Coexpression of potato type I and II proteinase inhibitors gives cotton plants protection against insect damage in the field

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Potato type I and II serine protease inhibitors are produced by solanaceous plants as a defense mechanism against insects and microbes. *Nicotiana alata* proteinase inhibitor (NaPI) is a multidomain potato type II inhibitor (pin II) that is produced at high levels in the female reproductive tissues of the ornamental tobacco, *Nicotiana alata*. The individual inhibitory domains of NaPI target the major classes of digestive enzymes, trypsin and chymotrypsin, in the gut of lepidopteran larval pests. Although consumption of NaPI dramatically reduced the growth and development of a major insect pest, *Helicoverpa punctigera*, we discovered that surviving larvae had high levels of chymotrypsin activity resistant to inhibition by NaPI. We found a potato type I inhibitor, *Solanum tuberosum* potato type I inhibitor (StPin1A), was a strong inhibitor of the NaPI-resistant chymotrypsin activity. The combined inhibitory effect of NaPI and StPin1A on *H. armigera* larval growth in the laboratory was reflected in the increased yield of cotton bolls in field trials of transgenic plants expressing both inhibitors. Better crop protection thus is achieved using combinations of inhibitors in which one class of proteinase inhibitor is used to match the genetic capacity of an insect to adapt to a second class of proteinase inhibitor.

chymotrypsin | resistance | Lepidoptera

Lepidopteran insects are one of the most important groups of crop pests in the world. In Australia, two of the major lepidopteran pests of cotton are *Helicoverpa punctigera* and *H. armigera* (1). *H. armigera* is the dominant pest and has developed resistance to a number of chemical pesticides (2). The only commercially available transgenes for control of these insect pests encode *Bacillus thuringiensis* (Bt) toxins and the Vip3Aa20 toxin (3). First-generation Bt crops expressing a single Bt toxin, Cry1AC, were highly successful. However, field-evolved resistance to Cry1Ac has been reported recently for populations of *H. zea* (4). Second-generation Bt crops containing two different Bt toxins are considered to be more robust, because the toxins bind to different targets in the larval midgut. However, cross-resistance has been demonstrated in the laboratory where feeding Cry2Ab to *Pectinophora gossypiella* (pink bollworm) caused a 420-fold increase in resistance to Cry1Ac (5). Stacking of insect resistance genes probably will be the industry standard for transgenic crops, and therefore, the discovery and development of insecticidal molecules with different modes of action is critical for long-term control of insect pests. Proteinase inhibitors (PIs) are a potential component of gene stacks for the protection of important agricultural crops against insect damage.

Plants have developed both physical and molecular strategies to limit consumption by insect pests while attracting insect pollinators. A classic example of plant–insect interactions is the production of potato type I inhibitor (pin I) and type II inhibitor (pin II) serine PIs by solanaceous plants responding to damage by lepidopteran larvae (6). PIs are expressed constitutively at high levels in reproductive tissues (7), whereas expression in leaves is relatively low until the leaves are damaged by chewing insects (8, 9). Signals produced by wounded plant cells as well as by molecules in insect saliva lead to rapid accumulation of pin II transcripts (10, 11). Early observations that PI accumulation was not restricted to the wounded leaves led to the identification of mobile signals, such as the peptide hormone systemin, that activate signaling pathways and induce the transcription of the PI genes in distal leaves (12). Furthermore, wounded plants produce volatile signals that attract parasitic and predatory insects (13) and induce PI production in neighboring, nonwounded plants to arm themselves before insect invasion occurs (14).

When plant PIs bind to the digestive proteinases of insects, they block the digestion of proteins, leading to developmental delays and increased mortality. Pin I and II inhibitors target the digestive serine proteinases trypsin and chymotrypsin, the major enzymes contributing to protein digestion in the gut of lepidopteran larvae (15). Most plants produce PIs for insect protection, but insects can adapt to PI ingestion by overproducing PI-sensitive proteases (16), and/or up-regulating the expression of proteases that are insensitive to the PIs produced by that plant (17–20), or inducing the production of PI-degrading enzymes (21, 22).

In this study we investigated the effect of ingestion of a pin I and II inhibitor on the growth of *Helicoverpa* spp. *Nicotiana alata* PI (NaPI) is a pin II inhibitor from *Nicotiana alata* that consists of four (6-kDa) trypsin inhibitors (T1–T4) and two (6-kDa) chymotrypsin inhibitors (C1 and C2) (23, 24). Ingestion of NaPI induced an NaPI-resistant chymotrypsin that was inhibited by a pin I inhibitor (StPin1A) from wounded *Solanum tuberosum* leaves. In our companion paper (25) we characterize the mechanism of the resistance of this chymotrypsin to NaPI. The combination of NaPI and StPin1A in artificial diet and transgenic plants was far more effective at reducing the growth and development of *Helicoverpa* spp. than either inhibitor alone.

Results

***H. punctigera* Larvae Contain Chymotrypsin Activity Resistant to NaPI.** To test the insecticidal activity of NaPI, *H. punctigera* larvae were fed a cotton leaf-based artificial diet containing 0.26% (wt/vol) NaPI. At day 21, there was 80% mortality in NaPI-fed larvae compared with 40% mortality in the control-fed larvae (Fig. 1*A*). Larvae raised on the NaPI diet weighed about 30 mg; larvae fed control diet weighed ≈100 mg (Fig. 1*B*).

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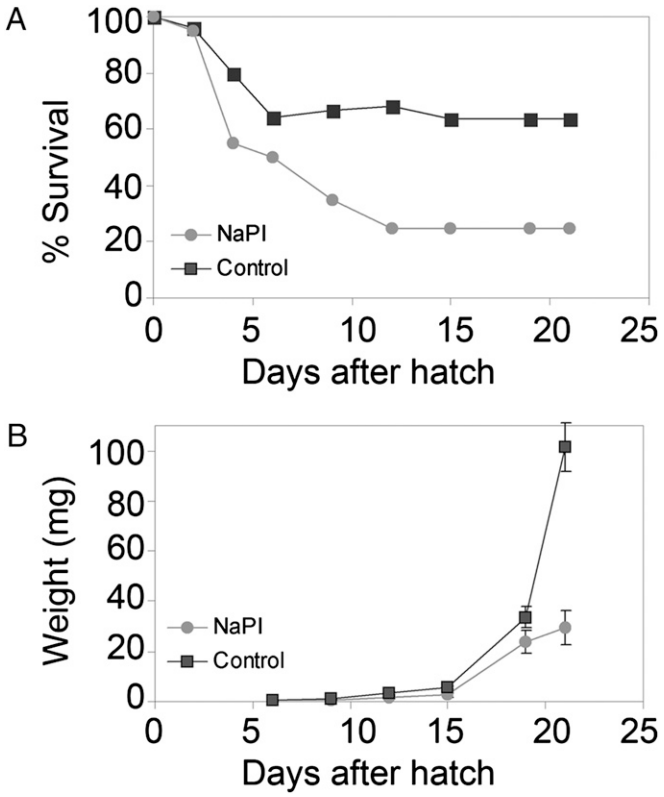

Fig. 1. Survival (*A*) and growth (±SEM) (*B*) of *H. punctigera* larvae raised on artificial cotton leaf diets containing 0.26% (wt/vol) NaPI.

Chymotrypsin and trypsin activity was measured in unfractionated gut extracts from surviving fifth-instar larvae. The in vivo effect of NaPI substantially lowered or abolished trypsin activity (Fig. 2), but chymotrypsin activity was either unaffected or enhanced. Although subsequent in vitro inhibition of chymotrypsin activity in gut extract from control larvae by NaPI was variable, NaPI did not inhibit any of the chymotrypsin activity in gut extracts of larvae that had consumed the NaPI (Fig. 2). This result suggested that larvae produce two classes of chymotrypsins: some that are inhibited by NaPI (NaPI-susceptible) and some that are not (NaPI-resistant). In a subsequent experiment, several commercially available PIs were tested against gut extracts from *H. punctigera* that had been depleted of NaPI-sensitive chymotrypsins by affinity chromatography (Table S1). The pin I inhibitor completely abolished all remaining chymotrypsin activity in the gut of these *H. punctigera* larvae.

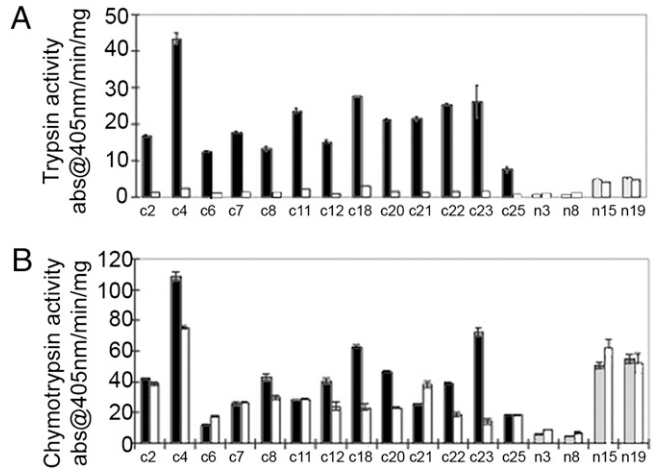

Fig. 2. Trypsin and chymotrypsin activity in larvae fed artificial diet containing NaPI. *H. punctigera* larvae (20) were raised from neonates to fifth instar on cotton leaf-based artificial diets with (n) or without (c) 0.26% (wt/vol) NaPI. Four larvae survived to fifth instar on the NaPI diet, whereas 13 control larvae survived. The presence of NaPI-insensitive enzymes in gut extracts from individual larvae was determined by (*A*) trypsin and (*B*) chymotrypsin activity assays before (black/gray bars) and after (white bars) the addition of NaPI in vitro. Units of protease activity are expressed as change in absorbance at 405 nm/min per milligram extracted protein (±SEM).

Chymotrypsin Activity in the Gut of Two *Helicoverpa* Species Is Abolished by a Type I Inhibitor from Potato. Commercial preparations of PIs often are contaminated (Fig. S1) and can contain several isoforms (26). To investigate the capacity of a pure pin I inhibitor to inhibit insect chymotrypsin activity, we obtained a pin I inhibitor cDNA using RNA from the wounded leaves of *S. tuberosum*. The recombinant pin I inhibitor, StPin1A, was expressed in *Escherichia coli* and purified to homogeneity for use in inhibition assays. We retested the gut extracts from both *H. punctigera* and a closely related species, *H. armigera*, and determined that the recombinant inhibitor StPin1A (0.2 μM) completely abolished all chymotrypsin activity in unfractionated gut extracts. In comparison, NaPI inhibited only 10% of total chymotrypsin activity at the same concentration (Fig. 3).

Ingestion of NaPI and StPin1A Impedes the Development of *H. armigera* Larvae. The discovery that StPin1A abolished the NaPI-resistant chymotrypsin activity led us to investigate whether the combination of StPin1A and NaPI would have a more marked effect on insect growth and development than either inhibitor on its own. To test this possibility, we placed *H. armigera* neonates on cotton leaf-based artificial diets with and without added PIs and recorded weight gain on days 5, 7, 9, and 11 (Fig. 4). At day 11, larvae fed diets containing NaPI or StPin1A weighed ≈50% and 40% less, respectively, than control larvae. In comparison, larvae fed an artificial diet containing both StPin1A and NaPI were on average 90% smaller than control larvae.

Coexpression of *NaPI* and *StPin1A* Improves Cotton Production Under Insect Pressure in the Field. To assess the potential of NaPI and StPin1A in plant protection, genes encoding these proteins were transferred into cotton plants for field-trial assessment. Transgenic

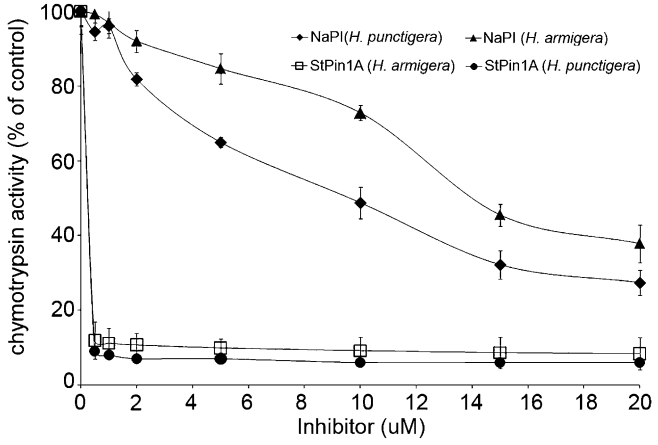

Fig. 3. Inhibition of chymotrypsin activity by NaPI and StPin1A. Unfractionated gut extract (1 μg protein) from *H. armigera* and *H. punctigera* larvae was incubated with increasing concentrations of native purified NaPI and recombinant StPin1A before the addition of chymotrypsin substrate (Succ-AAPFpNA). The residual activity is expressed as percent chymotrypsin remaining compared with controls. Error bars show SEM of three independent experiments performed in duplicate.

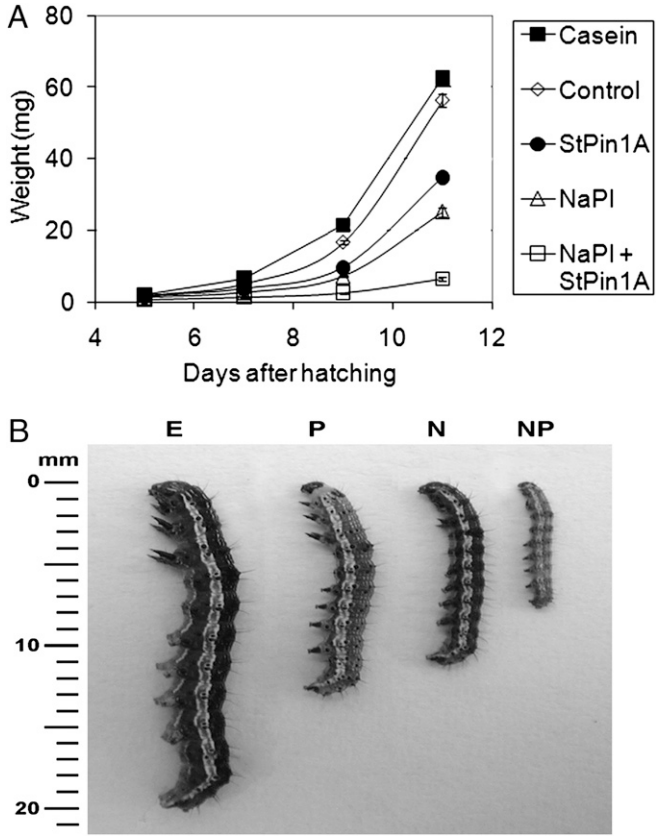

Fig. 4. Growth of *H. armigera* larvae on cotton leaf-based diets containing various PIs. Eggs were hatched, and neonates were transferred to artificial diets containing 0.3% (wt/vol) of each PI tested. (*A*) Larval weight gain was recorded every second day. The average weight (±SE) of 60 larvae per treatment is shown. (*B*) The average size of larvae recorded on day 11. E, casein; P, StPin1A; N, NaPI; NP, NaPI/StPin1A.

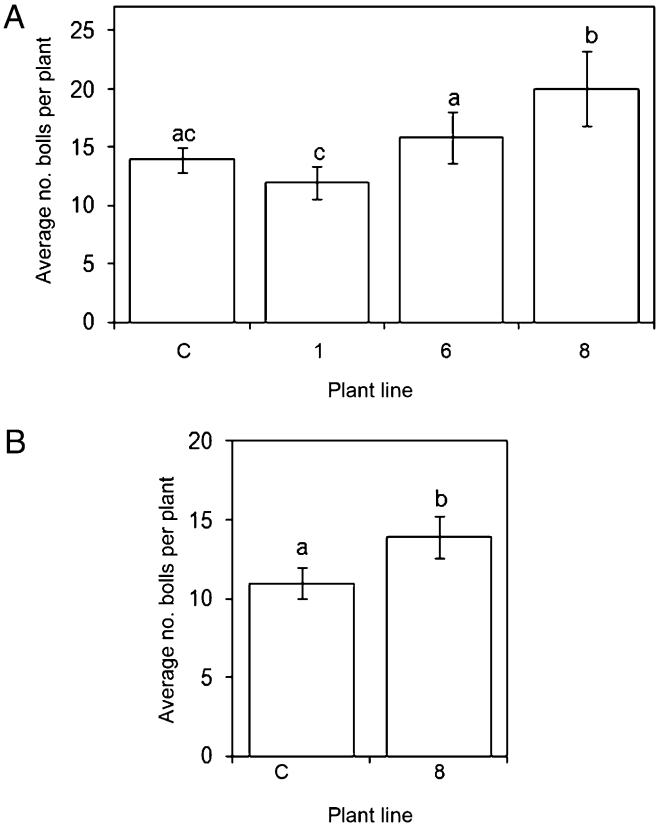

Fig. 5. Mean number of cotton bolls per plant on transgenic cotton plant line 1 (*NaPI*, homozygous), line 6 (*StPin1A*, homozygous), line 8 (*NaPI* + *StPin1A*, hemizygous/homozygous), and untransformed parent line (C; control). (*A*) In field trials conducted in 2004–2005, hemizygous line 8 had a significantly higher number of bolls than all other lines ($P < 0.001$). (*B*) During the 2006–2007 field trial, homozygous line 8 also recorded significantly higher cotton boll numbers than the control line ($P = 0.05$). Different lowercase letters indicate significantly different values, and error bars indicate 95% confidence intervals.

cotton plants expressing *NaPI* (line 1), *StPin1A* (line 6), or the combined *NaPI-StPin1A* (line 8) were tested for their performance in preventing plant damage caused by insect pests under field conditions in Queensland, Australia, during the 2004–2005 cotton-growing season. The major insect pests of cotton during the field trial period were *H. armigera* and *H. punctigera*, and the site was artificially infested with *H. armigera* eggs to maintain insect pressure. *NaPI* was highly expressed in the leaves of line 1 (1.0–1.3% of total soluble protein) and line 8 (0.4–0.6% of total soluble protein). Expression of *StPin1A* in line 8 was substantially lower than expression of *NaPI*, at 0.001–0.002% of total soluble protein. Cotton plants expressing both *NaPI* and *StPin1A* out-performed plants expressing these inhibitors alone, as measured by the number of cotton bolls per plant at maturity (Fig. 5*A*). Based on the strong performance of line 8 in the first field trial, homozygous line 8 cotton plants were produced for further testing in the 2006–2007 cotton-growing season in Queensland. Artificial insect infestation was not required because of the higher insect pressure; the major insect pests again were *H. armigera* and *H. punctigera*. Line 8, expressing *StPin1A* and *NaPI*, again out-performed the control plants, recording a statistically significant higher number of cotton bolls than the untransformed parental line (Figs. 5*B* and 6)and a higher lint weight per plant [27.8 ± 0.59 (SE) g for line 8; 22.9 ± 2.1 (SE) g for control line].

Discussion

In this study and previously (27), we showed that ingestion of the pin II inhibitor NaPI has a much more severe impact on growth and mortality of the cotton pest *H. punctigera* than it does on the more serious pest *H. armigera.* In an attempt to improve the efficacy of NaPI against *H. armigera*, we tested the combination of StPin1A, a potent inhibitor of *H. punctigera* and *H. armigera*

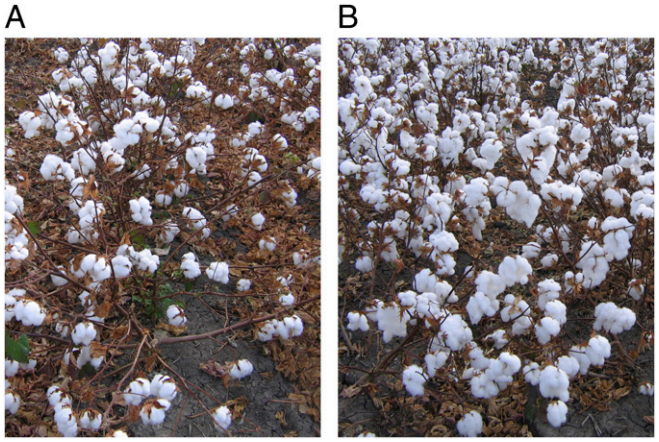

Fig. 6. Comparison of cotton bolls on field-grown transgenic cotton plants. (*A*) Coker 315 control plants have lower numbers of cotton bolls than (*B*) homozygous line 8 expressing *NaPI* and *StPin1A*.

chymotrypsins, with NaPI. Artificial diet containing both inhibitors led to a marked decrease in growth rate that was not obtained with either inhibitor in isolation.

Control larvae that had not been exposed to NaPI had variable levels of NaPI-insensitive chymotrypsin activity in their gut, suggesting that the encoding gene is expressed constitutively. It is possible that the gene is also up-regulated after exposure to diets containing the inhibitor, but this possibility needs to be verified by using more larvae and examining the levels of the gene transcript. Resistance to NaPI is likely to be multitiered, as described for the cowpea bruchid and a plant cysteine PI (21), of which one component is the genetic predisposition to have high levels of the NaPI-resistant chymotrypsin.

Previous studies have demonstrated changes in chymotrypsin gene expression (17, 28) and induction of PI-resistant chymotrypsins (17, 29, 30) in response to consumption of PIs. PI-resistant chymotrypsins have been characterized only by their activity, and there are no reports of inhibitors that target the PI-resistant activity. In the current study we show that trypsin activity was severely reduced in NaPI-fed larvae, but the presence of NaPI-resistant trypsins was not investigated further. PI-resistant trypsins have been identified in numerous lepidopteran species (17, 20, 31) and have been characterized more thoroughly than PI-resistant chymotrypsins (29, 32). Volpicella and colleagues (32) discovered that PI-susceptible and PI-resistant trypsins have different substrate preferences. To determine if NaPI-resistant trypsins are present in *Helicoverpa* spp., we would use the substrates described by Volpicella (32) to distinguish between resistant and susceptible enzymes.

Generally, PIs are screened for their inhibitory activity against insect gut extracts before in vivo testing using artificial diets or transgenic plants. Sometimes PIs that perform well during in vitro inhibition assays do not perform well in subsequent bioassays (16). Our study highlights the importance of working with homogenous preparations of PIs that are identical to the PIs that will be used as potential transgenes.

Transgenic cotton expressing *StPin1A* from potato and *NaPI* from tobacco showed improved performance over 2 y of field trials. Pin I and pin II inhibitors accumulate naturally in the leaves of solanaceous plants in response to damage by insects or mechanical injury (33). The use of transgenes encoding PIs from structurally distinct families thus is more likely to provide better plant protection under field conditions. Although the level of StPin1A in the hemizygous cotton plants was ≈1,000-fold lower than NaPI the presence of StPin1A still improved protection against insects in field trials. Combinations of PIs that target different classes of digestive proteases have shown promising results *in planta* (34, 35), but individual inhibitors also have been reported to improve plant resistance to insects (36, 37).

PIs have the potential to enhance the current Bt toxin technology because they target a broader range of pests, including nematodes and fungi (38). There is a major concern that the effectiveness of Bt will be negated if field-evolved Bt resistance (39) becomes a more widespread problem. A proposed management strategy for delaying insects' development of resistance to plant-protection transgenes, such as Bt toxins, is to deploy multiple insect-control genes (such as PIs) with different modes of action in a single plant (40). There is evidence that the combination of PIs with a sublethal-dose Bt toxin has a strong effect on the growth and development of insects (41).

Here we demonstrate the potential for using combinations of different classes of plant PIs to prevent crop damage caused by insects. The long-term aim of our approach is to select combinations of inhibitors that counter the genetic capacity of the target insect to produce various proteases under different pressures.

Materials and Methods

The synthetic substrate, *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-phenylalanine-4-nitroanilide (succ-AAPF-*p*NA), α-chymotrypsin from bovine pancreas (*N*α-Tosyl-L-lysine chloromethyl ketone hydrochloride treated), trypsin-agarose (N-*p*-Tosyl-L-phenylalanine chloromethyl ketone treated) and cyanogen bromide-activated Sepharose 4B were from Sigma-Aldrich. The NaPI series of 6-kDa chymotrypsin and trypsin inhibitors were purified from *Nicotiana alata* as described previously (23, 24). The Pin I inhibitor was from Calbiochem-Novabiochem. Recombinant StPin1A was supplied by Dr. Fung Lay of La Trobe University, Melbourne, Australia. All other PIs were from Sigma-Aldrich. Benzamidine agarose (35 μmol benzamidine/mL) was from MP Biomedicals.

Insect-Feeding Trials: Bioassay 1. *H. punctigera* feeding trials were conducted as described previously (42), except that the artificial diet was prepared with freeze-dried cotton leaves as described for a potato leaf artificial diet (43). The gut was removed from early fifth-instar larvae and was homogenized in gut extraction buffer (500 μL ice-cold 10 mM Tris-HCl, pH 8). Insoluble material was removed by centrifugation (13,000 × *g*, 5 min), and the supernatant was stored at −80 °C before use in enzyme assays.

Insect-Feeding Trials: Bioassay 2. *H. armigera* were raised on a cotton leaf-based artificial diet that was supplemented with NaPI (530 μM), StPin1A (530 μM), NaPI (530 μM) + StPin1A (530 μM), casein, or on an unsupplemented control diet. *H. armigera* neonates (60/treatment) were raised in individual microcentrifuge tubes (Sarstedt) with perforated lids. Weight gain was recorded on day 5 and every second day thereafter. Mortality was recorded for the first 5 d and then every second day thereafter. Artificial diet was replaced as required to provide a continuous supply. The larvae were kept at 25 °C with a 16-h/8-h light/dark cycle. *H. armigera* larvae were sourced from the Department of Primary Industries and Fisheries, Indooroopilly, Queensland, Australia. Statistical comparisons of larval weight were made by one-way ANOVA at a 99% confidence limit and Tukey–Kramer multiple-comparisons posttests, using StatPro software version 1.0 (Christopher Albright, http://www.kelley.iu.edu/albrightbooks/Free_downloads.htm).

Partial Purification of the NaPI-Insensitive Chymotrypsin for Inhibition Assays. The midgut from 100 fourth-instar larvae were pooled and homogenized in 50 mL of ice-cold extraction buffer [10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10% (wt/vol) glycerol, 2% (wt/vol) polyvinyl pyrrolidine, 0.01% (wt/vol) NaN_3] using a Sorvall Omnimixer. Insoluble material was removed by centrifugation (17,000 × *g*; 30 min; 4 °C) and filtration through Miracloth (Calbiochem). NaPI-sensitive proteases were removed by passage (×5) through an affinity column consisting of NaPI (C1, C2, T1–T4; 10 mg) cross-linked to cyanogen bromide-activated Sepharose 4B (1 g). The material that did not bind to the NaPI column was used to study the effect of a series of PIs on the activity of the NaPI-insensitive chymotrypsins.

Enzyme Activity and Inhibition by PIs. Gut extracts from individual *H. punctigera* larvae from Bioassay 1 were assayed at pH 10 in 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid using the chymotrypsin and trypsin substrates succ-AAPF-*p*NA and N-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BA-*p*NA), respectively. Gut extracts were preincubated with 80 nM of purified plant-derived T1 or C1 monomer for 30 min at 30 °C before the addition of substrate. The release of *p*NA was recorded at 405 nm on a SpectraMax 250 microtiter plate reader (Molecular Devices). Inhibition assays using StPin1A were performed using gut extracts from larvae raised on control diets.

Construction of the Binary Vectors and Transgenic Plant Lines. DNA encoding the sequence of the NaPI gene (GenBank accession number AF105340) (44) and the StPin1A gene (GenBank accession number FJ839694) were amplified by PCR and cloned between Cauliflower Mosaic Virus (CaMV) 35S promoter and terminator sequences (45). The expression cassettes were inserted into the pBIN19 binary vector (GenBank accession number U12540) (46) and named "pHEX2" (NaPI) or "pHEX6" (StPin1A).

The binary vectors pHEX2 and pHEX6 were used to produce transgenic cotton plants by *Agrobacterium*-mediated transformation as described in (47) with modifications. *A. tumefaciens* strain LBA4404 containing the vector was used to infect hypocotyl sections of *Gossypium hirsutum* L. cv. Coker 315. Embryogenic callus was selected on the antibiotic kanamycin. Plantlets were transferred to soil and after acclimatization were transferred to a greenhouse. Plant lines expressing *NaPI* or *StPin1A* were identified by protein immunoblot analysis. To produce homozygous plant lines, a segregation analysis of kanamycin resistance was performed on the progeny of self-pollinated primary transformants. The hemizygous transgenic plant line 8

(*NaPI-StPin1A*) was produced by crossing the homozygous plant line 1 (*NaPI*) with the homozygous plant line 6 (*StPin1A*).

Field Evaluation of Transgenic Plants. For the 2004–2005 field trial, the transgenic cotton lines 1 (homozygous), 6 (homozygous), and 8 (hemizygous) and the untransformed parent line Coker 315 were grown in the Darling Downs area of Queensland, Australia. Seed was planted by hand in three replicate plots, each plot containing 40 seed per variety. For the 2006–2007 field trial, the transgenic cotton line 8 and the untransformed parent line Coker 315 were grown in the Darling Downs region of Queensland, Australia. Seed was planted mechanically in four replicate plots per variety, each plot containing 80 seeds. Plant pests were monitored regularly throughout the growing season. In both trials, nonlepidopteran pests were controlled by the application of selective pesticides. Lepidopteran pests were controlled using a low-spray regimen consisting of three or four sprays. At weeks 6 and 12 in the 2004–2005 season, the expression of *NaPI* and *StPin1A* in the first fully expanded leaf from selected plants was determined by double-sandwich ELISA using polyclonal antibodies. At the completion of the trial, the number of mature open cotton bolls was counted on line 8, line 1, line 6 and Coker plants. Data were analyzed by ANOVA using SPSS statistics (SPSS Inc.).

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