The phenotypic consequences of proteinase inhibitor (PI) expression in Nicotiana attenuata, a molecular and ecological analysis.

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To Laura

Table of Contents

List of Manuscripts

Manuscript 1

Ecological costs and benefits of trypsin protease inhibitor production in *Nicotiana attenuata*

Grit A. Glawe, Jorge A. Zavala, André Kessler, Nicole M. van Dam, and Ian T. Baldwin

(Published: Ecology 2003, 84: 79-90)

Manuscript 2

Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*

Jorge A. Zavala, Aparna G. Patankar, Klaus Gase and Ian T. Baldwin

(Published: PNAS 2004, 101: 1607-1612)

Manuscript 3

Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as anti-herbivore defenses

Jorge A. Zavala, Aparna G. Patankar, Klaus Gase, Dequan Hui and Ian T. Baldwin

(Published: Plant Physiology 2004, 134: 1181-1190)

Manuscript 4

Fitness Benefits of Trypsin Proteinase Inhibitor Expression in *Nicotiana attenuata* Are Greater Than Their Costs When Plants Are Attacked

Jorge A. Zavala and Ian T. Baldwin

(Published: BMC Ecology 2004, 4:11)

We will see the entire plant world, for example, as a vast sea which is as necessary to the existence of individual insects as to the oceans and rivers are to the existence of individual fish, and we will observe that an enormous number of living creatures are born and nourished in this ocean of plants.

von Goethe, Johan Wolfgang

Ecology and genetics have always been uneasy bedfellows, despite their intrinsic complementarity; genetics is about what exists, ecology is about how it exists.

Berry, R.J. and Bradshaw, A.D.

Science is a voyage of discovery, and beyond each horizon there is another.

Hitching, Francis

The thesis that *we can learn from our mistakes* is a theory of reasons that assigns to rational arguments the modest and yet important role of criticizing our often mistaken attempts to solve our problems. And it is a theory of experience that assigns to our observations the equally modest and almost equally important role of tests which may helps us in the discovery of our mistakes. Though it stresses our fallibility it does not resign itself to skepticism, for it also stresses the fact that knowledge can grow, and that science can progress, just because we can learn from our mistakes.

Popper, Karl

1. Introduction

The cost-benefit paradigm is central to functional biology and to ecological and evolutionary theory because fitness costs and benefits associated with a trait determine its equilibrium value in a population. A motivation for incorporating cost into models of evolution is that costs of resistance against natural enemies can account for the common observation that, although organisms exhibit considerable genetic variation for resistance, they are not maximally resistant to attack by natural enemies (Berembaum et al. 1986; Dirzo and Harper 1982). If the resistance trait has fitness benefits in the population and does not incur any cost then selection should lead to fixation of the beneficial allele(s), reducing the variability (Simms and Rausher 1987). Alternatively, when the fitness-benefit of the trait also has a cost, selection should favor an intermediate frequency of the trait in the population because the benefit of the trait may vary under different environmental conditions, increasing the variability (Cipollini et al. 2003; Mauricio 1998; Simms and Rausher 1987).

The cost-benefit balance of defense traits affects both animals and plants. Some small invertebrates in fresh water and marine environments respond to predators through morphological modifications such as the production of helmets in daphnia (Havel and Dodson 1987), heavier shells in barnacles (Lively 1986) and spines in bryozoans (Harvell 1986). These induced morphological changes reduced growth and/or fecundity. When predators are not present, unarmored individuals have a fitness advantage. Resistance against natural enemies has costs as well as its obvious benefits on fitness in insect-parasite, insect-parasitoid and plant-insect systems (Baldwin 1998; Kraaijeveld et al. 2002; Milks et al. 2002).

In particular, most models of evolution of plant resistance to herbivores make the assumptions that resistance has fitness cost (Gulmon and Mooney 1986; Simms and Rausher

1987). Herbivores can reduce seed production and other correlates of plant fitness, and this reduction can result in natural selection for either constitutively expressed or inducible plant defenses (Karban and Baldwin 1997; Marquis 1984; Stamp 2003). Current theory predicts that one benefit of induced defenses is to optimize the plant's allocation to defense, growth and reproduction (Karban and Baldwin 1997). Although defenses might benefit plants in the presence of herbivores, plant resistance to herbivores can be costly in the absence of plant enemies and inducible expression of resistance traits allow plants to forgo the potential fitness cost of resistance traits when they are not needed (Agrawal 1998; Baldwin 1998; Cipollini et al. 2003; Hare et al. 2003; Strauss et al. 2002; Zangerl 2003).

Evidence for the existence of resistance costs and benefits from studies using plant species with constitutive and inducible defenses is increasing (Bergelson and Purrington 1996; Cipollini et al. 2003; Heil and Baldwin 2002; Zangerl 2003). However, conclusive evidence attributing fitness cost to a particular defense trait has been elusive, but recent studies have made significant advances (Cipollini 2002; Tian et al. 2003). One of the main difficulties to test this paradigm is that attribution of fitness consequences to expression of a particular defense trait in an environment either with or without herbivory is onerous, because genes that control the expression of defensive traits may have pleiotropic effects on fitness traits (Elle et al. 1999). Ideally, one should assess the costs and benefits of inducible defenses in plants that differ only in the expression of genes that control (induced) resistance but are otherwise genetically identical (Bergelson and Purrington 1996). Transformation technology provides a means of manipulating traits with unparalleled precision. Although the benefits of plant traits that provide resistance against herbivores are expected to equal or exceed their cost (Coley et al. 1985; Feeny 1976; Rhoades and Cates 1976), very few direct tests have been done. While costs and putative

benefits of defense traits have been studied in separate experiments, their currencies are usually not comparable (i.e., plant fitness for the cost; herbivore performance for the benefits). Tests of the cost-benefit model using the same currency are few (Baldwin 1998).

Proteinase inhibitors.

One of the most studied plant defensive compounds is proteinase inhibitors (PI) as growth retardants for insects, particularly phytophagous insects, and for other organism as well (Carozzi and Koziel 1997). PIs are ubiquitous in nature, and appear to have a very significant role of protection of the cell against the proteinases of pests and pathogens (Carozzi and Koziel 1997; Ryan 1990). In this protective role they are often induced locally by wounding or by systemic signals (Green and Ryan 1972; McManus et al. 1994; Orozco-Cardenas et al. 1993; Ryan 2000) or are present in storage organs at high levels (Gatehouse et al. 1979; Rackis and Anderson 1964). For a PI to be effective in targeting an insect proteinase, it should have a tight affinity for its target proteinase and thus inactivate the proteinase (Laing and McManus 2002). This prevents the target insect from digesting protein. Further evidence that is often cited for the role of PIs as protectants in plants is the demonstration of the efficiency of these proteins when expressed in transgenic plants (Hilder et al. 1987; Johnson et al. 1989; McManus et al. 1999; McManus et al. 1994). While such studies do show that accumulation of these proteins can confer insect resistance to the transformants, surprisingly, I know of no studies that have altered the expression of an endogenous *pi* gene to examine its defensive function.

Insects adapt to plant PI ingestion through three main mechanisms (Jongsma and Bolter 1997). 1) Through overproduction of insect target proteinases, in order to overcome the levels of PI provided through the diet (Broadway et al. 1986; Marwick et al. 1998). This is thought to

increase the metabolic demands on the organism and may lead to slow growth and possibly death (Laing and McManus 2002). 2) The insect can develop a degree of immunity to the PI by evolving a proteinase with a low affinity to the PI. For example, potato tuber moth (*Phthimaea operculella*), a successful pest on potato, was less inhibited by PIs from potato when compared with a range of other lepidopteran pest of host other than potato (Christeller et al. 1992). It might be suggested that any insect that is capable of successfully living off a plant must be at least partially resistant to the PIs produced by the plant (Laing and McManus 2002). 3) The short-term induction of PI resistant proteinases by the insect (Gruden et al. 1998; Jongsma et al. 1995), although this has not been observed in *Helicoverpa armigera* larvae feeding on an artificial diet (Gatehouse et al. 1997). However, the relationship between PI expression and defense can have counter responses, such as, in response to ingestion of high PI leaves insects increase their rate of leaf consumption (Winterer and Bergelson 2001). Together, these studies lead to the suggestion that a key adaptation for herbivory is for the insect to be at least partially resistant to the PIs produced by the plant, and point to the significance of PIs as protectants in higher plants.

***Nicotiana attenuata* as a model system**.

Nicotiana attenuata [Torr. Ex Wats. (synomonous with *Nicotiana torreyana* Nelson and Macbr.)], a diploid, largely selfing, native tobacco inhabiting the Great Basin Desert, was selected as a model system for two reasons: 1) it exhibits a large amount of morphological and chemical phenotypic plasticity that appears to be adaptive, and 2) it has evolved to grow in the primordial agricultural niche: the immediate post-fire environment (Baldwin 2001). These characteristics of the life history of *N. attenuata* make it particularly useful to test the cost-benefit model for induced defenses. *N. attenuata* is an ephemeral member of the annual

community in burned areas and synchronizes it growth with the post-fire environment by producing dormant seeds that germinate in response to cellulose combustion product(s) found in wood smoke (Baldwin et al. 1994). Such synchronization allow this species to exploit the ephemeral, but nutrient-rich, herbivore- and competition-poor environments that commonly exists after fires (Baldwin 2001). As a consequence of this germination behavior, seeds germinate into (N)-rich soils (Lynds and Baldwin 1998) and hence have selected for rapid growth to deal with an intense intraspecific competition when water availability is high (Baldwin 2001). This temporal window of growth opportunity is quite short, for as post-fire succession proceeds, and herbivores including *Manduca sexta* which is adapted to feed on *N. attenuata* and *Tupiocoris notatus* decrease plant fitness (Baldwin 2001).

N. attenuata has a number of well-described herbivore-induced direct and indirect defenses (Baldwin 2001), which increase the fitness of plants under attack in natural populations (Baldwin 1998; Kessler and Baldwin 2001). Closely related plant species, such as *N. tabacum* (Jongsma et al. 1994), *N. alata* (Heath et al. 1997), and *N. plunbaginafolia* (Ausloos et al. 1995), are known to produce several types of inducible PIs, all of which belong to the serine PIs (Koiwa et al. 1997). Hence, is expected that PIs, such as trypsin proteinase inhibitors (TPI), play an important defensive role in addition to nicotine in *N. attenuata* (Baldwin 2001). The within-plant pattern of systemic TPI induction at the rosette stage of growth suggested that the signal(s) triggering remote TPI induction follows a source-sink relationship: regardless of ontogenetic stage, if young sink leaves are damaged TPI levels increase only locally, while older leaves are less sensitive to leaf damage and produce a less local intense wound signal but increases the TPI levels systemically in younger leaves (van Dam et al. 2001).

Some studies have quantified the costs and benefits of constitutive and inducible defenses in *N. attenuata* treating plants with jasmonates such as jasmonic acid (JA). JA provides a convenient way to disassociate chemical defense and leaf loss, because JA causes the induction of several well documented defensive pathways without the removal of leaf tissue(Baldwin 1996; Gatehouse 2002). Although JA-elicited plants in the field realize a higher fitness when they are under attack, this resistance comes at a substantial fitness cost if plants are not attacked (Baldwin 1998). The mechanisms responsible for these large fitness costs were explored in laboratory experiments. When untreated plants competed in the same pot with JA-elicited plant, untreated plants realize opportunity benefits with a large increase in lifetime seed production at the expense of the seed production of the neighboring JA-elicited plants (van Dam and Baldwin 1998; van Dam and Baldwin 2001). The fitness cost of JA elicitation increased with N supply and was associated with greater ability to compete for below-ground N resources and increase in allocation of acquired ^{15}N to seed production (van Dam and Baldwin 2001).

It remains undetermined as to whether slowing of growth, with its concomitant loss of competitive ability, is required for JA-elicited induced resistance (Baldwin 2001). JA decreases transcripts of a number of photosynthetic-related genes (Hermsmeier et al. 2001) and this down-regulation may be required to free up resources for defense-related processes (Baldwin 2001). Direct genetic manipulation of a particular resistance trait, such as TPI allows researchers to determine both whether defense traits are intrinsically costly and its benefits, as well as whether the benefits of the trait outweigh their costs. The cost-benefit paradigm is a useful heuristic tool to generate testable hypotheses about the function of TPI and the fitness consequences in *N. attenuata* plants. The following collection of papers examines the costs and benefits of TPI

expression in *N. attenuata*. The general objective of the study and the following 3 hypothesis were addressed in the four manuscripts:

GENERAL OBJECTIVE:

Determine costs and benefits of TPI expression, and whether the benefits outweigh their costs in *Nicotiana attenuata.*

HYPOTHESES:

1- TPI expression in *N. attenuata* is costly for plant fitness when plants are not attacked.

Prediction 1: *N. attenuata* genotypes with either low or no TPI growing next to high TPI-producing genotypes are taller with earlier flowering and produce a higher number of lifetime seed capsules.

2- *N. attenuata*'s TPI decreases the performance of *Manduca sexta* larvae and plant colonization of *Tupiocoris notatus.*

Prediction 2a: *Manduca sexta* larvae fed on genotypes with either low or no TPI expression grow faster, have higher survivorship and produce heavier pupae than those fed on high TPI-producing genotypes.

Prediction 2b: *Tupiocoris notatus* has higher colonization preference for genotypes with either low or no TPI expression than genotypes with high TPI expression.

3- The putative fitness benefits of TPI expression outweigh their costs by decreasing *M. sexta* larval mass.

Prediction 3a: Unattacked genotypes with either low or no TPI expression produce more seed capsules than genotypes with high TPI levels, and after *M. sexta* attack genotypes with either low or no TPI expression produced less seed capsules than genotypes with high TPI levels.

Prediction 3b: High TPI mediate a decrease in larval mass and behavioral change in *M. sexta* that is associated with fitness benefits for the plant.

Manuscript 1

Ecological costs and benefits of trypsin protease inhibitor production in *Nicotiana attenuata*

Grit A. Glawe, Jorge A. Zavala, André Kessler, Nicole M. van Dam, and Ian T. Baldwin

(Published: Ecology 2003, 84: 79-90)

I compared a *N. attenuata* genotype with normal TPI production (WT) competing with a genotype collected in Arizona (A) that lost the ability to produce TPI. This experiment gave me the first indication that constitutive and inducible TPIs have fitness cost in *N. attenuata* and decrease the larval performance of *Manduca sexta,* a natural herbivore. Manuscript 1 is a joint effort by all authors, whereby Grit Glawe and I are responsible for the realization of the plant competition experiment with both genotypes of *N. attenuata*. This experiment gave us the first indication that TPI production has fitness cost in *N. attenuata*. I am responsible for the Northen blot shown in the paper and Grit Glawe and André Kessler are responsible for the field experiments. All authors were involved in the discussion of the data for the final version of the paper, while Grit Glawe, Nicole M. Van Dam and Ian T. Baldwin prepare the first draft of the manuscript (which was optimized after discussions with Grit Glawe and Ian Baldwin).

Manuscript 2

Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*

Jorge A. Zavala, Aparna G. Patankar, Klaus Gase and Ian T. Baldwin

(Published: PNAS 2004, 101: 1607-1612)

I down regulated in the WT and over-expressed in the A genotypes TPI production by transgenic technology. Using these genotypes, I found that TPI expression in *N. attenuata* decrease plant fitness when plants compete with conspecifics and this difference increased when MeJA was applied to the leaves, confirming that TPI expression incurs large fitness cost when *N. attenuata* is not attacked by herbivores. The idea for the competition experiment with *N. attenuata* genotypes in which TPI expression was reduced in a TPI-producing genotype (WT) and restored (S++) in a natural genotype from Arizona (A) was from Ian T. Baldwin. I am responsible for the selection and development of the transgenic genotypes and planning, data collection, chemical and statistical analysis, while compilation of the manuscript was a joint effort by Jorge A. Zavala

and Ian T. Baldwin (I wrote the first draft, which was optimized after discussions with the co-author). Klaus Gase is responsible for the design and development of the plasmid used in plant transformation and Aparna G. Patankar and Jorge A. Zavala are responsible for the Southern blot showed in the paper.

Manuscript 3

Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as anti-herbivore defenses

Jorge A. Zavala, Aparna G. Patankar, Klaus Gase, Dequan Hui and Ian T. Baldwin

(Plant Physiology March 2004, in Press)

In this Manuscript, using the transformed and untransformed genotypes, I found that TPI decreased the performance and fecundity of *M. sexta* larvae, suggesting that TPI is an effective defense against *M. sexta*. In this paper we used the same genotypes used in Manuscript 2. I am responsible for data collection, chemical and statistical analysis, while planning of the experiment and compilation of the manuscript was a joint effort by Jorge A. Zavala and Ian T. Baldwin (I wrote the first draft, which was optimized after discussions with the co-author). Klaus Gase and Aparna G. Patankar are responsible for sequencing and analysis of the PI precursors and Dequan Hui is responsible for screening a cDNA library of *N. attenuata*.

Manuscript 4

Fitness Benefits of Trypsin Proteinase Inhibitor Expression in *Nicotiana attenuata* Are Greater Than Their Costs When Plants Are Attacked

Jorge A. Zavala and Ian T. Baldwin

(Submitted: PLoS)

Unattacked low TPI-producing genotypes produced more seed capsules than did plants with high TPI levels. Caterpillar attack reduced seed capsule production in all genotypes and reversed the pattern of seed capsule production among genotypes, demonstrating that the fitness benefits of TPI production outweigh their costs when plants are attacked. In this paper we used the same genotypes used in Manuscript 2. I am responsible for data collection, chemical and statistical analysis, while planning of the experiment and compilation of the manuscript was a joint effort

by Jorge A. Zavala and Ian T. Baldwin (I wrote the first draft, which was optimized after discussions with the co-author).

Manuscript 1

(Published: Ecology 2003, 84: 79-90)

Ecological costs and benefits of trypsin protease inhibitor production in *Nicotiana attenuata*

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Running head: costs and benefits of PI production

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Abstract

Genotypes of the wild tobacco *Nicotiana attenuata* from different geographic regions in North America vary considerably in the level of constitutive and inducible protease inhibitors (PIs), a potent direct defense, as well as in the production of herbivore-induced volatiles that function as indirect defense. One genotype collected from Arizona (A) that was found to lack the ability to produce PIs at a transcriptional level, had decreased volatile production, but exhibited nicotine and growth responses that were not distinguishable from genotypes collected in Utah (U). In field trials with naturally occurring herbivores and in lab experiments with *Manduca sexta* larvae, the A genotype was damaged more and sustained greater herbivore growth than the U genotype. When A and U genotypes were grown in competition, the A genotype produced significantly more seed capsules than the U neighbor. Moreover, jasmonate elicitation, which dramatically increased PI production in only the U genotype, reduced lifetime fitness measures of the U genotype more than of the A genotype, demonstrating for the first time that PI production is correlated with a fitness cost. The loss of both a direct and indirect defense suggests a functional linkage between these types of defense.

Key words: chemical polymorphism, costs, induced defenses, intraspecific plant competition, genetic/ phenotypic variation, *Nicotiana attenuata*, phenotypic plasticity, protease inhibitors, volatile emission.

Introduction

Plants respond to herbivore attack with the induction of direct and indirect chemical defenses (Karban and Baldwin 1997). Resistance is known to vary among individuals from the same species and this variation is thought to be maintained by trade-offs between the benefits of reduced herbivory and the costs of resistance (Coley et al. 1985, Herms and Mattson 1992, Karban and Baldwin 1997). For example, inducible expression of resistance is thought to be a 'cost-savings' measure that allow plants to forgo the costs of defense when it is not needed and time the expression of resistance traits with the need for defense (Baldwin 1998). Studies of resistance polymorphisms within and among populations suggest that this interplay of costs and benefits constitutes an important selection pressure. Variation in cyanogenesis in *Trifolium repens* appears to depend on mollusk density, which generates the fitness benefit (Dirzo and Harper, 1982a, b) and the probability of frost, which makes the defense costly (Daday, 1954). Resistance costs may arise from processes internal to the plant, such as from the allocation of fitness-limiting resources to defense metabolite production, and from processes external to the plant, for example when resistant plants are unable to attract pollinators. Evidence for both external and internal costs are found in studies on induced defenses in *Nicotiana attenuata*, which invests 8% of its whole-plant nitrogen budget into nicotine production alone after herbivore attack and withdraws nicotine from the outer parts of its flowers when advertising for pollinator services (Baldwin, 2001).

Evidence for the existence of resistance costs from studies using constitutively- and inducibly-expressed resistance is increasing (Bergelson and Purrington 1996, van Dam and Baldwin 2001, Heil and Baldwin 2002). The interpretation of these studies, however, is not trivial. For example, genes that control the expression of defensive traits may have pleiotropic effects on fitness traits (Elle et al. 1999). Similarly, fitness differences between induced and

uninduced plants may be due to pleiotrophic effects of the elicitor used to activate the induced resistance, rather than the expression of the resistance traits themselves (Creelman and Mullet 1997). Ideally, one should assess costs and benefits of inducible defenses in plants that differ only in the genes that control the expression of induced resistance but otherwise are genetically identical (Bergelson and Purrington 1996). Although agricultural plant species have been transformed to alter signaling pathways that regulate aspects of induced resistance, appropriate controls are frequently lacking in these studies and more generally, agricultural species are less useful for the analysis of ecological processes involved in the evolution of induced defenses (Heil and Baldwin 2002). To date, no naturally occurring genotypes have been identified that are polymorphic for an inducible defense.

An additional complication is that the expression of resistance traits is often dependent on environmental conditions. This contingency may have been the cause of failures to detect costs of resistance (e.g., Brown 1988). Resistance costs thus may be more readily detected when plants are grown under environmental stresses, which simulate those that predominate in natural plant populations (Bergelson 1994, Karban and Baldwin 1997). For example, the fitness costs of jasmonate-induced defenses were only observed when *N. attenuata* plants were grown in competition with other uninduced plants (van Dam and Baldwin 1998, 2001).

N. attenuata, a post-fire annual wild tobacco inhabiting the Great Basin Desert, has a number of well-described herbivore-induced direct and indirect defenses (Baldwin, 2001). In addition to nicotine, the plants also produce serine protease inhibitors (PIs) after herbivore attack (van Dam et al., 2001b). PIs are among the best-studied defensive chemicals in plants (Ryan 1990, Jongsma et al. 1994, Koiwa et al. 1997, Heath et al. 1997). PIs have been found to reduce herbivore growth in plants that were transformed with a heterologous PI gene (Hilder et al.1987, Johnson et al. 1989). Genetic manipulations of wound signal transduction cascades (systemin, jasmonic acid) that resulted in increased PI expression also increased insect resistance (McGurl et al. 1992, Howe et al. 1996), but these signaling cascades regulate

many other traits in addition to PIs (Bergey et al. 1996). Surprisingly, we are not aware of any study that has altered the expression of an endogenous PI gene to examine its defensive function.

Unlike nicotine induction, which is not inhibited by nitrogen stress (Ohnmeiss and Baldwin, 1994) and is only inhibited by unnatural growing conditions in some species (e.g. pot-bound roots: Baldwin, 1988), PI accumulation is contingent on plant growth parameters and nutrient supply. Van Dam et al. (2001b) found diurnal fluctuations in PI activity in leaves of *N. attenuata* rosette plants and an increase in PI activity with plant development. Nutrient stress apparently constrained both constitutive as well as induced levels in *Brassica napus* when plants were grown at high densities (Cipollini and Bergelson 2001).

In addition to eliciting the production of direct defenses, herbivore attack to *N. attenuata* plants elicits the release of a bouquet of volatile organic carbons (VOC), which function as a potent indirect defense. VOC emission can reduce the plants' herbivore load by more than 90 % in nature, because it attracts a generalist predator and reduces herbivore oviposition rates (Kessler and Baldwin 2001). In comparison to the resource demands of induced nicotine production, the resource allocation to VOC production is trivial (Halitschke et al. 2000). The composition of the VOC release is known to vary among *N. attenuata* genotypes collected from different geographic locations, with the release of cis-α-bergamotene being the most consistently released component of the herbivore-induced VOC bouquet (Halitschke et al. 2000). Hence, if costs are responsible for maintaining the variation in VOC components, these costs are likely to be 'ecological costs' rather than resource-allocation costs.

In this paper we survey the variation in direct and indirect defenses of *N. attenuata* collected across the species' geographical range. We find nicotine induction to be invariable, while both PI activity and the composition of the induced VOC emissions are highly variable between plants from different geographical sites. Here, we report the first naturally occurring

genotype that is unable to produce trypsin PIs constitutively or after herbivore- or jasmonate-elicitation. We show that the inability to produce PIs proteins is due to an inability to produce PI mRNA. The PI-deficient and -producing genotypes were found to differ in their acceptability and susceptibility to four naturally occurring herbivores in the field. We compared the fitness of the PI-deficient and -producing genotypes in a competitive experiment, with and without MJ-elicitation, to examine the fitness costs associated with inducible and constitutive PI production. These experiments show that PI induction is correlated with a fitness cost when PI-producing plants grow in competition with plants that lack PI production.

Methods

Plant growth. Seed collections of *Nicotiana attenuata* Torr. Ex Wats. (Solanaceae) populations were made across western North America. Seeds used in this study were from 1996 field collections from three different geographic regions (genotypes: (1) Arizona (A) - bulk collection from a 20-plant population near Flagstaff, (2) California (C) – bulk collection from a 50-plant population near Benton, and (3) Utah (U) – bulk collection from a 1000-plant population near Apex Mine, SW Utah. Seeds of these 3 genotypes were from the pooled collection of 20-30 plants from 3 (A and C) or 2 generations (U) of growth and selfing in the glasshouse. Seeds used in the experiments therefore represent a sample of the genetic variation present at the collection locality with minimal differences due to maternal effects.

Seeds were germinated in diluted liquid smoke solution as described in Baldwin et al. (1994). After 14 d, seedlings for the phenotypic comparison were transferred to communal hydroponic chambers and 6-7 d later to 1L individual pots with No-N solution supplemented with 2mmol/L KNO_3 (Ohnmeiss and Baldwin 1994). Every week, plants received an additional 1mmol KNO_3 and every 2 weeks, the complete hydroponic solution was replaced.

Seedlings for the competition experiment were transplanted to 2 L pots containing a 1:1 peat-Perlite "High N" mixture (van Dam and Baldwin 1998). Two seedlings of similar size and appearance were planted 7 cm apart in each pot. Seedlings that were used for the feeding experiment were transplanted into individual 1 L pots containing high N peat-Perlite mixture. All plants were placed in a climate chamber at 32ºC/ 16 h L, 27ºC/ 8 h D, 65% relative humidity with 1000-1500 µmol m^{-2} s^{-1} PPFD at plant growing level. For the field experiments, seedlings were transplanted to root-trainers (Hummert, Earth City, Missouri, USA) containing the peat-Perlite mixture and grown in a shade-house for about 14 d before being transplanted to an experimental garden at the Lytle Preserve, Santa Clara, Utah. Plants that provided the leaf material for the choice tests were transplanted into individual 2 L pots and remained in the shade-house.

Plant elicitation. The application of jasmonic acid (JA) or its methyl ester methyl jasmonate (MJ), and oral secretion (OS) is known to elicit the synthesis of herbivore-induced defense metabolites in *N. attenuata*, such as nicotine (Winz and Baldwin 2001), PIs (van Dam et al. 2001b) or VOC (Halitschke et al. 2000). MJ was used to characterize the induction of nicotine and trypsine (Tryp) PIs in the different genotypes: 20 µl of lanolin containing 250 µg MJ was applied to two fully expanded leaves of each plant (Baldwin and Schmelz 1996). Control plants were treated similarly with 20 µl pure lanolin. For VOC induction, plants were elicited with *Manduca sexta* oral secretions (OS) applied to a standardized mechanical wound (Halitschke et al. 2000). Two fully expanded leaves per plant were damaged by rolling a fabric pattern wheel 10 times over the leaf surface to create rows of puncture wounds into which 15 µl OS from 4^{th}-5^{th} instar larvae was applied (McCloud and Baldwin 1997). Controls remained untreated. Volatile collection commenced 24 h after treatment. In the competition experiment, plants were elicited with either 150 µg (*) or 250 µg (**) MJ dissolved in 20 µl lanolin, which was applied as above 10 d after transplantation to 2 L pots.

Protein, TrypPI, nicotine, and volatiles measurements. Protein and TrypPI concentrations were measured as described in van Dam et al. (2001b). PI activity is expressed as nmol mg^{-1} protein. Nicotine concentrations were measured by HPLC as described in Keinaenen et al. (2001) and expressed as mg g^{-1} FM (fresh mass). The herbivore-induced VOC released from whole plants was trapped on activated charcoal traps for 8 hours, 24 h after elicitation and measured by GC-MS as described in Halitschke et al. (2000). The most consistently released component of the plant's VOC release, cis-α-bergamotene, was quantified by external standards and expressed in ng per h and L of air sampled.

Phenotypic comparison of genotypes

Comparison of developmental and reproductive parameters. Twenty-five plants of each genotype (A, C and U) were grown in individual plant hydroponic chambers for four weeks. Every 5 d, 5 randomly-selected plants from each genotype were harvested and measured. The following fitness correlates were measured: (i) whole plant biomass, (ii) root mass, (iii) shoot mass, (iv) stalk length at first day of flowering, (v) first day of flowering, (vi) number of branches, (vii) number of flowers at day 25 and, (viii) number of seed capsules at day 25.

*Comparison of direct and indirect defensive compounds***.** For TrypPI and nicotine analysis, 10 soil-grown rosette-stage plants of each genotype were selected two weeks after their transfer to 1 L pots and randomly assigned to two treatment groups, control and MJ (see *Plant elicitation*). Four d after treatments, the shoots were harvested to determine concentrations of PIs and nicotine. For volatile measurements, 14 plants of each genotype were grown for 10 d in 1 L hydroponic chambers and received on the treatment day an additional 1 mmole KNO_3.

Field experiments

Field experiments were conducted to examine behavior and performance of naturally occurring herbivores on A and U genotypes. We chose the herbivores that were the most common consumers of *N. attenuata* in natural populations during the 2000 field season at Lytle Preserve, Santa Clara, SW Utah.

A. *Choice tests.* Choice-tests (*bioassay)* with the following field collected insects were used to examine herbivore preference between the genotypes: (1) adult *Trichobarus mucrorea* (Coleoptera: Curculionidae), a weevil that feeds on leaves of several Solanaceae, such as *Datura wrightii* and *N. attenuata,* (2) adult *Epithrix* spp. (Coleoptera: Chrysomelidae, Halticinae), a flea beetle specialist herbivore on tobacco that can cause severe damage at high population densities (Deseö et al. 1993), and, (3) adult *Trimerotropis* spp. (Orthopteroidea: Caelifera), a generalist grasshopper which opportunistically feeds on *N. attenuata*. Insects used in the bioassay were collected 24 h before the experiments started. *T. mucrorea* and *Trimerotropis* spp. were collected at the Lytle Preserve, Beaver Dam Wash, and *Epithrix* spp. from a population of *N. attenuata* growing in a 1-year old burn on Apex Mine, Utah. All insects were transported to the laboratory and placed in polystyrene containers (500 ml) without any food until the next day. Three days prior to start of each bioassay, potted plants in the elongation stage from both genotypes were elicited with 250 µg of MJ in 20 µl lanolin applied to the adaxial surface of two rosette leaves to get a maximal induction of PIs. On day 3, the second stalk leaf from all plants was harvested for protein and PI analyses while the first stalk leaf was collected for the choice-test bioassays. Two same-sized leaves from A and U genotypes, were placed 5 cm apart in a polystyrene container lined with wet paper towels to prevent wilting of the leaves, with either: (1) 3 *T. mucrorea*; (2) 5 *Epithrix* spp.; and (3) 1 *Trimerotropis* spp. Containers were kept in a trailer at ambient temperature and after 48 h, the amount of leaf area damaged was measured with graph paper. Seven replicate choice-arenas were used for each herbivore species.

B. *Colonization experiment.* We monitored the accumulation of leaf bugs *(Tupiocoris notatus*, Hemiptera: Miridae) on *N. attenuata* plants recently transplanted into an experimental garden. Commonly, *T. notatus* is the first insect species colonizing wild tobacco seedlings in the years following fires. Forty plants of the 2 genotypes were randomly planted in furrows, at a distance of 50 cm within and between the furrows. Plants were fertilized with water soluble NPK (14:14:13) every 7 d. In contrast to the choice tests, plants in the experimental garden were not treated with MJ, because we wanted to test the preference of herbivores for both genotypes displaying their constitutive phenotype. After 2 weeks of transplanting, the accumulation of *T. notatus* was measured by counting adult individuals on each single plant.

Feeding experiment

Because PIs are known to decrease protease activity in larval lepidoptern guts and reduce larval growth rates (Heath et al. 1997), we measured larval development on U and A plants. Eggs of *Manduca sexta* L. (Lepidoptera: Sphingoidae) were obtained from Carolina Biological Supply Company (Burlington, NC, USA) and placed in square polysterene containers (200 ml) with a clear lid, lined with a moist tissue. The containers were kept in climate chambers at 28ºC, 65% r.h. and a 16:8 hours L:D photoperiod until larvae had hatched. Freshly hatched *M. sexta* larvae were placed individually on 10 soil-grown rosette plants of each genotype. To prevent larvae from moving between plants, 1 L pots were placed 30 cm apart and each pot was shrouded with cardboard, providing a barrier to larval movement. Larval mass and instar were measured on the 2^{nd}, 5^{th} and 7^{th} day after hatching. Each plant provided ample leaf material to supply one larva for the duration of the experiment.

Competition experiment

To examine whether inducible and constitutive PI production incurs a fitness cost, we measured the correlation of plant fitness with PI levels in A and U plants that were not elicited, or elicited with either 150 µg (*) or 250 µg (**) MJ. We used a competition design which has been optimized to detect fitness effects of jasmonate-induced resistance in *N. attenuata* (van Dam and Baldwin 1998, 2001, Voelckel et al. 2001). Ten replicate pairs of plants were assigned to the following 12 treatment groups: untreated controls (1) A – A; (2) U – U; (3) A – U, and induced treatments (see *Plant elicitation*) (4) A –A*, (5) A – A**, (6) U – U*, (7) U – U**, (8) A* -U, (9)A** - U, (10) A – U*, (11)A – U** and (12) A* - U*. Every 2nd day, the pots were watered with 250 ml demineralised water for 6 weeks to mimic the typically growth period in the plant's natural environment. Four d after induction, the source-sink transition leaf was harvested for PI and nicotine analysis. The following fitness estimates were recorded from each plant: (i) stalk length after start of elongation for a period of 17 d and on the last day of watering, (ii) the first day of flowering (when the first flower had fully opened), and (iii) the number of seed capsules two weeks after last watering day. The number of capsules per plants reflects the lifetime reproductive output in *N. attenuata* under natural or greenhouse conditions (Baldwin 1998, Baldwin et al., 1998).

RNA gel blot analysis. The lack of TrypPI activity in the A genotype prompted us to examine the accumulation of PI mRNA. For RNA analysis, 20 shoots of each genotype, 10 from the 150 µg MJ treatment and 10 from the untreated controls were harvested from plants of the competition experiment 3 d after induction. Shoot tissues were pulverized in liquid nitrogen using a mortar and pestle. From this powder, total RNA was extracted using the acid guanidin thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), which was modified by Ogawa (1999) and explained in detail in Winz and Baldwin (2001). RNA was quantified spectrophotometrically at 260, 280 and 320 nm. RNA samples (10 µg) were size-fractionated by 1.2% (w / v) agarose formaldehyde gel electrophoresis and northern blotted

onto nylon membrane (GeneScreenPlus; NEN-DuPont, Boston) as described in the manufacturer's protocol. Ethidium bromide staining of the gel prior to blotting revealed rRNA bands, which served as the loading control (Fig. 2 inset). The PI gene of *N. attenuata* encodes a protein with seven repeat domains, each with a potential reactive site for either chymotrypsin (2 domains) or trypsin (5 domains) and putative signal peptides (K. Gase, D. Hui, A. Patankar, J. Zavala, and I.T. Baldwin in preparation). ^{32}P-labeled probes specific for PI were prepared by PCR using ["-^{32}P] dCTP in the reaction with the corresponding isolated PI plasmid as template and a primer pair allowing the amplification of the multi domain zone of the PI gene. The area of PI mRNA signal was quantified with an Image Quant version 5.1 (Molecular Dynamics, Amersham Pharmacia Biotech, Buckinghamshire UK).

Statistical analysis

Data were analyzed with Statview (Statview, SAS, Institute Inc., Cary, NC, USA). In all experiments PI activity values and volatile release values were log transformed before analysis to meet requirements of normality. The PI and protein data were analyzed by ANOVAs followed by Fisher's protected LSD *post-hoc* comparisons in all experiments, if the ANOVAs main effects were significant. P-values from ANOVAs were corrected with the sequential Bonferroni correction for multiple comparisons whenever appropriate (Holm 1979). Differences in leaf damage in the choice tests were assessed by paired t-tests. Differences in colonization rates of leaf bugs were analyzed with repeated measures ANOVA (RM-ANOVA). Larval mass data were analyzed with RM-ANOVA and a G-test was used to compare frequency distribution of larval instars. Data of developmental parameters and reproductive output from the competition experiment were analyzed by Wilcoxon signed-rank matched-pairs tests (WILCOX) for all comparisons to test for significant differences of competing plant pairs of both genotypes in one pot. Data of the mean differences and the percentage of mean differences (all proportions were arcsine square root transformed before

analysis) in seed capsule number between the different plant pairs were analyzed by ANOVAs. The difference in capsule production between plants in one pot was calculated as x-y (capsule production on the plant with the most seed capsules is considered as x, capsule production of the least productive plant is y), and the percentage difference between plants in one pot was calculated as 100% - (y/x*100%). These values were averaged per treatment combination to obtain the mean differences and percentage mean differences.

Results

Developmental and reproductive parameters. A repeated measures comparison of total plant dry mass (DM) at all harvests found no significant differences among the three genotypes ($F_{2,44} = 2.62$, $P = 0.1173$), but at 25 d, plants of the C genotype were significantly smaller than those of the A and U genotypes (Fig. 1, Table 1). C genotypes had shorter stalks and more branches than plants of the A and U genotype (Table 1), which gave C plants a bushier appearance. Moreover, plants of the C genotype were the first to flower, and as a consequence of the greater number of branches, they produced the greatest number of seed capsules. Plants of A and U genotypes were remarkably similar in their developmental and reproductive parameters, differing only in the initiation of flowering (Fig 1, Table 1). Plants of the A genotype on average started flowering 5 d earlier than those of the U genotype.

Defensive compounds. Plants from the C genotype did not differ significantly in any of the measured chemical defenses from plants of the U genotype (data not shown) and hence we report only comparisons between the A and U genotypes.

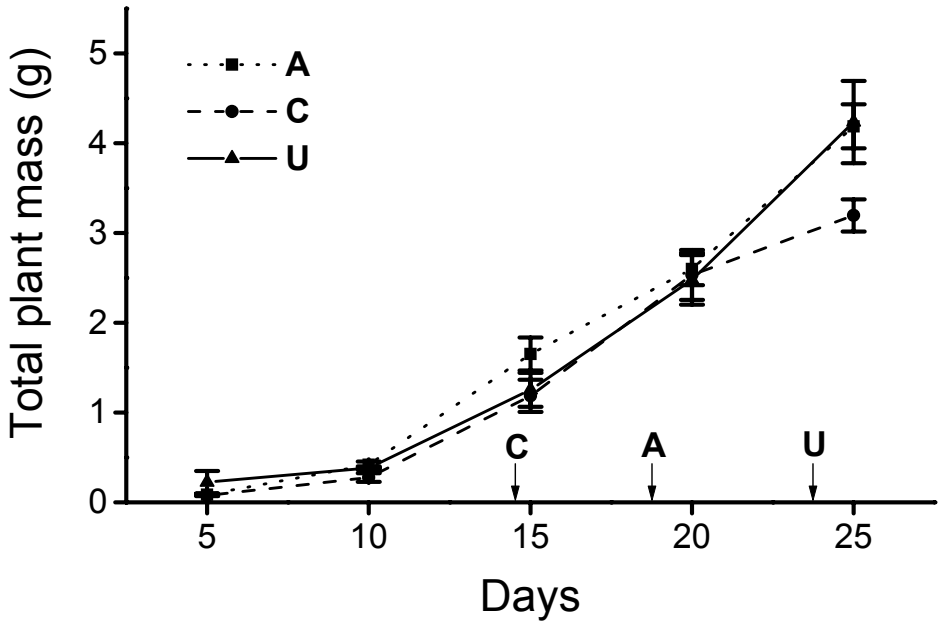

Fig. 1. Mean (± SEM) dry whole-plant biomass of hydroponically-grown plants of *Nicotiana attenuata* genotypes collected from Arizona (A), California (C), and Utah (U). Arrows indicate day of first flower production.

TABLE 1. Differences at day 25 between the 3 genotypes of *Nicotiana attenuata* (Arizona, A; California, C; and Utah, U) in developmental parameters and reproductive output (*P*-values from one-way ANOVAs after Bonferroni correction for multiple comparisons). Bold type depicts significant differences at $P<0.05$. (DM = dry mass)

	Genotype comparison		
	A/ C	A/ U	C/ U
DM shoot (g)	P = 0.0530	P = 0.5953	P = 0.1354
DM root (g)	P = 0.1731	P = 0.3353	**P = 0.0305**
DM whole plant (g)	**P = 0.0475**	P = 0.9157	**P = 0.0391**
Stalk length (mm)	**P = 0.0052**	P = 0.2890	**P = 0.0401**
Number of branches	**P = 0.0001**	P = 0.0532	**P = 0.0001**
Days to first flower	**P < 0.0001**	**P < 0.0001**	**P < 0.0001**
Number of flowers	P = 0.5362	P = 0.5004	P = 0.2077
Number of seed capsules	P = 0.2386	P = 0.1934	**P = 0.0225**

Nicotine: No significant differences were found in either constitutive or MJ-induced nicotine content between both genotypes (ANOVA, genotype effect, $F_{1,8} < 0.755$; *P's* > 0.05). Both genotypes exhibited a similar and significant increase in nicotine contents after MJ elicitation (Fig. 2a; ANOVA, MJ effect $F_{1,8} = 39.137$, $P = 0.0002$).

PIs and protein: All plants of the U genotype showed detectable constitutive PI activity and elicitation with MJ increased this activity up to 4.5-fold (Fig. 2b; ANOVA, $F_{1,8} = 34.346$, $P = 0.0004$). In contrast, all A genotype plants completely lacked constitutive or MJ inducible TrypPI activity (Fig. 2b). Protein levels were not significantly different between genotypes. Northern blot analysis of the accumulation of TrypPI mRNA in the U genotype revealed a 5-fold increase in PI mRNA after MJ elicitation (Fig. 2b). In contrast, PI transcripts were not detectable in A plants, not even after MJ-induction (Fig. 2b). These results demonstrate that MJ-induced PI activity is accompanied by increased PI mRNA production and that the lack of PI activity in the A genotype results from a lesion in PI transcript accumulation.

VOC: Mechanical wounding and OS application significantly increased whole-plant VOC emissions in U plants, as illustrated by the dramatic increase in cis-α-bergamotene release (ANOVA $F_{3,24}=4.253$, $P = 0.0152$; Fisher's PLSD, P = 0.0176). However, OS-elicitation of plants of the A genotype did not significantly increase VOC emission (Fig. 2c; Fisher's PLSD, $P = 0.9466$). Overall, cis-α-bergamotene emission of the U genotype was significantly higher (up to 50-fold) than that of the A genotype (Fisher's PLSD, $P = 0.0057$).

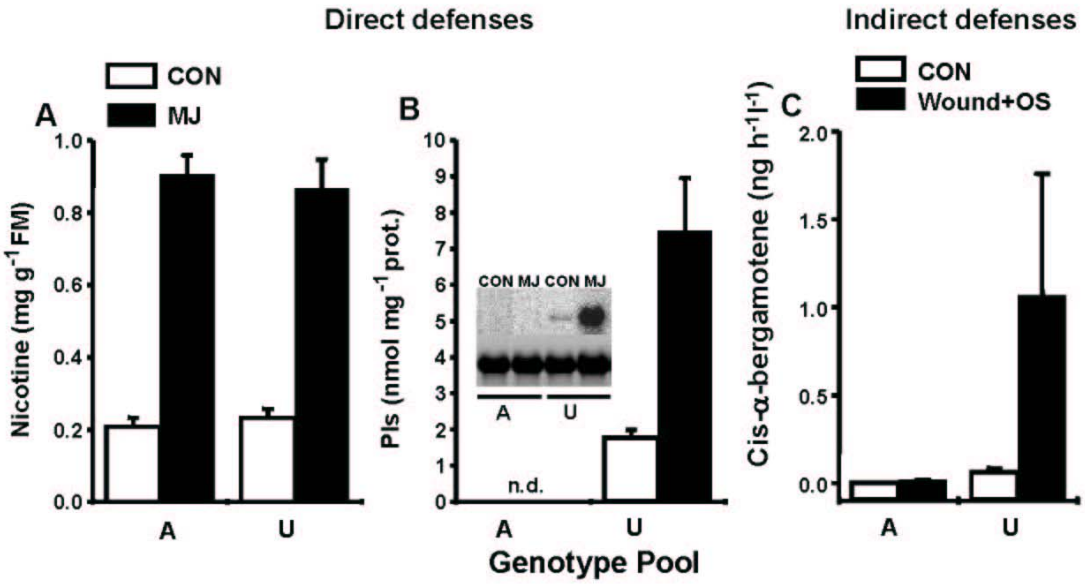

Fig. 2. Arizona (A) and Utah (U) genotypes of *Nicotiana attenuata* differ enormously in their inducible direct and indirect defenses: **A.** Constitutive and methyl jasmonate (MJ)-induced nicotine contents (mean ± SEM) 4 d after elicitation with 250 µg MJ. **B.** PI levels (mean ± SEM) for constitutive and MJ-induced TrypPIs 4 d after elicitation with 250 µg MJ. Inset: Northern analysis of transcripts for uninduced controls (CON) and 150 µg MJ-induced plants (upper band, PI mRNA: 1.5 kb, lower band, rRNA: 3.4 kb). The lower band serves as a loading control, the same amount of RNA was loaded in each lane. (n.d. = not detectable) **C.** Mean (± SEM) cis-α-bergamotene trapped from individual plants grown in whole plant chambers 24 h after a single leaf (source-sink transition leaf) was wounded and treated with 20 µl of *M. sexta* oral secretion (OS). Cis-α-bergamotene is known to be the most consistent component of *N. attenuata*'s herbivore-induced volatile releases from all genotypes.

Utah field experiments. A. *Choice tests.* Together the three herbivores removed significantly more leaf area from A genotype leaves than from U genotype leaves (Fig. 3a; paired t-test, $t_{18} = 2.916$, $P = 0.0276$). However, when compared per species, only the weevil *T. mucrorea* fed significantly more (up to 2.5-fold) on leaves of the A genotype (Fig. 3a; $t_6 = 2.969$, $P = 0.025$).

B. Field colonization. About 10 d after transplantation to the field, *T. notatus* adults began appearing on plants, where they were observed to feed and mate. After 2 weeks, plants of the A genotype had significantly more (up to 2.5-fold) of these leaf bugs than plants of the U genotype (Fig. 3b; ANOVA, $F_{1,34} = 24.342$, $P < 0.0001$).

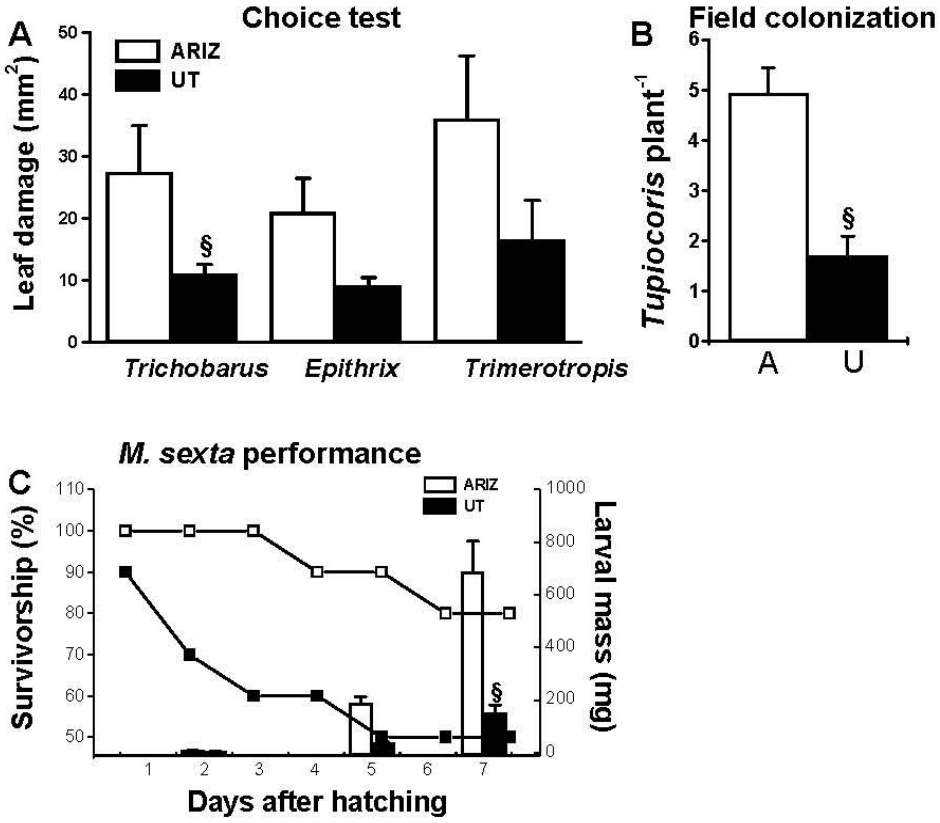

Fig. 3. The Utah (U) genotype is more resistant to feeding from four insect species than the Arizona (A) genotype. **A.** Choice (measures mm^2 leaf eaten) of *Nicotiana attenuata* first stalk leaves from A and U plants of the following three naturally occurring herbivores in choice tests: *Trichobarus mucrorea*, *Epithrix* spp., and *Trimerotropis* spp. Leaves were from bolting plants, which had been elicited with 250 µg methyl jasmonate (MJ) applied to two rosette-stage leaves. Values are the mean (± SEM) of 7 replicate trials for each herbivore. **B.** Mean (± SEM) number of *Tupiocoris notatus* per plant 2 weeks after A and U genotypes of *N. attenuata* were transplanted into the field in a random spatial design. **C.** Percentage survivorship and larval mass of *Manduca. sexta* larvae feeding on soil-grown plants from hatching to 7 days. Values are the mean (± SEM) of ten replicates for each genotype. (***P<0.005).

Laboratory feeding experiment. M. sexta larval development, larval mass and survivorship were significantly different between caterpillars fed on the two *N. attenuata* genotypes (Fig. 3c; G-test for frequency distribution of larval instars, $G_4 = 11.54$, $P = 0.021$; RM-ANOVA on larval mass, $F_{1,18} = 22.974$, $P = 0.0001$). Larvae fed on U plants consistently had lower survivorship (50 % by day 7) and larval mass (approximately 58 % lower by day 7) than those fed on A plants for the duration of the experiment (Fig. 3c). By day 7, all 10 larvae on the A genotype had reached the 4th instar, while on the U genotype, only 1 out of 10 larvae had reached the 4th instar, 8 the 3rd, and 1 the 2nd instar. As expected, larval feeding for 7 d on plants significantly induced TrypPI production in the U genotype (ANOVA, $F_{1,18} = 24.785$, $P < 0.0001$) but again, no PI activity was detected in the A genotype. Protein concentrations decreased by 30 % in both genotypes during the 7 d experiment.

Competition experiment. In order to estimate the fitness consequences associated with constitutive and inducible TrypPI production, we calculated the mean differences and the percentage mean differences in seed capsule production for several treatment groups in the competition experiment (Table 2). Unelicited plants of the A genotype were less sensitive to peer competition than U plants. Although stalk lengths of U and A genotypes did not differ when they were grown in individual pots (Table 1), the stalks of plants in AA pots were significantly taller than those of UU pots (RM-ANOVA, $F_{1,19} = 12.811$, $P = 0.002$). Capsule production, however, did not differ between these treatments (ANOVA, $F_{1,19} = 2.17$, $P = 0.1571$). When untreated A plants competed with uninduced U neighbors in the same pot, they had not only significantly taller stalks, but also flowered significantly earlier and produced significantly more seed capsules than their U neighbor (Table 2, Fig. 4a; WILCOX, $P_{stalk} = 0.0051$, $P_{flower} = 0.0093$, $P_{capsules} = 0.0033$). The difference in seed production between unelicited plants of the two genotypes in competition arose principally from a decrease in reproductive output of the U genotype, rather than an increase in the output of the A genotype (Fig. 4a).

TABLE 2. Absolute and relative mean differences in lifetime seed capsule production from pairs of developmentally synchronized plants from the Arizona (A) or Utah (U) genotypes of *Nicotiana attenuata* that were either uninduced or induced with 150 (*) or 250 (**) µg of methyl jasmonate and grown in the same pot (see Fig. 4 for primary data). *P*-values are from one-way ANOVAs after arcsine square root transformation. Bold type depicts significant differences at $P<0.05$.

Competitors	Mean diff. in capsule number	P	% Mean diff. in capsule number	P
A U	17.000 ± 3.925	**0.0033**	46.072 ± 6.554	**<.0001**
A* U*	23.625 ± 7.744	**0.0157**	56.887 ±10.390	**0.0002**
A A*	16.889 ± 4.228	**0.0109**	35.164 ± 5.602	**<.0001**
A A**	23.456 ± 4.660	**0.0098**	44.762 ± 4.489	**<.0001**
U U*	13.700 ± 6.556	**0.0080**	46.625 ± 7.818	**<.0001**
U U**	16.200 ± 3.463	**0.0069**	61.101 ± 7.325	**0.0003**
A U*	25.700 ± 4.228	**0.0051**	67.948 ± 6.506	**<.0001**
A U**	34.000 ± 2.564	**0.0041**	79.830 ± 5.030	**0.0002**
A* U	8.095 ± 4.936	0.0995	41.647 ±19.765	0.2061
A** U	17.368 ± 5.310	0.0584	14.788 ±25.104	0.5482

MJ elicitation of both genotypes when plants were competing with unelicited plants of the same genotype (Fig. 4b) reduced stalk length only for the U genotype (WILCOX, $P_{U\text{-}U*} = 0.0284$, $P_{U\text{-}U**} = 0.0051$), and delayed flowering (all P's < 0.05) and decreased seed capsule production in both genotypes (all P's < 0.05). The absolute decreases in capsule production in the A genotype (16-23 capsules) were larger than the absolute differences in the U genotype (14 -16 capsules), but the proportional differences were greater in the U genotype (Table 2). Interestingly, the difference in the A genotype treatments resulted from both a decrease in the elicited pair member (representing a cost of elicitation) as well as an increase in capsule production of the unelicited pair member (representing an opportunity benefit). Both costs and opportunity benefits of elicitation were approximately equal and proportional to the degree of

elicitation (Fig 4b; Table 2). In contrast, the difference in the U genotype treatments resulted only from a decrease in capsule production of the elicited member of the pair (Fig 4b). The difference between the two genotypes in their ability to realize an opportunity benefit profoundly influenced the fitness outcome of competition between plants of different genotypes that were differentially induced with MJ (Fig 4c). Elicitation of A plants competing with uninduced U plants did not significantly reduce development or seed output compared to their neighbor (Fig. 4c). Although A plants that were elicited with the highest dose of MJ produced 17 capsules less than their unelicited U competitor, this difference was only marginally significant ($P = 0.0584$; Table 2). In contrast, elicited U plants competing with unelicited A plants, had significantly shorter stalks, flowered later, and produced fewer capsules (26-34 capsules, a 68-80% relative difference; Fig. 4c; Table 2; WILCOX, all P's < 0.05). When both plants were elicited (A*U*), the outcome of the competition was similar to that of AU treatments, but with a larger (by 6 seed capsules) advantage for the A* genotype over U* (Table 2; Fig. 4A,C). Because nicotine induction is similar in both genotypes, this increased fitness costs of induced U* plants may reflect the costs of inducing PIs -and possibly also the costs correlated with VOCs production- specific to the U genotype.

In summary, when unelicited A plants compete with unelicited U plants, the A plants produce on average 17 additional capsules, which is similar to the difference in capsule production when U plants compete with highly elicited U plants (16.2 capsules; Table 2). A plants, but not U plants, realize opportunity benefits when growing next to induced plants which account for approximately half of the fitness differences between induced and uninduced pairs. When a highly elicited U-genotype plant competes with an unelicited A genotype plant, the difference is 34 capsules, half of which (17 capsules) can be attributed to the fitness benefit realized by the A genotype and the other half (17 capsules) agrees well with the estimates of the fitness costs borne by elicited and unelicited U genotype plants.

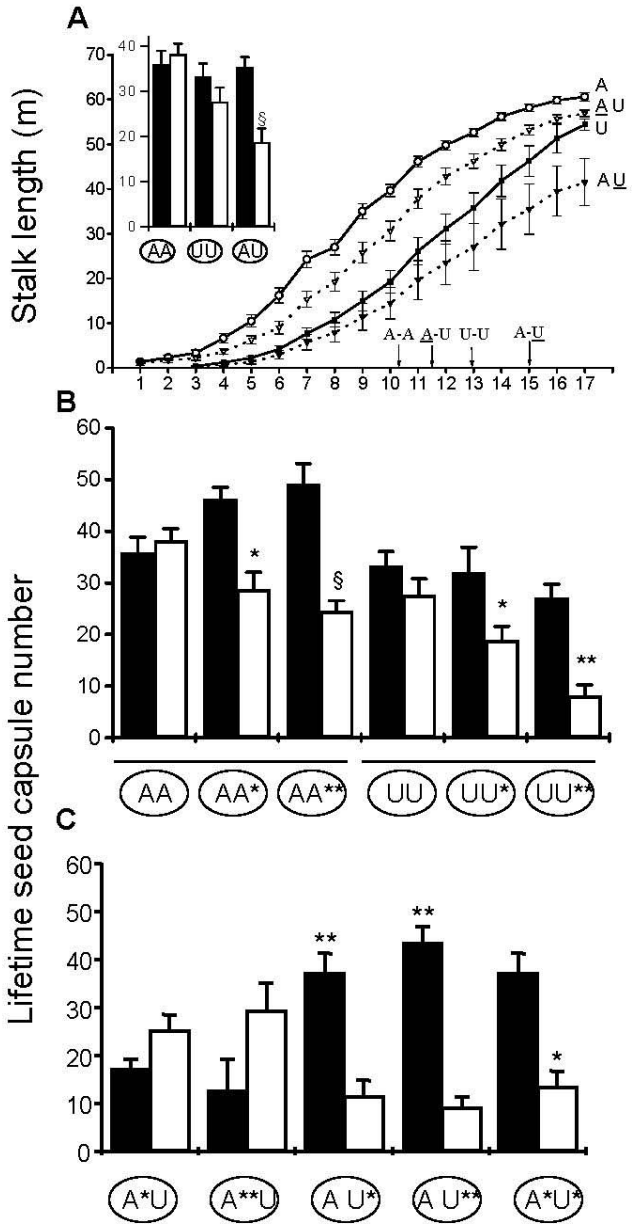

Fig. 4. PI-deficient *Nicotiana attenuata* plants realize a significant fitness benefit when grown under intense intraspecific competition. Plants from the Arizona (A) and Utah (U) genotype were either uninduced or induced with 150 (*) µg or 250 (**) µg of methyl jasmonate (MJ) and grown in the same pot (* within circular pot symbols) in 12 different treatment groups. For all treatment groups, stalk length, days to first flower, and lifetime seed capsule production were analyzed as fitness estimates. **A.** Treatments without MJ elicitation: mean (± SEM) stalk length, starting on the day with measurable stalk growth for 17 subsequent days and mean (± SEM) lifetime seed capsule production (inset). Arrows depict the mean day of first flowering. (Significant differences: stalk length on day 17, $P_{ARIZ-UT}$ = 0.0290; first flower, $P_{ARIZ-UT}$ = 0.0093). Treatments with MJ elicitation: **B and C.** Mean (± SEM) lifetime seed capsule number produced by plants in different treatment combinations. Number of asterisks above columns indicates level of significant differences between members of a pair (*P<0.05, **P<0.01, ***P<0.005). In C (but not A and B) the A genotype is depicted by solid bars and the U genotype by open bars.

Discussion

Genotypes from different geographical regions clearly differ in morphology, phenology and defensive chemistry. The California genotype flowers earlier and attains a smaller, bushier growth-form compared to the genotypes collected from Utah and Arizona, which, in turn, are phenologically and morphologically very similar (Fig. 1). The C genotype was collected from a disturbed roadside habitat, while both A and U genotypes were collected from populations growing in juniper-pine habitats that had burned the previous year. Possibly, the earlier flowering C genotype may realize a higher fitness in the microclimatic and disturbance regimes of road-side habitats, while later flowering genotypes may have an advantage in one-year old burns, with their modest herbivore loads (Baldwin 1998) and high soil nitrogen levels (Lynds and Baldwin 1997).

Dramatic differences in the chemistries mediating both direct (PIs) and indirect (VOCs) defenses were found between the two morphologically similar U and A genotypes. The PI-deficient and VOC-impaired A genotype is the first reported natural ecotype to lack components of both an inducible direct and indirect defense. PI production is a widespread character in the Solanaceae (Koiwa et al. 1997), but the extent to which it is genetically polymorphic trait is unknown. Additionally, the A genotype does not increase cis-α-bergamotene emissions after elicitation, although this VOC previously was the most consistently released component of herbivore-induced volatile bouquet from a number of *N. attenuata* genotypes (Halitschke et al. 2000). It is unclear whether the lack of VOC induction is, similar to the lack of PI protein accumulation, associated with a deficiency in mRNA accumulation. Ongoing molecular work is addressing the mechanisms responsible for the lack of PI transcript accumulation and how this correlates with the lack of VOC emissions.

The two chemically-distinct but morphologically similar genotypes from Utah and Arizona were used to examine the costs and benefits associated with PI production. The

results of our bioassays and field colonization experiments (Fig 3A,B) strongly implicate the difference in PI levels as being responsible for the differences in resistance between the two genotypes, because they did not differ in either induced or constitutive nicotine production. This is consistent with studies reporting that transformation of agricultural varieties with heterologous PIs can provide durable resistance against leaf-feeding herbivores (Johnson et al. 1989, Wolfson and Murdock 1990, Broadway and Colvin 1992, Jongsma et al. 1994, Heath et al. 1997, but see Winterer and Bergelson 2001). The dramatically lower performance of *M. sexta* larvae on plants of the U genotype (Fig 3C) is likely a response to both constitutive and induced PI activities. Moreover, *M. sexta* feeding decreased protein contents in leaf extracts of both genotypes, which may have exacerbated the effects of the protease inhibiting activities of the PI (Duffey and Stout 1996). However conclusive evidence that PIs are directly responsible for the observed effects will require the silencing of the endogenous PI gene in the U genotype or expressing the PI gene from the U genotype in A-genotype plants.

The VOCs, on the other hand, at the concentrations released from plants, are not known to have direct effects on feeding herbivores, and their role in resistance appears to be in attracting predators and parasitioids to the feeding herbivores or reducing oviposition by adults (Dicke 1994, De Moraes et al. 1998, 2001, Kessler and Baldwin 2001). The experimental enhancement of cis-α-bergamotene emissions in the field is known to dramatically attract a generalist predator (Kessler and Baldwin 2001), but whether plant genotypes that lack the ability to release this and other VOCs are unable to attract predators when attacked is unknown. Since the volatiles that potentially attract herbivores to their host plants are unknown for the insects used in our bioassays, their choices may reflect an avoidance of plants releasing VOCs to avoid potentially higher predation pressure as well as PI laced tissues. Predator avoidance may have contribute to the strong preference of *Tupioccoris* for the A genotype in the field plantation, however it is unlikely to account for

the preferences of excised leaves from A plants in the sealed chambers of the choice tests, where VOC concentrations differences would be unlikely.

While our experiments clearly show that producing PIs is correlated with insect resistance our results also demonstrate that there is a strong opportunity cost associated with PI production. The competitively-mediated cost of constitutive and inducible PI production resulted in an opportunity benefit for the PI deficient A genotype growing adjacent to a PI producing U genotype. Plants of the U genotype, in contrast, were not able to realize an opportunity benefit when growing in competition with an MJ-treated plant of either genotype. The fitness consequences associated with both constitutive and inducible PI production are large: the observed 17 capsule differences represent approximately a 46% decrease in reproductive output (Fig 4; Table 2). The fitness consequences of inducible PI production can be estimated by comparison of differences in seed capsules between AU and A*U* treatments. Elicitation of both genotypes increased the difference between plants in one pot by 6 capsules, a fitness cost of approximately 20%. Again tests of the hypothesis that PI production is directly responsible for the observed fitness differences await competition experiments with A genotype plants transformed to express PIs and U genotype plants with silenced PI genes.

Our results differ from those obtained in earlier experiments with tomato in which PI accumulations was elicited with chitin injections (Brown 1988). In these experiments, seed output of chitin-treated plants was indistinguishable from that of untreated control plants, despite the large difference in inhibitor contents. Tomato appears to be particularly well buffered against decreases in seed and fruit production (Thaler 1999) and the plants were grown in individual containers with a high nitrogen supply rates. Thus plants actually might only incur the cost of inhibitor production when competing with others, making the costs of PI production are mainly opportunity costs (van Dam and Baldwin 1998, 2001).

Because PI production is a heritable trait (van Dam, unpublished results) and involves both costs and benefits, natural selection may act on PI levels in *N. attenuata*. The geographic location, e.g. altitude, of plant populations may be of importance for the level of a chemical defense, because it is linked with the intensity of herbivore pressure that plants experience (Daday 1954, Carey and Wink 1994, Salmore and Hunter 2001). The Utah and Arizona populations are separated by the Grand Canyon and more than 500 km in distance and 2 km in height above sea level. Hence the two populations are likely to have minimal direct gene flow between them.

Given this geographic isolation, it is tempting to speculate that the linkage between PI (or other unmeasured direct defenses) and VOC production is adaptive, as PI expression frequently slows the growth rate of insect herbivores by making their digestive processes less efficient (Winterer and Bergelson 2001, Cloutier et al. 2000, Mochizuki et al. 1999, Charity et al. 1999). Hence the fitness benefits of PI expression may result from extending the period during which larvae can be successfully attacked by natural enemies. Moreover, expression of VOCs without direct defenses that slow the growth of herbivores may represent a liability if these VOCs allow potential herbivores to locate host plants (Kalberer et al. 2001). Alternatively, the correlated loss of herbivore-induced VOC and PI production in the A genotype may be caused by random effects, such as genetic drift or founder effects (Maynard Smith1989).

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Manuscript 2

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Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*

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Abstract

Plant trypsin proteinase inhibitors (TPIs) are potent herbivore- and jasmonate-induced defenses, but support for the commonly-invoked explanation for their inducible expression, namely their associated fitness costs, has been elusive. To determine whether the expression of TPIs incurs fitness costs, we expressed 175 bp of the 7-domain *pi* from *Nicotiana attenuata* in an anti-sense (AS) orientation in a TPI-producing genotype (WT) of *N. attenuata* to reduce TPI expression. Moreover, we expressed the full-length 7-domain *pi* in a sense orientation (S) under control of a constitutive promoter to restore TPI activity in a natural genotype unable to produce TPIs due to a mutation in its endogenous *pi* gene. Lifetime reproductive output was determined from high- and low-TPI producing plants of the same genetic background with and without jasmonate (JA) elicitation and grown in the same pot to simulate natural competitive and nutrient regimes. Transformants with either low or no TPI activity grew faster, taller, flowered earlier and produced more seed capsules (25-53%) than did neighboring TPI-producing genotypes and JA elicitation increased TPI production and decreased seed capsule production further. Growth under high light levels only marginally reduced these fitness costs. Results were similar regardless whether TPI activity was suppressed or restored by transformation: the larger the difference in TPI activity between neighbors, the larger the difference in seed capsule production ($R^2 = 0.57$). TPI production is costly for a plant's components of fitness when grown under realistic competitive regimes and is consistent with the hypothesis that inducibility evolved as a cost-saving mechanism.

Introduction

Although defenses might benefit plants in the presence of herbivores, plant resistance to herbivores can be costly in the absence of plant enemies (1-4). This cost-benefit paradigm has motivated most of the theory about the evolution of plant defenses against herbivores (5-8) but conclusive evidence attributing fitness costs to a particular defense trait has been elusive, but recent studies have made significant advances (9, 10). The paradigm has been difficult to test for two reasons: 1) fitness costs, which can be measured as reductions in either male (11) or female (1, 9, 12) reproductive function, arise from many different types of compromises that could result from the expression of defense traits; 2) the fitness costs of a defense trait must be disentangled from the costs of genetically correlated traits (13-15).

The fitness costs of defense can arise from processes both internal to the plant, such as when fitness-limiting resources are allocated to defenses which cannot be rapidly re-allocated to growth and reproduction (16) or auto-toxicity (2, 17, 18) as well as external processes (ecological costs) which occur when defense expression results in reduced pollination, attracts enemies, or impairs the expression of other resistance traits (3, 19, 20). Fitness measures integrate a plant's performance in a given environment and consequently should be measured under conditions commonly found in the plant's natural environment (20, 21). For example, the large reductions in lifetime seed production associated with jasmonate (JA)-elicited herbivore resistance in *Nicotiana attenuata* were only found when plants were grown with competitors (22-24), one of the dominant selective factors for this species, which synchronizes its germination from long-lived seed banks after fires in the Great Basin Desert (12). Hence costs may not be

apparent in experiments on isolated plants grown under optimized conditions; this contingency makes negative evidence for fitness costs difficult to evaluate.

While experimental work with natural populations ensures realism in the measurement of potential costs, demonstrating that a fitness costs can be attributed to the expression of a defense is difficult in genetically heterogeneous natural populations (2). Ideally, one should determine the cost of defenses in plants that differ only in the gene that controls the expression of a resistance trait but are otherwise genetically identical (25). Many defense traits are elicited after herbivore attack and inducible expression is thought to allow plants to forgo the costs of defense when they are not needed, namely in environments without pests or pathogens. Numerous studies (reviewed in 2, 3) have exploited inducible expression as a means of controlling for, or homogenizing, the genetic background of plants and have measured plant fitness before and after eliciting resistance in plants in herbivore-free environments. The discovery that herbivore attack elicits the JA cascade in many species, and that exogenous JA treatments elicits induced resistance without the wounding that normally accompanies herbivore attack, has motivated studies to measure the fitness costs of JA-induced responses (1, 10, 26-29). However due to pleiotropic effects of the elicitors, the observed fitness differences do not arise solely from the expression of the resistant trait (12, 30) and therefore these studies are likely to overestimate the fitness costs of resistance.

These experimental difficulties can be addressed with mutants defective in the endogenous production of the defense elicitors, but most studies focusing on molecular aspects of resistance signaling do not report factors such as growth rate or seed set (20). A recent exception to this trend is a study that used the *jar1-1* mutant in *Arabidopsis*

thaliana, which is deficient in JA signaling and expression of proteinase inhibitors, but surprisingly found greater reductions in seed production after JA elicitation in the mutant line than in the Col wildtype lines (10). Transformation technology provides a novel approach to manipulating plant resistance traits. Recently, an elegant study that rigorously controlled for potential differences in genetic background demonstrated that the presence of a particular R-gene (*RPM1*) that confers resistance against particular strains of *Pseudomonas syringae* pathogens decreased reproductive output by 9 % in *Arabidopsis thaliana* (9). The R gene protein functions as the receptor for the pathogen elicitors, the *AvrRpm1* or *AvrB* proteins, but the responses elicited by this pathogen recognition system responsible for the decrease in reproductive output are unknown. The *A. thaliana* genome contains more than 100 R genes and it is unlikely that the expression of each results a 9% fitness reduction.

Here we used *Nicotiana attenuata* to examine the fitness consequences of trypsin proteinase inhibitor (TPI) production, an established defense against a variety of different herbivores (24, 31). We compared the components of fitness of *N. attenuata* genotypes with either low or no TPI production with that of TPI-producing genotypes in competitive experiments in which plants were either elicited or not with methyl jasmonate (MeJA) applications to increase TPI production and other insect resistance traits. We compared two independently transformed *N. attenuata* lines in which the expression of the *pi* gene was down-regulated by antisense expression of a 175 bp fragment of the *N. attenuata pi* gene with two lines independently transformed with empty vector constructs, which had fitness and PI production not distinguishable from untransformed wildtype (WT) plants of the same genetic background (an inbred line

collected from Utah). We additionally compared the fitness of an untransformed *N. attenuata* genotype collected from Arizona (A), which has a mutation in the endogenous 7-domain *pi* gene and does not produce *pi* transcripts or TPI activity, with A plants transformed with the full length cDNA of the 7-domain *pi* gene in a sense orientation under control of a constitutive promotor, which produced TPIs at 60% of the level found in MeJA-elicited WT Utah genotype plants. These constructs allowed us to compare the fitness consequences of TPI expression both by silencing endogenous TPI production in TPI-producing genotypes and by restoring TPI production in the mutant A genotype by expressing a functional *pi*. Our data demonstrate that constitutive and inducible TPI production incurs large fitness costs when plants compete against plants of the same genetic background that lack the ability to produce TPIs. Previous work with the A genotype (24) and on-going research with all genotypes used in this study (32) demonstrates that TPI expression profoundly determines herbivore resistance.

Materials and Methods

Plant material and transformation, and DNA Isolation and Southern Hybridization. Are described in the Supplemental Information (www.pnas.org).

*Fitness consequences of TPI expression***.** We used a competition design optimized to detect the fitness consequences of jasmonate-induced resistance in *N. attenuata* and simulates the soil mineral nutrition and competition levels that typify this plant's natural habitat (12). In this experimental setup, plants compete for below-ground resources (23) as they do in their natural habitat, and are synchronized in their germination and early growth, so that all fitness-based differences result from differences in performance during

competitive growth (22-24; Suppl Information). Seeds were germinated in diluted liquid smoke solutions as described in (33). Two seedlings of similar size and appearance were transplanted 7 cm apart in 2 L pots in a glasshouse in the conditions described in (24; Suppl Information) with minimum 800 – 900 μmol m^{-2} s^{-1} PPFD supplied by 450 W Na-vapor HID bulbs.

Genotypes (C1, C2: empty vector transformed WT; AS--, AS-: WT transformed with a construct containing the *pi* gene in an antisense orientation; A: Arizona genotype that completely lacks the ability to produce TPIs; S++: A transformed to express the functional *pi*) were grown in 3 different combinations which represented three separate experiments: AS-- competing with C1 (AS-- vs C1), AS- competing with C2 (AS- vs C2) and A competing with S++ (A vs S++). The choice of particular AS-C pairs were randomly chosen, as informed by the results of a preliminarily experiment.

A preliminary competition experiment was conducted to determine whether the two empty vector transformed lines (C1, C2) differed from each other in lifetime seed production: 9 replicate pairs of C1-C2 plants were grown in a glasshouse under minimum 1000 - 1300 μmol m^{-2} s^{-1} PPFD irradiance. No significant differences in seed capsule production were found in the experiment: C1 (94 ± 7.1) competing with C2 (103 ± 10.6; paired *t* test, $t_{8\text{-C1-C2}} = 0.585$; P = 0.57).

In each of the three experiments, individual plants were either uninduced (CON) or elicited with 150 μg of MeJA (*) and pairs of plants were assigned to the following 3 treatment groups: 1) CON-CON (AS-- vs C1; AS- vs C2; A vs S++), 2) MeJA-MeJA (AS--* vs C1*; AS-* vs C2*; A* vs S++*) and 3) CON-MeJA (AS-- vs C1*; AS- vs C2*; A vs S++*). Either pure lanolin paste (20μl) or lanolin paste containing 150 μg of

methyl jasmonate (MeJA) in 20µl was applied to the node +1 (one position older than the source-sink transition leaf) leaf of each plant as described in (34) 11 days after transplanting to 2 L pots. Each experiment had 14 replicate pairs for each of the three treatment groups: 4 randomly selected pairs were destructively harvested for chemical characterization, while the remaining 10 were used for growth and fitness characterization. The entire AS- vs C2 experiment was replicated under the same growth conditions, while the entire AS-- vs C1 experiment was replicated under higher irradiance (minimum of 1000 - 1300 µmol m^{-2} s^{-1} PPFD) supplied by 600 W Na-vapor HID bulbs. As an additional check on the choice of pairing, 7 replicate pots of AS- vs C1 for the CON-CON treatment were established.

Treated leaves were harvested 24 h after induction for Northern blot analysis of TPI mRNA accumulation as described in (24, 35) in 4 replicate plants from each genotype and treatment and pooled. These plants were excluded from subsequent analysis. Constitutive and MeJA-induced TPI activity and nicotine were determined from all remain replicates at the rosette stage. Leaves growing at node 0 (source-sink transition leaf) were harvested 3 days after elicitation and protein concentrations and TPI activity was measured by radial diffusion assay and expressed as nmol mg^{-1} as described in (34). Nicotine concentrations were measured by HPLC as described in (36) and expressed as mg g^{-1} FM (fresh mass).

To compare the lifetime reproductive performance among genotypes, we recorded for each plant: i- stalk length starting on the day with measurable stalk growth (14 days after transplanting) for 22 subsequent days, ii- the day of first flowering (when the first flower had fully opened), and iii- the number of seed capsules 51 d after transplanting.

Watering was stopped 15 d prior to mimic the typically growth period in the plant's natural environment. The number of capsules per plant reflects the lifetime reproductive output in *N. attenuata* under natural or glasshouse conditions (1, 26).

Statistical Analysis. Data were analyzed with Statview (Statview, SAS, Institute Inc., Cary, NC, USA). The TPI and nicotine were analyzed by ANOVAs followed by Fisher's protected LSD *post-hoc* comparisons in all experiments. Data of reproductive output from the competition experiments were analyzed by paired *t*-tests for all comparisons of competing plant pairs in one pot. Differences in stalk elongation between competitors were analyzed with repeated-measures ANOVA. Data of the mean differences and the percentage of mean differences in seed capsule number (all proportions were arcsine square root transformed before statistical analysis to correct non-normality) between the different plant pairs were analyzed by ANOVAs. The difference in capsule production between plants in each pot was calculated as x-y (capsule production from the plant with higher seed capsules is considered as x and capsule with lower seed capsule production as y). The percentage differences between plants in seed capsule production were calculated as 100 % - (y/x*100%). These values were averaged per treatment combination to obtain the mean differences and percentage mean differences.

Results

*Characterization of transgenic plants***.** In order to silence the expression of *N. attenuata*'s *pi* gene, WT was transformed with pNATPI1 (Suppl Information) containing a 175 bp of *N. attenuata*'s *pi* gene in an anti-sense orientation under the control of CaMV

35S promoter. Two initial transformants (T_0), AS-- and AS-, were selected for low and intermediate TPI expression as determined from an activity assay (34). Southern analysis (Suppl Fig. 2) and segregation ratios (3:1 for both lines) for NTC resistance revealed that both AS-- and AS- contained one copy of T-DNA at one locus. A genotype of *N. attenuata* collected from Arizona (A), which completely lacks the ability to produce TPI (24), was transformed with pRESC2PIA2 (Suppl Information) containing the full-length *N. attenuata pi* gene in the sense orientation under the control of CaMV 35S promoter. Southern analysis (Suppl Fig. 2) and segregation ratios (3:1) of hygromycin resistance revealed 1 copy of T-DNA present at one locus. To examine the constitutive and inducible levels of TPI mRNA of the transformed lines, a Northern blot analysis was performed on total RNA from transformed lines (AS--, AS-, S++, C1 and C2) and untransformed genotypes (WT and A). Unelicited untransformed WT and lines transformed with empty vector constructs (C1 and C2) revealed a 1.4 kb TPI transcript which increased four fold 24 h after elicitation with 150 µg of MeJA (Fig. 1A). While TPI transcripts were not detectable in AS-- plants, not even after MeJA-elicitation, intermediate levels were found in AS- line (Fig. 1A). The difference in TPI expression between the two lines is likely due to differences in transgene insertion sites. TPI mRNA in A genotype, which lack the ability to produce TPIs (24), was not detectable, and constitutive and inducible levels in A genotype plants which were transformed with the full-length *N. attenuata pi* gene in the sense orientation (S++), were similar to the constitutive levels found in WT plants (Fig. 1A). TPI mRNA accumulation correlated well with TPI activity levels.

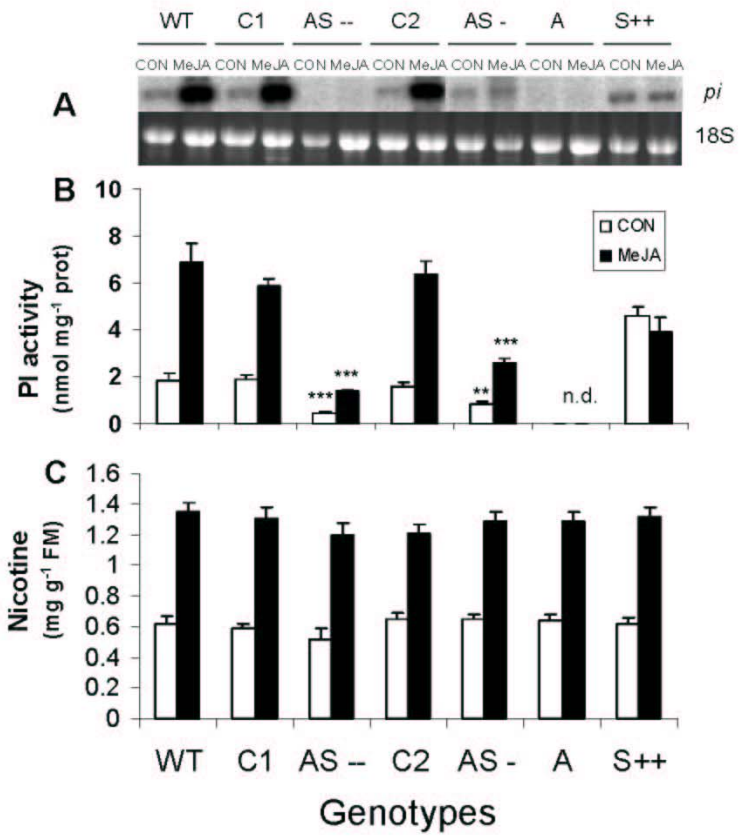

Fig. 1. Northern blot analysis of trypsin proteinase inhibitor (TPI) mRNA and concentrations of two direct defenses (nicotine and TPI activity) in: untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT), two homozygous T_3 independently transformed lines of the Utah genotype that had been transformed either with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--, AS-), or with an empty vector construct (C1, C2); untransformed plants of the Arizona (A) genotype and plants of a homozygous T_3 transformed line of the Arizona genotype transformed with a construct containing the full-length *pi* gene in a sense (S++) orientation. Methyl jasmonate (MeJA: 150 µg) in a lanolin paste or pure lanoline (CON) was applied to leaves growing at node +1 (one position older than the source-sink transition leaf: node 0) at the rosette stage 11 days after transplanting. Asterisks indicate the level of significant differences between members of pairs (*P<0.05, **P<0.001, *** P<0.0001). **A.**

RNA gel blot analysis of *pi* gene transcripts in +1 leaves of unelicited control (CON) and MeJA-elicited plants 24 h after elicitation (upper band, TPI mRNA: 1.4 kb, lower band, 18S rRNA: 3.4 kb). **B**. TPI activity (mean ± SEM) in leaves at node 0 in CON and MeJA-elicited plants 3 d after elicitation (n.d. = not detectable). **C.** Nicotine concentrations (mean ± SEM) in leaves at node 0 in control (CON) and MeJA-elicited plants 3 d after elicitation.

Leaf TPI activity was determined before and 3 d after elicitation with 150 μg of MeJA in transformed and untransformed genotypes. Compared to the constitutive levels of TPI activity in the WT, C1 and C2 plants (which did no differ significantly; $F_{2,92}$ = 0.593; P = 0.55), levels in AS-- and AS- plants were 77 % and 50 % lower, respectively (Fig. 1B; $F_{4,166}$ = 14.397; P < 0.0001). Elicitation with MeJA increased TPI activity 3.6 fold in WT, C1, C2 plants, while AS-- and AS- TPI levels were 22 % and 40 % of their respective controls (Fig. 1B; $F_{4,166}$ = 38.851; P < 0.0001). MeJA-elicitation did not alter ($F_{1,55}$ = 1.007; P = 0.31) TPI activity in S++ plants, which remained at approximately 61 % of the induced WT plants (Fig. 1B; $F_{1,55}$ = 7.742; P = 0.007). As expected, the untransformed A genotype showed no TPI activity even after induction with MeJA (Fig. 1B).

To facilitate comparison between constitutive and inducible TPI activity measures between members of competitor pairs and the fitness differences between competitors, we calculated the TPI activity difference between pairs. While MeJA elicitation increased the difference of TPI activity between pairs from 1.4 ± 0.2 to 4.5 ± 0.7 nmol $prot^{-1}$ in AS-- vs C1 (3.2 fold) and from 0.7 ± 0.2 to 3.7 ± 0.5 nmol $prot^{-1}$ in AS- vs C2 (5.1 fold), no difference was found in the A vs S++ pair (from 4.6 ± 0.8 to 3.9 ± 1.6 nmol $prot^{-1}$). The difference of TPI activity at the constitutive level in AS-- vs C1 pair was higher (2 fold) than in AS- vs C2 pair ($F_{1,74}$ = 5.025; P = 0.02), while after elicitation the difference of

TPI activity in the AS-- vs C1 pair was only 20 % higher than that of AS- vs C2 ($F_{1,74}$ = 1.156; P = 0.28). Transformation with *pi* genes did not affect constitutive and inducible nicotine production, another nitrogen-intensive direct defense of *N. attenuata* (12). No significant differences were found in either constitutive or MeJA-induced nicotine content among genotypes (Fig. 1C; $F_{6,98\text{-CON}}$ = 0.925; P = 0.48; $F_{6.98\text{-MeJA}}$ = 0.59; P = 0.73).

Fitness consequences of differential TPI expression. We measured stalk lengths during elongation, seed capsule number, the day of first flowering and calculated the mean differences and the percentage mean differences in seed capsule production for pairs with the same elicitation in order to estimate consequences of constitutive and inducible TPI production for components of fitness. Growth under higher irradiance levels increased the number of seed capsules produced and the difference in capsule production between pairs (by 68-51 capsules in AS-- vs C1, with a 17 capsule increase in the difference), but the relative difference in seed capsule production was similar between different irradiance environments (Fig. 3; Table 1; Suppl Fig. 3; Suppl Table 1). When untreated AS--, AS- lines and A genotype competed with uninduced C1, C2 and S++ neighbors, respectively, they not only grew faster and produced significantly taller stalks, but flowered earlier (Fig. 2A, B and C; repeated measures ANOVA, $F_{1,18\text{-AS1-C1}}$ = 4.686; P = 0.04; $F_{1,18\text{-AS2-C2}}$ = 9.175; P = 0.007; $F_{1,18\text{-A-S++}}$ = 256.227; P<0.0001) and produced significantly more seed capsules (Fig. 3A, B and C; Suppl Fig. 3 A and B) than their neighbors. The choice of C and AS pairs did not influence the results. Similar results was found when the AS- competed with C1; AS- plants produced more seed capsules (39.2 ±

2.6) than C1 lines (29.8 ± 1.3) with 9.4 capsules difference between neighbors (paired *t* test, $t_6 = 2.361$; P = 0.056).

Table 1. Absolute and relative mean differences in lifetime seed capsule production from pairs of developmentally synchronized plants from homozygous T_3 independently transformed lines of a WT genotype of *Nicotiana attenuata* which had been transformed with constructs containing a *pi* gene fragment in an anti-sense orientation (AS--, AS-), or an empty vector construct (C1, C2); untransformed plants of the Arizona (A) genotype and plants of the Arizona genotype transformed with constructs containing the full-length *pi* gene in a sense (S++) orientation. Two plants were grown in the same pot in 3 different pair combinations (AS-- vs C1, AS- vs C2 and A vs S++). Plants were either treated with 150 µg of MeJA in 20 µl of lanolin paste (*) to elicit jasmonate-induced defenses or treated with 20 µl of pure lanolin paste as controls; n = 10 per treatment and pair combination. P-values are from paired *t*-Test comparisons; relative values were arsine square root transformed prior to analysis.

Competitors	**Mean diff. in Capsule number**	**P**	**% Mean diff. in capsule number**	**P**
AS-- vs C1	16.8 ± 2.9	0.0003	33.2 ± 4.3	0.0001
AS--* vs C1*	20.8 ± 0.9	<0.0001	54.2 ± 1.8	<0.0001
AS1-- vs C1*	38.4 ± 2.5	<0.0001	64.9 ± 4.5	<0.0001
AS- vs C2	10.6 ± 1.8	0.0032	25.7 ± 3.0	0.0063
AS-* vs C2*	14.0 ± 1.0	<0.0001	36.8 ± 2.8	<0.0001
AS- vs C2*	19.0 ± 3.2	0.0005	43.3 ± 5.4	<0.0001
A vs S++	70.2 ± 10.7	0.0001	53.4 ± 5.3	<0.0001
A* vs S++ *	60.3 ± 3.8	<0.0001	60.7 ± 3.5	<0.0001
A vs S++ *	136 ± 5.5	<0.0001	75.5 ± 3.2	<0.0001

The highest absolute and relative difference of seed capsule production was observed in the A vs S++ pair (70.2 capsules; 53.4 %) and the lowest in the AS- vs C2 (10.6 capsules; 25.7 %, and 9.5 capsules; 25.0 %) pair (Table 1; Suppl Table 1). Delay in first flower production within pairs was 1 d in AS-- vs C1, 2 d in AS- vs C2 and 3 d in A vs S++ (Fig. 2A, B and C; ANOVA, $F_{5,54\text{-Flower}} = 8.879$; $P < 0.0001$).

When both competitors were elicited with MeJA, stalk length (Fig. 2A, B and C; repeated measures ANOVA, $F_{1,18\text{-AS1-C1}} = 10.411$; $P = 0.004$; $F_{1,18\text{-AS2-C2}} = 2.661$; $P = 0.1$; $F_{1,18\text{-A-S++}} = 36.751$; $P<0.0001$) and seed capsule production (Fig. 3A, B, and C; Suppl Fig. 3A and B) decreased in both competitors, and the relative difference between competitors in seed capsule production were amplified in comparison to the differences observed when neither competitor was elicited: 21.0 % and 20.2 % increases in the medium and high light replicates of AS--* vs C1*; 11.1 % and 16.1 % increases for the two replicate experiments of AS-* vs C2* (Table 1; Suppl Table 1). These increases in fitness costs are commensurate with MeJA-elicited increases in TPI production (Fig. 1B). The smallest increases in MeJA-elicited fitness costs were observed in S++ genotypes (ANOVA, $F_{2,27} = 2.027$; $P = 0.15$; a 7.3 % increase in A* vs S++*; Fig. 3C). MeJA elicitation did not increase TPI production in S++ plants, because *pi* is under control of a constitutive promotor in these plants. After MeJA elicitation, flowering was delayed by 1 d in all cases (Fig. 2A, B and C).

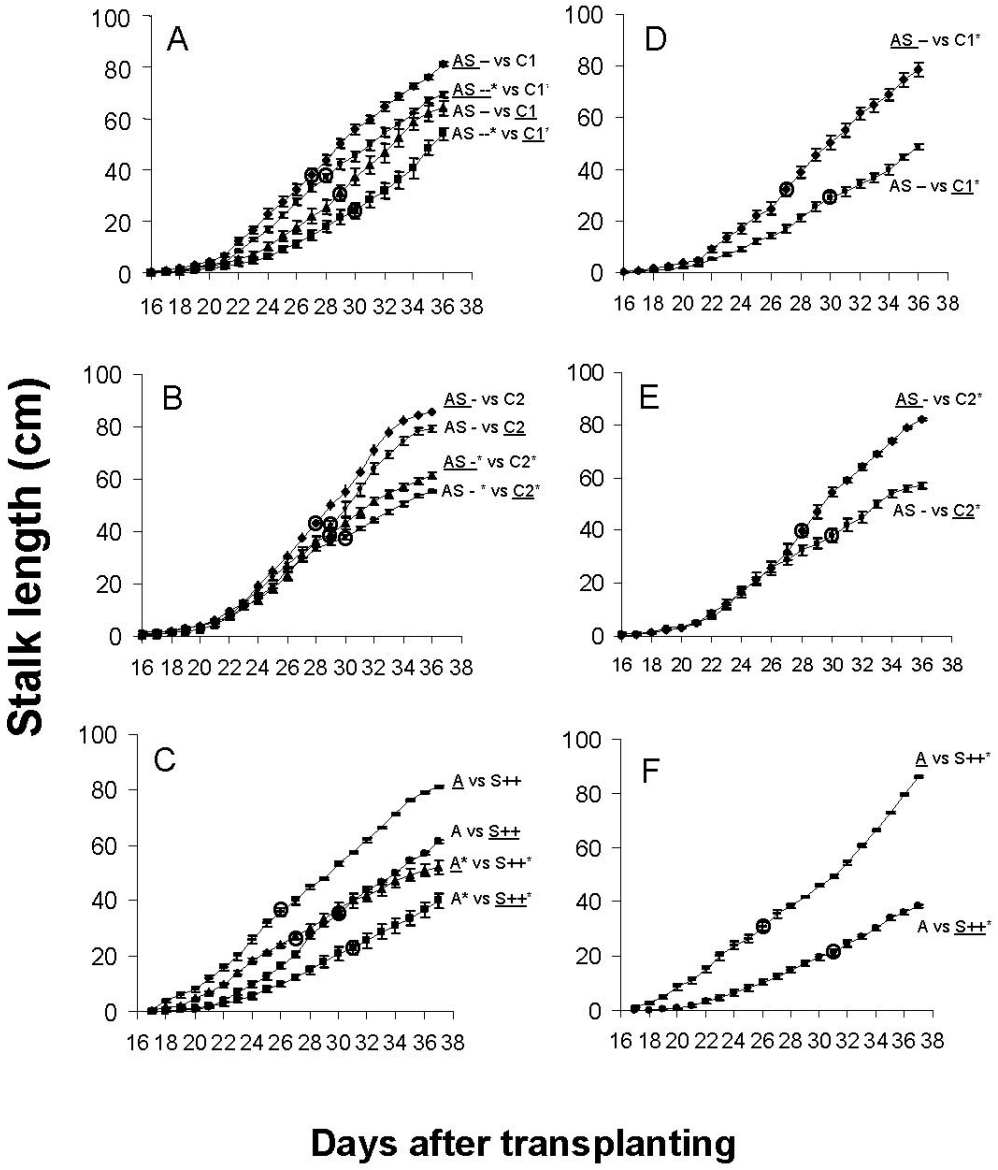

Fig. 2. Growth and flowering time of *N. attenuata* genotypes differing in TPI production (see Fig. 1 caption for abbreviations) grown in competition with each other and were either uninduced or elicited with 150 µg (*) of MeJA. Stalk lengths (mean ± SEM) of the genotype (underlined) starting on the day with measurable stalk growth for 22 subsequent days; circles depict the mean day of first flowering. *Left panels*: Both competitors from the same pot had the same treatment: either MeJA (*) or control. *Right panels*: Competitors from the same pot had different treatments: either MeJA (*) or untreated. Stalk length (mean ± SEM) of: AS-- and C1 genotypes **(A and D);** of AS- and C2 genotypes **(B and E);** of A and S++ genotypes **(C and F)**.

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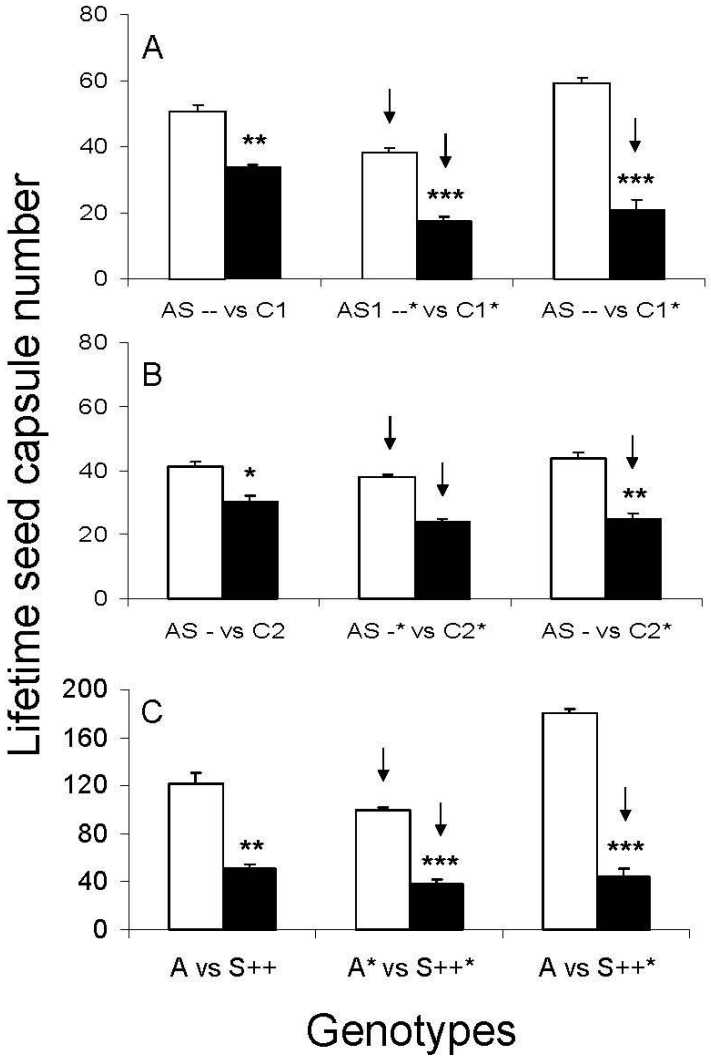

Fig. 3. Mean (± SEM) lifetime seed capsule number produced by *N. attenuata* genotypes differing in TPI production (see Fig. 1 caption for abbreviations) grown in competition with each other and were either uninduced or elicited with 150 µg (*) of MeJA. Arrows depict the genotype that was elicited with MeJA and asterisks indicate the level of significant differences between members of pairs (*P<0.05, **P<0.001, *** P<0.0001). **A.** Mean (± SEM) capsule number of AS-- (open bars) and C1 (solid bars) genotypes. **B.** Mean (± SEM) capsule number of AS- (open bars) and C2 (solid bars) genotypes. **C.** Mean (± SEM) capsule number of A (open bars) and S++ (solid bars) genotypes.

Elicitation of only the control plants (C) from either AS-- vs C1 or AS- vs C2 or the A plants of the A vs S++ pairs reduced stalk length and seed capsule production of the elicited plant, and resulted in the greatest fitness differentials between competitors (Fig. 2D and E; repeated measures ANOVA, $F_{1,18\text{-}AS1\text{-}C1} = 26.13$; $P < 0.0001$; $F_{1,18\text{-}AS2\text{-}C2} = 100.098$; $P < 0.0001$; Fig. 3A and B; Suppl Fig. 3A and B). These large differences in lifetime seed production were due to both the costs of TPI production and the opportunity benefit realized by the unelicited neighbor. Unelicited plants growing adjacent to induced plants produce more seed than do uninduced plants growing adjacent similarly uninduced plants due to greater resource acquisition of the uninduced plants (22). When unelicited A competed with elicited S++ plants, the absolute difference of seed capsule production (136) was 2 fold greater than when competing with unelicited S++ (70.2) and this difference arose from an increase in 60 seed capsules (a 33 % increase) in the A genotype, rather than a decrease in the output of the S++ genotype, representing an opportunity benefit (Fig. 3A; Table 1).

In order to determine the effect of TPI production on seed capsule production, we regressed the differences in TPI activity between neighbors against the relative differences in seed capsule production between neighbors and found that a logarithmic function ($Y = 9.9787 \text{ Ln (PI)} + 34.32$; $R^2 = 0.5799$; Fig. 4; $P<0.01$) represented the best fit. The relationship suggests that the higher the difference in TPI activity between neighbors, the higher the relative differences in seed capsule production. For this analysis we only included plants from pots where both competitors were either unelicited or elicited with 150 μg of MeJA.

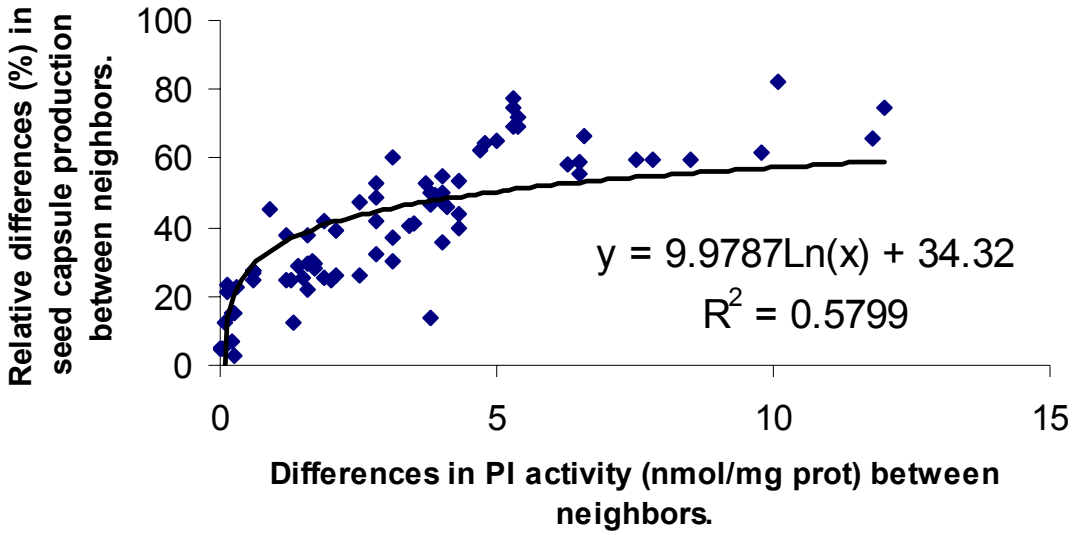

Fig. 4. Relative differences in seed capsule production (as %) between neighbors of *N. attenuata* genotypes that had been transformed with constructs containing the *pi* gene in an antisense orientation or an empty vector construct, untransformed plants of the Arizona genotype and plants of the Arizona genotype transformed with constructs containing the full-length *pi* gene in a sense orientation, regressed against the differences in trypsin proteinase inhibitors (TPI) activity (nmol/mg protein) between neighbors. The analysis included only plant pairs in which both competitors received the same treatment, either uninduced or induced with 150 µg of methyl jasmonate. Line represent a regression fitted to the points (Y = 9.9787 Ln (PI) + 34.32; R^2 = 0.5799).

Discussion

Our experiments demonstrate that TPI is directly responsible for the observed fitness differences between neighbors. Across all experiments, the larger the difference in TPI activity between neighbors, the larger was the difference in seed capsule production (Fig. 4). Plants with high constitutive TPI levels (C1, C2 and S++) growing next to plants with low TPI levels (AS--, AS- and A), realized a large reduction in lifetime seed production: 26 and 25 % in AS- vs C2, 33 and 28 % in AS-- vs C1 and 53 % in A vs S++ (Fig. 4; Table 1; Suppl Table 1). Not only were the qualitative results entirely

reproducible across all replicates of the experiments, but the quantitative measures of the relative fitness consequences of TPI production were also remarkably similar between experiments, regardless of whether *pi* expression was silenced or restored. For example, when both competitors were elicited in the AS-- vs C1 experiment, which resulted in an average difference in TPI activity between neighbors equivalent to the difference in TPI activity measured between the A vs S++ pairs (4.6 nmol $prot^{-1}$; Fig. 1B), the relative fitness differences were the same (53-54% differences; Table 1). The rank order of fitness differences within the different silencing experiments tracked the differences in TPI production; the fitness differences in the AS-- experiments with low TPI production (16 and 33 capsules) were greater than those observed in the AS- experiments which had intermediate TPI production (from 9 to 10 capsules; Fig. 3; Table 1; Suppl Table 1). Elicitation of both competitors with MeJA increased the differences in TPI production and consistently increased the realized fitness differences between competitors (AS- vs C2: 4 and 1 capsules; AS-- vs C1: 4 and 13 capsules; Table 1, Suppl Table 1). Since seed capsule production did not differ between controls (C1 vs C2) and WT (C1 vs WT) and the fitness differences between AS- competing with either C1 or with C2 were not different (10 capsules), the observed fitness differences cannot be attributed to particular pairing combinations. We conclude from these results that TPI production is intrinsically costly when plants compete for below-ground resources with conspecifics, as they commonly do in nature.

Why TPI production is so costly for the reproductive performance of competitively-growing plants remains an open question. TPI production may make demands on a plant's nitrogen (N) budget that a plant could otherwise allocate to growth

and reproduction. In addition, such demands might decrease the allocation of N to other N-intensive defenses. However, we found no evidence that TPI expression had any effect on either constitutive or inducible nicotine production (Fig. 1C), a N-intensive defense that can utilize 6% of *N. attenuata*'s whole-plant N budget (26). Whole-plant nicotine pools in *N. attenuata* are stable, increase under N-limited growth and are not metabolized and re-used for growth. In contrast, PIs are thought to be metabolized and decrease under competitive- and N-limited growth in other plant systems (37), suggesting that an investment of fitness-limiting resources to PIs can be adjusted to internal resource levels. We found no evidence that competitive growth decreased TPI activity in any line (Fig. 1B and J. A. Zavala and I.T. Baldwin unpublished data). Similarly, increases in the irradiation levels to the competing plants did not alleviate the fitness differences associated with differential TPI production. Growth under high light levels increased reproductive output of competing plants but the fitness and growth differentials were retained (Table 1; Suppl Table 1). In an earlier experiment that examined the mechanisms responsible for the fitness costs of MeJA elicitation in WT *N. attenuata* plants, found that increases in below-ground N supply accentuated fitness consequences of elicitation rather than decreasing them (23). These results suggest that the simple allocation of fitness-limiting resources to TPI production cannot directly account for their costs. However, the changes in metabolism associated with or required to support increases in TPI production may contribute to the observed fitness differences.

Growth and fitness differences between competing plants in our experimental design result in part from differences in the rate of harvesting below-ground resources. Slow-growing plants do not harvest resources as rapidly as fast-growing plants, providing

an opportunity benefit for the fast-growing plants (22, 23, 27). Unelicited WT plants growing next to MeJA-elicited WT neighbors acquire more $^{15}NO_3$ from the soil, grow faster and allocate more ^{15}N to seed production than do unelicited plants growing next to competitively similar, unelicited plants (23). Interestingly, such opportunity benefits were not observed in the experiments with plants with silenced TPI production, but were clearly apparent in the A vs S++ experiment, in which A plants growing next to elicited S++ plants realized an opportunity benefit of 60 capsules, an 33% increase in reproductive output, over A plants growing next to unelicited S++ plants (Fig. 3C). These results suggest that production of TPIs does not directly interfere with a plant's ability to take up soil N (as MeJA elicitation clearly does), because if it did, the neighboring plant would be able to capitalize on this unclaimed soil resource and increase growth and reproductive output. Alternatively, the silencing of TPI production may somehow interfere with a plant's ability to capitalize on this opportunity benefit, but this seems unlikely given the large fitness increases associated with the silencing of TPI production. MeJA elicitation decreases the transcription of photosynthetic-related genes (30, 38, 39) and this down-regulation may be required to free up resources for defense related processes. Transcriptional analysis of the AS lines will be required to determine if the down-regulation of growth-related transcripts after elicitation are comparable to those of TPI producing lines.

Hence alternative physiological explanations for the costs associated with TPI production are needed. Proteinase inhibitors have been suggested to play an endogenous regulatory role to protect cells from proteinase activity in unwanted locations (40). It is possible that the large quantities of TPI required for defense also inhibit enzyme activities

that support rapid growth. However until the physiological underpinnings of rapid growth required for competitive prowess are better understood, this hypothesis will be difficult to test.

The fitness costs of TPI production measured in this laboratory study included only one of the plant's natural ecological interactions: intra-specific competition. When plants grow with their full complement of natural ecological interactions, these costs are likely to be balanced by the fitness benefits resulting from the defensive utility of TPI expression (24). However ecological costs might also be incurred that result from the complicated interactions with other species (2, 16), such as the decrease the attractiveness of the pollinators (2, 41). Since TPI expression frequently slows the grow rate of insect herbivores by making their digestive processes less efficient (40, 42), the fitness benefits of TPI expression may result from extending the period during which larvae can be successfully attacked by natural enemies. Hence without the attraction of natural enemies, TPI expression may not increase plant fitness in environments with herbivores. Interestingly, the most important natural enemy of *N. attenuata* herbivores, *Georcoris pallens*, is attracted to herbivore-attacked plants by volatile signals released from the plant (43) which are elicited by the same signals that elicit TPI production (34, 44, 45) A. Roda and I. T. Baldwin, unpublished results) and hence these direct and indirect defense traits are coordinately expressed in WT plants. The A genotype, in addition to not producing TPIs, also does not release an important predator-attracting component of the herbivore-induced volatile blend: cis-α-bergamotene (24). Since volatile release may also attract other herbivores, it is possible that AS plants, with their down-regulated TPI production but intact volatile release (J. A. Zavala and I. T. Baldwin, unpublished results)

may incur additional fitness costs beyond those attributable to the down-regulation of PI based defenses. These results underscore the relevance of an important assumption in life history theory: that defense expression incurs fitness costs.

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Supplemental Information

I. Construction of antisense and sense transformation vectors:

Vector Construction: For antisense expression, a 175 bp fragment of the chromosomal *pi* gene of *N. attenuata* containing only coding sequence (GenBank accession number AY184823) was PCR amplified using primers: PIA1-34 (5'-GCGGCGGGTCACCGTACTTTAGTGATGATGGAAC-3'), PIA2-32 (5'-GCGGCGCCATGGCTTACAACCCTTCGTGCCTG-3') and chromosomal DNA from *N. attenuata*, genotype DI92 collected from Utah (1), as template. After digestion with *Bst*EII and *Nco*I, the fragment was cloned in pNATGUS3 (2) and cut with the same enzymes. On the T-DNA of the resulting binary plant transformation vector pNATPI1 (9.0 kb) the *pi* gene fragment was resident in antisense orientation downstream from 35S promoter and upstream from terminator both from Cauliflower Mosaic Virus (CaMV), thus enabling the transcription of *pi* antisense RNA.

In order to express the full-length *pi* from the Utah genotype of *N. attenuata* into the genotype collected from Arizona which lacks the ability to produce TPIs at a transcriptional level (3), we created an efficient binary *N. attenuata* transformation vector with rescuing functionality and containing as little superflous DNA as possible. In the following steps, the essential functional DNA elements were assembled as easily exchangeable boxes: The 1.8 kb fragment of pUC19 (4) digested with *Aat*II and *Afl*III was ligated with the 1.0 kb *Aat*II-*Afl*III fragment obtained by PCR with primers NPT3-73 (5'-GCGGCGACATGTAAGCTTGGCGCGCCGGATCCGTATACCCCCCCGCATGCATAATTGTGGTTTCAAAATCGGC-3'), NPT4-49 (5'-GCGGCGGACGTCGGTACCCCCGAATTCTAGGTACTAAAACAATTCATCC-

3') and template pBI121-ASPMT (5). To the resulting plasmid pRESC1 (2.9 kb), carrying the ColE1 origin of replication and the *bla* and *nptIII* resistance genes, the pVS1 replicon was added as 4.0 kb *Acc*I-*Sph*I-fragment obtained from pCAMBIA-1301 (GeneBank accession number AF234297), yielding pRESC2 (6.8 kb). Right border of T-DNA was inserted as 0.2 kb *Bcl*I-*Bgl*II-fragment from pUCNAT2 (2), resulting in pRESC4 (7.0 kb). Left border of T-DNA was inserted as 0.3 kb *Kpn*I-*Eco*RI-fragment obtained by PCR with primers RESC1-32 (5'-GCGGCGGAATTCGATCACAGGCAGCAACGCTC-3'), RESC2-44 (5'-GCGGCGGGTACCGGATCCGGCGCGCCAGTACATTAAAAACGTCC-3') and template pCAMBIA-1301, producing pRESC5 (7.3 kb). Insertion of the nopaline synthase (NOS) terminator as 0.3 kb AatII-BamHI fragment PCR synthesized with primers RESC4-34 (5'-GCGGCGGGATCCAATTCCCGATCTAGTAACATAG-3'), RESC3-49 (5'-GCGGCGGACGTCATTAATCCCGGGGGTACCAGCTCGAATTTCCCCGATC-3') and template pCAMBIA-1301 led to pRESC6 (7.6 kb). To obtain CaMV 35S promoter and CaMV terminator as suitable constructs, the 8.0 kb *Xho*I-*Pvu*II fragment of pCAMNAT2 (2) was ligated to the *Xho*I digested 0.6 kb PCR-fragment obtained with Vent DNA Polymerase, primers RESC5-31 (5'-GCGGCGCTCGAGTCAAGAGTCCCCCGTGTTC-3'), RESC6-29 (5'-CCTCTAGAGCTTCATGGAGTCAAAGATTC-3') and template pCAMBIA-1301, forming pCAMNAT4 (8.5 kb). The CaMV transcription signals from this plasmid were inserted as 1.0 kb partial *Ase*I-*Kpn*I fragment into pRESC6, giving pRESC7 (8.6 kb). To clone the hygromycin resistance marker *hptII* (from pCAMBIA-1301) driven by the NOS promoter, the 3.8 kb *Bam*HI-*Bcl*I fragment from pUCNAT2 was circularized, yielding pUCNAT3, and cut with *Hin*dIII and *Bst*YI. The resulting 0.3

kb NOS promoter fragment was cloned in pUC18 (6) to form pUCNOS1 (3.0 kb). After *Kpn*I-*Bgl*II digestion the 1.0 kb *hgtII* PCR-fragment synthesized with primers RESC9-48 (5′-GCGGCGAGATCTGGATCGTTTCGCATGAAAAAGCCTGAACTCACCGCG-3′), RESC10-33 (5′-GCGGCGGGTACCCTATTTCTTTGCCCTCGGACG-3′) and template pCAMBIA-1301 was inserted into this vector, yielding pHYG1 (4.0 kb). The construction of the new plant transformation vector pRESC20 (9.8 kb; Supplemental Fig. 1) was completed by fusing the 8.4 kb and 1.4 kb *Kpn*I-*Xba*I fragments of pRESC7 and pHYG1, respectively.

The full length 7-domain repeat proteinase inhibitor gene of *N. attenuata* (GeneBank accession number AF542547) originating from a cDNA library prepared from *Manduca sexta* larvae attacked shoot material and residing on pPI1/14 (7) was amplified with primers PIA9-33 (5′-GCGGCGCTCGAGATGGCTGTTCACAGAGTTAGC-3′), PIA10-35 (5′-GCGGCGGGTCACCTTAGGAAACAGCAACCCTAGAC-3′) and cloned as 1.4 kb *Bst*EII-*Xho*I fragment in the 9.7 kb fragment of pRESC20 cut with the enzymes. The resulting plant transformation vector pRESC2PIA2 (11.1 kb) carried the full length *pi* gene driven by CaMV 35S promoter and CaMV terminator in the T-DNA, thus allowing the synthesis of TPIs in the previously TPI-deficient genotype of *N. attenuata* (Arizona).

II. Plant material and transformation:

Nicotiana attenuata Torr. Ex Wats. (Solanaceae) used in this study were grown from seeds collected from either Utah (1) or Arizona (3) and inbred 10 and 4 generations respectively. In order to silence the expression of *N. attenuata*'s *pi* gene

in the genotype collected in Utah (WT), WT was transformed with pNATPI1, which contains the Nourseothricin (NTC) resistance gene *sat-I* as a selectable marker, via an *Agrobacterium*-mediated transformation procedure designed for transformation of *N. attenuata* (2). To determine the segregation ratios, T_1 seeds from transformed plants were sterilized and germinated in a petri-dish. Half of one cotyledon of 8-10 day old seedlings were removed and placed on callus induction media with NTC (250 mg/l). Positive (WT cotyledons on callus induction media without antibiotics) and negative controls (WT cotyledons on callus induction media with antibiotics) were included. Cotyledons that exhibited vigorous callus growth within 4-7 days identified transformed seedlings and were planted in soil and grown in a glasshouse. Three lines were selected that showed 3:1 segregation ratios in the T_1 and these were bred to obtain homozygous lines and screened for TPI activity. Homozygous lines of T_2 seedlings showed 100% resistance to antibiotic media and 2 homozygous T_3 lines, AS-- and AS- in which constitutive and inducible TPI activity was lower than that of WT, were selected. Two homozygous T_3 independently transformed lines of WT plants (C1 and C2) that had been transformed with an empty vector construct **(**Supplemental Fig. 1; lacking only the *pi* gene fragment**)** and had TPI activity equivalent to that of WT plants, were selected as controls for the competition experiments. Southern analysis (see Supplemental Fig. 2) confirmed that all T_3 lines were single-copy independent transformants.

To provide an additional test of the fitness costs of TPI production, we used a genotype of *N. attenuata* collected from Arizona (A), which has MeJA-inducible nicotine production identical to that found in WT plants, but completely lacks the ability to produce TPIs, or accumulate TPI mRNA (3). Using a series of classical crossing designs between A and WT plants, the ability to produce PIs was determined

to be inherited as a dominant Mendelian trait and that homozygous A plants contain two non-functional recessive alleles, whereas heterozygous plants or homozygous dominant plants both are able to produce PIs (8). More recently, the mutation in the 7-domain repeat *pi* of A plants has been characterized and found to be located in the 5'signal peptide, resulting in a premature stop codon (J. Wu and I.T. Baldwin unpublished data). Plants of the A genotype were transformed with a binary transformation vector pRESC2PIA2 containing the full-length 7-domain *N. attenuata pi* gene from the WT genotype in the sense orientation under control of a CaMV 35S promotor and the hygromycin resistance gene *hpt*-II. T_1 seeds from transformed plants were sterilized and germinated in a petri-dish with hygromycin (35mg/ml) with negative and positive controls. Hygromycin resistant seedlings were planted in soil and transferred to the glasshouse. These lines showed a simple Mendelian segregation for a single copy of the transgene, which was confirmed by Southern analysis (see Supplemental Fig. 2). Constitutive and inducible levels of TPI activity in 5 homozygous T_3 transformed lines were determined and all had TPI activity comparable to that of elicited WT plants. One of these A lines (S++) with 60% of the activity of MeJA-elicited WT plants was selected for study. All non-transformed plants of the Arizona genotype (A) had no detectable TPI activity.

III. *Nicotiana attenuata*'s natural history and the design of competition experiments:

Nicotiana attenuata, a diploid, largely selfing, native tobacco of North America, has evolved to grow in the primordial agricultural niche: the immediate post-fire environment. *N. attenuata's* habitat selection is in large part determined by its particular germination behavior. *N. attenuata* "chases" fires in the Great Basin

Desert by synchronizing its germination from long-lived seedbanks with the immediate post-fire environment. The dormant seeds respond to a combination of germination stimulants found in wood smoke (9) and inhibitors from the unburned litter of the dominant vegetation (10, 11). As a consequence of this germination behavior, seeds germinate synchronously into nitrogen (N) rich soils (12) and hence have been selected for rapid growth when water availability is high and for intense intra-specific competition. In nature, *N. attenuata* is typically found for 1-2 years after fires and occurs in large single-species stands.

Since we are interested in determining whether TPI production was associated with fitness costs attributable to differences in growth performance (rather than the fitness costs resulting from higher-order ecological interactions-- see Introduction and Discussion), we designed the competition experiments so as to mimic the environmental conditions of *N. attenuata*'s native habitat that are known to profoundly determine growth performance in nature: intra-specific competition, water, light, and N-supply. We grew plants in N-P-K rich soils (that mimicked the levels typical of the post-fire environment (12), provided ample water for 37 days of growth (an interval characteristic of a growing season in SW Utah), provided high PAR fluence in the glasshouse by supplementing natural light with Na vapor HID lights, and growth with an intra-specific competitor. This competition design has been tested in three prior publication (3, 13, 14) and found to capture the fitness costs of JA elicitation observed in a field experiment (1).

In short, two developmentally synchronized seedlings from the different TPI producing genotypes were transplanted into 2-L pots containing soil with 1.19:1.22:1.25g N:P:K per pot, watered daily for 37-days, and grown under 16H days with a minimum of either 800-900 μmol m^{-2} s^{-1} PPFD (intermediate) or 1000-1300

μmol m^{-2} s^{-1} PPFD (high). Actual daytime values were frequently 2000 μmol m^{-2} s^{-1} PPFD, the level commonly measured in Utah (I.T. Baldwin unpublished data). Plants started flowering 26 days after transplanting into the 2L pots (Fig. 3) and capsules were counted 15 days after the last watering, when all plants had dried and senesced.

IV. Plant growth and additional preliminary experiments:

A preliminary competition experiments was conducted to determine whether the empty vector transformed line (C1) differed from untransformed (WT) plants in lifetime seed production: 5 replicate pairs of the C1 vs WT plants were grown under a minimum 800 – 900 μmol m^{-2} s^{-1} PPFD. No significant differences in seed capsule production were found in the experiment: WT (36.0 ± 2.9) competing with C1 (37.6 ± 3.4; $t_{4\text{-WT-C1}} = 0.387$; P = 0.71).

Since the transformed (AS--, AS-, C1, C2, and S++) and untransformed genotypes (A and WT) did not differ with respect to other direct defense traits, such as nicotine production (Fig. 1), this is an ideal system in which to examine the cost of TPI expression. The AS (AS--and AS-) lines and empty vector control (C1 and C2) lines were produced from the WT ecotype and the S++ line was produced from the A ecotype, which cannot produce TPI due to a natural mutation. As a consequence, in order to determine the cost of TPI production, we grew genotypes with the same genetic background, which differ only in TPI production, in the same pot. In each of the three experiments, individual plants were either uninduced (CON) or elicited with 150 μg of MeJA (*) and pairs of plants were assigned to the following 3 treatment groups: 1) CON-CON (AS-- vs C1; AS- vs C2; A vs S++), 2) MeJA-MeJA (AS--* vs C1*; AS-* vs C2*; A* vs S++*) and 3) CON-MeJA (AS-- vs C1*; AS- vs C2*; A vs S++*).

V. DNA Isolation and Southern Hybridization:

DNA was isolated using CTAB (15) and was quantitated spectrophotometrically at A260 nm. Twenty µg of DNA was digested with the enzyme *Eco*RI (for the Utah genotypes: WT, AS--, AS-, C1 and C2) and with *Hin*dIII (for the Arizona genotypes: A and S++), electrophoresed on 1 % agarose gel (in 1X TAE buffer), blotted on nylon membrane (NEN Life Science Products, USA) according to the protocol of (16) and hybridized with radiolabelled probe from the NOS terminator of the vector plasmid pRESC 20 (Supplemental Fig. 1) generated by PCR using the primers NOT 2-23 5' CCCCGATCGTTCAAACATTTGGC 3' and NOT 1-29 5' CCCGATCTAGTAACATAGATGACACCGCG 3' . Hybridization was detected using a Phosphoimager (FLA 3000 Fujifilm, Japan) and quantified with Aida Image Analyzer (v. 3.11; Fujifilm, Japan).

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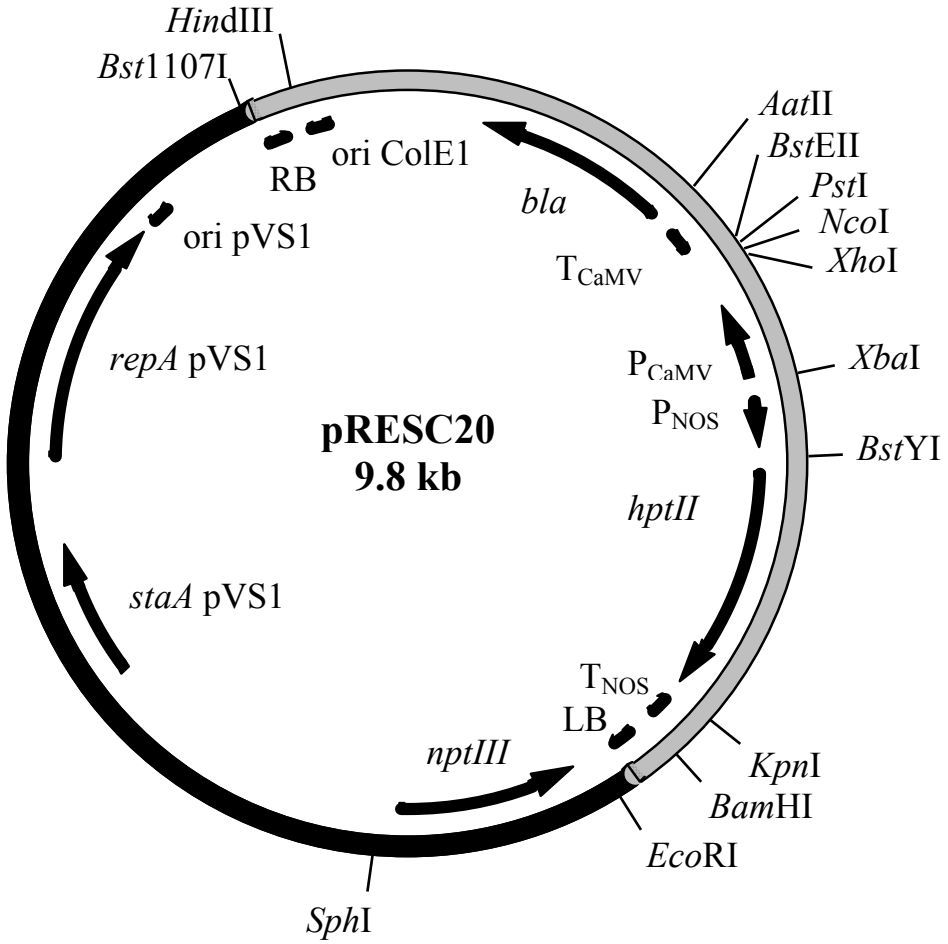
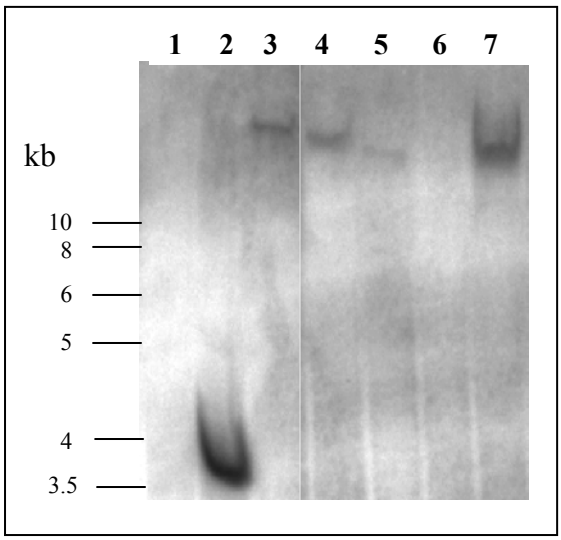
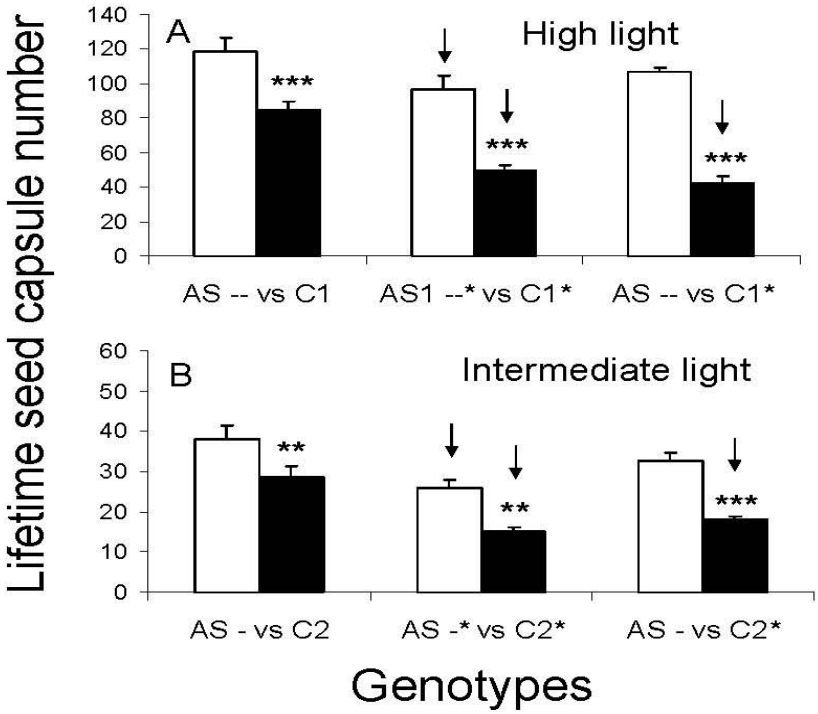


Supplemental Fig. 1: *Nicotiana attenuata* transformation vector pRESC20 (9.8 kb) with *hptII* as plant selectable marker gene. Displayed sites are unique (except *Aat*II, *Bst*YI, *Sph*I) and mark the borders of functional elements. *Bst*EII, *Pst*I, *Nco*I and *Xho*I are cloning sites for full-length *pi* from the Utah genotype of *Nicotiana attenuata*. Functional elements on T-DNA (grey): LB/RB, left/right border of T-DNA; P_{NOS}/T_{NOS}, promoter/terminator of nopaline synthase gene; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301; P_{CaMV}/T_{CaMV}, 35S promoter/terminator of cauliflower mosaic virus; *bla*, beta-lactamasease gene from pUC19; ori ColE, pUC19 origin of replication. Functional elements outside T-DNA (black): ori pVS1, *repA* pVS1, *staA* pVS1, origin of replication, replication protein gene, partitioning protein gene from plasmid pVS1; *nptIII*, aminoglycoside phosphotransferase of type III from *Streptococcus faecalis*.



Supplemental Fig. 2: Southern blot of the genomic DNA of untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT; lane 1), two homozygous T_3 independently transformed lines of the Utah genotype that had been transformed either with a construct containing 175 bp of the *pi* gene in an antisense orientation (AS--, AS-; lanes 2 and 3), or with an empty vector construct (C1, C2; lanes 4 and 5); digested with *Eco*RI and of untransformed plants of the Arizona (A; lane 6) and transformed plants of Arizona (S++; lane 7) digested with *Hin*dIII and hybridized with a radiolabelled probe from the NOS terminator of the vector plasmid pRESC 20 (Supplemental Fig. 1)**.**



Supplemental Fig. 3: Mean (± SEM) lifetime seed capsule number produced by pairs of *N. attenuata* plants of genotypes differing in TPI production (see Fig. 1 caption for abbreviations). Pairs of plants were grown in competition with each other in the same pot and were either uninduced or elicited with 150 µg (*) of methyl jasmonate (MeJA). Arrows depict the genotype that was elicited with MeJA and asterisks indicate the level of significant differences between members of pairs (*P<0.05, **P<0.001, *** P<0.0001). **A.** Mean (± SEM) capsule number of AS-- (open bars) and C1 (solid bars) genotypes. Plants were grown under high light conditions (minimum 1000 - 1300 µmol m^{-2} s^{-1} PPFD irradiance). **B.** Mean (± SEM) capsule number of AS- (open bars) and C2 (solid bars) genotypes grown under normal light conditions (minimum 800 – 900 µmol m^{-2} s^{-1} PPFD).

Supplemental Table 1. Absolute and relative mean differences in lifetime seed capsule production from pairs of developmentally synchronized plants from homozygous T_3 independently transformed lines of a WT genotype of *Nicotiana attenuata* which had been transformed with constructs containing 175 bp of the *pi* gene in an anti-sense orientation (AS--, AS-), or an empty vector construct (C1, C2). Two plants were grown in the same pot in 2 different pair combinations (AS-- vs C1 and AS- vs C2). Plants were either treated with 150 µg of MeJA in 20 µl of lanolin paste (*) to elicit jasmonate-induced defenses or treated with 20 µl of pure lanolin paste; n = 10 per treatment and pair combination. The AS-- vs C1 pairs were grown under a minimum of 1000 -1300 µmol m^{-2} s^{-1} PPFD and the AS- pairs under a minimum of 800 – 900 µmol m^{-2} s^{-1} PPFD. P-values are from paired *t*-Test comparisons; relative values were arsine square root transformed before analysis.

Competitors	**Mean diff. in Capsule number**	**P**	**% Mean diff. in capsule number**	**P**
AS-- vs C1	33.7 ± 4.5	<0.0001	28.4 ± 2.3	<0.0001
AS--* vs C1*	47.3 ± 7.3	<0.0001	48.6 ± 3.4	<0.0001
AS1-- vs C1*	64.6 ± 4.7	<0.0001	60.3 ± 4.1	<0.0001
AS- vs C2	9.5 ± 1.8	0.0008	25.0 ± 4.0	0.0002
AS-* vs C2*	10.6 ± 3.2	0.0009	41.1 ± 5.9	0.0004
AS- vs C2*	14.6 ± 1.3	<0.0001	44.5 ± 2.7	<0.0001

Manuscript 3

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Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuata* demonstrates their function as anti-herbivore defenses

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ABSTRACT

Evidence for the *in planta* defensive function of trypsin protease inhibitors (TPIs) comes from observations of enhanced herbivore resistance after heterologous TPI expression or the manipulation of signal cascades that activate numerous defense responses including TPI production; no studies have altered the expression of an endogenous *pi* gene to examine defensive function. We isolated two genes with 7- and 6-repeat TPI domains from *Nicotiana attenuata* from the potato PI-II family. To determine whether endogenous TPIs in *N. attenuata* function defensively against the native herbivores, *Manduca sexta* and *Tupiocoris notatus*, we expressed 175 bp of the 7-domain *pi* from *N. attenuata* in an anti-sense (AS) orientation in a TPI-producing genotype (WT) to reduce TPI expression and expressed the full-length 7-domain *pi* in a sense orientation (S) under control of a constitutive promoter to restore TPI activity in a natural genotype from Arizona (A) unable to produce TPIs. Constitutive and inducible TPI production in two AS lines were diminished by 80-90 % and 33-52 %, respectively, and sense-expression (S++) restored 67% of the activity found in WT after caterpillar attack in the TPI-deficient A genotype. *M. sexta* larvae fed on genotypes with either low or no TPI activity grew faster, had higher survivorship and produced heavier pupae than those that fed on genotypes with high TPI activity. *T. notatus* showed higher preference for genotypes with either low or no TPI activity than for genotypes with high TPI levels. We conclude that endogenous TPIs are an effective defense against these native herbivores.

Additional Keywords: herbivory, induced defenses; host plant resistance; insect resistance

INTRODUCTION

Plant proteinase inhibitors (PIs) are polypeptides or proteins that occur naturally in a wide range of plants and are considered to be an essential part of the plant's natural defense system against herbivores (Ryan, 1990; Jongsma and Bolter, 1997; Schuler et al., 1998). Evidence for the *in planta* defense function of PIs comes from two types of experiments: 1) heterologous expression of *pi* genes (Hilder et al., 1987; Johnson et al., 1989; McManus et al., 1994; Duan et al., 1996) and, 2) manipulation of signal cascades affecting PI elicitors (e.g., Orozco-Cardenas et al., 1993). PIs have been found to reduce herbivore growth in plants that were transformed with a heterologous *pi* gene in at least 45 studies (e.g., Hilder et al., 1987; Johnson et al., 1989; McManus et al., 1994; Duan et al., 1996). Surprisingly, we know of no studies that have altered the expression of an endogenous *pi* gene to examine its defensive function.

While heterologous expression studies have demonstrated reduced herbivore performance, 2 complications temper the extrapolation of these results to the conclusion that PIs are natural defenses against herbivores that normally feed on plants (Johnson et al., 1989; McManus et al., 1994; Broadway, 1996). First, as a result of the co-evolutionary interaction between insects and their natural hosts, the gut proteases of specialist insects are frequently resistant to the PIs of their host plants (Broadway, 1995; Jongsma et al., 1995; Winterer, 2002). Hence when an insect feeds on a transgenic plant expressing a novel *pi* gene, the results may not reflect the effect of endogenous PIs (e.g., Gatehouse et al., 1999; De Leo et al., 2001; Winterer and Bergelson, 2001) because the larvae are not adapted to the novel PI. Second, the effects of PIs on insect performance can be strongly influenced by other nutritional and defensive factors resulting in PI-diet

and -plant interactions (Broadway, 1997; McManus et al., 1999). For example, when tobacco and potato, transformed with the same heterologous *pi* gene that resulted in similar trypsin proteinase inhibitor (TPI) contents, were challenged with *Spodoptera litoralis* larvae, the tobacco plants killed 95 % of the attacking larvae, while the potato plants killed only 36 % (Marchetti et al., 2000). PI-diet interactions are clearly seen in artificial diet studies in which manipulations of PI content do not reflect changes in insect performance on plant material (Broadway, 1996). An additional complication for the interpretation of the heterologous expression studies is that high levels of endogenous TPIs can be elicited by herbivore attack (Green and Ryan, 1972; Jongsma et al., 1994) and these endogenous inhibitors may interact with introduced inhibitors.

The second line of evidence for PI defensive function comes from experiments in which transgenic suppression of the wound signal cascades that elicit PI production enhances the performance of herbivores compared to those herbivores that feed on plants with intact signaling (McGurl et al., 1992; Orozco-Cardenas et al., 1993; Howe et al., 1996; Royo et al., 1999). These signaling cascades regulate many other traits in addition to PIs (Bergey et al., 1996), and it is not possible to attribute the changes in performance observed in these studies solely to changes in PI expression.

Ideally, one should determine the benefits of an endogenous *pi* in plants that differ only in a gene that controls the expression of a resistance trait but are otherwise identical (Bergelson and Purrington, 1996). There are at least two different ways to test the defensive function of an endogenous *pi* gene: 1) produce a defenseless plant (one deficient in defense signaling) and over-express the *pi* gene; or 2) down- or up-regulate the *pi* gene, keeping the other defense traits and their signaling intact. The first approach

is a weaker test of the defensive value of TPIs than the second and has similarities to tests of defensive function by heterologous expression because the function of the *pi* is examined without the context of the other defenses present in the plant. Interactions with other defenses may profoundly determine the defensive function of TPIs and these interactions would be lost in the first approach. Here we provide a test of the defensive value of endogenous TPIs with the second approach in a native tobacco species.

Nicotiana attenuata Torr. Ex Wats., a postfire annual inhabiting the Great Basin Desert, has a number of well-described herbivore-induced direct and indirect defenses (Baldwin, 2001), which increase the fitness of plants under attack in natural populations (Baldwin, 1998; Kessler and Baldwin, 2001). In addition to nicotine, the plants also produce TPIs after herbivore attack (van Dam et al., 2001) which reduce the performance of *Manduca sexta* larvae (Glawe et al., 2003), a specialized lepidopteran herbivore from which three trypsin-like cDNA sequence have been cloned (Peterson et al., 1994). Mirids (*Tupiocoris notatus*) are the second most commonly found herbivore on *N. attenuata* (Glawe et al., 2003), and feed by a "lacerate and flush" mechanism, in which the stylets and watery saliva are used to lacerate and flush out pockets of cells (Miles, 1972). Recently, a chymotrypsin-like protease was detected in the salivary glands of the mirids (Colebatch et al., 2001) and one chymotrypsin-like cDNA sequence has been sequenced from the midgut (Colebatch et al., 2002).

Here, we report the cloning of 2 multi-domain TPI genes belonging to potato PI-II family from *N. attenuata* plants collected in Utah, and provide a critical test of whether endogenous TPIs in *N. attenuata* function defensively against two specialist herbivores, *M. sexta* and *T. notatus*. We conducted bioassay experiments comparing hornworm larval

performance and mirid colonization preference for plants with either low or no TPI activity. We used two independently transformed *N. attenuata* lines in which the expression of the *pi* gene was down-regulated by antisense expression of a 175 bp fragment of the *N. attenuata pi* gene (AS--, AS-), one line independently transformed with an empty-vector construct (C) and untransformed wildtype plants (WT) of the same genetic background (an inbred line collected from Utah). In addition, we used a natural *N. attenuata* genotype collected from Arizona, which has a mutation in the endogenous 7-domain *pi* gene and does not produce *pi* transcripts or TPI activity (A) and which we transformed with the full-length cDNA of the 7-domain *pi* gene in a sense orientation under control of a constitutive promotor (S++), so that it produced TPIs at 67% of the level found in the wildtype Utah genotype after caterpillar attack.

RESULTS

N. attenuata produces a 7-domain TPI belonging to potato PI-II family

Earlier results with PI activity assays of *N. attenuata* plants revealed that TPI activity is strongly increased after MeJA elicitation (van Dam and Baldwin, 2001). To isolate the TPI gene(s) responsible for the MeJA elicited TPI activity, N-terminal sequencing of partially purified extracts revealed a high degree of similarity with the *N. alata* multidomain PIs (Atkinson et al., 1993). A set of homologous primers were synthesized and used in PCR of chromosomal DNA of *N. attenuata*. The amplified product (pUCPI2/14) was sequenced and used to screen the cDNA library of caterpillar-attacked *N. attenuata* leaves (Hermsmeier et al., 2001) to isolate the full-length clones. Four clones were isolated and sequenced and all 4 yielded the identical sequence. The

largest clone (pPI1/14) was 1546 bp long with a 1368 bp ORF (Suppl. Fig. 1: Genbank Accession Number AF 542547). The TPI precursor contains a signal peptide at the N-terminal followed by seven repeat domains encoding TPIs and a vacuolar targeting sequence (VTS) at the C-terminal. The 7 domains are linked by a 5 amino acid linker peptide EEKKN (Fig. 1).

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  1 MAVHRVSFLALLLLFGMSMLVSNVEHADA                                SP
 30                                  KACPRNCDGRIAYEICPRSEEKKN T7 N-ter
 54 DRICTNCCAGTKGCKYFSDDGTFICEGESDPRNPKACPRNCDGRIAYGICPRSEEKKN T1
112 DRICTNCCAGTKGCKYFSDDGTFICEGESDPRNPKACPRNCDGRIAYEICPRSEEKKN T2
170 DRICTNCCTGTKGCKYFSDDGTFVCEGESDPRNPKACPRNCDGRIAYEICPRSEEKKN T3
228 DRICTNCCAGTKGCKYFSDDGTFICEGESDPRNPKACPRNCDGRIAYEICPRSEEKKN T4
286 DRICTNCCAGTKGCKYFSDDGTFICEGESDPRNPKACPRNCDGRIAYEICPRTEEKKN T5
344 DRICTNCCAGTKGCKYFSDDGTFVCEGESDPRNPKACPRNCDERIAYGICPRTEEKKN T6
402 NQICTNCCAGTKGCNYFSANGTFICEGES                                 T7 C-ter
431                              EYVSKVDEYVHEVENDLQKSRVAVS 455 VTS
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Figure 1. Organization of the PI gene isolated; (GenBank accession number AF 542547). The gene contains a 29 aa signal peptide region (SP) followed by 6 or 7 repeated trypsin inhibitor domains (T1- T6 or T7) followed by vacuolar targeting region (VTS). The box delineates the aas of the TPI reactive site; the 8 conserved cysteines (labeled with a dot) and the conserved proline (labeled with a diamond on the first repeat) are all essential for the conformation of PI-II repeat domains (Antcheva et al., 2001). The missing repeat from the 6-repeat gene is marked with an underline and the signal peptide of this gene differs only by a methionine (overlined) to leucine substitution. Bold letters signify the aas differing among the repeats.

Multiple sequence alignments of a single PI repeat (T2) with the corresponding repeats of potato PI-II family members found in other Solanaceous species reveals a high degree of amino acid identity, including the conserved cysteines involved in the 4

disulfide bridges and the conserved proline (Fig. 1) that characterize all 77 known PI-II repeat sequences (Antcheva et al., 2001; Barta et al., 2002). The precursor PI has 93% homology with TPI isolated from *N. alata* stigmas (Atkinson et al., 1993).

N. attenuata TPI belongs to a multigene PI family

Genomic DNA of *N. attenuata* was completely digested by *Eco*RI, *Hind*III, *Eco*RV and *Ssp*I enzymes and hybridized with a radiolabeled plasmid containing the repeat domain of the *N. attenuata* PI precursor (Suppl Fig. 2). The analysis revealed at least 2 bands and suggested that TPIs exist in a multigene family in *N. attenuata*.

To isolate the additional gene(s), primer pairs corresponding to the 5' and 3' regions were synthesized and used in RT-PCR of mRNA extracted from MeJA-elicited leaves. The resultant clone was sequenced and found to encode a six-domain PI highly homologous to the earlier isolated PI. In addition to the deletion of one repeat domain, the 'new' PI also differed by a single amino acid in the signal peptide region (Fig. 1).

TPI activity and transcripts

In order to determine the constitutive and caterpillar-inducible levels of TPI mRNA of the transformed lines, Northern blot analysis was performed on total RNA from transformed lines (AS-, AS--, S+, S++, C and AC) and untransformed genotypes (WT and A). Analysis of unattacked leaves by larvae from untransformed WT and the line transformed with empty vector construct (C) revealed a 1.4 kb TPI transcript which increased 4-fold 24 h after the larvae started to feed on the leaf (Fig. 2A). While TPI transcripts were not detectable in AS--, not even after caterpillar damage, intermediate

levels were found in the AS- line (Fig. 2A). TPI mRNA in A as well as in the AC line (A independently transformed with an empty-vector construct), which lacks the ability to produce TPIs (Glawe et al., 2003), was not detectable. Constitutive TPI mRNA levels in A plants transformed with the full-length *N. attenuata pi* gene in the sense orientation and low levels of TPI (S+) were not detectable, but in a line with high TPI activity (S++), transcript levels were similar to the constitutive levels found in WT plants (Fig. 2A and B). TPI mRNA accumulation correlated well with TPI activity levels.

Endogenous leaf TPI activity was determined before and 3 d after larvae started to feed on the node +1 leaf of plants from transformed and untransformed genotypes. Compared to the constitutive levels of TPI activity in the WT and C plants (which did not differ significantly; $F_{1,38}$= 0.04; P = 0.8434), levels in AS-- and AS- plants were 90 % and 33 % lower, respectively (Fig. 2A; $F_{3,76}$= 90.640; P<0.0001). Caterpillar damage increased TPI activity 4-fold in WT and C plants, while AS-- and AS- TPI levels were also increased after caterpillar damage and TPI levels in these two genotypes were 17 % and 48 % of those found in attacked WT plants (Fig. 2A; $F_{3,76}$= 49.434; P<0.0001). Caterpillar attack did not alter TPI activity in S++ and S+ plants ($F_{1,38\text{-}S++}$= 3.744; P = 0.06; $F_{1,38\text{-}S+}$= 0.015; P = 0.9044), which remained at approximately 67 % and 4 % of the induced WT plants, respectively (Fig. 2A and B; $F_{2,57}$= 122.655; P<0.0001). As expected, the untransformed A and the transformed AC genotypes showed no TPI activity even after caterpillars had fed on the plant for 24 h (Fig. 2B). Protein levels were not significantly different among genotypes.

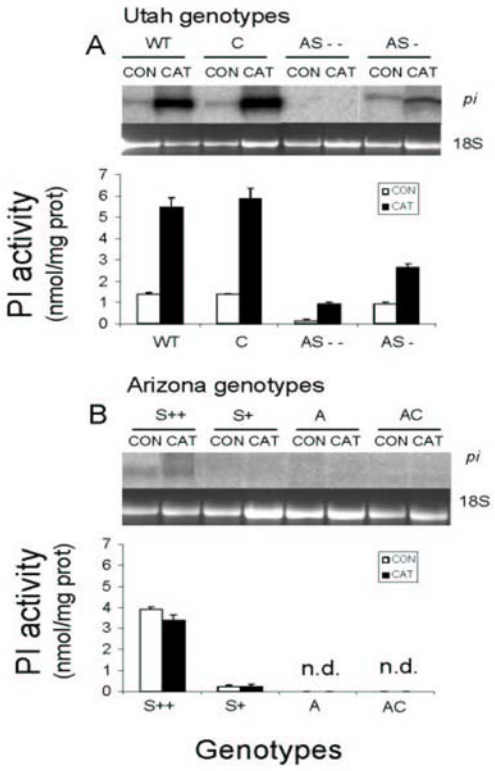

Figure 2. Northern blot analysis of trypsin proteinase inhibitor (TPI) mRNA and TPI activity (mean ± SEM) in leaves growing at node +1. RNA gel blot analysis of *pi* gene transcripts of unelicited control (CON) and *Manduca sexta*-elicited (CAT) plants 24 h after the start of feeding (upper band, TPI mRNA: 1.4 kb, lower band, 18S rRNA: 3.4 kb). TPI activity (mean ± SEM) in CON and CAT-elicited plants 3 d after elicitation (n.d. = not detectable in A genotype). **A.** untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT), two homozygous T_3 independently transformed lines of the Utah genotype that had been transformed either with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--, AS-), or with an empty vector construct (C). **B.** untransformed plants of the Arizona (A) genotype, and plants of the Arizona genotype transformed with a construct containing the full-length *pi* gene in a sense orientation (S+, S++), or with an empty vector construct (AC). *M. sexta* caterpillars fed on leaves growing at node +1 (one position older than the source-sink transition leaf: node 0) at the rosette stage.

Since chymotrypsin can be inhibited by TPI (Moura and Ryan, 2001), we measured the qualitative TPI and chymotrysin proteinase inhibitor (CPI) activities of untransformed (WT and A) and transformed (AS-- and S++) genotypes by radial diffusion assay after 3 days of W + OS treatment (Suppl. Table 1). W + OS increased TPI activity in WT plants, but did not affect TPI activity in the S++ genotype, which was high either before or after induction (Suppl. Table 1). CPI activity in both WT and S++ genotypes was low even after elicitation with W + OS (Suppl. Table 1). The AS-- genotype showed low TPI and CPI activities in all treatments, while the A genotype did not show any activity even after induction with W + OS (Suppl. Table 1).

Real-time PCR analysis was used to quantify the increases in the TPI transcripts in response to caterpillar elicitation. Both WT and C plants showed similar responses to caterpillar attack; TPI mRNA expression increased 7-fold after 24 h of caterpillar damage in the attacked leaf (+1) and 4-fold in the undamaged systemic leaf (-1), compared to undamaged WT plants (Fig. 3A). Constitutive expression of TPI mRNA was lower in AS-- (30 % of the undamaged WT) than in AS- (50 % of the undamaged WT; Fig. 3B). After caterpillars fed on antisense plants for 24 h, the expression of TPI mRNA increased 2- and 1.5-fold in AS-- and 5-and 2-fold in AS- lines in the local and systemic leaves, respectively, compared to undamaged WT (Fig. 3B). Caterpillar attack had a small effect on TPI mRNA transcripts in both S++ (2-fold) and S+ (0.5-fold) genotypes during the 48 h feeding period, compared to the levels found in undamaged WT leaves growing at the same nodal positions (Fig. 3C). Surprisingly, 24 h after caterpillar damage, the relative expression of TPI mRNA in the systemic leaves decreased transiently in the S++

genotype to only 1.1-fold of that found in the undamaged WT (Fig. 3C), perhaps due to general metabolic stress resulting from caterpillar attack.

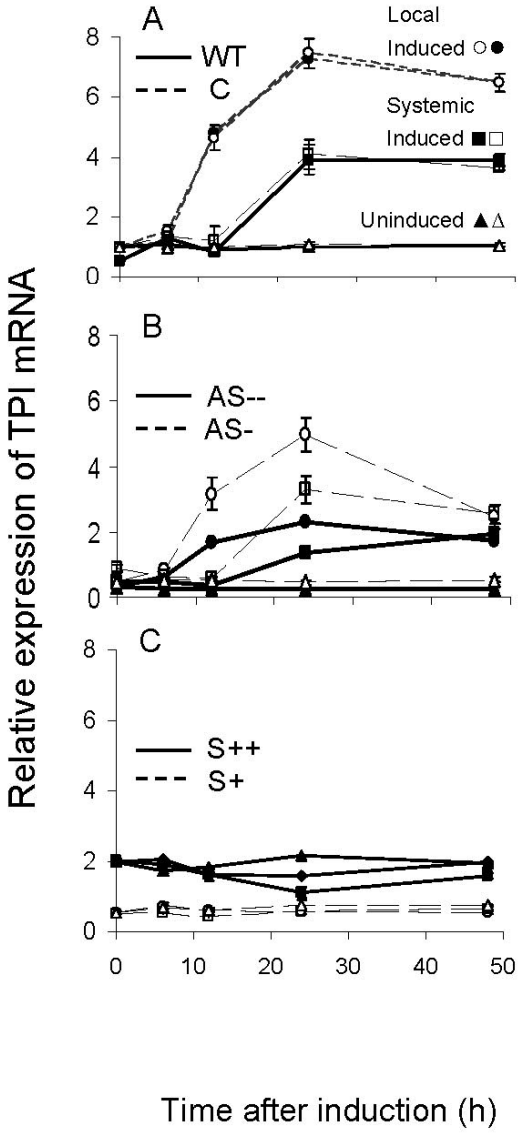

Figure 3. Fold induction (mean ± SEM) of the TPI transcripts by real time PCR in local (+1; ○●) and systemic (-1; □■) leaves after *M. sexta* elicitation relative to that of unelicited (control; Δ▲) WT leaves from 3 replicate plants harvested and analyzed separately at 0, 6, 12, 24 and 48 h after elicitation. **A.** WT and C genotypes. **B.** AS-- and AS- genotypes. **C.** S++ and S+ genotypes.

Endogenous TPIs as a defense against herbivores

To determine whether endogenous TPIs in *N. attenuata* function defensively against *M. sexta* and *T. notatus*, we assessed caterpillar performance and colonization preference of *T. notatus* on transformed and untransformed genotypes with either low or no TPI activity (A, AC, S+, AS-- and AS-) and high TPI activity (WT, C and S++). *M. sexta* larval mass gain per day, survivorship and pupal mass differed significantly between caterpillars fed on genotypes with either high or low TPI activity (repeated measures ANOVA on larval mass gain, $F_{4,190\text{-A- AS-- AS- WT-C}} = 5.069$; $P = 0.0023$; $F_{4,170\text{-A-AC-S+-S++-WT}} = 3.910$; $P = 0.0102$; survivorship analysis Table 1; ANOVA on pupal mass, $F_{4,51\text{-A- AS-- AS- WT-C}} = 32.370$; $P < 0.0001$; $F_{4,51\text{-A-AC-S+-S++-WT}} = 8.613$; $P < 0.0001$; Fig. 4A and B). Within the first group of genotype comparisons (A, AS--, AS-, WT, C), larvae fed on genotypes with either low or no TPI activity (repeated measures ANOVA, $F_{2,\ 130\text{-A-AS-- AS-}} = 1.234$; $P = 0.3075$) grew faster, had higher survivorship (Table 1) and produced heavier pupae than those fed on genotypes with high TPI activity (repeated measures ANOVA, $F_{1,\ 60\text{-C-WT}} = 0.024$; $P = 0.8787$; Fig. 4A). Between the first and second days after caterpillar attack, the larval mass gain per day was 3.89 ± 0.41 (AS--), 4.22 ± 0.45 (AS-), 1.42 ± 0.31 (WT), 3.71 ± 0.33 (A) and 1.57 ± 0.23 mg d^{-1} (C); data obscured by symbols (Fig. 4A). Larvae fed on AS- lines showed intermediate percentage survivorship (Table 1); during the first 3 d, survivorship was similar to that on AS-- lines, but after day 3 when plants had accumulated TPIs in response to larval feeding, survivorship was similar to that of larvae fed on WT and C genotypes (Fig. 4A).

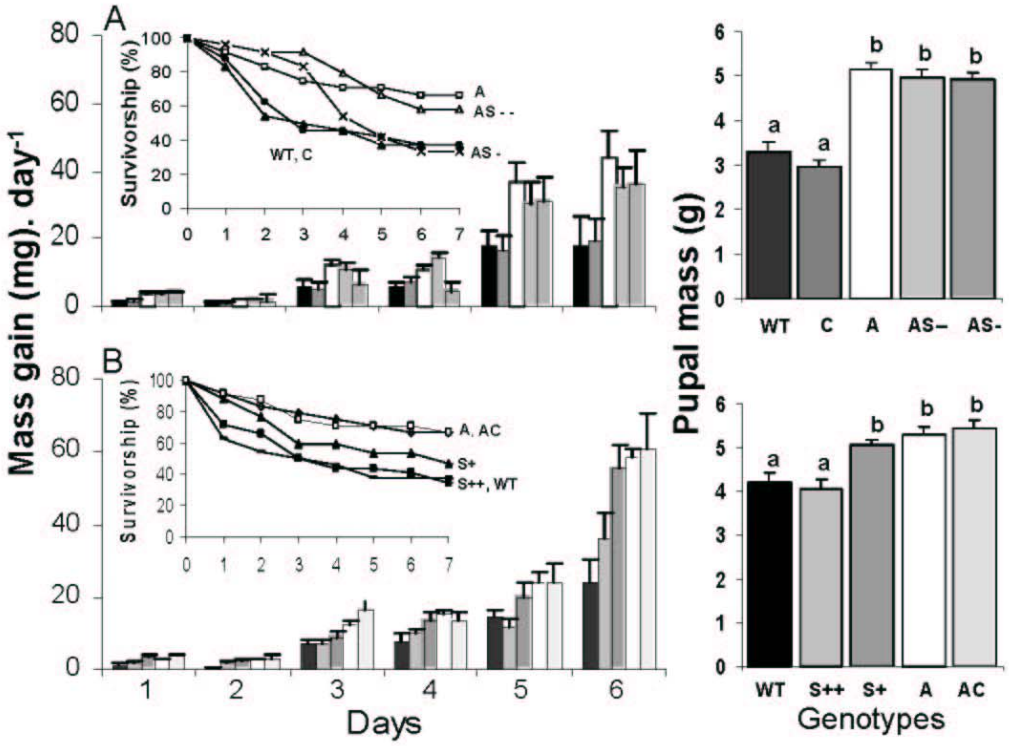

Figure 4. *M. sexta* mass gain per day (mean ± SEM), percentage survivorship and pupal mass (mean ± SEM), neonates started to feed on +1 leaves. **A.** A, AS--, AS-, WT and C genotypes. **B.** A, AC, S+, S++ and WT genotypes. Caterpillars were weighed and counted daily for 7 consecutive days (n = 24 caterpillars in each treatment). Larval mass gain per day was calculated as (final larval mass – initial larval mass). Bars with the same letter are not significantly different at $P < 0.05$ as determined by a one way ANOVA.

T**able 1:** Global and pairwise survivorship analysis of *M. sexta* larvae that fed on untransformed wild type *N. attenuata* plants (WT), two homozygous T_3 independently transformed lines transformed either with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--, AS-), or with an empty vector construct (C); untransformed plants of the Arizona (A) genotype, and A plants transformed with a construct containing the full-length *pi* gene in a sense orientation (S+, S++), or with an empty vector construct (AC).

	Chi-Square	d.f.	**P**		Chi-Square	d.f.	**P**
Global	46.229	4	**<0.0001**	Global	51.418	4	**<0.0001**
Pairwise comparisons				Pairwise comparisons			
AS-- vs AS-	9.001	1	**0.0027**	S++ vs S+	4.409	1	**0.0358**
AS-- vs WT	26.354	1	**<0.0001**	S++ vs WT	0.514	1	0.4734
AS-- vs A	0.252	1	0.6157	S++ vs A	22.059	1	**<0.0001**
AS-- vs C	24.372	1	**<0.0001**	S++ vs AC	22.059	1	**<0.0001**
AS- vs WT	4.824	1	**0.0281**	S+ vs WT	7.900	1	**0.0049**
AS- vs A	6.291	1	**0.0121**	S+ vs A	7.000	1	**0.0082**
AS- vs C	3.969	1	**0.0464**	S+ vs AC	7.000	1	**0.0082**
WT vs A	21.697	1	**<0.0001**	WT vs A	28.998	1	**<0.0001**
WT vs C	0.042	1	0.8372	WT vs AC	28.998	1	**<0.0001**
A vs C	19.886	1	**<0.0001**	A vs AC	0.146	1	0.7026

Bold type depicts significant differences at $P<0.05$.

Similar responses were found within the second group of genotype comparisons (A, AC, S+, S++ and WT). Larvae fed on S+ genotypes showed intermediate percentage survivorship, while larvae fed on A and AC showed the highest and those fed on S++ and WT genotypes the lowest percentage survivorship (Fig. 4B; Table 1). Pupal mass and larval mass gain were higher on A, AC and S+ (repeated measures ANOVA, $F_{2,\ 85\text{-A-AC-S+}} = 0.249$; $P = 0.7824$) than on S++ and WT genotypes (repeated measures ANOVA, $F_{1,\ 85\text{-S++-WT}} = 0.421$; $P = 0.5253$; Fig. 4B). Between the first and second days after caterpillar attack, the larval mass gain per day was 1.88 ± 0.46 (S++), 3.37 ± 0.63 (S+), 1.42 ± 0.23 (WT), 2.80 ± 0.33 (A) and 3.97 ± 0.31 (AC) mg d^{-1}; data obscured by symbols (Fig. 4B).

T. notatus is usually the first insect species to colonize *N. attenuata* in its native habitat, that is, the first growing season after fires in the Great Basin Desert (Glawe et al., 2003). When pairs of *N. attenuata* genotypes differing in TPI levels (AS-- vs WT; AS-- vs C; A vs S++) were exposed to a *T. notatus* colony for 24 h, adults and nymphs showed a higher preference for genotypes with either low or no TPI activity than for genotypes with high TPI levels (Wilcoxon Signed Rank test $_{\text{AS-- WT}}$; $P = 0.0022$; test $_{\text{AS-- C}}$; $P = 0.0022$; test $_{\text{A-S++}}$; $P = 0.0022$; Fig. 5A). After 3 d of *T. notatus* colonization, the number of insects per plant doubled, but the preference for genotypes with either low or no TPI activity did not change (Wilcoxon Signed Rank test $_{\text{AS-- WT}}$; $P = 0.0029$; test $_{\text{AS-- C}}$; $P = 0.002$; test $_{\text{A-S++}}$; $P = 0.002$; Fig. 5A).

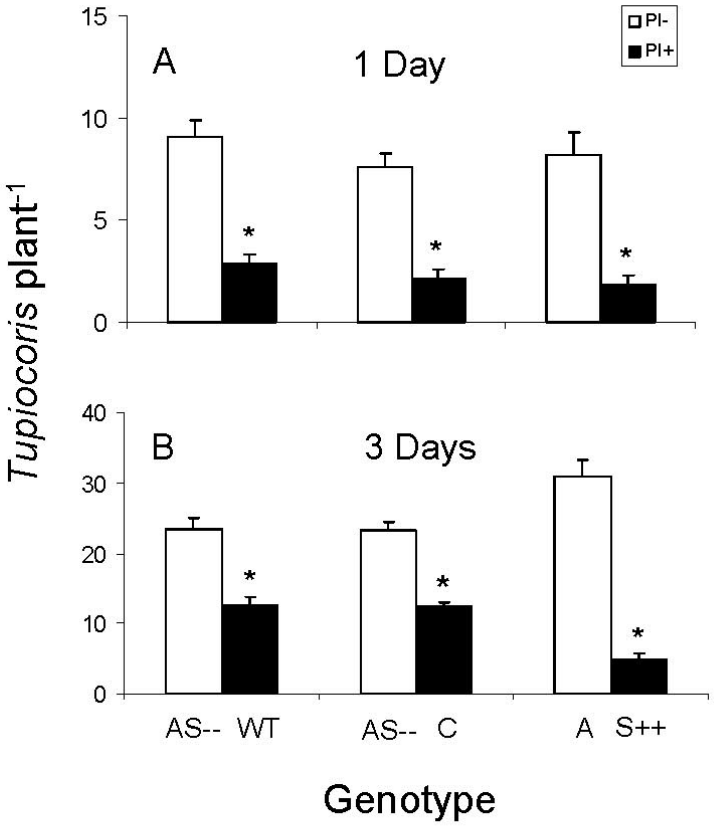

Figure 5. Number of adult and nymphs *Tupiocoris notatus* (mean ± SEM) per plant on AS-- vs WT, AS-- vs C and A vs S++ pairs. Number of mirids one day **(A)** and 3 days **(B)** after genotypes were exposed to the insects are presented. Asterisks indicate the level of significant differences between genotypes (*P<0.05); genotypes with either no or low TPI activity are depicted by open bars and those with high levels of TPI activity with solid bars.

DISCUSSION

We isolated two genes from *N. attenuata* coding for PI precursors that belong to the potato PI-II family. One codes for a 455 aa protein with a 7-repeat TPI domain, while the other codes for a 396 aa protein with a 6-repeat TPI domain, both having a N-terminal signal peptide (Nielsen et al., 1997) and a C-terminal putative vacuolar targeting sequence (Nielsen et al., 1996; Fig. 1; Suppl. Fig. 1). The *N. attenuata* PI precursor shares a conserved aa sequence with that of *N. alata* (93%) (Atkinson et al., 1993) and *N. glutinosa* (84%) (Choi et al., 2000) PIs, including the Ala-Lys cleavage site for signal peptide cleavage and the position of the Cys residues in the repeat domains for the formation of disulphide linkages. Previously 2-, 4-, 6- and 8-domain PI-II precursors have been identified in other Solanaceous plants (Graham et al., 1985; Atkinson et al., 1993; Choi et al., 2000; Miller et al., 2000) and the diversity of repeats is thought to have evolved by crossing-over events within the genomes (Barta et al., 2002). How the 7-domain repeat arose is unclear, but two 3-domain PI-II family members have been identified in tomato and tobacco (Taylor et al., 1993; Balandin et al., 1995) and single repeat members have been identified in other plant families (Barta et al., 2002). It is possible that unequal crossing over among even-numbered members, or fusions of odd and even numbered members produced the 7-repeat domain member.

Transgenic manipulation of the ability to produce TPIs allowed us to determine whether endogenous TPIs function defensively in response to attack from native, and presumably adapted, herbivores. We used the 7-repeat TPI gene to both down-regulate (1.5-and 3.5-fold) and restore (50 % and 2-fold of the uninduced WT) the expression of the *pi* gene in the WT and A genotypes of *N. attenuata*, respectively (Fig. 2). TPI activity

followed the patterns of TPI mRNA (Fig. 2 and 3) and increased 2-fold, 4 d after caterpillar attack (van Dam et al., 2001) with highest activity values after 3 d (J.A. Zavala and I.T. Baldwin unpublished data), and with the highest TPI mRNA expression after 24 h (Fig. 3). Since the transformed (AS--, AS-, C, S+, S++ and AC) and untransformed genotypes (A and WT) did not differ with respect to other defense traits, such as nicotine production (Zavala et al., 2003), this is an ideal system in which to examine defensive function of TPI expression. *M. sexta* larvae fed on genotypes with either low or no TPI activity (A, AC, S+, AS-- and AS-) grew faster, had higher survivorship and produced heavier pupae than those that fed on genotypes with high TPI activity (WT, C and S++; Fig. 4). Similarly, *T. notatus* preferred genotypes with either low or no TPI and CPI activity (AS-- and A) to genotypes with high TPI and CPI levels (WT, C and S++; Fig. 5). These results are qualitatively and quantitatively consistent with those from previous work on A and WT genotypes (Glawe et al., 2003) and demonstrate that endogenous TPI functions defensively against native herbivores.

The defensive function of TPIs begins with their affinity for insect proteinases (Laing and McManus, 2002), which generates the expectation of a positive quantitative relationship between the level of TPI expression and the protection they afford. However, the relationship between TPI expression and defense is complicated by the numerous counter-responses that insects have evolved. Insects, for example, in response to ingestion of high TPI leaves are known to increase their rate of leaf consumption (Cloutier et al., 2000; Winterer and Bergelson, 2001), the secretion of total proteolytic digestive enzymes (Broadway, 1997) or of particularly TPI-insensitive proteinases (Jongsma et al., 1995). If the insect responds by increasing its consumption rate, the net

fitness effect of TPI expression may not favor the plant. TPI expression is frequently found to slow herbivore growth (Charity et al., 1999), and the fitness benefit of this direct defense can be greatly enhanced when simultaneously expressed with indirect defenses that increase the mortality rate of these slow-growing herbivores. In *N. attenuata*, TPI expression is coordinated with the release of volatile signals that attract the generalist predator *Geocoris pallens* to feeding larvae (Kessler and Baldwin, 2001). This voracious predator is size-selective, preferentially attacking eggs and larvae in the first three instars. The up-regulation of TPIs by herbivore attack slows the growth of larvae and keeps them in stages that are more vulnerable to the predator. Interestingly, the volatile signals are elicited by the same signals that elicit TPI production (Halitschke et al., 2000; Halitschke et al., 2001; van Dam et al., 2001), underscoring the coordination of these direct and indirect defenses. TPIs affect not only herbivore performance, but can also affect the natural enemies of the herbivores. When the Colorado potato beetle (*Leptinotarsa decemlineata*) feeds on transgenic potato plants that express cysteine PI, which increase the PI levels of the prey, these sequestered PIs do not influence the digestion of the predatory stinkbug (*Perillus bioculatus*) because the predator compensates by synthesizing *de novo* serine-type proteinases (Bouchard et al., 2003; Bouchard et al., 2003).

TPI expression alone is known to increase the mortality rate of herbivores, particularly for neonate larvae (McManus and Burguess, 1995; Heath et al., 1997; Charity et al., 1999; McManus et al., 1999; Marchetti et al., 2000; De Leo and Gallerani, 2002). In this study, larvae that ingested plants with high TPI content had not only decreased growth rates but also a lower survivorship (approximately 40 % difference) than larvae

fed on low TPI genotypes. The effects of constitutive differences in TPI expression between genotypes is clearly seen in the weight gain of caterpillars during the first two days of feeding: larval mass gain per day was 2-fold higher in AS-, AS-- and A than in WT and C genotypes (Fig. 4A) and in A, AC, and S+ than in S++ and WT genotypes (Fig. 4B). These early differences translated into significantly different pupal masses 22 days later, which in turn, is an accurate proxy for fecundity in lepidoptera (Haukioja and Neuvonen, 1985; Awmack and Leather, 2002; De Leo and Gallerani, 2002; Klemola et al., 2003). For the high TPI expressing lines (WT, C, S++) survivorship decreased (Fig. 4A, B) in tandem with the decreases in growth rate. Interestingly, for the larvae fed on the genotype with intermediate TPI levels (AS-: 0.94 nmol. mg $prot^{-1}$), the decrease in survivorship is correlated with inducible TPI expression, which attained maximum values 3 days after attack. Survivorship for these larvae during the first 3 d was similar to survivorship of those feeding on the low TPI (AS--) line, but after day 3 (TPI induction; 2.63 nmol. mg $prot^{-1}$) survivorship was similar to that of larvae fed on WT and C genotypes. These results suggest that a threshold amount of TPI expression is required to effect changes in larval survivorship and corroborate results from experiments with *Helicoverpa armigera* fed on artificial diets containing soybean TPI and on transgenic tobacco plants expressing giant taro TPI on (Johnston et al., 1993; Wu et al., 1997). In the AS- lines, TPI elicitation killed larvae with low mass, which increased the average mass of the survivors (Fig. 4A). The intermediate constitutive TPI levels in AS- plants may have allowed heavier larvae to adapt their digestive system to a diet rich in TPIs, while smaller larvae were unable to adapt. The process of adaptation may involve replacing the inhibited trypsin with the secretion of new trypsins, which are insensitive to the particular

TPIs of the diet (Broadway, 1995; Jongsma et al., 1995). Our results suggest that the sensitivity threshold for TPI is likely to differ from insect to insect and that the developmental stage of the insect when first exposed to the inhibitor may also determine the defensive value of TPIs for plants.

In summary, this research demonstrates that despite the ongoing evolutionary interaction between *N. attenuata* and its herbivores, TPIs remain an effective defense against both *T. notatus* and *M. sexta*.

MATERIALS AND METHODS

Plant material and transformation

Nicotiana attenuata Torr. Ex Wats. (Solanaceae) used in this study were grown from seeds collected from either Utah (Baldwin, 1998) or Arizona (Glawe et al., 2003) and inbred 10 and 4 generations, respectively. In order to silence the expression of *N. attenuata*'s *pi* gene in the genotype collected in Utah (WT), WT was transformed by an *Agrobacterium*-mediated transformation procedure with pNATPI1, which contains 175 bp of *N. attenuata*'s 7-repeat domain *pi* gene in an anti-sense orientation, as described in (Zavala et al., 2003). One homozygous T_3 independently transformed line of WT plants (C) which had been transformed with an empty vector construct (lacking only the *pi* gene fragment**)** and whose TPI activity resembled that of WT plants, was selected as a control for the bioassay experiments. Southern gel blot analysis confirmed that all T_3 lines were single-copy independent transformants (Zavala et al., 2003).

A genotype of *N. attenuata* collected from Arizona (A), has MeJA-inducible nicotine production identical to that found in WT plants, but completely lacked the ability

to produce TPIs or accumulate TPI mRNA (Glawe et al., 2003). More recently, the mutation in the 7-domain repeat *pi* of A plants has been characterized and found to be located in the 5'signal peptide, resulting in a premature stop codon (J. Wu and I.T. Baldwin unpublished data). Since we never detected TPI activity with radial diffusion assay in A genotype, nor have we detected TPI mRNA transcripts with either Northern blots (van Dam et al., 2001; Glawe et al., 2003) or RT-PCR, we suggest that this transcript is rapidly silenced. Plants of the A genotype were transformed with a binary transformation vector pRESC2PIA2 containing the full-length 7-domain *N. attenuata pi* gene from the WT genotype in the sense orientation under control of a CaMV 35S promotor (Zavala et al., 2003). Several T_1 lines harboring a single copy of the transgene (Zavala et al., 2003) were screened for TPI activity; one of those lines (S) expressing either low TPI activity (heterozygous; S+) or high TPI activity (homozygous; S++) at T_1 was used in the experiments. S++ plants were bred to homozygosity in the T_2 and used again in the experiments, confirming the previous results. One homozygous T_2 independently transformed line of A plants (AC: see Supplemental Information) which had been transformed with an empty vector construct (lacking only the *pi* gene fragment**)** and had TPI activity equivalent to that of A plants (not detectable), was selected as a control. All of these transformed and untransformed genotypes were used in the bioassay experiments.

Isolation of the TPI gene

Based on the *N. alata* TPI sequence (Atkinson et al., 1993), primers PI2-FOR (5' CTGATCCTAGAAATCCAAAGGC 3') and PI2-REV (5'

GCATATTCAGATTCTCCTTCAC 3') were designed and used in PCR of chromosomal DNA of *N. attenuata*. The product was cloned in pUC19 cut with *Sma*I (pUCPI2/14), sequenced, and used to screen a cDNA library (Hermsmeier et al., 2001) of *Manduca sexta*-attacked leaves of *N. attenuata* plants for isolating a full-length clone of the PI gene.

Feeding bioassay experiments.

In order to determine the effect of either down-regulation or restored expression of the *pi* gene in *N. attenuata* on caterpillar mass gain, survivorship and pupal mass, two feeding experiments with different combinations of genotypes were performed either: 1) with AS lines (AS-- and AS-), untransformed genotypes (WT and A) and a transformed WT line with empty vector construct (C), or 2) with A lines transformed to express the functional *pi* (S+ and S++), untransformed genotypes (WT and A) and a transformed A line with empty vector construct (C). Seeds were germinated in diluted liquid smoke solutions as described in (Baldwin et al., 1994). Seedlings were transplanted in 1-L pots in a glasshouse in the conditions described in (Glawe et al., 2003) with 800 – 900 μmol m^{-2} s^{-1} PPFD supplied by 450 W Na-vapor HID bulbs. Eggs of *Manduca sexta* L. (Lepidoptera: Sphingidae) were obtained from Carolina Biological Supply Company (Burlington, North Carolina, USA) and placed in plastic containers (200 mL) on a moist tissue. The containers were kept in climate chambers at 28°C and 65 % relative humidity and a 16:8 h light:dark photoperiod until the eggs hatched. Neonates were placed individually on the node +1 (one position older than the source-sink transition leaf; (van Dam et al., 2001) leaf of 24 soil-grown rosette plants of each genotype. Larvae mass and

number were determined daily for 7 days after hatching and pupae were weighed 25 days after hatching. In order to ensure that caterpillars reached the pupal stage with sufficient leaf material, larvae between the third and fourth instars were transferred to new plants from the same genotype. Constitutive and TPI activity induced by caterpillar damage were determined from 20 replicates during rosette stage growth. Leaves growing at node +1 were harvested 3 days after the larvae started to feed, and protein concentrations and TPI activity were measured using a radial diffusion assay and expressed as nmol mg^{-1} as described in (van Dam et al., 2001).

To determine whether *N. attenuata* TPI inhibits chymotrypsin, 10 rosette stage plants were either unwounded or wounded with a pattern wheel over the source-sink transition leaf surface (W) and 20 µl of *M. sexta* oral secretion (OS) was applied to the fresh puncture wounds (W + OS) (Halitschke et al., 2000). Source-sink transition leaf from unwounded and W +OS was harvested 3 days after induction for both TPI and CPI analysis. The qualitative determination of TPI and CPI activities was measured using a radial diffusion assay as described in (van Dam et al., 2001).

Colonization experiments.

In order to determine the colonization preference of *Tupiocoris notatus* (Hemiptera: Miridae), 3 pairs of genotypes with either low or no TPI activity and high TPI levels (AS-- vs WT; AS-- vs C; A vs S++) were placed in a glasshouse adjacent to *N. attenuata* plants infested with a *T. notatus* colony. We monitored the accumulation of the insects on the plants at 24 h and 3 days after the pairs of genotypes placement. These experiments were repeated twice.

TPI mRNA expression analysis.

Leaves attacked by larvae during the first 24 h (CAT) and leaves from plants without larvae (CON) from the same position (+1) were harvested for Northern blot analysis of TPI mRNA accumulation as described in (Winz and Baldwin, 2001; Glawe et al., 2003) in 4 replicate plants from each genotype and pooled. These plants were excluded from subsequent analysis.

For real-time PCR analysis leaves growing at nodes +1 and -1 (one position younger than the source-sink transition leaf) from CAT and +1 from CON treatments were harvest from 3 replicate plants at 0, 6, 12, 24 and 48 h after the larvae started to feed. The relative expression of TPI mRNA was compared to that of undamaged WT. The isolated RNA was quantified spectrophotometrically and diluted to 300 ng/µl. The diluted RNA was reverse-transcribed (Applied Biosystems) and 10 ng of the reverse transcribed template was used in a 25 µl PCR reaction containing 1x universal mix (Eurogentec, Belgium), 300nM forward (5' TCAGGAGATAGTAAATATGGCTGTTCA 3') and reverse primers (5' ATCTGCATGTTCCACATTGCTTA 3') and 300nM of FAM-labelled Taqman® probe (5' TCCTTGCTCTCCTCCTCTTATTTGGAATGTCT 3') with 18s RNA (Eurogentec, Belgium) as internal standard. Thermal cycling and detection was performed on a ABI Prism 7700 Sequence Detector.

Statistical analysis.

Data were analyzed with Stat View, Version 5.0 (SAS, 1998). The TPI, protein and pupal mass were analyzed by ANOVAs followed by Fisher's protected LSD *post-hoc* comparisons in all experiments. For the survivorship analyses, we used the log-rank test for the global hypothesis of equality of survival distribution for *M. sexta*, and performed the same test with a pairwise ranking of data using only two groups at a time (Zavala et al., 2001). Larval mass gain per day was calculated as (final larval mass – initial larval mass) and data were analyzed with repeated measures ANOVA. Differences in colonization preference were analyzed by Wilcoxon Signed Rank test.

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Supplemental Section.

I. DNA Isolation and Southern Hybridization:

Prior work revealed 1 copy of T-DNA present at one locus in transformed lines of the WT genotype that had been transformed either with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--, AS-), or with an empty vector construct (C), as well as in lines of the Arizona genotype transformed with a construct containing the full-length *pi* gene in a sense orientation (S+, S++) (Zavala et al., 2003). The genotype of *N. attenuata* collected from Arizona (A), which completely lacks the ability to produce TPIs (Glawe et al., 2003), was transformed with pRESC2PIA2 (Zavala et al., 2003), with an empty vector construct (lacking only the *pi* gene fragment; AC), and Southern analysis revealed 1 copy of T-DNA in all transformed lines used (Suppl Fig. 3; Zavala et al., 2003). The Southern analysis confirmed our initial PCR analysis in which the transformed plants revealed a 260 bp band corresponding to the NOS terminator and no signal in the WT plants (data not show).

DNA was isolated using CTAB (Rogers and Bendich, 1988) and the amounts were quantified spectrophotometrically at A260 nm. Twenty μg of DNA was digested with the *Hin*dIII, electrophoresed on 1% agarose gel (in 1X TAE buffer), blotted on nylon membrane (NEN Life Science Products, USA) according to the protocol of Sambrook et al., 1989 and hybridized with a radiolabelled probe either from a plasmid containing the repeat region (Suppl. Fig. 1) of the PI gene (pUCPI2/14; see below) generated by PCR using the primers SMA1-19 (5' GAATTCGAGCTCGGTACCC 3') (Suppl. Fig. 2) and SMA2-20 (5' GTCGACTCTAGAGGATCCCC 3') or from the NOS terminator of the vector plasmid pRESC 20 (Zavala et al., 2003) generated by PCR using

the primers NOT 2-23 5' CCCCGATCGTTCAAACATTTGGC 3' and NOT 1-29 5' CCCGATCTAGTAACATAGATGACACCGCG 3' (Suppl. Fig. 3). Hybridization was detected using a Phosphoimager (FLA 3000 Fujifilm, Japan) and quantified with Aida Image Analyzer (v. 3.11; Fujifilm, Japan).

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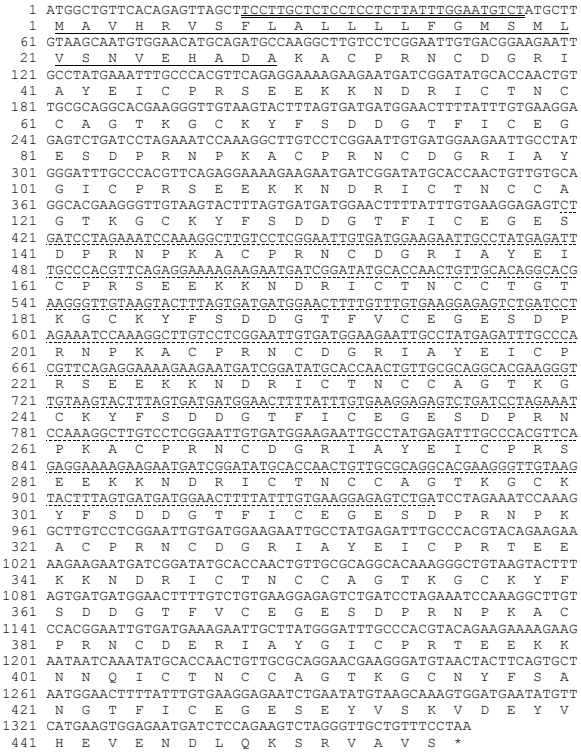
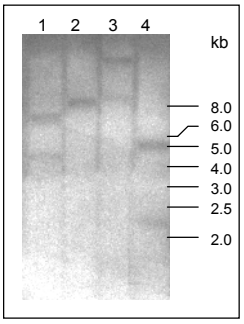
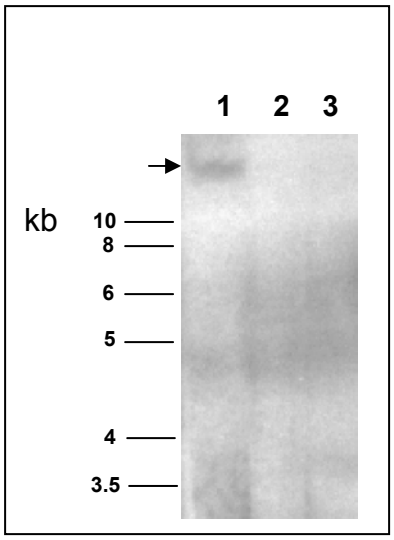


Supplemental Figure 1. DNA sequence of the 7-repeat *Nicotiana attenuata* PI precursor gene isolated from the cDNA library. The sequence of the Taqman probe used for real time PCR is underlined with a double line while that of the signal peptide (SP) is underlined with a single line. The region contained in the plasmid pUCPI2/14 (see Experimental procedures) used as a probe for hybridizations is underlined with a dotted line.



Supplemental Figure 2. Southern gel blot of the genomic DNA of *Nicotiana attenuata* plants of the WT genotype digested with *Eco*RI (lane1), *Hin*dIII (lane2), *Eco*RV (lane3) and *Ssp*I (lane4) and hybridized with radiolabelled plasmid containing *N. attenuata* PI repeat (pUCPI2/14) (Supplemental Fig. 1).



Supplemental Figure 3. Southern blot of the genomic DNA of transformed plants of Arizona with an empty vector construct (lacking only the *pi* gene fragment**)** (AC; lane 1), of untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT; lane 2), and of untransformed plants of the Arizona genotype (A; lane 3) digested with *Hin*dIII and hybridized with radiolabelled probe from the NOS terminator of the vector plasmid pRESC20**.**

Supplemental Table 1. Comparative qualitative activities of trypsin proteinase inhibitor (TPI) and chymotrypsin proteinase inhibitor (CPI) after 3 days of either unwounded or wounded and *M. sexta* oral secretion (W + OS), of either untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT), two homozygous T_3 independently transformed lines of the Utah genotype that had been transformed either with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--), or untransformed plants of the Arizona (A) genotype, and plants of a homozygous T_2 transformed line of the Arizona genotype transformed with a construct containing the full-length *pi* gene in a sense orientation (S++). TPI and CPI activity in A genotype were not detectable (-).

Genotype	Treatments	TPI	CPI
Utah			
WT	unwounded	+	+
WT	W + OS	++	+
AS --	unwounded	+	+
AS --	W + OS	+	+
Arizona			
A	unwounded	-	-
A	W + OS	-	-
S ++	unwounded	++	+
S ++	W + OS	++	+

Manuscript 4

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Fitness Benefits of Trypsin Proteinase Inhibitor Expression in *Nicotiana attenuata* Are Greater Than Their Costs When Plants Are Attacked

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Summary

The commonly invoked cost-benefit paradigm, central to most of the functional biology, explains why one phenotype cannot be optimally fit in all environments; yet it is rarely tested. Trypsin proteinase inhibitors (TPIs) expression in *Nicotiana attenuata* is known to decrease plant fitness when plants compete with unattacked conspecifics that do not produce TPI and also to decrease the performance of attacking herbivores. In order to test whether the putative benefits of TPI production outweigh its cost, we compared the lifetime seed production of *N. attenuata* genotypes of the same genetic background with low or no TPI to that of genotypes with high TPI levels on which *M. sexta* larvae were allowed to feed freely. Unattacked low TPI-producing genotypes produced more seed capsules than did plants with high TPI levels. Caterpillar attack reduced seed capsule production in all genotypes and reversed the pattern of seed capsule production among genotypes, demonstrating that the fitness benefits of TPI production outweigh their costs, when plants are attacked. *M. sexta* larvae attacking genotypes with high TPI activity consumed more TPI, less protein, and didn't move to the high-fitness value young leaves; their lower masses were negatively correlated ($R^2 = 0.56$) with seed capsule production per plant.

Introduction

The cost-benefit paradigm is central to functional biology and to ecological and evolutionary theory because fitness costs and benefits associated with a trait determine its equilibrium value in a population. If the trait offers fitness benefits to the population rather than costs, then selection should lead to beneficial allele(s) being fixed, which reduces variability (Simms and Rausher 1987). Alternatively, when the fitness benefit of the trait also has a cost, intermediate frequencies of the trait are favored because the benefit varies (Cipollini et al. 2003; Mauricio 1998; Simms and Rausher 1987). For example, the cost-benefit balance determines the fitness value of group living in low-ranking females as mediated by maternal care in matrilineally organized social groups of spotted hyenas (*Crocuta crocuta*) (Hofer and East 2003) and in small forest rodents (Bujalska and Grum 1989; Eccard and Ylonen 2003a; Eccard and Ylonen 2003b; Gerlach and Bartmann 2002). In addition, the cost-benefit balance of parental care and the decision to court or defend can affect the fitness of some animal species (Kraak and Weissing 1996; Santangelo et al. 2002; Strohm and Marliani 2002; Zink 2003). Resistance against natural enemies has costs as well as obvious benefits for fitness, as has been shown in insect-parasite, insect-parasitoid, and plant-insect systems (Baldwin 1998; Kraaijeveld et al. 2002; Milks et al. 2002).

Herbivores can reduce seed production and other correlates of plant fitness, and this reduction can result in natural selection for either constitutively expressed or inducible plant defenses (Karban and Baldwin 1997; Marquis 1984; Stamp 2003). Current theory predicts that one benefit of induced defenses is to allow a plant to optimize its allocation of limiting resources to defense, growth, and reproduction (Karban

and Baldwin 1997). Although defenses might benefit plants in the presence of herbivores, plant resistance to herbivores can be costly in the absence of enemies, and inducible expression of resistance traits allow plants to forgo or, to pay the potential fitness cost of resistance traits when they are needed (Agrawal 1998; Baldwin 1998; Cipollini et al. 2003; Hare et al. 2003; Strauss et al. 2002; Zangerl 2003).

Evidence for the existence of resistance costs and benefits from studies using plant species with constitutive and inducible defenses is increasing (Bergelson and Purrington 1996; Cipollini et al. 2003; Heil and Baldwin 2002; Zangerl 2003). However, attribution of fitness consequences to expression of a particular defense trait in an environment either with or without herbivory is onerous, because genes that control the expression of defensive traits may have pleiotropic effects on fitness traits (Elle et al. 1999). Ideally, one should assess the costs and benefits of inducible defenses in plants that differ only in the expression of genes that control (induced) resistance but are otherwise genetically identical (Bergelson and Purrington 1996). Transformation technology provides a means of manipulating traits with unparalleled precision. Although the benefits of plant traits that provide resistance against herbivores are expected to equal or exceed their cost (Coley et al. 1985; Feeny 1976; Rhoades and Cates 1976), very few direct tests have been done. While costs and putative benefits of defense traits have been studied in separate experiments, their currencies are usually not comparable (i.e., plant fitness for the cost; herbivore performance for the benefits). Tests of the cost-benefit model using the same currency are few (Baldwin 1998).

All ecological interactions can be viewed as the net outcome of a series of cost-benefit maximizations in which both players respond to the variability in each others'

defense traits. For example, there is enormous within-plant heterogeneity of defensive secondary metabolites. This heterogeneity could motivate within-plant movement of herbivores, so that they eat leaves of low fitness value rather than leaves of high plant fitness value, or it could motivate herbivores to move off plants and onto neighboring competitors (Denno and McClure 1983; van Dam et al. 2001a). Herbivores, in turn, can both readjust their metabolism to cope with the secondary metabolites as well as adjust their feeding positions to maximize their performance (Cloutier et al. 2000; Denno and McClure 1983; Jongsma et al. 1995). We present here a cost-benefit analysis of a plant-insect interaction in which the costs and benefits of a defensive protein are evaluated in the currency of plant fitness.

Nicotiana attenuata [Torr. Ex Wats. (synomonous with *Nicotiana torreyana* Nelson and Macbr.)], a postfire annual native tobacco inhabiting the Great Basin Desert, has a number of well-described herbivore-induced direct and indirect defenses (Baldwin 2001), which increase the fitness of plants under attack in natural populations (Baldwin 1998; Kessler and Baldwin 2001). Trypsin proteinase inhibitors (TPI) play an important defensive role in addition to nicotine (Baldwin 2001). We isolated cDNA from *N. attenuata* that coded for a TPI precursor belonging to the potato PI-II family with a 7-repeat TPI domain. The normal constitutive expression of this gene increases 4-fold after herbivore attack (Glawe et al. 2003; Zavala et al. 2004b).

The elicitation of TPI expression in *N. attenuata* is constrained by ontogeny and leaf age (van Dam et al. 2001b), as is true for nicotine (Ohnmeiss and Baldwin 2000) and volatile emissions (Halitschke et al. 2000). The within-plant pattern of systemic TPI induction at the rosette stage of growth suggested that the signal(s) triggering remote TPI

induction follows a source-sink relationship: regardless of ontogenetic stage, if young sink leaves are damaged TPI levels increase only locally, while older leaves are less sensitive to leaf damage and produce a less local intense wound signal but increases the TPI levels systemically in younger leaves (van Dam et al. 2001b). The spatial and temporal variability in *N. attenuata*'s ability to deploy certain defenses against herbivores can be correlated with the relative fitness values of leaves growing at particular nodes. Removal of young and mature leaves at the elongation stage in *N. sylvestris* had a greater negative effect on fitness than did the removal of old leaves, but not at either the rosette or flowering stages, demonstrating the different fitness values of leaves growing at different nodes on a plant. Damage to younger leaves increases nicotine contents more than damage to older leaves does, suggesting that defense allocation is proportional to the fitness value of the tissue, as predicted by Optimal Defense (OD) theory (McKey 1974; Ohnmeiss and Baldwin 2000; Rhoades and Cates 1976; Stamp 2003).

Manduca sexta moths, a specialized lepidopteran herbivore, prefer elongating *N. attenuata* plants to rosette-stage plants for ovipositing and place eggs on leaves in the middle section of the stem (from S1 to S3; Figure 1; Kessler and Baldwin 2002). TPIs of *N. attenuata* leaves reduce the growth of *M. sexta* larvae (Glawe et al. 2003; Zavala et al. 2004b). However, insects may adapt to high TPI levels, replacing the inhibited trypsin with the secretion of trypsins that are insensitive to the particular TPIs of the diet (Broadway 1995; Jongsma et al. 1995). Intra-plant movement of the first instar larvae is very rare but common in the second- to fourth-larval instars (Kessler and Baldwin 2002). Larvae are particularly sensitive to jasmonate-induced defenses during the third instar (approx. 11 days after hatching), and can be motivated to move between adjacently

growing plants (van Dam et al. 2001a) by the plant's induced defense. When *M. sexta* larvae were placed on MeJA-induced plants, larvae left the induced plants 1-3 days earlier than did larvae placed on uninduced plants, which dramatically reduced the leaf area consumed and larval weight gain (van Dam et al. 2000).

TPI expression in *N. attenuata* is known to decrease lifetime seed production in unattacked but competing plants (Glawe et al. 2003) and to decrease herbivore performance in attacked plants (Glawe et al. 2003; Zavala et al. 2004). Whether the TPI-mediated decrease in herbivore performance translates into a fitness benefit for the plant is unknown. In other systems, plants expressing high PI levels caused herbivores to grow more slowly, but they compensated by eating more tissue, a potential fitness detriment for the plant (Winterer and Bergelson 2001). Here we provide a critical test of whether the fitness benefits of TPI expression outweigh their costs.

We compare lifetime seed production of *N. attenuata* genotypes with either low or no TPI production to that of TPI-producing genotypes on which *M. sexta* larvae were allowed to feed freely for 11 days. TPI and protein content were measured in all genotypes at all leaf positions. *M. sexta* larval mass and movement were recorded, and we calculated and simulated the amount of TPI and protein consumed by the larvae from the larval movement and the TPI and protein concentration at each leaf position from each genotype. We used two independently transformed *N. attenuata* lines in which the expression of the *pi* gene was down-regulated by antisense expression of a 175 bp fragment of the *N. attenuata pi* gene (AS--, AS-), and untransformed wildtype plants (WT) of the same genetic background (an inbred line collected from Utah). In addition, we used a natural *N. attenuata* genotype collected from Arizona, which has a mutation in

the endogenous 7-domain *pi* gene and does not produce *pi* transcripts or TPI activity (A). We transformed this genotype with the full-length cDNA of the 7-domain *pi* gene in a sense orientation under control of a constitutive promotor (S++), so that after 11 days of caterpillar attack it produced TPIs at 74 % of the level found in the stem leaves of the wildtype Utah genotype. Our analysis demonstrates that the fitness benefits of TPIs production outweigh their cost.

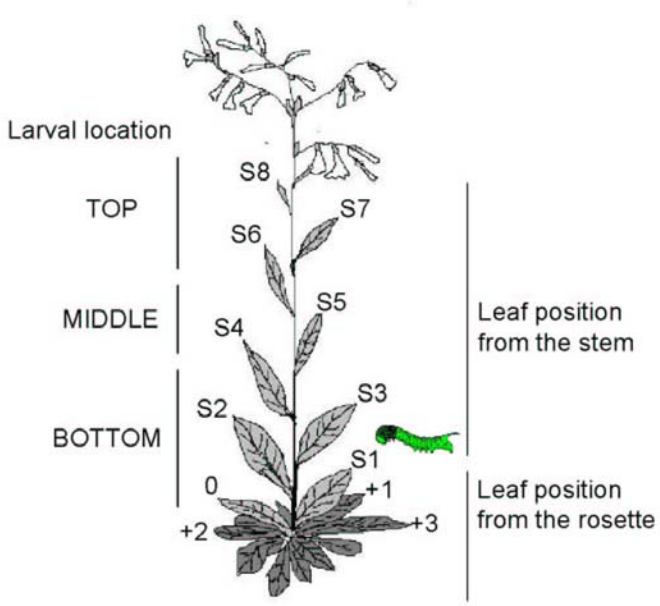

Figure 1. Sketch of *Nicotiana attenuata* plant showing different leaf positions on either the rosette or the stem (Kessler and Baldwin 2002) and larval location. Larva depicts the leaf growing at node S1 on which a single *M. sexta* neonate was placed.

Results

Spatial and temporal distribution of plant TPI/protein contents

In order to determine the effect of caterpillar attack on TPI activity, measurements were made from all rosette and stem leaves before, and 4 and 11 d after larvae started to feed on S1 leaves (Figure 1) from transformed (AS--, AS-, and S++) and untransformed (WT and A) genotypes (Figure 2 and Supplemental Figures 1-4). All genotypes had high within-plant heterogeneity of TPI activity and protein contents. Constitutive TPI levels in all genotypes on day 0 (before larvae started to feed) were higher in rosette leaves than in stem leaves ($F_{1,70\text{-AS--}} = 217.13$; $P < 0.0001$; $F_{1,70\text{-AS-}} = 357.76$; $P < 0.0001$; $F_{1,70\text{-WT}} = 209.89$; $P < 0.0001$; $F_{1,70\text{-S++}} = 4.27$; $P = 0.042$), while protein content showed the opposite pattern, with higher levels in stem leaves than in rosette leaves ($F_{1,70\text{-AS--}} = 331.82$; $P < 0.0001$; $F_{1,70\text{-AS-}} = 256.68$; $P < 0.0001$; $F_{1,70\text{-WT}} = 289.67$; $P < 0.0001$; $F_{1,70\text{-S++}} = 1.87.16$; $P < 0.0001$; Supplemental Figure 1-4) which persisted through the samplings performed on day 4 and 11 (data not shown). A-genotype plants had a similar pattern in protein content (data not shown; $F_{1,70\text{-A}} = 245.51$; $P < 0.0001$). Caterpillar attack increased levels and within-plant heterogeneity of TPI activity. Larval damage to WT plants increased TPI activity 2.5-fold in S1 leaves ($F_{1,14} = 197.079$; $P < 0.0001$) and 1.7-fold in unattacked ($F_{1,110} = 17.341$; $P < 0.0001$) stem leaves, and did not alter TPI activity in older rosette leaves 4 d after neonates started to feed ($F_{1,62} = 0.042$; $P = 0.8389$; Figure 2 and Supplemental Figure 1). By day 11, TPI activity had increased in WT S1 leaves 4-fold ($F_{1,22} = 183.304$; $P < 0.0001$), 2.5-fold in the stem leaves (S avg; $F_{1,334} = 337.072$; $P < 0.0001$; Figure 2), and also marginally (1.1-fold) on the rosette leaves ($F_{1,94} = 8.666$; $P < 0.0041$; Supplemental Figure 1).

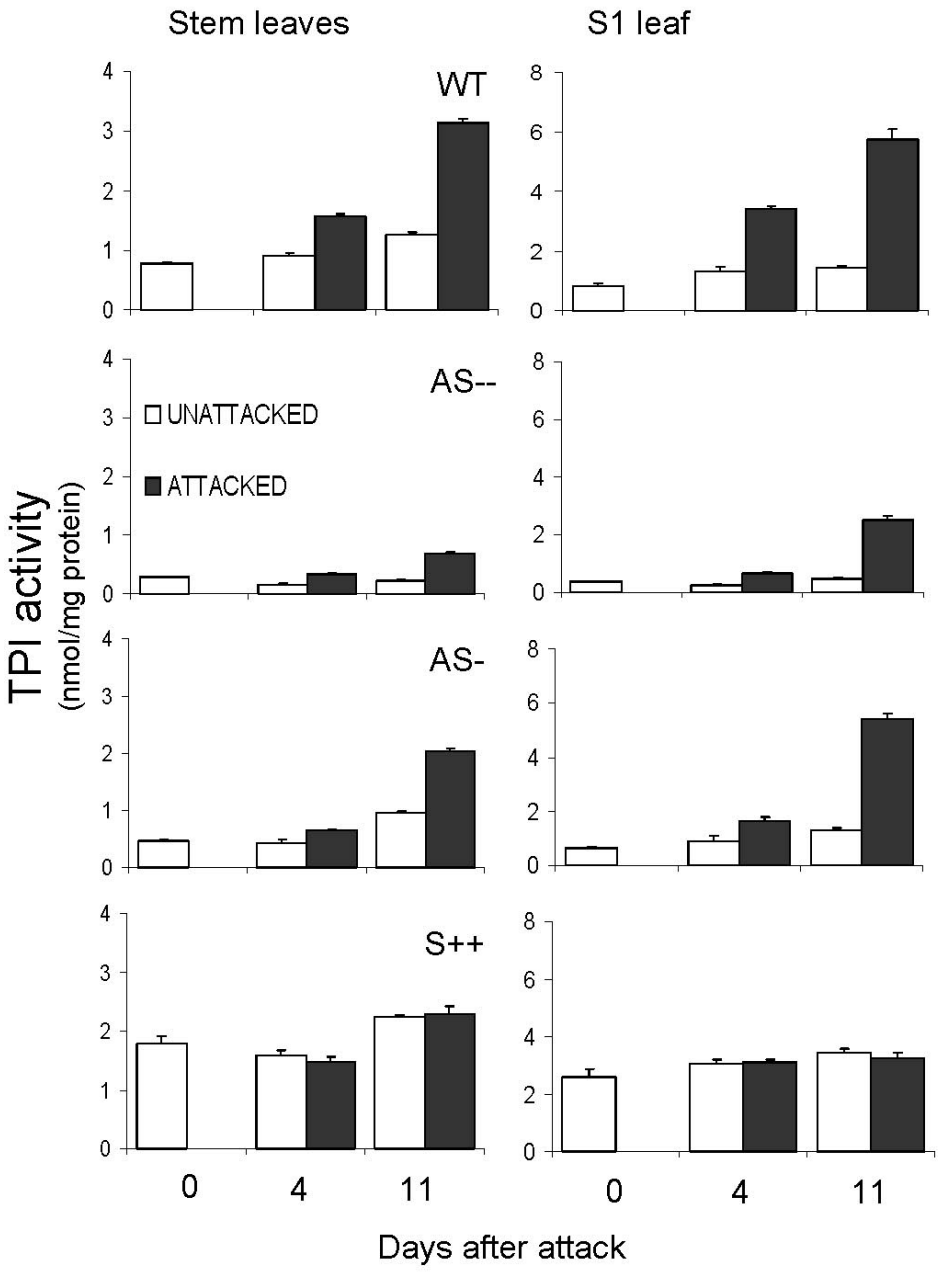

Figure 2. TPI activity (mean ± SEM) from stem leaves and the leaf growing at node S1 of untransformed wild type *Nicotiana attenuata* plants of the Utah genotype (WT); two homozygous T_3 independently transformed lines of the Utah genotype that had been transformed with a construct containing a 175 bp *pi* gene fragment in an antisense orientation (AS--, AS-); plants of a homozygous T_3 transformed line of the Arizona genotype transformed with a construct containing the full-length *pi* gene in a sense (S++) orientation before attack (day 0); and either unattacked or attacked by *M. sexta* larvae 4 and 11 d after neonates started to feed on the leaf at S1 position.

Levels and within-plant heterogeneity of TPI activity were either intermediate or low in AS compared to WT plants after larval damage. After 4 days of caterpillar attack, TPI levels in AS-- and AS- genotypes were 60 % and 40 % lower than those of unattacked WT ($F_{1,190\text{-AS--total}} = 62.48$; $P<0.0001$; $F_{1,190\text{-AS-total}} = 23.46$; $P<0.0001$; Supplemental Figures 2 and 3). Caterpillar attack increased TPI activity 2.4-fold in S1 leaves in AS plants, attaining values that were 19% and 48% in AS-- and AS- plants, respectively of that in attacked WT plants ($F_{1,14\text{-AS--S1}} = 630.391$; $P<0.0001$; $F_{1,14\text{-AS-S1}} = 193.239$; $P<0.0001$; Figure 1); TPI levels in the stem leaves were 37% and 55% of that found in induced WT plants ($F_{1,190\text{-AS--}} = 89.862$; $P<0.0001$; $F_{1,190\text{-AS-}} = 42.165$; $P<0.0001$; Figure 2). By day 11, TPI levels in stem leaves were 22% in AS-- and 65% in AS- of the WT levels ($F_{2,501} = 225.561$; $P<0.0001$; Figure 2).

As expected, caterpillar attack did not affect either levels or within-plant heterogeneity of TPI activity of S++ plants. Compared to the constitutive levels of TPI activity in the WT, levels in S++ plants on day 4 were 30% higher ($F_{1,190\text{-total}} = 23.8$; $P<0.0001$; Supplemental Figure 4). Caterpillar attack did not alter TPI activity in S++ plants ($F_{1,430\text{-total}} = 0.179$; $P = 0.06723$; Supplemental Figure 4) which remained at approximately 90% of the induced WT plants in the S1 leaf and 1.1-fold at the plant level (averaged across all measured leaf positions; $F_{1,14\text{-S++-S1}} = 3.881$; $P = 0.0689$; $F_{1,190\text{-total}} = 0.833$; $P = 0.3626$; Figure 2). By day 11 d, TPI activity in S++ plants were 56% in the S1 leaf ($F_{1,22} = 48.821$; $P<0.0001$) and 74% in stem leaves of the induced WT levels ($F_{1,430} = 72.349$; $P<0.0001$; Figure 2). As expected, the untransformed A genotype showed no TPI activity even after caterpillars had fed on the plant for 4 or 11 d. Protein levels did not differ significantly among genotypes.

Within-plant movement of *M. sexta* larvae

To determine the effect of TPI on within-plant movement of *M. sexta* larvae, we measured the position of each larva on each plant daily. Caterpillars on low TPI genotypes left the S1 leaf and moved to the bottom of the plant earlier than those feeding on high TPI genotypes (Figure 3). While larvae on WT plants started to move from the S1 leaf to the bottom of the plant after 5 d, larvae on AS-- plants started to move 2 d earlier (day 3), and those that fed on AS- plants started to move 1 d earlier (day 4; Figure 3). This early larval movement resulted in more larvae on the top and middle parts of plants from the AS-- genotype (51 %, 77 %, and 80%) than on WT plants (11 %, 37 %, and 49 %) during subsequent days (days 6-8; Mann-Whitney *U*-test; $P < 0.0001$; Figure 3). Interestingly, by day 11, 65 % of the larvae on WT plants were on the top and 19 % were on the bottom, while on AS-- plants, 30 % were on the top and 48 % on the bottom, and on AS- plants 44 % were on the top and 36 % on the bottom (Mann-Whitney *U*-test; $P_{WT\text{-}AS\text{--}TOP} = 0.0013$; $P_{WT\text{-}AS\text{--}BOTTOM} = 0.038$; $P_{WT\text{-}AS\text{-}TOP} = 0.011$; $P_{WT\text{-}AS\text{-}BOTTOM} = 0.35$; Figure 3). This caterpillar behavior is a result of higher defoliation levels of plants with either no or low TPI compared to those with high TPI levels (J.A. Zavala, pers. obs.).

Similar movement patterns were found in larvae on S++ and A genotypes. Larvae on A plants moved earlier (day 4) from the leaf at node S1 and toward the middle and top of the plant compared to larvae on S++ plants (Figure 3). This earlier movement was reflected in the number of larvae on the middle and top of the plant from day 6 to 9 with a greater percentage in A (23 %, 65 %, 69 %, and 84 %) than in S++ (8 %, 16 %, 28 %, and 56 %) genotypes (Mann-Whitney *U*-test; $P < 0.0001$; Figure 3). On day 11, there were no differences in the number of caterpillars between A and S++ plants at the bottom

and at top of the plant (Mann-Whitney *U*-test; $P_{A\text{-}S++\ TOP} = 0.35$; $P_{A\text{-}S++\ BOTTOM} = 0.16$; Figure 3).

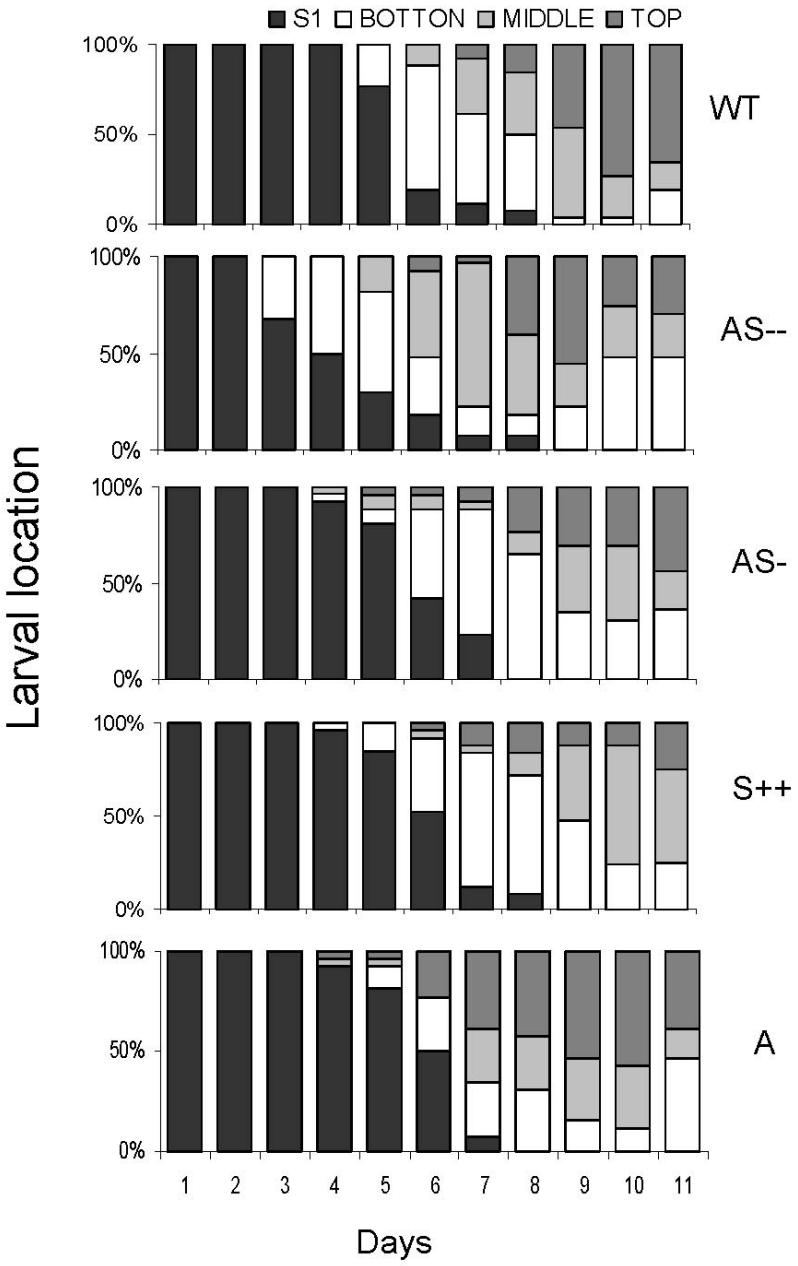

Figure 3. Relative number of *M. sexta* larvae on different plant locations on WT, AS--, AS-, S++, and Arizona (A) genotypes during 11 days on leaves growing at node S1, or the bottom, middle or top part of the plant (Figure 1). A single *M. sexta* neonate was placed on the leaf growing at node S1 and larval movement was monitored.

Calculated and simulated TPI and protein consumed by *M. sexta* larvae

We calculated (C) the amount of TPI and protein consumed by *M. sexta* larvae during the first, second, and third instars from each larvae's instar-specific feeding site and amount (Supplemental Table 1a and b). Plant genotype strongly influenced the C amount of TPI and protein consumed. C total and TPI consumed during the first, second and third instars were the highest for larvae on WT and the lowest for larvae on AS-- plants ($F_{2,76\text{-Total}} = 888.64$; $P < 0.0001$; $F_{2,76\text{-First}} = 28419.39$; $P < 0.0001$; $F_{2,76\text{-Second}} = 442.87$; $P < 0.0001$; $F_{2,76\text{-Third}} = 671.26$; $P < 0.0001$; Supplemental Figure 5). During the second instar, larvae on AS-- plants consumed the highest C amount of protein, larvae on WT plants, the lowest, but no differences were found between genotypes during the first and second instars ($F_{2,76\text{-First}} = 1.87$; $P = 0.161$; $F_{2,76\text{-Second}} = 87.14$; $P < 0.0001$; $F_{2,76\text{-Third}} = 2.14$; $P = 0.123$; Supplemental Figure 5). As expected, the C total amount of protein consumed was higher on larvae fed on AS-- than those fed on either AS- or WT genotypes ($F_{2,76\text{-Total}} = 11.62$; $P < 0.0001$; Supplemental Figure 5). Larval mass of caterpillars fed on WT, AS--, and AS- genotypes was affected by the amount of TPI but not by protein consumed ($F_{2,76\text{-11d}} = 10.248$; $P = 0.0001$).

Similar results were found when larvae fed on S++ and A genotypes. Second instar larvae on A consumed more protein than those on S++ plants, but no differences were found during the first and third instars ($F_{1,49\text{-Second}} = 152.99$; $P < 0.0001$; $F_{1,49\text{-Third}} = 1.038$; $P = 0.31$; Supplemental Figure 5). The C total amount of protein consumed was higher for larvae on A than on S++ genotypes ($F_{1,49\text{-Total}} = 7.81$; $P = 0.0074$; Supplemental Figure 5). Larval mass of caterpillars on A and S++ genotypes was affected by the amount of TPI and protein consumed ($F_{1,49\text{-11d}} = 49.803$; $P < 0.0001$).

We estimated the effect of the differences in larval movement by simulating TPI and protein consumption by transposing movement and consumption patterns from untransformed (WT and A) to transformed (AS--, AS-, and S++) plants as explained in the supplemental section (Supplemental Figure 6 and Table 1a and b). Patterns of larval movement on WT plants (S_{WT}) did not alter TPI consumed on the AS (AS-- and AS-) genotypes when WT movement data were transposed to larvae on AS genotypes (P_{AS--} = 0.98; P_{AS-} = 0.09); the highest values were found in the C WT genotype ($F_{4,126}$ = 545.49; P< 0.0001; Supplemental Figure 6). WT daily movement patterns decreased S_{WT} protein consumed from AS-- genotype plants ($F_{4,126}$ = 11.69; P< 0.0001; Supplemental Figure 6). Larval movement on AS-- plants increased TPI and protein consumed on WT plants ($F_{4,127\text{-TPI}}$ = 473.85; P< 0.0001; $F_{4,127\text{-Protein}}$ = 8.12; P< 0.0001; Supplemental Figure 6).

Larval movement on A plants did not change the amount of TPI consumed on S++ genotype plants ($F_{1,49}$ = 1.52; P = 0.22) but did increase the amount of protein consumed ($F_{2,74}$ = 7.46; P = 0.0011; Supplemental Figure 6); larval movement on S++ plants did not change protein consumed on A genotype ($F_{2,73}$ = 3.87; P = 0.22; Supplemental Figure 6). In summary, when larval movement patterns on low TPI plants were transposed to high TPI genotypes, protein and TPI consumption increased. Transposing WT movement patterns to AS-- genotype decreased the amount of protein consumed.

Fitness consequences of TPI expression for plants attacked by *M. sexta* larvae

To determine whether expression of TPIs increases *N. attenuata*'s fitness when plants are attacked by *M. sexta* larvae, we measured caterpillar mass on and capsule

number per plant from transformed and untransformed genotypes with either low or no TPI activity (A, AS--, and AS-) and high TPI activity (WT and S++). Larval mass of caterpillars fed on low TPI genotypes were higher than those fed on genotypes with high TPI activity ($F_{4,35\text{-}4d} = 20.099$; $P<0.0001$; $F_{4,195\text{-}11d} = 8.626$; $P<0.0001$; Figure 4), those that fed on either WT or S++ ($F_{1,14\text{-}4d} = 0.022$; $P = 0.9883$; $F_{1,78\text{-}11d} = 0.166$; $P = 0.6845$) or AS-- or A ($F_{1,14\text{-}4d} = 0.0155$; $P = 0.9044$; $F_{1,78\text{-}11d} = 1.603$; $P = 0.2093$) did not differ (Figure 4).

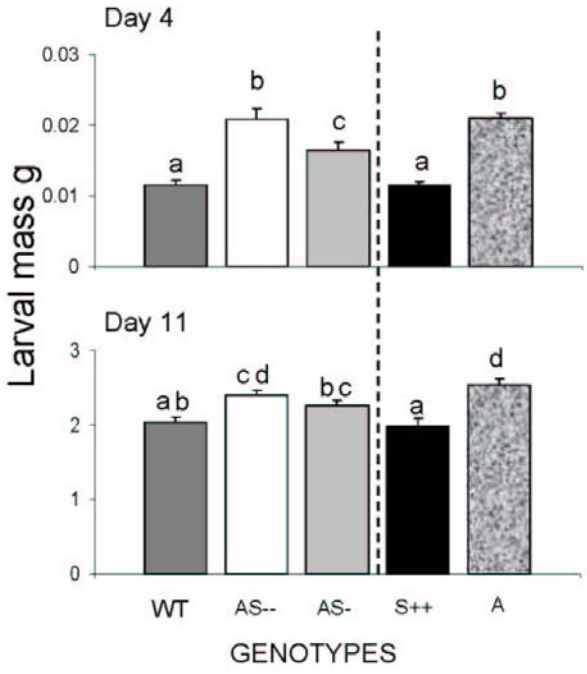

Figure 4. *M. sexta* mass (mean ± SEM) at 4 and 11d after neonates started to feed on leaves at S1 position (elongation stage) from WT, AS--, AS-, S++, and Arizona (A) genotypes. Bars with the same letter are not significantly different at $P< 0.05$ determined by one-way ANOVA.

We measured lifetime seed capsule number per plant on unattacked and attacked plants and calculated the mean differences and the percentage mean differences between treatments in order to estimate fitness consequences of constitutive and inducible TPI production. As expected, mean capsule number in unattacked plants was higher on genotypes with either low or no TPI activity (A and AS--) than on genotypes with intermediate and high TPI activity (WT, S++, and AS-; Figure 5), which reflects the fitness cost of TPI production. Eleven days of caterpillar attack reduced seed capsule production per plant in all genotypes and reversed the pattern of seed capsule production among high and low TPI-containing genotypes.

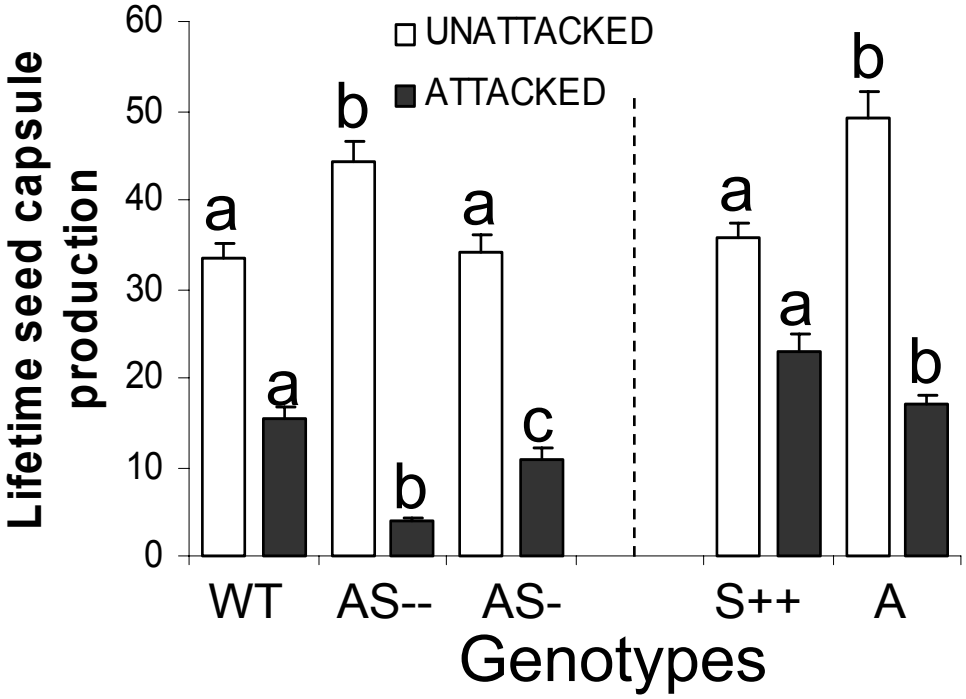

Figure 5. Mean capsule number from WT, AS--, AS-, S++, and A genotypes that were either unattacked or attacked by *Manduca sexta* larvae for 11 days. Bars with the same letter within a group are not significantly different at $P < 0.01$ determined by one-way ANOVA.

Within the group of transformed (AS-- and AS-) and untransformed (WT) unattacked plants from the Utah genotype, mean capsule number was higher on the genotype with low TPI activity (AS--) than on genotypes with intermediate and high TPI activity (AS- and WT; $F_{2,81} = 8.639$; $P = 0.004$; Figure 5); however after 11 d of caterpillar attack, mean capsule number, absolute and relative mean difference in capsule number were the highest on WT and the lowest on AS-- genotypes ($F_{2,81} = 25.309$; $P<0.0001$; Figure 5 and Table 1). Within the Arizona genotypes, mean capsule number of unattacked plants was higher on the genotype with no TPI activity (A) than on the genotype with high TPI activity (S++; $F_{1,54} = 16.484$; $P = 0.0002$; Figure 5). However, when plants were attacked, mean capsule number as well as absolute and relative mean difference in capsule number were higher on S++ than on A genotypes ($F_{1,54} = 7.967$; $P = 0.0067$; Figure 5 and Table 1).

Table 1. Absolute and relative mean differences between treatments in seed capsule production from either untransformed wildtype (WT) or homozygous T_3 independently transformed lines of a WT genotype of *Nicotiana attenuata* which had been transformed with constructs containing the *pi* gene in an anti-sense orientation (AS--, AS-); absolute and relative mean differences between untransformed plants of the Arizona (A) genotype and plants of the Arizona genotype transformed with constructs containing the full-length *pi* gene in a sense (S++) orientation, that were either unattacked or attacked by *Manduca sexta* larvae for 11 days.

Genotypes	Mean diff. in capsule number	% Mean diff. in capsule number	P
WT	18.04	54.18	<0.0001
AS--	40.36	91.35	<0.0001
AS-	23.14	68.14	<0.0001
S++	12.64	35.47	<0.0001
A	32.25	65.58	<0.0001

P-values are from one-way ANOVAs between treatments

In order to determine the effect of caterpillar attack on seed capsule production per plant, we regressed caterpillar mass against seed capsule production per plant from transformed and untransformed genotypes and found that a linear equation (Y = -12.773 (g) + 42.229; $R^2 = 0.5642$; Figure 6; $P<0.0001$) represented the best fit. The relationship suggests that the higher the *M. sexta* larvae mass, the lower the seed capsule number production per plant.

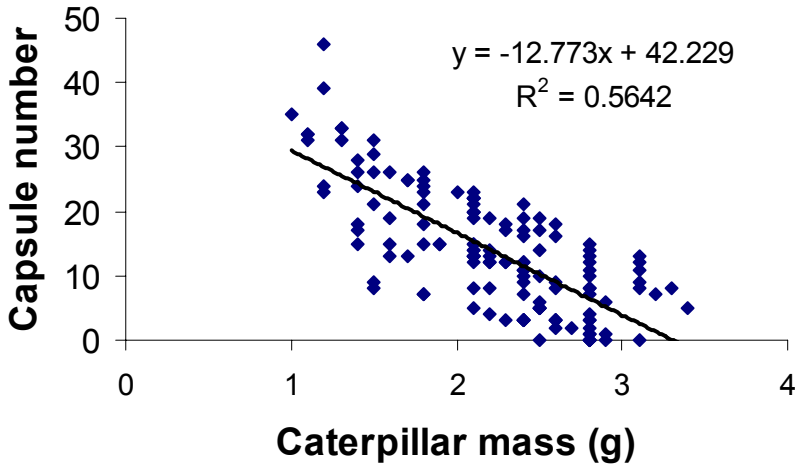

Figure 6. Seed capsule production per plant of *N. attenuata* genotypes (WT, AS--, AS-, S++ and A), regressed against *M. sexta* larvae mass (g) 11 d after neonates started to feed on the leaf at S1 position (elongation stage). Line represents a regression fitted to the points (Y = -12.773 (g) + 42.229; $R^2 = 0.5642$).

Discussion

Our experiments demonstrate that the benefits of TPI expression in *N. attenuata* outweigh their costs when plants are attacked. Unattacked plants with low constitutive TPI levels produced more seed capsules (AS--: 44, AS-: 34 and A: 49 capsules) than did plants with high TPI levels (WT: 33 and S++: 35 capsules), and 11 days of *M. sexta*

attack reduced seed capsule production per plant in all genotypes and reversed the pattern of seed capsule production with higher reductions in AS (AS--: 91 % and AS-: 68 %) and A (65 %) than in WT (54 %) and S++ (35 %) plants (Figure 5 and Table 1). This differential reduction in seed capsule production amongst genotypes correlated negatively with larval mass. Across all genotypes, the larger the larval mass, the lower the number of capsules per plant (Figure 6). This result is consistent with previous demonstrations that endogenous TPIs function defensively against native herbivores (Zavala et al. 2004b) and with the predictions of the Optimal Defense theory, namely that 1) defense is costly, and 2) plants parts with a high fitness value are more heavily defended (McKey 1974; Ohnmeiss and Baldwin 2000; Rhoades and Cates 1976). Moreover, the results highlight the heuristic value of the cost-benefit paradigm for functional studies.

We manipulated TPI production by transformation. Antisense expression of the *pi* gene reduced constitutive and caterpillar induced TPI levels in AS-- and AS- genotypes (by 35-80% of the activity of WT) in S1 and stem leaves without influencing protein contents. Caterpillar attack increased TPI levels 2-2.5-fold in either WT or AS genotypes (Figure 2; Supplemental Figures 1-3) but the absolute levels were substantially lower in the AS genotypes. Transformation of the A genotype with a functional TPI gene under the control of a constitutive promoter (S++ genotype) produced TPI levels that were 74% of the activity found in caterpillar attacked WT plants (Figure 2; Supplemental Figure 4). Because these transformed lines did not differ in any other measured defense traits (Zavala et al. 2004a), they provided valuable tools to examine the defensive function of TPIs by constraining plant responses to herbivore attack and observing unconstrained herbivore behavior. In this way, the dymamics of the plant responses, or the lack thereof,

can be visualized in the herbivore behavior. Low constitutive TPI expression may increase proteolytic enzyme activity in the guts of neonates, digestion efficiency and the growth rates (Zalucki et al. 2002); Figure 4). This increase in larval growth rate translates into increases in pupal mass, which is an accurate proxy for fecundity in Lepidoptera (Awmack and Leather 2002; De Leo et al. 2001), but may also profoundly influence larval movement.

Given the large within-plant heterogeneity in food quality, it is reasonable to expect a complex resource-oriented larval behavior that changes with instars (Browne 1993; Denno and McClure 1983). Moving has been shown to be costly during the first 3 instars (Kessler and Baldwin 2002; van Dam et al. 2001a), but these costs are thought to decrease with size (Scriber and Slansky 1981). Larvae with larger mass (on either low or no TPI-producing genotypes) left the S1 leaf 1-2 days earlier than did those with lower mass (on high TPI-producing genotypes; Figure 3). The heavier larvae moved earlier than lighter larvae to young leaves which typically have higher levels of protein and water contents (Denno and McClure 1983; Scriber and Slansky 1981; Stamp and Bowers 1990). Over the 11d of the experiment, larvae fed on high TPI genotypes consumed 3-4 fold more TPI and 25 % less protein than did larvae feeding low TPI genotypes (Figures 3 and 4; Supplemental Figure 5). These results suggest that a high TPI content keeps caterpillars from feeding on the high-protein younger leaves at the top of the plants possibly by decreasing larval mass and thereby their ability to move. Larval movement influences the caterpillar's ability to compensate for variation in diet quality.

By moving, caterpillars can exploit the high within-plant heterogeneity in food quality to compensate for nutritional imbalances. For example, *Helicoverpa zea* larvae

feed on multiple plant structures to balance their amino acid requirements (Felton 1996). *M. sexta* larvae fed low protein and nutritionally unbalanced diets compensated not only for the decreased protein intake (Woods 1999) but also for unbalanced nutrition by selecting diets high in the missing nutrients which increased larval growth rates (Simpson et al. 1988; Thompson et al. 2001). Growth depends on nutrient ratios, and insects may use behavioral and post-ingestive mechanisms to compensate for nutrient imbalances (Raubenheimer and Simpson 1993; Raubenheimer and Simpson 1999). To estimate the consequence of TPI-induced movement, we transposed the larval location data of caterpillars from those on low- to high-TPI genotypes, and found increased larval protein (10 %) and TPI (12 %) consumption (Supplemental Figure 6). Transposing daily larval location data in the opposite direction decreased (by 10 %) protein consumed but did not influence TPI consumption (Supplemental Figure 6). These results suggest that caterpillars adjust their feeding positions to minimize TPI consumption and maximize protein intake. Hence the high natural TPI levels prevent caterpillars from feeding on high-quality younger leaves, which may have a high fitness value for the plant (Felton 1996; Mattson 1980; Ohnmeiss and Baldwin 2000).

The interaction between *N. attenuata* and *M. sexta* starts with moth oviposition at the bottom region of the plant; ovipositioning is influenced by temperature, food quality and quantity, and predation risk (Kessler and Baldwin 2002). Plants respond by increasing TPI levels, which decreases larval mass and survivorship (Zavala et al. 2004b), and by indirectly increasing volatile emission, which alters oviposition choices and attracts the generalist predator *Geocoris pallens* to feeding larvae (Kessler and Baldwin 2001). *Geocoris* is size selective and preferentially attacks eggs and larvae in the

first three instars. The up-regulation of TPIs by herbivore attack slows larval growth and keeps larvae in stages that are more vulnerable to the predator, thus increasing larval mortality (Williams 1999). Interestingly, the volatile signals that function as indirect defenses by attracting *Geocoris* to feeding larvae are elicited by the same signals that elicit TPI production (Halitschke et al. 2000; Halitschke et al. 2001; van Dam et al. 2001b), providing the mechanism of coordination among these defense system. Once larvae reach a mass that can compensate for the cost of movement, they move upward within the host plant and feed preferentially on young leaves with high levels of protein and nicotine, which increases larval mass and decreases plant fitness (Kessler and Baldwin 2002; Ohnmeiss and Baldwin 2000; Woods 1999). Other generalist herbivores on *N. attenuata*, namely noctuid larvae and weevil beetles, usually attack older leaves that are lower in nutrients as well as nicotine (Baldwin 2001; Kessler and Baldwin 2002; Ohnmeiss and Baldwin 2000). Nicotine is not an efficient defense against *M. sexta*, because this insect is adapted to feed on *N. attenuata* and larvae can detoxify nicotine (Appel and Martin 1992; Campo del and Renwick 1999; Snyder et al. 1993). Moreover, its attack down-regulates nicotine production which could be sequestered by the herbivore and maybe co-opted and used as a defense against parasitoids (Barbosa et al. 1991; Winz and Baldwin 2001). Hence the plant relies on other defenses when attacked by *M. sexta* larvae: TPIs, for example, decrease larval mass and prevent caterpillars from feeding on leaves with high fitness value for the plant. By eliciting only of those defenses that confer resistance to the herbivore eliciting the response (targeting) rather than the entire defensive repertoire may minimize the cost of resistance (Zangerl 2003). We conclude that despite the ongoing evolutionary interaction between *N. attenuata* and *M.*

sexta, TPI-mediated decrease in herbivore performance translates into fitness benefit for the plant.

Material and Methods

Plant material and transformation. *Nicotiana attenuata* Torr. Ex Wats. (synomonous with *Nicotiana torreyana* Nelson and Macbr.; Solanaceae) used in this study were grown from seeds collected from either Utah (Baldwin 1998) or Arizona (Glawe et al. 2003) and inbred 10 and 4 generations, respectively. In order to silence the expression of *N. attenuata*'s *pi* gene in the genotype collected in Utah (WT), WT was transformed by an *Agrobacterium*-mediated transformation procedure with pNATPI1, which contains 175 bp of *N. attenuata*'s 7-repeat domain *pi* gene in an anti-sense orientation (AS), as described in (Glawe et al. 2003). Southern gel blot analysis confirmed that all T_3 lines were single-copy independent transformants (Zavala et al. 2004a).

A genotype of *N. attenuata* collected from Arizona (A), with methyl jamonate (MeJA)-inducible nicotine levels identical to that found in WT plants, but completely lacking the ability to produce TPIs or accumulate TPI mRNA (Glawe et al. 2003). More recently, the mutation in the 7-domain repeat *pi* of A plants has been characterized and found to be located in the 5'signal peptide, resulting in a premature stop codon (J. Wu and I.T. Baldwin unpublished data). Plants of the A genotype were transformed with a binary transformation vector pRESC2PIA2 containing the full-length 7-domain *N. attenuata pi* gene from the WT genotype in the sense orientation under control of the constitutive CaMV 35S promotor (Zavala et al. 2004a). Several T_3 lines harboring a

single copy of the transgene (Zavala et al. 2004a) were screened for TPI activity, and all had TPI activity comparable to that of elicited WT plants. One of these A lines (S++) with 60% of the activity of MeJA-elicited WT plants was selected for study. Arizona non-transformed plants (A) had no detectable TPI activity. All of these transformed and untransformed genotypes were used in the experiments.

Bioassay experiments and plant fitness determination. In order to determine the effect of *M. sexta* herbivory on the fitness of *N. attenuata*'s genotypes using either down-regulation or restored expression of the *pi* gene, a single *M. sexta* neonate was placed on the leaves growing at node S1 (Figure 1) of 48 soil-grown plants in elongation stage of AS lines (AS-- and AS-), on A line transformed to express the functional *pi* (S++), and on untransformed genotypes (WT and A). Larvae were allowed to move and feed freely on plants for 11 days. Their mass was determined 4 and 11 days after hatching. Larval movement on the plant during this time was monitored, and larval location on the plant classified as follows: S1 (leaf where larvae started to feed), bottom (from 0 to S3 leaf position), middle (from S4 to S5 leaf position), and top (from S6 to S9 leaf position; Figure 1). Eggs of *Manduca sexta* L. (Lepidoptera: Sphingidae) were obtained from Carolina Biological Supply Company (Burlington, North Carolina, USA) and placed in plastic containers (200 mL) on a moist tissue. The containers were kept in climate chambers at 28°C and 65 % relative humidity under a 16:8 h light:dark photoperiod until the eggs hatched.

Seeds were germinated in diluted liquid smoke solutions as described in (Baldwin et al. 1994). Seedlings were transplanted in 1-L pots in a glasshouse under the conditions described in (Zavala et al. 2004a) with 1000 – 1300 μmol m^{-2} s^{-1} PPFD supplied by 450

W Na-vapor HID bulbs. To compare the lifetime reproductive performance among genotypes after being either unattacked or attacked by *M. sexta* larvae, we recorded the number of seed capsules per plant from 28 plants of each genotype and treatment two weeks after last watering day. Daily watering stopped 21 d after neonates started to feed on the leaf, in order to mimic the drying and termination of growth in the plant's nature habitat, the Great Basin Desert. The number of capsules per plant reflects the lifetime reproductive output (seeds) in *N. attenuata* under natural or glasshouse conditions (Baldwin 1998; Baldwin et al. 1998).

Constitutive and TPI activity induced by caterpillar damage were determined from stem and rosette leaves before the larvae were placed on the leaf at node S1 (8 replicates; Figure 1), and 4 (8 replicates) and 11 (12 replicates) days after the larvae started to feed. During the last harvest TPI activity was also determined on axillary leaves from S1 to S4 nodes. Protein concentrations and TPI activity were measured and expressed as nmol mg^{-1} as described in (van Dam et al. 2001b). Larvae TPI and protein consumption were calculated and simulated as explained in the supplemental section.

Statistical analysis. Data were analyzed with Stat View, Version 5.0 (SAS, 1998). The TPI, protein and larval mass, and calculated and simulated values were analyzed by ANOVAs followed by Fisher's protected LSD *post-hoc* comparisons in all experiments. Differences in larval number on plants were analyzed with the Mann-Whitney *U*-test.

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Supplemental Section.

Calculation of TPI and protein consumed by *M. sexta* larvae.

In order to determine the amount of TPI and protein consumed by *M. sexta* larvae during the experiment, we estimated leaf TPI and protein values for days 1, 2, and 3 with daily leaf concentrations extrapolated from measurements at day 0 and day 4, and for days 5 - 10 with measurements at day 4 and day 11 for all rosette and stem leaf positions (Supplemental Figures 1-4). With these concentration values, the location of the larvae each day on the plant (Figures 1 and 3), and information from the literature about the average leaf area consumed by a *M. sexta* larvae during the first, second, and third instars (Madden and Chamberlin 1945), we calculated the amount of TPI and protein consumed (C) for larvae feeding on each genotype (Supplemental Figure 5). These C values are products of the larvae's instar-specific feeding site, amount, and TPI and protein content of each leaf position consumed (Supplemental Tables 1a and b). C values were obtained as follows: protein consumed = leaf protein content (mg/g) x larvae consumption (g) and for TPI consumed = leaf TPI content (nmol/g) x larvae consumption (g).

In order to determine the effect of larval movement on TPI and protein consumption, we simulated transformed (S_T) and untransformed (S_{WT} and S_A) genotypes' TPI and protein consumption by transposing either daily larval location data (S_{WT} and S_A) or leaf TPI and protein content (S_T) from untransformed (WT and A) to transformed (AS--, AS-, and S++) genotypes (Supplemental Tables 1a and b). For example, to calculate the S_T value in combination with WT either TPI or protein content and larvae location on AS-- genotype, we used the TPI and protein contents of the WT and calculated the TPI

and protein consumption using the larval location on AS-- plants (Supplemental Table 1a).

Supplemental Table 1. Combination of TPI and protein from different *N. attenuata* genotypes and larval location of different genotypes that have either calculated (C) or simulated (S) values. **a**. WT, AS-- and AS- genotypes. **b.** S++ and A genotypes. C values resulted from larval location and either TPI or protein from the same genotype. S_{WT} and S_A values resulted from transposing larval location data from untransformed (WT and A) to transformed (AS--, AS-, and S++) genotypes. S_T values resulted from transposing either TPI or protein contents from untransformed (WT and A) to transformed (AS--, AS- and S++) genotypes.

TPI and protein from genotypes

a

Larval location	**WT**	**AS--**	**AS-**
WT	C	S_{WT}	S_{WT}
AS--	S_T	C	
AS-	S_T		C

b

	S++	**A**
S++	C	S_T
A	S_A	C

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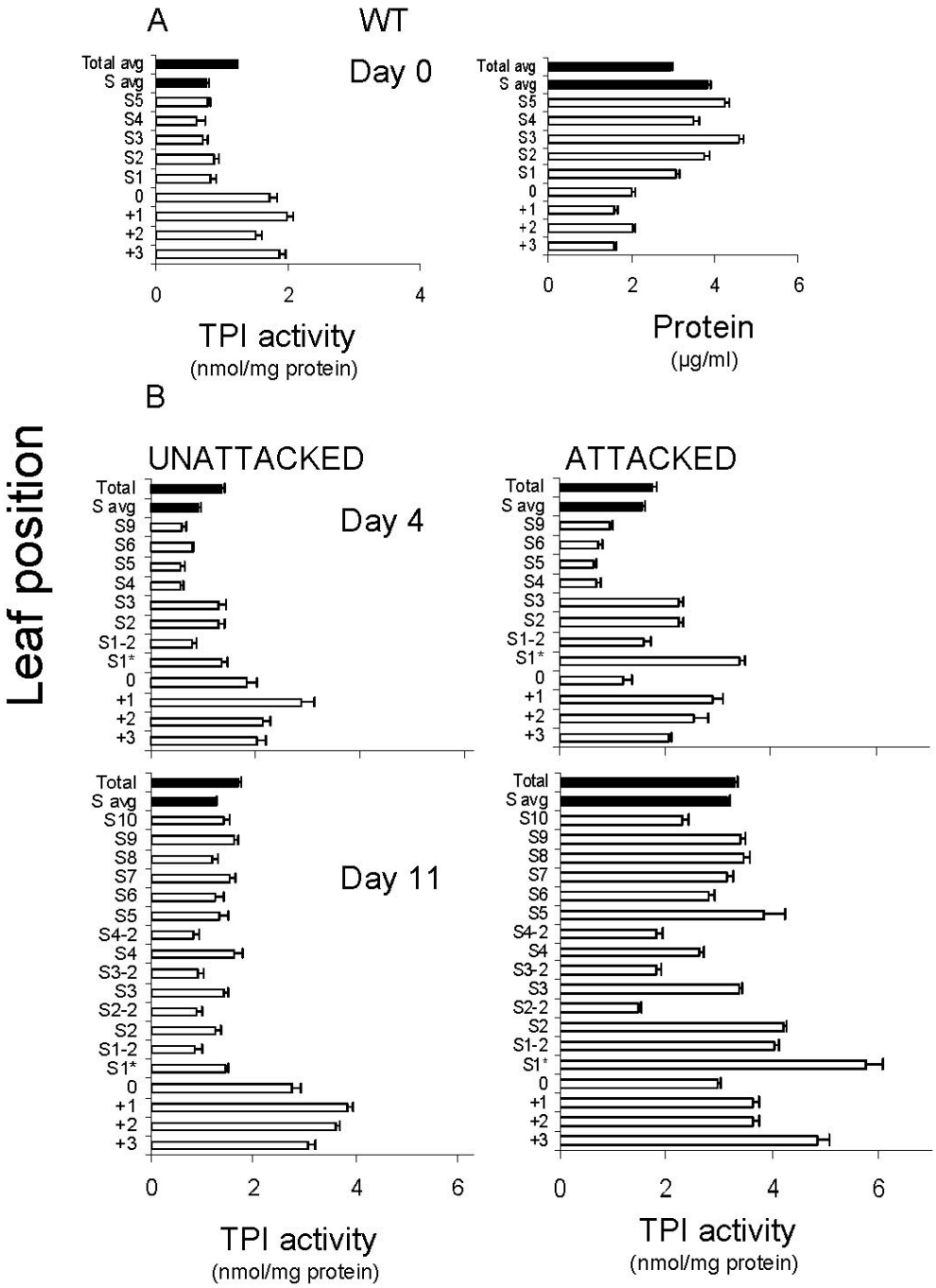
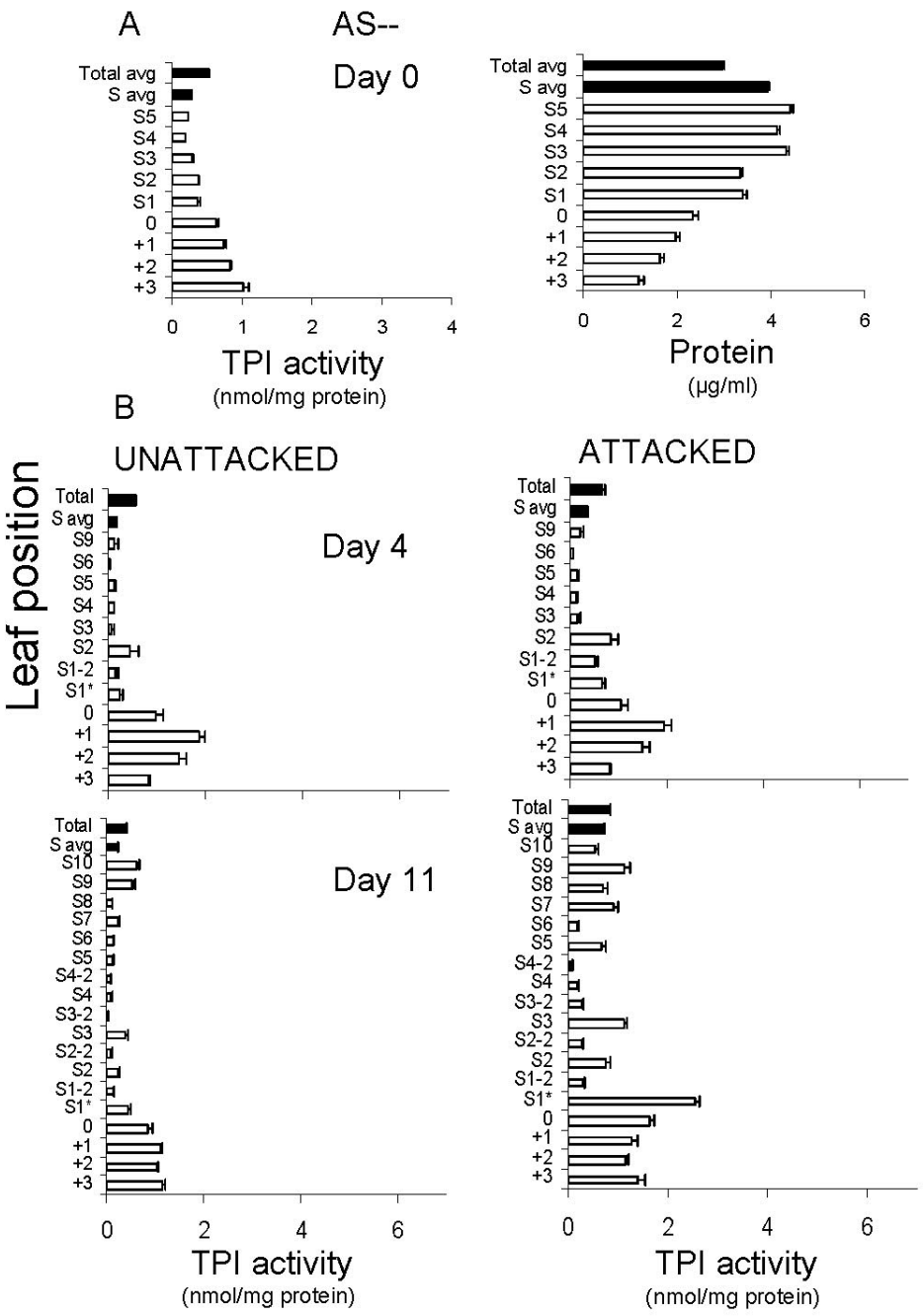
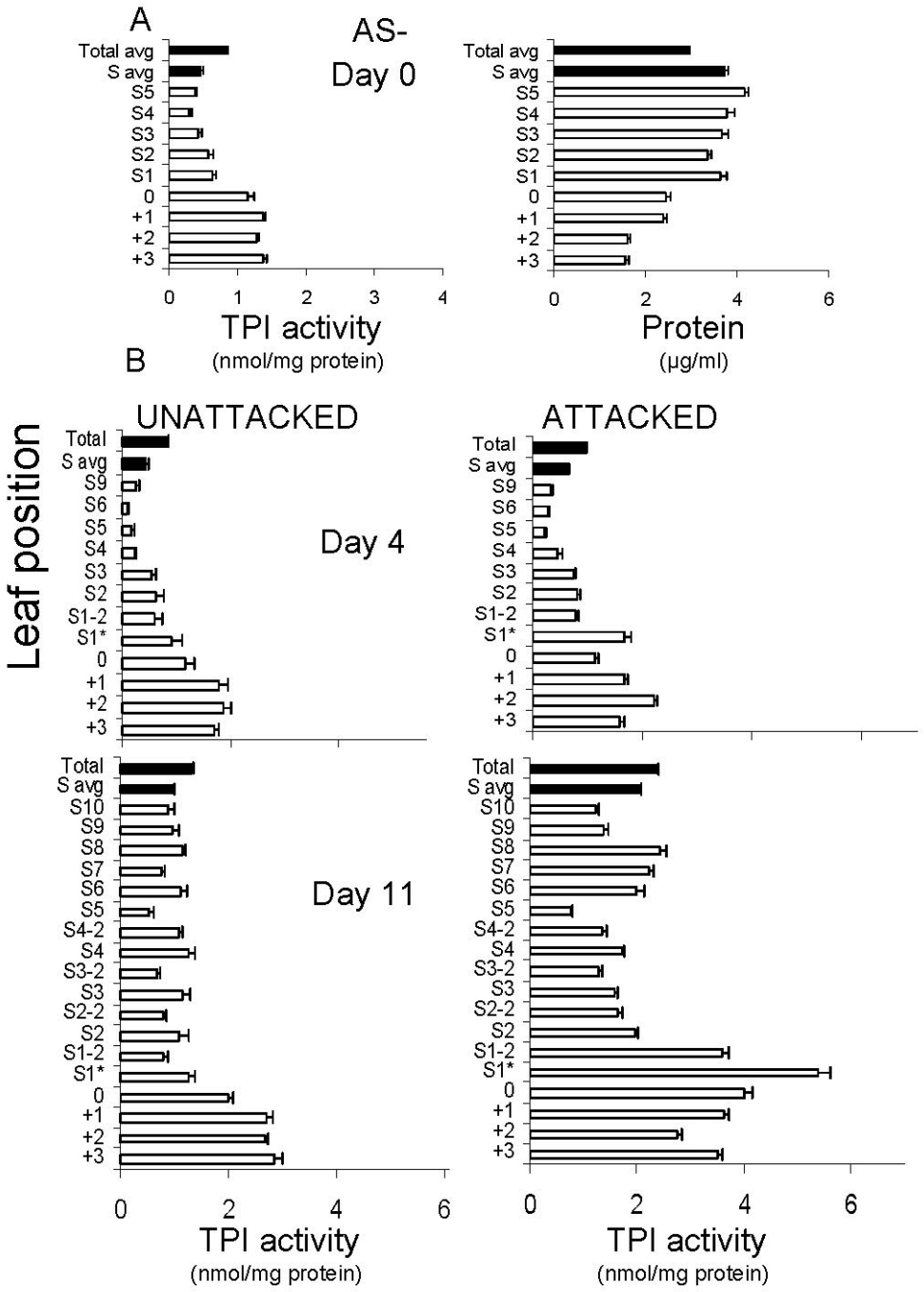


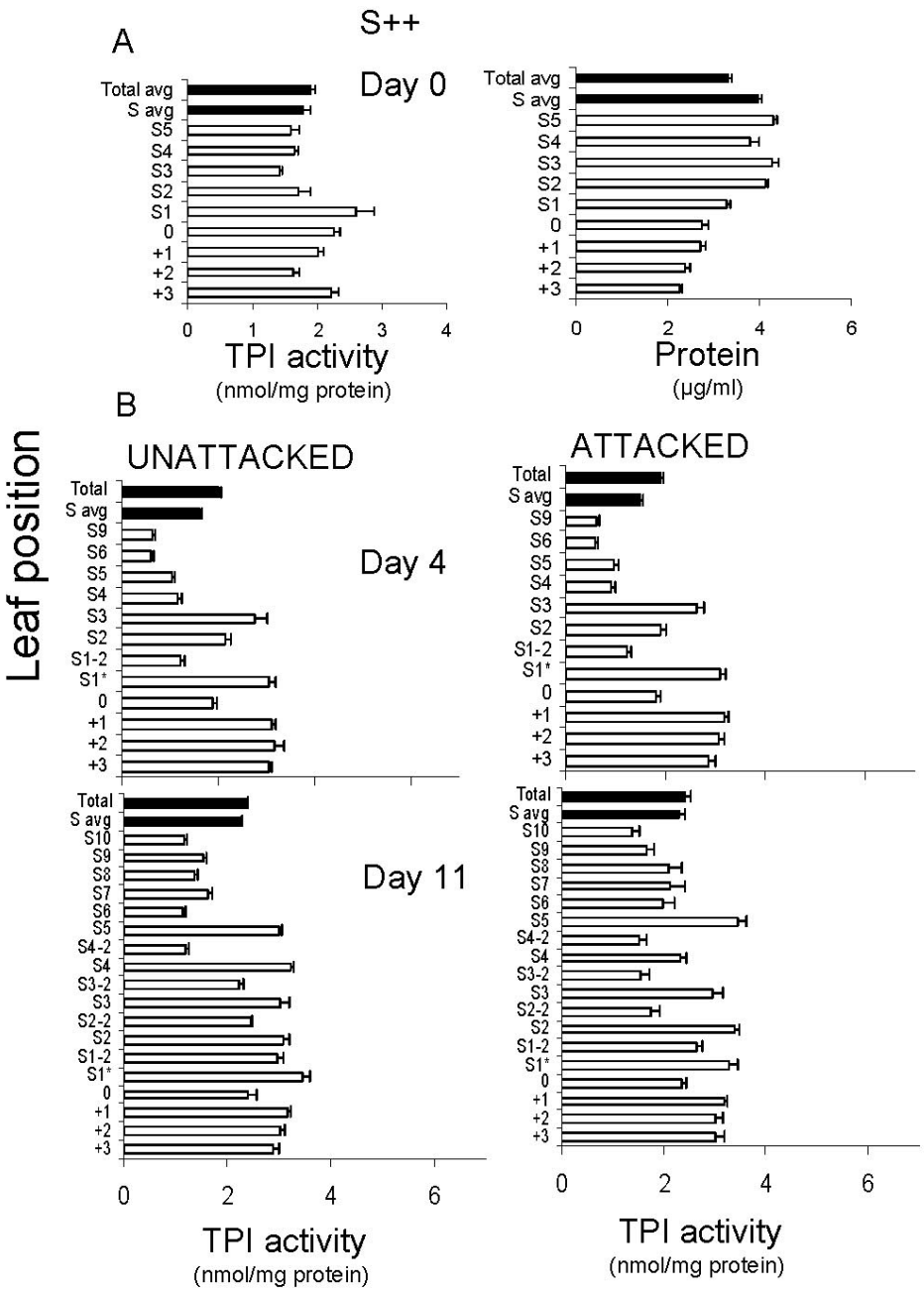
Supplemental Figure 1. TPI activity (mean ± SEM) and protein content from different leaf positions of WT plants at the elongation stage before *M. sexta* larvae attack. **B.** TPI activity (mean ± SEM) from different leaf positions of the WT plants at the elongation stage either unattacked or attacked by *M. sexta* larvae 4 and 11 d after neonates started to feed on the leaf at S1 position (*). Solid bars show the average of TPI activity and protein content either of rosette and stem leaves (Total avg) or of stem leaves only (S avg). See Figure 1 for graphic depiction of leaf position.



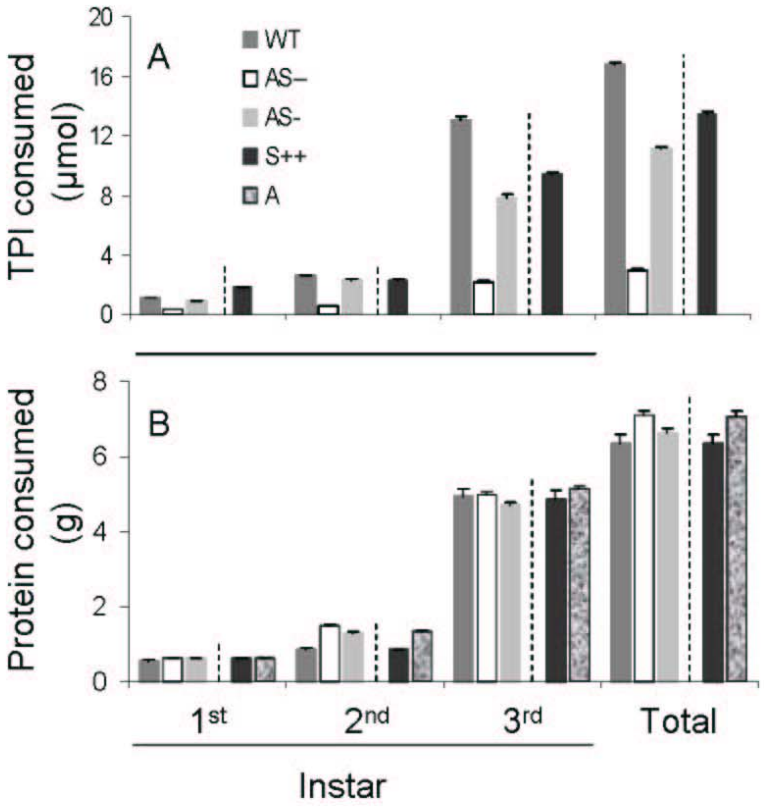
Supplemental Figure 2. TPI activity (mean ± SEM) and protein content from different leaf positions of AS-- plants at the elongation stage before *M. sexta* larvae attack. **B.** TPI activity (mean ± SEM) from different leaf positions of the AS-- plants at the elongation stage either unattacked or attacked by *M. sexta* larvae 4 and 11 d after neonates started to feed on the leaf at S1 position (*). Solid bars show the average of TPI activity and protein content either of rosette and stem leaves (Total avg) or of stem leaves only (S avg). See Figure 1 for graphic depiction of leaf position.



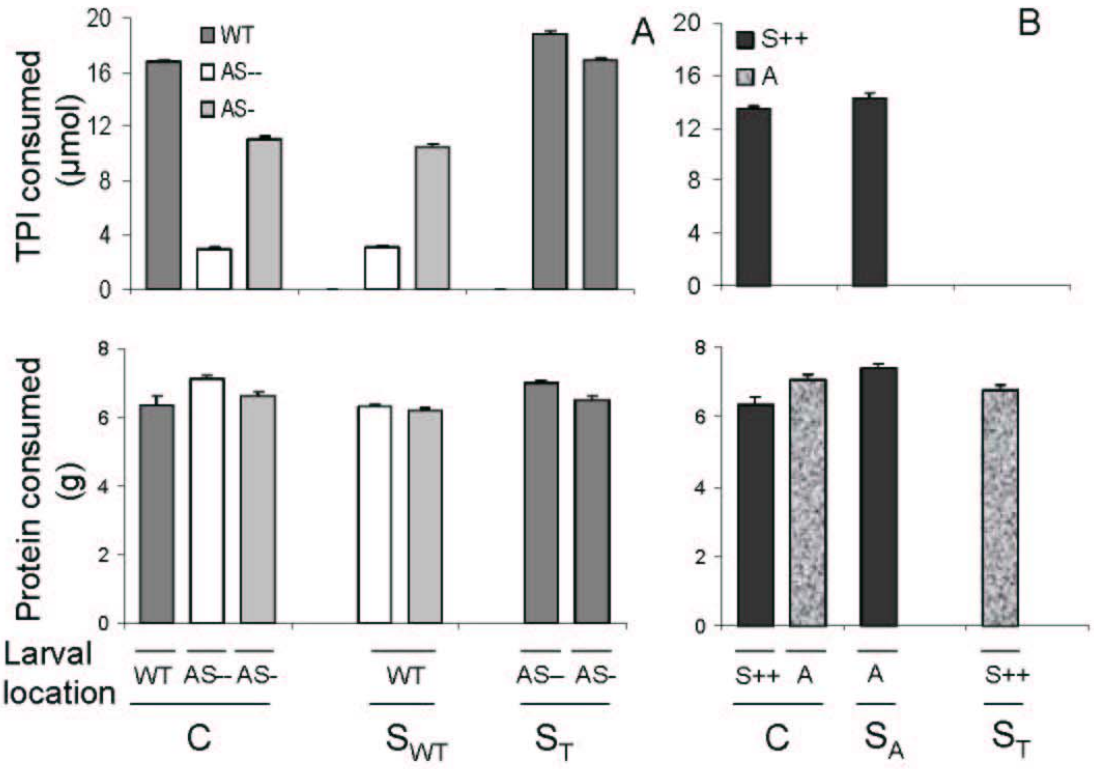
Supplemental Figure 3. TPI activity (mean ± SEM) and protein content from different leaf positions of AS- plants at the elongation stage before *M. sexta* larvae attack. **B.** TPI activity (mean ± SEM) from different leaf positions of the AS- plants at the elongation stage either unattacked or attacked by *M. sexta* larvae 4 and 11 d after neonates started to feed on the leaf at S1 position (*). Solid bars show the average of TPI activity and protein content either of rosette and stem leaves (Total avg) or of stem leaves only (S avg). See Figure 1 for graphic depiction of leaf position.



Supplemental Figure 4. TPI activity (mean ± SEM) and protein content from different leaf positions of S++ plants at the elongation stage before *M. sexta* larvae attack. **B.** TPI activity (mean ± SEM) from different leaf positions of the S++ plants at the elongation stage either unattacked or attacked by *M. sexta* larvae 4 and 11 d after neonates started to feed on the leaf at S1 position (*). Solid bars show the average of TPI activity and protein content either of rosette and stem leaves (Total avg) or of stem leaves only (S avg). See Figure 1 for graphic depiction of leaf position.



Supplemental Figure 5. Calculated TPI and protein consumed by *M. sexta* larvae fed on WT, AS--, AS-, S++, and A genotypes during the first, second and third instars. These calculated values are products of larvae's instar-specific feeding site, amount, and TPI and protein content of each leaf position consumed and are extrapolated from measurements from days 0 and 11.



Supplemental Figure 6. Calculated (C) and simulated (S) TPI and protein consumed by *M. sexta* larvae. **A.** Genotypes and larval location of WT, AS--, and AS- . **B.** Genotypes and larval location of S++ and A. C values resulted from larval location and either TPI or protein from the same genotype. S_{WT} and S_A values resulted from transposing larval location data from untransformed (WT and A) to transformed (AS--, AS-, and S++) genotypes. S_T values resulted from transposing either TPI or protein contents from untransformed (WT and A) to transformed (AS--, AS- and S++) genotypes (Supplemental Tables 1a and b).

2. Discussion.

2.1. Contrasting the predictions.

Hypothesis 1- Trypsin proteinase inhibitor (TPI) expression in *N. attenuata* is costly for plant fitness when plants are not attacked.

Prediction 1: *N. attenuata* genotypes with either low or no TPI growing next to high TPI-producing genotypes are taller with earlier flowering and produce a higher number of lifetime seed capsules. In Manuscript 1 and 2, genotypes with either low or no TPI growing next to high TPI-producing genotypes were taller with earlier flowering and produced a higher number of lifetime seed capsules, demonstrating that TPI is directly responsible for the observed fitness differences between genotypes. Hence I accept prediction 1.

Hypothesis 2- *N. attenuata*'s TPI decreases the performance of *Manduca sexta* larvae and plant colonization of *Tupiocoris notatus.*

Prediction 2a: *Manduca sexta* larvae fed on genotypes with either low or no TPI expression grow faster, have higher survivorship and produce heavier pupae than those fed on high TPI-producing genotypes. In Manuscript 1, 3 and 4, *Manduca sexta* larvae fed on genotypes with either low or no TPI expression grew faster, had higher survivorship and produced heavier pupae than those fed on high TPI-producing genotypes, demonstrating the TPI expression in *N. attenuata* decrease *M. sexta* performance. Hence I accept prediction 2a.

Prediction 2b: *Tupiocoris notatus* has higher colonization preference for genotypes with either low or no TPI expression than genotypes with high TPI expression. In Manuscript 1 and 3, *Tupiocoris notatus* had higher colonization preference for genotypes with either low or no TPI expression than genotypes with high TPI expression, demonstrating that TPI expression in *N. attenuata* decrease *T. notatus* colonization. Hence I accept prediction 2b.

Hypothesis 3- The putative fitness benefits of TPI expression outweigh their costs by decreasing *M. sexta* larval mass.

Prediction 3a: Unattacked genotypes with either low or no TPI expression produced more seed capsules than genotypes with high TPI levels, and after *M. sexta* attack genotypes with either low or no TPI expression produced less seed capsules than genotypes with high TPI levels. In Manuscript 4, unattacked low TPI-producing genotypes produced more seed capsules than did plants with high TPI levels. Caterpillar attack reduced seed capsule production in all genotypes and reversed the pattern of seed capsule production among genotypes, demonstrating that the fitness benefits of TPI production outweigh their costs, when plants are attacked. Hence I accept prediction 3a.

Prediction 3b: High TPI mediate a decrease in larval mass and behavioral change in *M. sexta* that is associated with fitness benefits for the plant. In Manuscript 4, *M. sexta* larvae attacking genotypes with high TPI activity consumed more TPI, less protein, and did not move to the high-

fitness value young leaves; their lower masses were negatively correlated ($R^2 = 0.56$) with seed capsule production per plant. Hence I accept prediction 3b.

Since all predictions were accepted, I accept all hypotheses.

2.2 Testing the cost-benefit paradigm.

Tests of the cost-benefit paradigm require both a detailed reductionist view of plant function and an equally sophisticated understanding of ecological function (Baldwin 2001). Arizona and Utah genotypes are from different geographical regions and the only known differences between them are direct defenses (TPI) and indirect (volatiles) defenses. However, since these two genotypes are from different populations, they most probably have different genetic backgrounds. Transgenic manipulation of TPI production allowed us to test the cost-benefit paradigm on *N. attenuata* and separate the TPI function from other plant traits. By expressing the *pi* gene in antisense orientation constitutively, caterpillar induced TPI levels were reduced by 30-90% of the activity of WT. In addition, transformation of the A genotype with a functional TPI gene under control of a constitutive promoter (S++ genotype) produced TPI level that were from 60 % to 80 % of the activity found in the caterpillar attacked WT plants. Because the transformed and untrasformed genotypes did not differ with respect to other defense traits, such as nicotine production, this is an ideal system in which to examine three important question for the cost-benefit paradigm in *N. attenuata*; a) whether TPI expression is an effective defense against its herbivores (*T. notatus* and *M. sexta*), b) whether the defensive function of TPI is

costly to the plant in terms of fitness when is not attack, and c) whether the benefits of TPI expression outweigh its costs.

Many studies have shown that TPI decrease larval growth and survivorship, but the evidence comes from heterologous expression of PI and manipulation of signal cascades affecting PI elicitors (e.g. Hilder et al. 1987; Orozco-Cardenas et al. 1993). When insects feed on a transgenic plant expressing a novel *pi* gene, the result may not be the same as endogenous PIs because the larvae are not adapted to the novel PI and the interaction between the expression of PI and other traits of the plant (nutritional and defensive factors) can affect larval performance. Hence, these studies do not reflect the real function of an endogenous TPI from a plant and the possible adaptation of insects to plant PIs by readjusting their metabolism or behavior (Cloutier et al. 2000; Jongsma et al. 1995; Winterer and Bergelson 2001). The second line of evidence comes from studies in which transgenic suppression of the wound signal cascades that elicit PI production, but in these experiments is not possible to attribute the changes in caterpillar performance to PI because many other traits may have been regulated (Howe et al. 1996; McGurl et al. 1992). In this study, *M. sexta* larvae fed on high TPI producing genotypes grew slower, had lower survivorship and produced lighter pupae than those fed on genotypes with low TPI levels. Similarly, *T. notatus* preferred genotypes with low or no TPI to genotypes with high TPI levels. This research demonstrates that despite the ongoing evolutionary interaction between *N. attenuata* and its herbivores, TPIs remain an effective defense against both *T. notatus* and *M. sexta,* but this defense can be costly in the absence of plant enemies (Baldwin 1998).

A central assumption in evolutionary ecology is that organisms make trade-offs in allocating limited resources to different functions (Cipollini 2004). Resources that are allocated to defense cannot be rapidly reallocated to growth and reproduction (Bazzaz et al. 1987; Herms

and Mattson 1992; Karban and Baldwin 1997). Our competition experiments demonstrate that TPI production is intrinsically costly when plants compete for below-ground resources with conspecifics, as they commonly do in nature. Across all experiments, the larger the difference in TPI activity between neighbors, the larger the difference in seed capsule production. TPI production may make demands on a plant's nitrogen (N) budget that a plant could otherwise allocate to growth and reproduction. In addition, competitive growth did not decrease TPI activity in any genotypes as found in other plant systems (Cipollini and Bergelson 2001). The differences in growth and fitness between genotypes can be attributed to changes in metabolism required to support TPI production and to differences in the rate of harvesting below-ground resources. Slow-growing plants do not harvest resources as rapidly as fast-growing plants, providing an opportunity benefit for the fast growing plants (van Dam and Baldwin 1998; van Dam and Baldwin 2001). This opportunity benefit was evident when untreated plant competed with MeJA-treated plant; MeJA elicitation decreases the transcription of photosynthetic-related genes (Halitschke et al. 2003; Hermsmeier et al. 2001), and this down regulation may be required to release resources for defense-related processes. TPI expression decreased the fitness in unattacked plants, but the benefits of TPI production can outweigh its cost.

Costs and putative benefits of defense traits have been studied in separate experiments, where their currencies are usually not comparable (i.e., plant fitness for the cost; herbivore performance for the benefits). Tests of the cost-benefit model using the same currency are few (Baldwin 1998). In this study, the cost-benefit analysis of plant-insect interaction in the currency of plant fitness demonstrates that the benefits of TPI expression in *N. attenuata* outweigh their costs when plants are attacked. Unattacked plants with low constitutive TPI levels produced more seed capsules than did plants with high TPI levels, and 11 days of *M. sexta* attack reduced

seed capsule production per plant in all genotypes and reversed the pattern of seed capsule production with higher reductions in low TPI-producing genotypes in high TPI-producing genotypes. This differential reduction in seed capsule production amongst genotypes correlated negatively with larval mass and suggested that despite the ongoing evolutionary interaction between *N. attenuata* and *M. sexta*, TPI-mediated decrease in herbivore performance translates into fitness benefit for the plant. This study showed that larvae respond to different TPI levels in the plant; caterpillar adjusted their feeding positions to minimize TPI consumption and maximize protein intake. These results are consistent with the predictions of the Optimal Defense theory, namely that 1) defense is costly, and 2) plants parts with a high fitness value are more heavily defended (McKey 1974; Ohnmeiss and Baldwin 2000; Rhoades and Cates 1976). Moreover, the results highlight the heuristic value of the cost-benefit paradigm for functional studies.

The test of the cost-benefit paradigm of TPI production measured in this laboratory study included only plant intraspecific competition and plant-insect interactions with two herbivores. When plants grow with their full complement of natural ecological interactions, the cost-benefit balance of TPI production can be different. For example, ecological costs might be incurred that result from the complicated interactions with other species, such as decreased attractiveness to pollinators (Karban and Baldwin 1997; Strauss et al. 2002). In addition, TPI can affect not only herbivores' performance but, can also affect the natural enemies of herbivores that may sequester TPI, such as *Geocoris pallens* which feeds on *M. sexta* larvae (Bouchard et al. 2003). General insects that feed on leaves with low nicotine but high TPI content can grow more slowly, but compensate by eating more tissue, a potential fitness detriment for the plant (Winterer and Bergelson 2001). The costs-benefits balance found in this study should be tested in *N. attenuata*'s natural environment and the mechanisms responsible for this ecological

sophistication can be explained by transcriptome analysis. However, without an intimate understanding of plant's natural history, the transcriptional responses will remain functional obscure (Baldwin 2001).

2.3 Future perspectives

Why TPI production is so costly for the reproductive performance of competitively growing plants remains an open question. One challenge will be to understand the metabolic cost of TPI production and its interaction with other traits that could influence fitness. At the gene expression level, microarray analysis could be used to coordinate regulation of gene responsive to herbivores and competitors more broadly than is possible using other methods. This technology would allow us to determine which genes are either up- or down-regulated when plants are grown with or without a competitor and when the transformed plants with different TPI production levels are either induced or uninduced.

3. Conclusions:

3.1 Conclusion (English)

Our competition experiments demonstrate that TPI production is intrinsically costly when *N. attenuata* plants compete for below-ground resources with conspecifics, as they commonly do in nature. The differences in growth and fitness between genotypes plants can be attributed to changes in metabolism required to support TPI production and to differences in the rate of harvesting below-ground resources.

This research demonstrates that despite the ongoing evolutionary interaction between *N. attenuata* and its herbivores, TPIs remain an effective defense against both *T. notatus* and *M. sexta.* High plant TPI levels reduced *M. sexta* performance and fecundity and *T. notatus* colonization.

Our experiments demonstrate that the benefits of TPI expression in *N. attenuata* outweigh their costs when plants are attacked. Unattacked plants with low constitutive TPI levels produced more seed capsules than did plants with high TPI levels, *M. sexta* attack reversed the pattern of seed capsule production with higher reductions in low TPI-producing genotypes than in high TPI-producing genotypes. Despite the ongoing evolutionary interaction between *N. attenuata* and *M. sexta*, TPI-mediated decrease in herbivore performance translates into fitness benefit for the plant. These results are consistent with the predictions of the Optimal Defense theory and highlight the heuristic value of the cost-benefit paradigm for functional studies.

3.2 Zusammenfassung (Deutsch)

Unsere Konkurrenzexperimente zeigen, daß die TPI-Produktion für *N. attenuata* Kosten erzeugt, wenn sie mit Pflanzen der gleichen Spezies um die Ressourcen im Boden konkurriert, wie es meist in der Natur der Fall ist. Die Unterschiede im Wachstum und der Fitneß zwischen den Genotypen kann auf Veränderungen im Metabolismus als Grundlage für die TPI-Produktion sowie auf unterschiedliche unterirdische Ressourcenverwertbarkeit zurückgeführt werden.

Diese Untersuchungen zeigen, daß TPIs trotz ständiger evolutionärer Interaktion zwischen *N. attenuata* und ihren Herbivoren eine effektive Verteidigung gegen sowohl *T. notatus* und *M. sexta* bleiben. Pflanzen mit hohen Gehalten an TPIs verringerten die Entwicklung und Fruchtbarkeit von *M. sexta* und die Kolonisation durch *T. notaus*.

Unsere Experimente zeigen, daß die Vorteile der TPI-Expression in *N. attenuata* ihre Kosten überkompensieren, sobald die Pflanzen befallen werden. Nicht befallene Pflanzen mit geringen konstitutiven TPI-Gehalten produzierten mehr Samenkapseln als Pflanzen mit hohen TPI-Gehalten. *M. sexta*-Befall kehrte die Verteilung der Samenkapseln um, mit einer größeren Verringerung in Genotypen mit niedriger TPI-Produktion als in Genotypen mit hoher TPI-Produktion. Trotz der evolutionären Interaktion zwischen *N. attenuata* und *M. sexta* resultiert eine durch TPIs induzierte Entwicklungshemmung der Herbivoren in einem Fitneßvorteil für die Pflanze. Diese Ergebnisse stimmen mit der Prognose der Theorie der optimalen Verteidigung überein und unterstreichen den heuristischen Wert des Kosten-Nutzen Paradigmas für funktionale Studien.

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Selbständigkeitserklärung

Entsprechend der Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertig habe.

Personen, die bei der Auswahl und Auswertung des Materials und bei der Herstellung der Manuskripte behilfich waren sind am Beginn eines jeden Manuskripts angegeben.

Die Hilfe eines Promotionsberaters wurde nicht in Anspruch genommen.

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