).

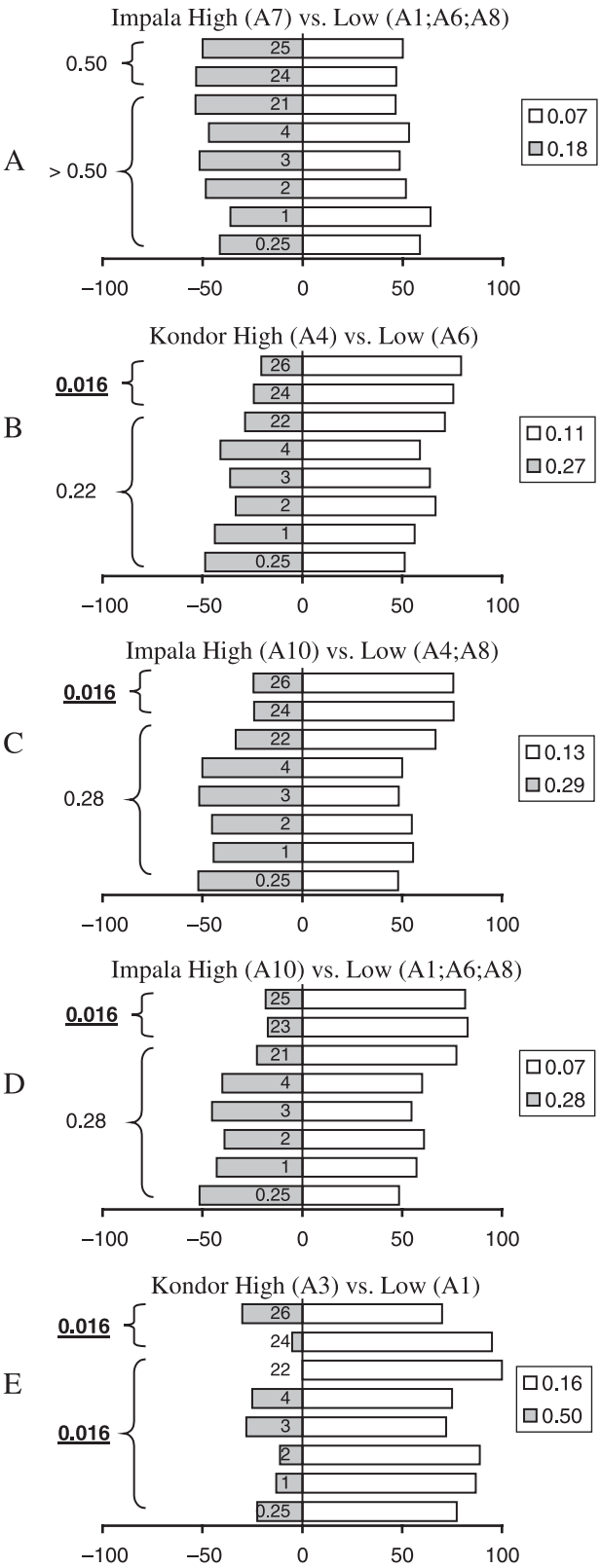

Figure 3 Results of five different thrip dual choice assays (A–E) on transgenic lines expressing stefin A. The plant lines and potato varieties are indicated at the top of each graph. On the *x*-axis, the percentage of thrips on the low (white) and high (grey) expressing plants are plotted against time (h) on the *y*-axis. On the right, the levels of expression measured by dot blot analysis as a percentage of total soluble protein are shown. The calculated *P* values of the one-sided Wilcoxon signed rank test for the early (0–6 h after the start of the experiment) and late (21–26 h after the start of the experiment) periods are given on the left of each panel. In bold and underlined are the statistically significant values: $P < 0.05$.

compared with stefin A (0.34% TSP difference) to induce a highly significant effect on WFTs. The graph in Figure 5A shows that, for equistatin, the threshold level of detection was about 1% TSP difference. Below this threshold, WFT females did not show a preference for the discs with low expression.

The accumulated proteins kininogen domain 3 and cystatin C in potato did not show any effects in WFT choice assays in the tested concentration range, with expression level differences of up to 1.6% TSP for cystatin C and 1.4% TSP for kininogen domain 3 (Figure 5B).

Thus, in transgenic potato plants, stefin A is the most deterrent inhibitor for female thrips, followed by equistatin, whereas kininogen domain 3 and cystatin C do not deter WFTs.

Discussion

WFTs are a serious polyphagous pest both in the field and in greenhouses on many different crops, and have developed resistance to many synthetic insecticides (Jensen, 2000). The identification of compounds or proteins that, in addition to being toxic/antimetabolic, will change the behaviour of an adult insect by causing it to leave the plant in search for better substrates is of great applied and scientific interest. Such deterrent or repellent effects potentially reduce the likelihood of pests developing resistance. Methyl jasmonate (MJ)-induced defence responses in plants are known to provoke changes in food preferences of insect herbivores (Thaler *et al*., 2001; van Dam *et al*., 2000). However, the complexity of the induced responses, such as digestive inhibitors (e.g. PIs), enzymes (e.g. polyphenol oxidases) and a range of secondary metabolites (e.g. nicotine), makes the precise assignment of the plant compounds involved in provoking the behavioural responses problematic.

In this study, we have demonstrated that transgenic plants over-expressing cysteine PIs are strongly deterrent to WFTs, and can rapidly affect behaviour less than an hour after feeding. Very strong deterrent effects were found for stefin A, followed by equistatin at 10–20-fold higher expression levels. Kininogen domain 3 and cystatin C did not yield any deterrent effect. This difference from the other two inhibitors

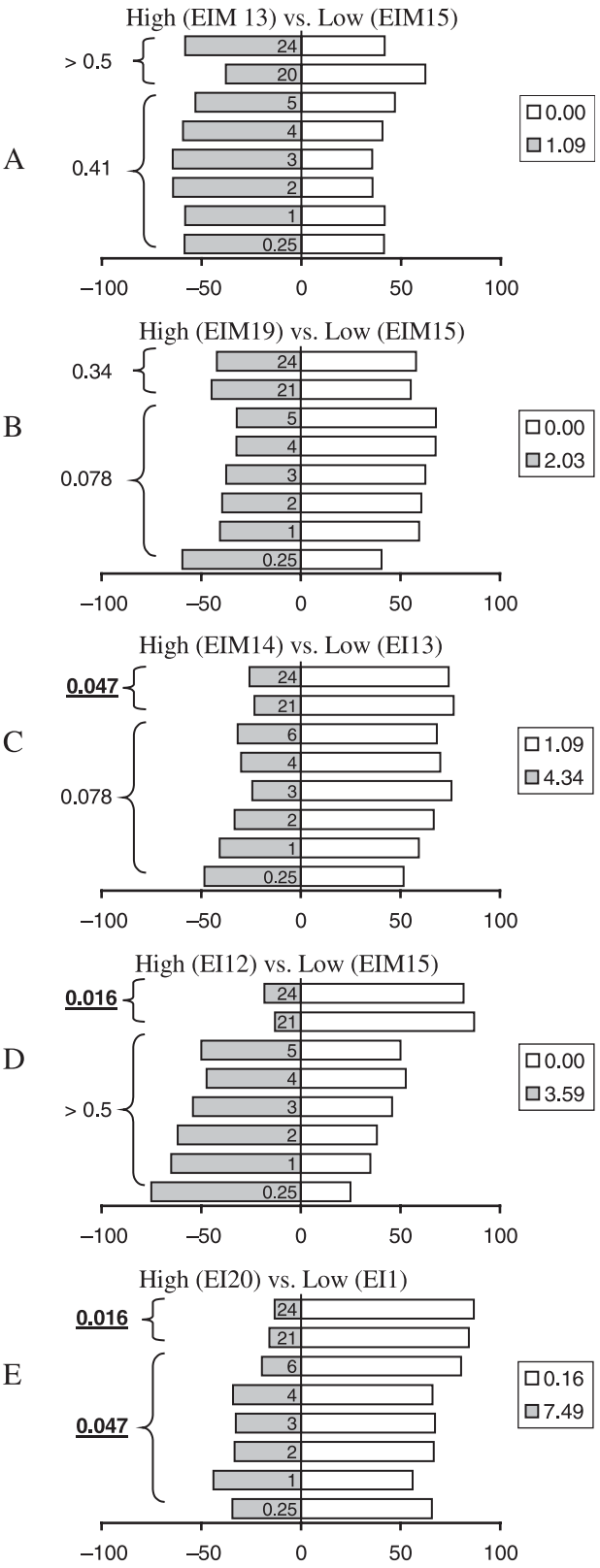

Figure 4 Results of five different thrip dual choice assays (A–E) on transgenic lines expressing the *EI* (AF184891) and *EIM* (AY166597) genes, both of which encode an identical equistatin protein. For details, refer to the legend of Figure 3.

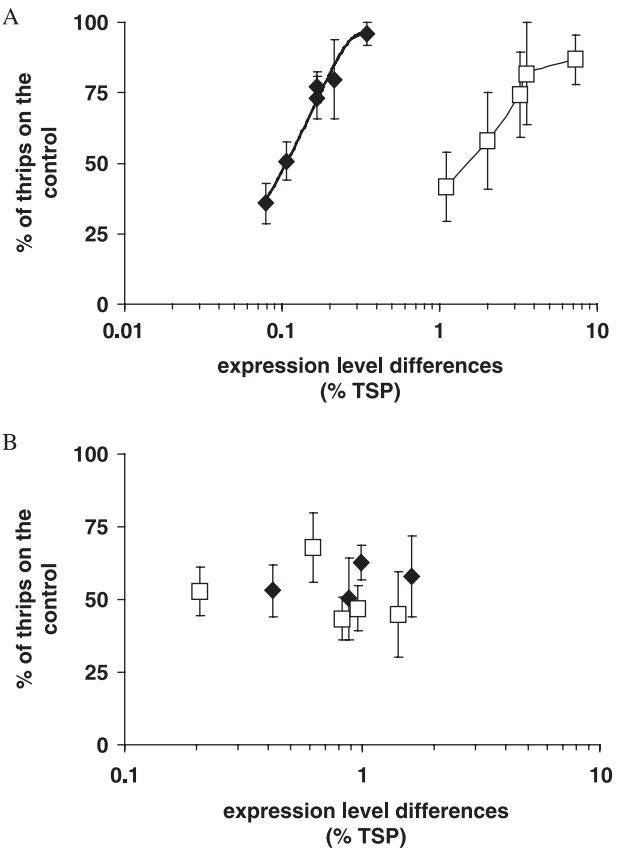

Figure 5 Summary of the results of all conducted thrip choice assays. Choice data obtained after 24 h were used. They were correlated with the expression level differences between tested plants (% total soluble protein, TSP). (A) Stefin A (◆); equistatin (□). (B) Cystatin C (◆); kininogen domain 3 (□).

did not correlate with the *in vitro* inhibition of WFT proteases. *In vitro* equistatin was the most potent PI (> 90% inhibition at 10 nM), followed by kininogen domain 3, stefin A, cystatin C (> 90% at 100 nM) and potato cystatin (~60% at 100 nM).

A straightforward explanation for the lack of correlation between the potency of the inhibitor and the degree of deterrence is not possible with the present data. Due to the small size of the insects, the *in vitro* studies were carried out with extracts of complete adult insects and not with dissected guts. Insect haemolymph is known to contain inhibitors of gut proteases, and the surplus proteolytic activity in the extract may therefore not correctly represent the actual proteolytic activity in the gut. The ranking of the effectiveness of the inhibitors on the basis of the *in vitro* experiments may therefore be incorrect. In addition, it is not known whether WFTs will adapt with the induction of a novel complement of insensitive proteases in response to these inhibitors. It may be that kininogen domain 3 and cystatin C are not effective at

inhibiting these enzymes. Finally, it may be that the high level of expression required for the deterrent effects of equistatin is related to the degradation of the protein *in planta* or *in insecta*. When the transgenic plants were analysed by Western blot, three of the expressed inhibitors, except stefin A and potato cystatin, showed distinct smaller bands in addition to the expected band. For cystatin C and kininogen domain 3, this was most likely the result of *in vivo* degradation, as previously shown for equistatin (Outchkourov *et al*., 2003b). Previously, it has been shown that the degradation of PIs in insect guts could potentially affect the efficacy of PIs (Jongsma and Bolter, 1997; Michaud *et al*., 1995, 1996). When the integrity of the expressed inhibitor is already affected *in planta* by degradation, higher expression levels may be necessary to obtain a deterrent effect, as in the case of equistatin, or the anti-WFT effects may even be lost, as possibly the case in cystatin C and kininogen domain 3. Alternatively, it may be that the folding and biological activity of some of the inhibitors is impaired *in planta.* In the given case, our immunological quantification technique could only determine the amount of accumulated protein, but not the biological activity. Our previous research has demonstrated that, probably due to degradation, there is at least a threefold difference between the amounts of immunologically detectable and functionally active equistatin protein in transgenic potato (Outchkourov *et al*., 2003b). A quantitative comparison based on the inhibitory activity in feeding studies with purified proteins will help to resolve whether *in planta* or *in insecta* degradation or misfolding of the inhibitors plays a role in determining the deterrence of thrips, and whether the insects induce inhibitor-insensitive enzymes.

A possible approach to enhance the level of resistance of the transgenic plants is to obtain higher levels of accumulated stefin A inhibitor. The coding sequence of stefin A contains multiple AT-rich motifs that are known to have a negative influence on gene expression in plants (Horvath *et al*., 2000; Strizhov *et al*., 1996). Alternatively, strategies to improve the resistance of equistatin, cystatin C and kininogen 3 domain to degradation by substituting susceptible amino acids in the protein sequence could be attempted. We reported earlier the precise susceptible sites in equistatin to guide such a study (Outchkourov *et al*., 2003b).

The effects of cysteine PIs on WFT food preferences may have ecological implications. The active search of WFT adults for an optimal food substrate devoid of high levels of cysteine PIs makes sense, considering that earlier experiments indicated a high fitness cost (Annadana *et al*., 2002). Exposure to potato cystatin and equistatin reduced the fecundity of thrips in a few days by at least 50%. The availability of *Nicotiana alata* populations, which differ in their ability to express PIs, may enable studies to be made that quantify the effects of insect deterrence on seed yield (Glawe *et al*., 2003).

The mechanism which senses PIs, and affects the behaviour of WFTs, will most likely utilize a completely different signalling pathway compared with the well-known olfactory and gustatory signalling pathways. Effects of PIs on behaviour have also been observed with other inhibitor classes and other organisms. In the pea aphid, *Acyrthosiphon pisum*, host plant-derived chymotrypsin inhibitors were found to induce a behaviour of restlessness (Rahbe *et al*., 2003). In an experiment with human subjects, who were given a soup containing a high dose of trypsin inhibitors prior to their lunch, the calorie intake during the rest of the lunch was strongly reduced (Hill *et al*., 1990). In mammals, the inhibitors are known to induce the release of cholecystokinin, which stimulates pancreatic secretions and provokes a feeling of satiety (see Liddle, 1995 for a review). In insects, other hormones have been found to modulate protease secretion (Borovsky *et al*., 1990; Tortiglione *et al*., 2002). We expect similar hormones to determine the behaviour of WFTs on inhibitor-containing substrates.

Transgenic crop protection strategies favour a combination of indirect deterrent and direct toxic effects on both adults and larvae. In that way, the probability of resistance development in insect populations is reduced compared with a situation in which, for example, the adult moth is unable to identify a Bt toxin-expressing plant as a poor substrate for its offspring. Non-choice greenhouse experiments on transgenic potato plants expressing stefin A and a multidomain combination of different cysteine PIs have been carried out separately (Outchkourov *et al*. 2004). They confirm the results presented here, and by Annadana *et al*. (2002) with purified proteins, that cysteine PIs will strongly affect population development by a combination of deterrence and a reduction of WFT fecundity. Thus, cysteine PI genes appear to be interesting novel candidates to control WFTs in field and greenhouse crops, such as cotton and ornamentals, suffering from this pest.

Experimental procedures

Oligonucleotides

EIM-SP1: 5′-AAAAAA*ACATGT*CTCTTAGCCAGAACCAGG.
EIM-SP2: 5′-GTTAGACTTGGATCCATGGGACTAGCTTCA-GTTGAAGTGATAG.
EIM-MP1: 5′-TCCCATGGATCCAAGTCTAACGAAATGCC-AACAG.

EIM-MP2: 5′-AGATCTGAGCTCTTAAAGTTCGTCCTTTTGATCACCTCCACCTCCGCATGTGGGACGTTTGAATC.
PC1: 5′-CCCCCCCCATGGATCCAGCAATCGTAGGAGGCCTTG.
PC2: 5′-GGCCCCGGAGCTCCTATTGATCACCTCCACCTCCTGTACTATCATCACCAAC.
709: 5′-TAAATGGCGCCCATGGATCCCATGATACCTGGAGGCTTATCT.
710: 5′-TAAATGAGCTCCTATTGATCACCTCCACCTCCAAAGCCCGTCAGCTCGTCA.
711: 5′-TAAATGGCGCCCATGGATCCCTCTTCTCCGGGTAAACCGC.
712: 5′-TAAATGAGCTCCTATTGATCACCTCCACCTCCGGCGTCCTGACAGGTGGA.
713: 5′-TAAATGGCGCCCATGGATCCCGGGAAGGATTTTGTACAACCA.
714: 5′-TAAATGAGCTCCTATTGATCACCTCCACCTCCTGAGATCATTCCCAGTGGTTG.

Plant material and insects

Potato (*Solanum tuberosum*) cultivars Impala and Kondor were used for plant transformation with kininogen domain 3, stefin A, cystatin C and potato cystatin-containing constructs. In addition, potato (*Solanum tuberosum*) cv. Line V, previously transformed with the equistatin genes *EI* (AF184891) and *EIM* (AY166597), both encoding an identical equistatin protein (Outchkourov *et al*., 2003a), was used in the WFT choice bioassays. A population of WFTs, *Frankliniella occidentalis*, was reared on flowering chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Sunny Casa in a greenhouse at 25 °C.

Inhibition of the proteolytic activity of thrips

Recombinant cystatin C and kininogen domain 3 were expressed in *Escherichia coli* and purified as described earlier (Abrahamson *et al*., 1988; Auerswald *et al*., 1993). The production of stefin A is described in detail elsewhere (Romero *et al*., in preparation). In brief, a cDNA segment encoding human stefin A was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR) from human liver RNA in expression vector pHD389, following the same procedures as used for cystatin D expression (Freije *et al*., 1993). Equistatin was produced and purified as described by Outchkourov *et al*. (2002), and potato cystatin as described by Annadana *et al*. (2003). The concentration of the inhibitors was quantified by titration against papain using Z-Phe-Arg-pNA (Bachem, Switzerland) as a substrate, as described by Rogelj *et al*. (2000). The concentration of papain was determined by titration with E-64 (Sigma). The inhibition of the WFT proteolytic activity in extracts of adult insects was conducted as described by Annadana *et al*. (2002) using FITC-labelled albumin (Sigma).

Preparation of constructs for plant transformation

The pUCRBC-EIM construct (Outchkourov *et al*., 2003a) was modified to allow subcloning of the four other PI genes into an expression cassette with an identical N-terminal signal peptide derived from equistatin and the same C-terminal KDEL sequence for retention in the ER. For this purpose, cloning sites were created behind the signal sequence of equistatin and before the KDEL signal (see Figure 6 for a schematic overview). Briefly, PCR was performed using pUCRBC-EIM as template and primer pairs EIM-SP1/2 and EIM-MP1/2 to amplify the DNA sequence encoding the equistatin signal peptide (*SP*) and equistatin mature protein (*MP*) separately. The primers were designed to create or remove restriction sites in the coding part of equistatin. The obtained fragments were digested with *Afl*III-*Bam*HI for *SP*, and *Bam*HI-*Sac*I for *MP*, and cloned with a three-point ligation into pUCRBC (Outchkourov *et al*., 2003a), that was digested with *Nco*I-*Sac*I. The ligation of the *Afl*III sticky ends into the *Nco*I site of pUCRBC destroyed the existing *Nco*I and *Afl*III sites. This, together with the new restriction sites from the PCR, resulted in plasmid pUCRBC-EIM-SP (Figure 1). The coding part of kininogen domain 3 was amplified from pDR1311 (Auerswald *et al*., 1993) using primer pair 713/714. Stefin A was amplified from pHD389/CysAmod (Romero *et al*., in preparation) using primer pair 709/710. Cystatin C was amplified from pCysCmut283-Nco (Mason *et al*., 1998) using primer pair 711/712. Potato cystatin was amplified from pUCRBC-PC (Outchkourov *et al*., 2002) using primers PC1 and PC2. The obtained fragments (kininogen domain 3, stefin A, cystatin C and potato cystatin) were digested with *Nco*I-*Bcl*I and cloned into the pUCRBC-EIM-SP vector, after removing the coding sequence of equistatin mature protein using the same restriction sites, to create pUCRBC-K, pUCRBC-A, pUCRBC-C and pUCRBC-P, respectively. The integrity of the expression constructs was checked by nucleotide sequencing on an Applied Biosystems 370A sequencing machine. These four new expression cassettes containing the *rbcS1* promoter fused to four cysteine PIs were excised from the pUC-based vectors by digestion with *Asc*I-*Eco*RI and inserted into the multiple cloning site of the pBINPLUS vector (van Engelen *et al*., 1995) to create pRBC-K, pRBC-A, pRBC-C and pRBC-P.

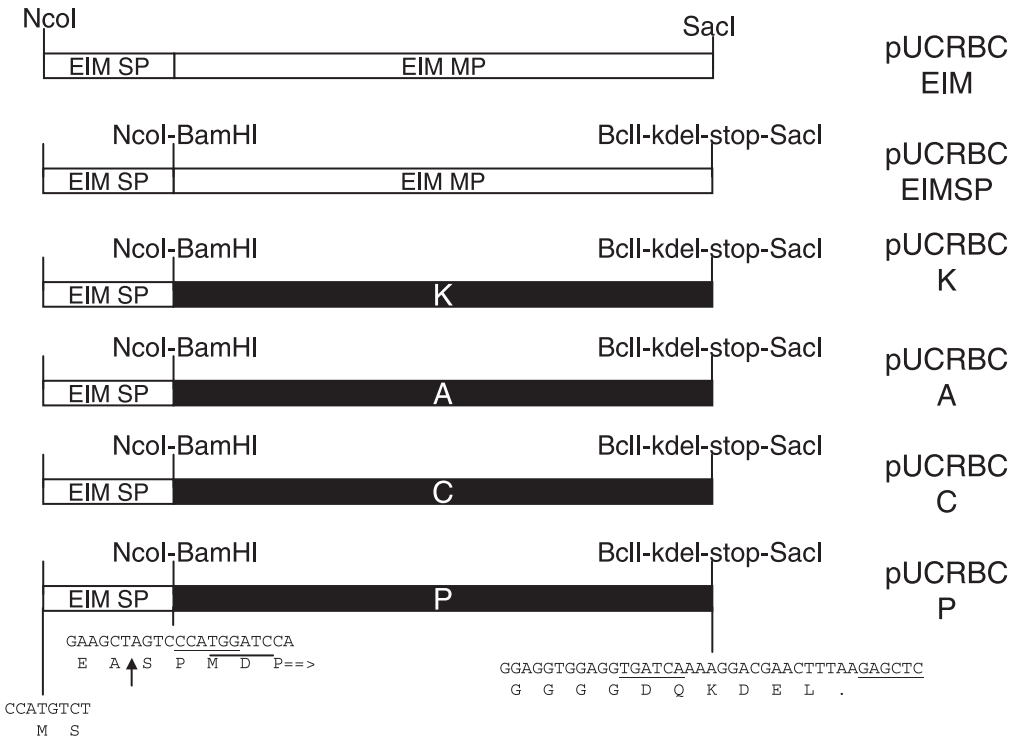

Figure 6 Schematic representation of the constructs used in this study. EIM SP, equistatin signal peptide; EIM MP, equistatin mature protein; K, kininogen domain 3; A, stefin A; C, cystatin C; P, potato cystatin. Representative DNA sequences for all of the constructs at the joining point, together with a protein translation, are shown at the bottom of the figure; restriction sites are underlined.

Transformation and regeneration

All pBINPLUS constructs were electroporated into electrocompetent *Agrobacterium tumefaciens* AGL0 (Lazo *et al*., 1991). Potato plants (*Solanum tuberosum cv.* Impala and Kondor) were transformed according to Hoekema *et al*. (1989) using tuber discs as a source of explants. Independent initial transformants (T1) were maintained as *in vitro* cuttings until needed. Four to six weeks prior to each insect bioassay experiment, plants were transferred to a greenhouse with supplementary high-pressure sodium light under a 16 h/8 h light/dark rhythm and a temperature regime of 21/18 °C.

Quantification and characterization of the expression of cysteine protease inhibitors in transgenic potato plants

Dot blot and Western blot immunological detection procedures were carried out as described in Outchkourov *et al*. (2003a) using rabbit: anti-equistatin, anti-potato cystatin (Eurogentec, Seraing, Belgium), anti-kininogen, anti-stefin A and anti-cystatin C (Abrahamson *et al*., 1986) as primary antisera, and anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Jackson Immuno Research, USA) as secondary antiserum.

Thrip choice assays

Four- to five-week-old primary transformants T1 of transgenic potato plants were used for the bioassays. Prior to each experiment, the expression levels of the transgenic plants were quantified by immunological dot blot detection. Adult female thrips of mixed ages were collected with an aspirator from a rearing greenhouse with chrysanthemum flowers as the host plant. Females are easily distinguished from males by their larger size. The age differences between females are potentially several weeks, but this effect was expected to be evened out with the replication of 6×10 insects. They were starved overnight, only provided with water, in Perspex ring cages (Murai, 1990) before experiments. The WFT females were briefly anaesthetised with CO_2 before transfer to Petri dishes using a fine brush. Dual choice assays were used to test the preference of thrips for leaf discs from potato plants with low vs. high PI expression. For this purpose, two leaf discs of 21 mm in diameter, punched from mature leaves at a similar position, were placed with the abaxial side up on a thin layer of 1% water agar in a Petri dish. In between the leaf discs was a small piece of filter paper (5 mm × 5 mm) on which 10 starved female thrips were released at 10.00 h. The Petri dishes were closed and sealed with parafilm to prevent

the thrips from escaping, and placed in a climate chamber (T = 22 °C; 16 h/8 h light/dark cycle; relative humidity, 70%). Petri dishes (six per treatment) were randomized to eliminate the effect of light or temperature gradients that could affect choice. The number of thrips on each leaf disc was recorded at different time points after the start of the experiment. If the leaf discs were exposed to 10 thrips for periods longer than 30 h, the level of damage to the susceptible leaf discs would start to influence the results. For this reason, the choice assays were limited to 1 day.

As the repeated measurements were taken from the same Petri dishes, the data were not independent. To circumvent this dependence, data from the multiple time point measurements were averaged into an early period (0–6 h after the start of the experiment) and late period (ranging from 21 to 26 h after the start of the experiment). For each of the two periods, the null hypothesis H_o (no preference of WFTs for leaf discs) was tested against the alternative hypothesis H_a (preference for leaf with low PI level) using a one-sided Wilcoxon signed rank test on the absolute differences of average numbers of WFTs on leaf discs for the low and high expressing groups (Hollander and Wolfe, 1973).

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