Costs and Benefits of Two Direct Defenses in *Nicotiana attenuata*: Nicotine and Trypsin Protease Inhibitors

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1. Introduction

1.1 On the bottom of the food chain: How plants survive

The world is green because plants persist in a world which is full of their natural enemies. As autotroph organisms, plants fix the energy of sunlight in organic compounds and as such they form the nourishing basis for almost all other organisms. All organisms either directly or indirectly consume plant-produced material (with the only exception being the few ecosystems based on archaebacteria). So how do plants survive the enormous predation pressure?

Unlike many animals, plants are sessile and cannot simply run away from those that aim to feed on them. Instead, plants have evolved other means of survival, including diverse defense and tolerance strategies, and this thesis focuses on those related to herbivore attack. Some mechanical plant defenses such as thorns, spines, hairs, and woody tissues are obvious. However, many plants produce chemical defenses such as toxic, antidigestive, or repellent substances (Bennett & Wallsgrove, 1994). All these examples of defenses are directly directed against the attacker, but some plants have evolved other strategies that make use of the natural enemies of the plants' herbivores. Such indirect defenses include plant structures that provide shelter (dormancies) or nutrition (e.g. nectaries) to predators or signals that are released to attract the predators when herbivores are present (Dicke, 1999; Heil *et al.*, 2001; Kessler & Baldwin, 2001). The latter example introduces another differentiation between defenses, namely, constitutive defenses that are continuously produced and induced defenses which are called on after a plant has been attacked. In the case of the volatiles released to attract predators, the need for inducibility is clear, as a constitutive emission would abolish the information of these signal compounds. However, many direct defenses are also produced after the herbivores start feeding, but this inducibility is bound to a delay of hours to days until the defense is established. That many plants still employ defenses inducibly despite this drawback of delayed resistance is most frequently explained with costs of defenses, which are minimized if they are only inferred when the defense is needed (see Karban & Baldwin (1997) and references therein). Such costs can arise from the resources that are allocated in defenses and consequently not available for growth and reproduction (allocation costs) as well as from interfering effects of defense compounds with the plant's primary metabolism (autotoxicity) or with the plant's ability to respond to other stresses (ecological costs). However, there are few alternative hypotheses that explain inducibility without requiring that

defenses are costly; one of these is the moving target theory, which argues that inducibility itself is a defense, because changing the nutritional quality of plant tissue decreases herbivore performance (Karban *et al.*, 1997).

Although costs and benefits of plant defenses have long been hypothesized (McKey, 1974; Feeny, 1975), the empirical evidence to date is rather weak. Many secondary metabolites produced by plants are thought to function as plant defenses because they have been shown to have anti-herbivore properties, but these metabolites could also serve other ecological and physiological functions, and the toxicity to herbivores may be just a side effect, of no real ecological relevance (Rausher, 1992). Therefore, the defensive function for many plant-produced toxins still needs to determined, even for those that have long been used as insecticides such as pyrethrines or nicotine (Schmeltz, 1971; McLaughlin, 1973). The difficulty of demonstrating the costs of defenses and how they arise is even more apparent than that of establishing their benefits. Most empirical support for costs of defenses comes from studies establishing correlations between the quantity of certain allelochemicals and plant resistance and fitness, by either making use of the genetic variation or by eliciting defense responses (reviewed in Bergelson & Purrington (1996); Heil & Baldwin (2002); Cipollini *et al.* (2003)). But correlations can not trace observed effects back to specific factors, because many plant traits vary between genotypes and after elicitation. During the past two decades, molecular tools have become available for ecological research and enabled a new experimental approach. Manipulating specific factors that are expected to be involved in defense responses allows their function as defenses and consequences for plant fitness to be tested (Zavala *et al.*, 2004b). The concepts of costs and benefits of defenses, the methods to examine them, and alternative hypothesis are discussed in manuscript I in more detail.

This thesis aims to test the hypothesis of costs and benefits of two major direct defenses of a native model plant using the molecular tool of gene silencing. This approach allows the consequences of the presence or absence of these two compounds to plant resistance, development, and fitness to be investigated under standardized conditions in the glasshouse as well as in the plant's native habitat.

1.2 "On/off-button" of genes: Using gene silencing to study plant defense

The method used in this thesis to test the function of putative defenses as well as their fitness costs for the plant is gene silencing via RNAi . The basic principle of RNAi is that in the presence of a complimentary sequence to that of a certain gene, the mRNAs of both form

a double-stranded RNA (dsRNA, Fig. 1) due to homologue base pairing (Waterhouse *et al.*, 1998). This dsRNA is cleaved by specific endonucleases, referred to as Dicer, into short pieces of the double-stranded RNA; these pieces are known as short interfering RNA (siRNA; Hamilton & Baulcombe, 1999). This siRNA binds to specific proteins to form the RNAi-silencing complex (RISC), which after unwinding of the double strand can bind to mRNA with a similar sequence and then cleave this mRNA (Zamore, 2001). The cleaved mRNA is no longer functional (aberrant RNA) and is either broken down to nucleotides by exonucleases or becomes the matrix for RNA-dependent RNA polymerases (RdRPs); these RdRPs synthesize a second strand and consequently new dsRNA is formed (Dalmay *et al.*, 2000). This feedback loop, together with the additional amplification of siRNA resulting from RdRP activity on unwinded siRNA, leads to the production of enormous amounts of the RiSC complex soon after the silencing process has started.

To achieve plants with mRNA complimentary to that of the target gene, plants can be transformed to integrate a part of the gene sequence in anti-sense (AS) orientation. The silencing process starts after the ASmRNA comes together with the mRNA of the gene to form dsRNA and thus both mRNAs are required in high amounts to increase the probability of their hybridization. The introduced ASmRNA is usually under control of a constitutive promoter and therefore highly expressed, but the endogenous mRNA may not be present in sufficient amounts. To increase the effectiveness of gene silencing Wesley *et al.* (2001) recommended that plants are transformed with inverted-repeat (IR) constructs which contain the sequence twice, one in sense and the other in reversed orientation, both of which are divided by an intron. After splicing of the resulting mRNA, both sequences immediately

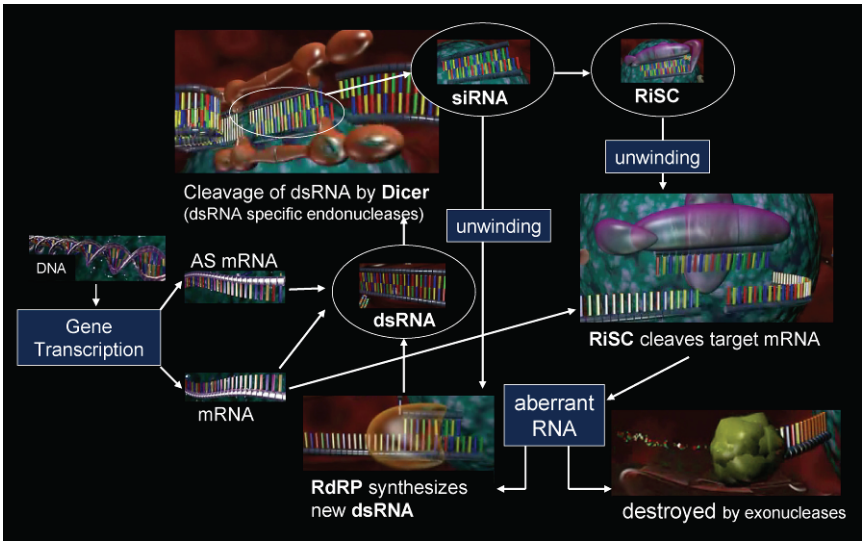

Figure 1: The mechanism of gene silencing by RNAi (RNA interference). Abbreviations: mRNA – messenger RNA, ASmRNA – antisense mRNA, dsRNA – double stranded RNA, siRNA – short interfering RNA, RiSC – RNAi silencing complex, RdRP – RNA-dependent RNA polymerase
Images: nature-online

hybridize to form dsRNA and the silencing process is started. Both methods are compared for their effectiveness in manuscript II.

Unlike studies that heterologously express defense-related genes, loss-of-function studies via gene silencing allow the endogenous function of a gene to be determined with the genetic and chemical background with which it has evolved. Comparing the herbivore resistance of plants silenced for a defense-related gene with untransformed wild-type (WT) plants allows testing how a putative defense trait benefits a plant – if at all –, as well as which herbivores a trait affects and under which conditions. Comparisons of the fitness of silenced and WT plants when herbivores are excluded can demonstrate whether, how, and under which conditions a plant incurs defense-related costs. Further, combining gene silencing with other approaches provides an elegant toolbox with which to test the costs and benefits of a certain trait relative to different aspects of ecological complexity. For example, combining gene silencing with elicitation experiments allows the role of a specific trait to be assessed within the whole suite of defense responses elicited by herbivores. By manipulating the same gene in different natural genotypes, the contribution of that trait to costs and benefits can be measured in the different genetic backgrounds to investigate the causes of genetic diversity. Finally, different genes can be silenced separately and together, allowing whether and how different traits interact in their functions to be tested.

To ensure that observed effects can be attributed to the silenced trait, side effects of the transformation procedure have to be excluded as a cause. Such side effects could arise from interrupting the genetic code at the site of insertion, from the vector used for transformation (particularly because frequently antibiotic resistances are used as selection markers), from the tissue culture procedures following the *Agrobacterium tumifaciens*-mediated transformation, and from polyploidyzation that is sometimes associated with transformation (Purrington & Bergelson, 1997; Bubner *et al.*, 2006). Therefore, the genetic and metabolic consequences of the transformation procedure should be well characterized. Transgenic lines should harbor a single insertion of the transgene, be homozygous, be verified for diminished transcript levels of the targeted gene, and not be altered in other defense-relevant metabolites. The insertion site

can be excluded as a cause of observed fitness effects by using multiple lines of independent transformation events. To control for the transformation vector and the procedure, plants transformed with empty vectors can be included in the analysis.

1.3 On the burn: The ecology of *Nicotiana attenuata*

Nicotiana attenuata Torr. ex Watson (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae) is a wild tobacco species native to Great Basin Desert in western North America. The so-called coyote tobacco is a post-fire annual (Fig. 2). Its seed germination is regulated by stimulants from burned wood and inhibitors from litter (Baldwin *et al.*, 1994; Lynds & Baldwin, 1998). As a consequence, *N. attenuata* grows in nitrogen-rich habitats with low inter-specific competition but due to the synchronized germination intra specific competition is high. Within a few years after a fire, the population vanishes as the ash content declines and strong inter-specific competitors immigrate to the area. The dormant seeds may wait up to 150 years for new fires in a seedbank that is well dispersed in this high erosion desert ecosystem.

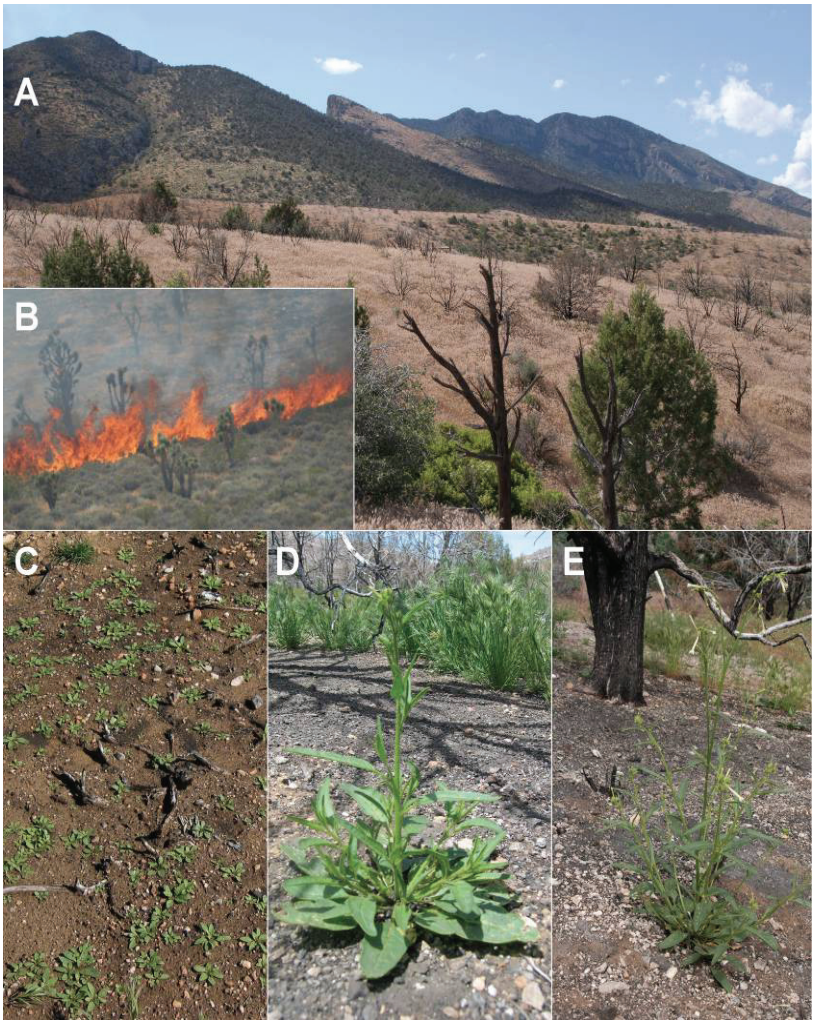

Figure 2: A Habitat of *Nicotiana attenuata* in the Great Basin Desert (near Santa Clara, Utah), **B** wildfire during summer 2005, **C** emerging rosettes of wild *N. attenuata* on a 1-year-old burn (from 2004), **D** elongating and **E** flowering transgenic plants transplanted into the wild population.
Photographs **B**: D. Kessler
A, C-E: A. Steppuhn

Though *N. attenuata* has an interesting life history, the question may arise, why use it as a model plant to study anti-herbivore defense? For molecular tools to be used on a species, the target organism has to be genetically well characterized, in particular, sequence information is necessary. The genetically best-investigated plants are crops. However, ecologically relevant interactions between plants and their natural enemies are best explored in native plants. *N. attenuata* is a Solanacae and as such, closely related to tomato, potato, and cultured tobacco, for all of which sequence information is generally available. Previous studies carried out in the Department for Molecular Ecology at the MPI-CE established a base of sequence information for genes related to defense (for example: Hermsmeier *et al.* (2001); Halitschke *et al.* (2003); Hui *et al.* (2003); Voelckel & Baldwin (2004)). Therefore, *N. attenuata* clearly satisfies both requirements: its genetic basis is well known and it encounters a variety of natural plant-insect interactions.

1.4 On the battlefield: *Nicotiana attenuata*'s herbivores and its defense strategies

Due to the post-fire ecology of *N. attenuata*, its herbivores always have to recolonize new populations, and therefore the community of natural enemies on is very variable between growing seasons and populations. *N. attenuata* is attacked by a wide range of herbivores, of diverse phylogenetic taxa, and of different feeding guilds with varying host ranges (Fig. 3). In the following, the most frequently observed herbivores will be briefly introduced.

Leaf-chewing herbivores include generalist and specialist herbivores of the insect orders Lepidoptera, Coleoptera, and Saltatoria as well as mammalian browsers. The larvae of two sphingid moths, *Manduca quinquemaculata* Haworth and *Manduca sexta* Linnaeus, which have specialized on nightshades such as *Nicotiana*, *Lycopersicon*, *Solanum*, and *Datura* species, are major defoliators of *N. attenuata*. Their geographic distribution, ranging from Canada to Argentina, matches that of *N. attenuata*. Adult moths are nocturnal, feed on the nectar of hawkmoth-pollinated flowers, and deposit single eggs on the underside of the leaves of their host plants. After on average 5 days, larvae emerge with about 1 mg body mass and normally pass through five instars within roughly twenty days, during which they increase their body mass thousandfold. Other lepidopteran larvae frequently observed on *N. attenuata* are *Spodoptera exigua* Hübner and cutworms of the genus *Agrotis,* generalist species that are distributed worldwide and feed on a broad range of host plants. Whereas *S. exigua* larvae that hatch from egg clutches of 50-150 feed like *Manduca* larvae and normally pass through five instars in ten to fourteen days, noctuid *Agroits* caterpillars (egg clutches

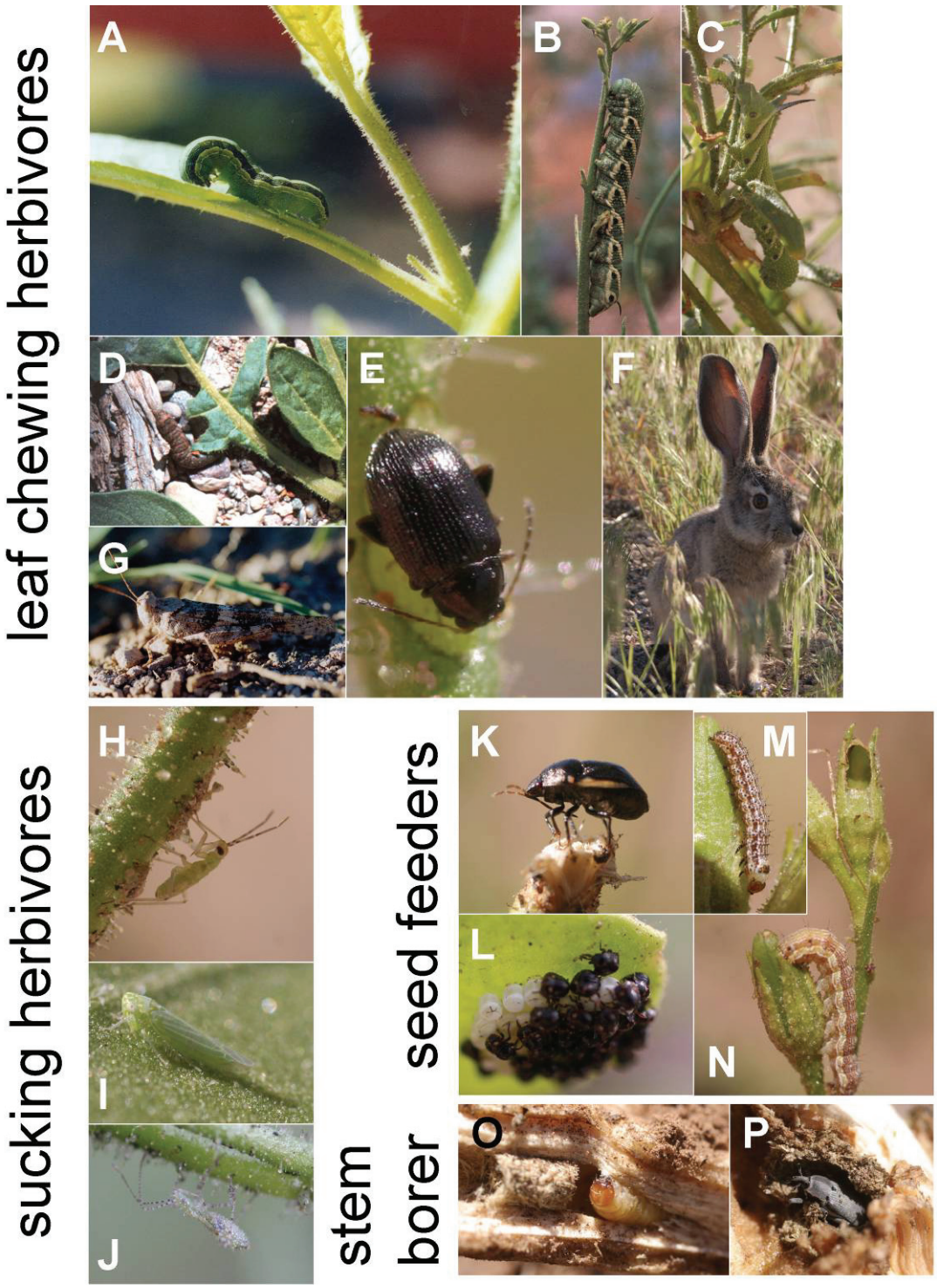

Figure 3: Herbivores present on *Nicotiana attenuata* during growing season 2005. Leaf-chewing herbivores: **A** *Spodoptera exigua* (beet army worm), **B** *Manduca quinquemaculata* (tomato horn worm), **C** *Manduca sexta* (tobacco horn worm), **D** *Agrotis ipsilon* (black cutworm), **E** *Epitrix subcrinita* (Western potato flea beetle), **F** *Lepus californicus* (black-tailed jack rabbit), and **G** *Trimerotropis* spp. (band-winged grasshoppers). Sucking herbivores: **H** *Tupiocoris notatus* nymph (suckfly), **I** *Empoasca* spp. (leaf hoppers), and **J** nymph of a *Berytidae* (stilt bug). Seed feeders **K,L** *Thyreocoridae* (negro bugs) and **M,N** *Heliothis virescens* (tobacco bud worm). **O** Larvae and **P** adult of stemboring *Trichobaris mucorea* (tobacco stalk borer). Photographs **A-P**: A. Steppuhn

of several dozen, 5-9 instars) are hidden in the soil and remove at night large portions from the leaves from their host plants. Further leaf-chewing herbivores include flea beetles such as *Epitrix subcrinita* Le Conte and *E. hirtipennis* Melsheimer , which bore small holes into the leaves and stems of *N. attenuata* and occur occasionally in high abundance, voracious grasshoppers of the genus *Trimerotropis*, and browsing mammals, such as, for example, *Lepus californicus*.

Sucking herbivores include generalist and specialist herbivores of the insect orders Hemiptera and Homoptera. The adults and nymphs of *Tupiocoris notatus* Distant, "lacerate and flush" feeders on the mesophyll, are the most abundant sucking herbivores on *N. attenuata*. Whereas *T. notatus* is a Solanaceae specialist, *Empoasca* leaf hoppers and *Berytidae* stilt bugs are generalist plant sap feeders and have been frequently observed on *N. attenuata*.

Other herbivores regularly observed are *Thyreocoridae* negro bugs and *Heliothis virescens* Fabricus bud worms, which mainly feed on the seeds of *N. attenuata*. Adults of *Trichobaris mucorea* Le Conte were frequently observed in the habitat of *N. attenuata* but only rarely seen feeding on the plants; in the 2005 field season we discovered that many stems had been infested by its larvae.

N. attenuata responds to herbivore attack by producing a variety of secondary metabolites, for example, flavonoids, phenolics (such as diterpene glycosides), toxic alkaloids (such as nicotine), antidigestive proteins (such as trypsin protease inhibitors, or TPIs), and volatile sesquiterpenes. Most of these allelochemicals are induced via the jasmonic acid (JA)-signaling cascade (Kessler & Baldwin, 2002; Roda & Baldwin, 2003). Evidence for the defensive value of JA-mediated traits comes from *N. attenuata*'s increased resistance to native herbivores in a wild population after elicitation with the methyl ester of JA and its diminished resistance when genetically silenced for JA biosynthesis (Baldwin, 1998; Kessler *et al.*, 2004). Much of the JA-mediated resistance can be accounted for by the elicitation of TPI production, as is demonstrated when the biosynthetic gene is silenced (Zavala & Baldwin, 2004; Zavala *et al.*, 2004a). Because the pyridine alkaloid nicotine can interact with the acetylcholine receptors in the nervous systems of animals, it is extremely toxic to most herbivores. Evidence for the defense value of nicotine arises from the agricultural practice of using nicotine sprays and genotypes of cultivated tobacco differing in nicotine levels (Jackson *et al.*, 2002) and the negative correlation between nicotine content and performance of *M. sexta* feeding on *Nicotiana sylvestris* with reduced nicotine production due to AS silencing of the regulatory enzyme for nicotine biosynthesis, *putrescine N-methyltransferase* (*pmt*)

(Voelckel *et al.*, 2001). Although nicotine is toxic to a wide range of organisms, adapted herbivores have evolved resistance to this alkaloid (Glendinning, 2002). The specialist herbivore *M. sexta* tolerates doses of nicotine that are fatal to unadapted herbivores but grows more slowly on high-nicotine diets (Appel & Martin, 1992; Wink & Theile, 2002). However, *M. sexta* might even be better defended by dietary nicotine against its parasitoid, *Cotesia congregata*, which suffers higher mortality when parasitizing larvae fed on high- rather than low-nicotine diets (Barbosa *et al.*, 1986; Thorpe & Barbosa, 1986). Even though parasitoids of *N. attenuata*'s herbivores have not been observed, predators play an important role in its defense.

In addition to the direct defenses of nicotine and TPI production, *N. attenuata* responds to herbivore attack with the release of volatile sesquiterpenes such as *cis*-α-bergamotene (Halitschke *et al.*, 2000), which function as an indirect defense. These sesquiterpenes attract predators of herbivores and thereby reduce herbivore load by over 90% (Kessler & Baldwin, 2001). The most abundant predator in *N. attenuata*'s habitat is *Geocoris pallens* Stäl but other predators are also frequently observed (Fig. 4).

The genotypes used in this thesis are derived from field-collected seeds from a population near Santa Clara, Utah, in 1998, and a population near Flagstaff, Arizona, in 1996 (Baldwin, 1998; Glawe *et al.*, 2003). Both ecotypes differ in their defensive phenotypes. Unlike the ecotype from Utah (UTE), the Arizona ecotype (AZE) lacks the ability to induce TPIs due to a nonsense mutation in the *pi* gene (Glawe *et al.*, 2003; Wu *et al.*, 2006). Furthermore, it does not release the volatiles that are prominent in the induced volatile bouquet of the UTE (Halitschke *et al.*, 2000; Glawe *et al.*, 2003).

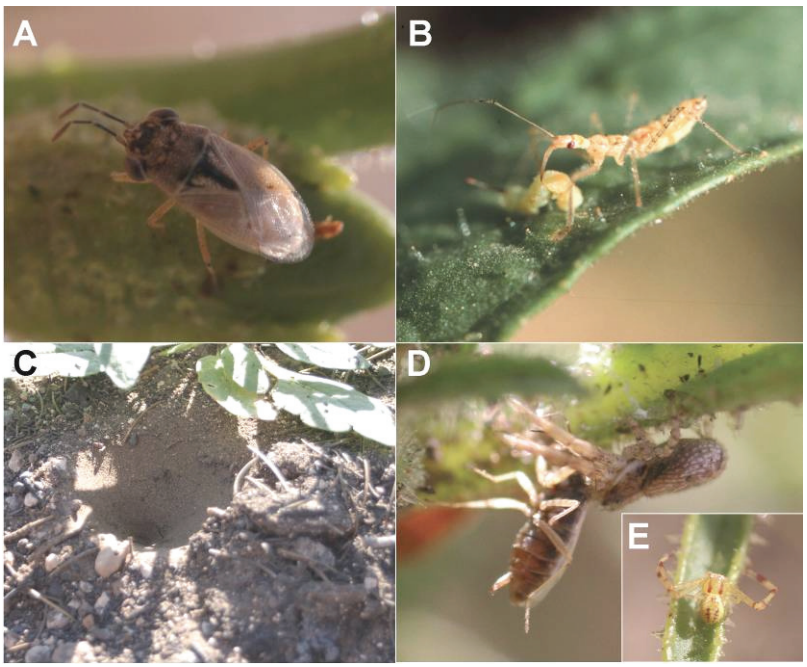

Figure 4: Predators present on *Nicotiana attenuata* during growing season 2005. **A** *Geocoris pallens* (big-eyed bugs) and **B** nymph of a *Redividae* (assassin bugs preying on a *Manduca sexta* larva), **C** pitfall of *Myrmeleontidae* larvae (antlion), **D** *Salticidae* (jumping spider preying on a *G. pallens*), **E** *Thomisidae* (crab spider). Photographs **A-E**: A. Steppuhn

1.5 On the books: Tests of the cost-benefit paradigm (thesis questions)

After the concepts of costs and benefits of defenses as well as the methods of examining them, and alternative hypotheses are reviewed in manuscript I, in manuscripts II, III, and IV the different hypotheses for *N. attenuata*'s nicotine and TPI production are tested using plants that are stably transformed to silence their biosynthetic genes, *pmt*, and *pi*. The resistance, development, and fitness of these silenced plants were compared with WT *N. attenuata* and investigated under standardized conditions in the glasshouse as well as in the plant's native habitat to answer the following questions:

- Is gene silencing with an IR-construct more efficient than with an AS-construct in *N. attenuata*?
- Does gene silencing of *pmt* and *pi* affect other traits within the primary and/or secondary metabolism and if so, how does this linkage occur?
- Does the production of nicotine and TPIs benefit *N. attenuata* plants by functioning defensively against herbivores in nature?
- To which herbivores do nicotine and TPI production provide resistance?

In addition to plants silenced for *pmt* and *pi* separately, a genotype was used that is deficient for both defenses as a result of being transformed twice. The use of this doubly transformed UTE and of AZEs silenced in nicotine or restored in TPI production due to sense expression of the *pi* gene offered new investigative possibilities. Nicotine and TPI production can be evaluated with and without the background of the other, and the UTE and AZE can be compared for differences in plant resistance and fitness, dependently and independently of nicotine and TPI production. Thus, the following questions could be addressed:

- Does the synchronized production of nicotine and TPIs affect each other in their function as anti-herbivore defenses?
- How can the AZE do without the production TPIs and volatiles that are effective defenses in the UTE?
- Do nicotine and TPI production differ in their cost-benefit-functions between the two ecotype of *N. attenuata*?

In several experiments the use of stably transformed plants silenced for *pmt* and/or *pi* gene was combined with other techniques, for example, virus-induced gene silencing (VIGS)

of the biosynthetic gene for JA biosynthesis, elicitation with the JA methyl ester, or pulse-chase studies with stable isotopes. These experiments addressed the following questions:

- What is the contribution of TPIs and nicotine to JA mediated defenses in both, the AZE and the UTE?
- Is the accumulation of a precursor responsible for increased anatabine levels in *pmt*-silenced plants?
- Does the production nicotine and TPI exhibit fitness costs in the absence of herbivores and if so, can these be attributed to resource allocation costs?

2. List of Manuscripts: Contents and Author's Contributions

Manuscript I

"Induced Defenses and the Cost-Benefit Paradigm"
A. Steppuhn and I.T. Baldwin
In: Induced Plant Resistance to Herbivory, ed. A. Schaller
Springer Berlin Heidelberg, in press
Section I – Introduction, Chapter 4

This review summarizes the classic concepts of costs of plant defense and the different approaches that were used to examine whether defenses are costly, with example studies. The advantages and weaknesses of the different approaches as well as the challenges resulting from the complexity of the plant responses to herbivores and other stresses are discussed. Further the manuscript suggests an updated view of the classic cost-benefit paradigm as well as an outlook for future research in the field. The book chapter provides an overview of the conceptional framework forming the basis of the experimental work of this thesis.

In close collaboration Ian T. Baldwin and I defined the contents and structure of the manuscript. I wrote the first draft of the manuscript and composed the figures and together with Ian T. Baldwin the manuscript was refined to its final version.

Manuscript II

"Nicotine's Defensive Function in Nature"
A. Steppuhn, K. Gase, B. Krock, R. Halitschke, and I.T. Baldwin
PLOS Biology 2004, 2: 1074-1080

This study establishes the defensive value of *Nicotiana attenuata*'s nicotine production in its native habitat by using transformant lines deficient for nicotine production. The silencing efficiencies of anti-sense and inverted-repeated constructs are compared and the latter is found to be more effective. Successful silencing of the *putrescine-N-methyl transferase* (*pmt*) gene, which encodes the regulatory enzyme of nicotine biosynthesis, decreased nicotine levels by more than 95% and led to the formation of anatabine up to 25% of the nicotine levels in wild-type (WT) plants. Using stable isotope-labeled nicotinic acid we demonstrated that

silencing *pmt* results in excess nicotinic acid, which dimerizes to form anatabine. Herbivores prefer the nicotine-deficient tranformants over the WT in choice tests and *Manduca sexta* larvae grow better on them. When planted in the native habitat, *pmt*-silenced plants received 3 times more leaf area damage from herbivores than did WT plants. Half of the canopy loss was caused by *Spodoptera exigua*.

The silencing plasmids were constructed by Klaus Gase (plant transformation: Michelle Lim). I screened and characterized the transformed lines and performed all extractions and analyses with the exception of the NMR analysis of anatabine, which was performed by Bernd Krock (in collaboration with Bernd Schneider). Ian T. Baldwin and I developed the experimental designs and I performed the experiments. Field release experiments were planned and performed in close collaboration with Rayko Halitschke, who also trained me in most of the molecular methods. Ian T. Baldwin and I wrote the manuscript, which was supplemented with information from Klaus Gase and Bernd Krock for the details of plasmid construction and NMR analysis, respectively.

Manuscript III

"Resistance Management in a Native Plant: Nicotine Prevents Herbivores from Compensating for Plant Protease Inhibitors"
A. Steppuhn, and I.T. Baldwin
Ecology Letters 2007, 10: 499-511

This manuscript demonstrates that producing nicotine and trypsin protease inhibitors (TPIs) provides a defensive synergism in *N. attenuata* when the plant is attacked by generalist herbivore *S. exigua*. To establish the effects of nicotine and TPIs with and without the background of the other on *S. exigua* growth, consumption, and conversion efficiency, we used transgenic plants silenced for the biosynthetic genes *pmt* and *pi* both separately and together. The experiments reveal that both metabolites function defensively against *S. exigua* larvae in WT plants, however, whereas nicotine alone reduced larval performance TPIs alone did not. In response to TPIs larvae started a compensatory feeding, which was prevented by nicotine in WT plants.

I screened and characterized the transformed lines (produced by Susan Kutschbach and Antje Wissgott) and performed all extractions and analyses. The experimental design was

developed by Ian T. Baldwin and me, and I performed the experiments. I wrote the first draft of the manuscript, which was refined by Ian T. Baldwin.

Manuscript IV

"Silencing Jasmonate (JA) Signaling and JA-Mediated Defenses Reveals Different Survival Strategies Between Two Ecotypes of *Nicotiana attenuata*"

A. Steppuhn, M. Schumann, and I.T. Baldwin

Submitted to Ecology Letters (12.07.2007)

In this manuscript we tested whether an ecotype of *N. attenuata* from Arizona (AZE) that lacks the herbivore-induced traits of trypsin protease inhibitor (TPI) production and the volatile signal cis-α-bergamotene, which function as major defenses in an ecotype from Utah (UTE), is less defended than the UTE. The AZE may save the costs of defenses or may have evolved different defense strategies. Using transient and stable plant transformation with silencing and sense constructs, we demonstrate differences between the ecotypes dependently and independently of the two JA-mediated defenses nicotine and TPIs, and conclude that the AZE evolved other traits including direct and indirect defenses as well as additional fitness-enhancing traits.

The experimental design was developed by Ian T. Baldwin and me and Merry Schuman, who performed and analyzed the VIGS experiments. I performed and analyzed the screening and characterization of the AZE *pmt*-silenced plants and the field experiments. The first draft of the manuscript was a joint effort by Merry Schumann and me, and was refined by Ian T. Baldwin.

Manuscript I

Induced Defenses and the Cost-Benefit Paradigm

A. Steppuhn[1] and I. T. Baldwin[1*]

In: Induced Plant Resistance to Herbivory, ed. A. Schaller
Springer in press
Section I – Introduction, Chapter 4

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92 References

Abstract

Defense costs are thought to be the *raison d'etre* for inducibility, by which costs are only incurred when a defense is needed. Costs can arise when resources are allocated to defenses and consequently not available for growth and reproduction, from the havoc that defenses might wreck with primary metabolism or the plant's ability to respond to other stresses, and from the damage caused by herbivores whose ability to resist plant defenses have been honed by constitutive defense expression. Though the cost-benefit paradigm is widely accepted, empirical evidence is rather slim. Elicitation studies, which elucidate fitness consequences of activating defense responses with elicitors, presented first conclusive evidence, but in these studies disentangling the costs of co-regulated responses is not possible. Quantitative genetics correlate changes in resistance and fitness among different genotypes, but defense-related traits can be genetically linked to other traits unrelated to defense. Mutant and transformant studies provide the strongest evidence for cost-benefit tradeoffs, because confounding effects of genetic linkage are minimized in isogenic lines. In addition to genetic linkage, defenses are linked to various aspects of primary and secondary metabolism and defense functions can be altered by other metabolites. The diverse linkages among responses to herbivory together with the complexity of internal and external factors that influence plant fitness suggest that cost-benefit functions are best examined as shifting in a multi-dimensional space bracketed by environmental conditions. Methodological advances allow the connections of the metabolic grid that shape phenotypes to be visualized and for the falsification of hypotheses about tradeoffs.

4.1 Introduction

Plants employ chemical defenses to protect themselves from attack by a variety of herbivores and pathogens. In many plants chemical defenses are deployed inducibly, that is, their production and accumulation increase dramatically after attack (Ryan, 1983; Karban and Baldwin, 1997). Different elicitation systems allow plants to elicit defenses that are most efficient against particular attackers (e.g. pathogens or herbivores of different feeding guilds (Kessler and Baldwin, 2004; Voelckel and Baldwin, 2004b, 2004a). But, due to the time lag between the first attack and the activation of the defense, plants remain unprotected for hours or even days until the defense is established. So why do plants put up with this risk? The selective advantage of induced over constitutively expressed defense traits, which lack the

detrimental delay, is commonly explained by the high cost of defenses. Consequently, the fitness benefit of forgoing these costs when resistance is not needed may outweigh the disadvantages of the delay.

Defenses can have physiological, ecological, and evolutionary costs. Physiological costs may involve allocation and autotoxicity. **Allocation costs** occur when fitness-limiting resources are tied-up in defenses, which may not be readily recycled and hence are unavailable for processes relevant to fitness such as growth or reproduction. **Autotoxicity costs** may occur because certain secondary metabolites can be toxic to the plant itself. Their constitutive expression may therefore impose a significant metabolic burden. Metabolites that are universally toxic can be very effective defenses, but they also represent a significant liability for the producer. Plants solve this "toxic waste dump" problem associated with chemical defense deployment by elaborating defenses that target tissues that don't occur in plants, such as neurotoxic alkaloids. **Ecological costs** result from the negative fitness effects of defenses on the complex interactions that plants have with their environment. These costs include the liability that results from producing toxins that are sequestered by adapted herbivores, protecting them from their own enemies. But the ecological costs can also be seen as opportunity costs: activating a particular defense response may compromise the activation of other defenses vis-à-vis other attackers or tolerance responses. Inducibility may minimize **evolutionary costs**, because constitutively deployed defenses may provide a stronger selection pressure for herbivores to evolve counter resistance. Given that the inducible defenses of plants greatly increase the complexity of the chemical environments that a plant's natural enemies are exposed to, the minimization of these evolutionary costs may be important.

Putative allocation costs play a central role in theories that attempt to explain the patterns of defense metabolite distribution within and among plants, but the assumption that defenses incur significant allocation costs have been difficult to test empirically. The optimal defense theory (McKey, 1974; Zangerl and Bazzaz, 1992) argues that the distribution of defense metabolites within the plant reflects an optimization of the fitness benefits over the costs of defense deployment: tissues with high fitness value to the plant and a high probability of attack receive preferential allocation. For example, the toxic alkaloid nicotine that is produced in the roots of *Nicotiana attenuata* is concentrated in young leaves, stems, and reproductive parts, whereas roots and old leaves have low levels (Ohnmeiss and Baldwin, 2000). Similarly, the distribution of induced defenses within the plant kingdom is commonly explained by the cost-benefit tradeoffs involved in defense traits. The apparence theory

(Feeny, 1976) predicts that slow-growing plants with long-lived tissues that are likely to be attacked by herbivores invest more in constitutive defenses compared to fast-growing species with short-lived tissues that are more likely to escape herbivory; such species optimize their fitness by expressing induced defenses, as the costs of defense are expected to be particularly onerous for fast-growing species. Consistent with this expectation, most species for which induced resistance has been reported are fast growers (Karban and Baldwin, 1997). Other theories, such as the carbon/nutrient (C/N) balance hypothesis (Bryant et al., 1983) and the substrate/enzyme theory (Bryant et al., 1983), and the growth/differentiation theory (Herms and Mattson, 1992), attempt to explain the inducibility of secondary metabolites in terms of nutrient (or substrate) availability either within the plant, throughout its ontogeny, or in its local environment. These "supply-side hypotheses" (Lerdau et al., 1994) postulate that the increases in secondary metabolites that commonly occur after herbivory do not result from a signal-mediated regulation of secondary metabolism, but rather as an indirect consequence of changes in overall metabolic balance. Although these theories are of little value as mechanistic models, their underlying concepts might help to explain the distribution of plant defenses with regard to their allocation costs. For example, carbon-intensive defenses indeed tend to increase when growth is constrained due to nutrient limitations which are thought to make carbon more available than nutrients; however, such changes may also result from adaptive responses by the plant to conditions which hinder the replacement of damaged tissues.

The hallmark of ecological costs is their environmental contingency, that is, they can only be seen in particular environments. Ecological costs may result from tradeoffs between resistance and susceptibility to different natural enemies and mutualists; between resistance and tolerance to herbivory; or between resistance to herbivores and the resulting effects on the plant's competitiveness. The latter is exemplified by the fact that large allocation costs can often only be observed when plants are grown with conspecific competitors, not without (e.g. in *N. attenuata* Baldwin and Preston, 1999). Tradeoffs between resistance and susceptibility to different natural enemies may be particularly important for some indirect defenses, such as the release of volatile "alarm" signals to attract the third trophic level, which for *N. attenuata* has been shown to be an exceptionally effective defense that can decrease herbivore abundance by up to 90% (Kessler and Baldwin, 2001). Although such small investments in volatile organic compounds (VOCs) are likely to exact only minor allocation costs (Dicke and Sabelis, 1992), a release of volatiles may increase a plant's apparency if herbivores co-opt the VOCs as signals when foraging for hosts (Takabayashi and Dicke, 1996; Halitschke et al.,

2007). Additionally, the simultaneous elicitation of direct and indirect defenses may incur ecological costs: for example, a toxic direct defense may poison organisms at the third trophic level which are attracted by the release of volatiles. Parasitoids have been found to be attracted to herbivore-induced VOCs, but as these predators spend their entire larval life bathed in the hemolymph of their hosts, they are exposed to all the plant-produced toxins ingested by the herbivore. For example, the tobacco hornworm *Manduca sexta* feeds on species of *Nicotiana* known to induce both VOCs and high levels of nicotine. *M. sexta* retains quantities of the toxin in its hemolymph that are sufficient to cause substantial mortality to its parasitoid wasp, *Cotesia congregata* (Barbosa et al., 1991). Although the herbivore-induced release of volatiles has been demonstrated to attract parasitoids in many laboratory settings, their real-world significance as a plant defense has only rarely been elucidated (but see Kessler and Baldwin, 2001; Halitschke et al., 2007). The potential for these ecological costs to influence the net fitness benefit of an induced response is great, underscoring the importance of testing the cost-benefit paradigm under environmentally realistic conditions.

In the past two decades, many studies have tested the fundamental assumption of the cost-benefit paradigm: that resistance traits are intrinsically costly. Various approaches have been used with differing degrees of success to control the genetic, physiological, and ecological complexity that may confound the relationship between defense expression and fitness.

4.2 The evidence for defense costs

4.2.1 Types of study

The cost-benefit paradigm, the foundation for most theories about the evolution of plant defenses, has been difficult to test, first, because of the complexity of internal and external processes that influence how defense traits may compromise male and/or female reproductive function. Second, the costs of a defense trait must be separated from those of genetically linked traits. The most conclusive evidence that plant defenses exact fitness costs originates from three types of studies: (1) elicitation studies, which measure the fitness consequences of activating defenses by applying signal molecules known to elicit defenses; (2) quantitative genetic studies, which correlate fitness with genetic variation in levels of defense metabolites within plant populations; and (3) mutant and transformant studies, which

evaluate the fitness consequences for plants that have been altered (overexpression or loss of function) in the expression of specific genes.

4.2.2 Elicitation studies

Most costs studies have elicited secondary metabolites production by wounding or herbivore attack or applied signaling molecules that mediate induced responses, and compared the fitness or performance of these elicited plants with that of unelicited control plants. Herbivore feeding, though biologically realistic, is difficult to standardize and the costs of induced responses are hard to separate from the fitness costs resulting from tissue loss. Mechanical wounding, which induces resistance in most plants, enables damage to be quantitatively, qualitatively, and temporally controlled. Puncture wounds, in particular, allows leaf area loss to be kept to a minimum, while still effectively eliciting defense responses. The first study which was able to detect the costs of induced defenses used different wounding regimes to elicit plant defense responses to different degrees, but all resulted in the same loss of tissue (Baldwin et al., 1990). However, the responses induced by mechanical wounding are not always the same as those induced by herbivore feeding (Halitschke et al., 2001; Halitschke et al., 2003 but see (Mithöfer et al., 2005).

Recently our understanding of the different signaling molecules mediating induced plant responses has increased, enabling the costs of induced defense to be uncoupled from the costs of tissue loss. Since the jasmonic acid (JA) signaling cascade is known to mediate many herbivore-induced responses (Creelman and Mullet, 1997; Wasternack et al., 1998; Halitschke and Baldwin, 2004), numerous studies of the costs of induced responses have applied JA or its methyl ester (MeJA; van Dam and Baldwin, 1998; Agrawal et al., 1999; Cipollini and Sipe, 2001; Redman et al., 2001) to elicit responses, and this approach has enabled the cost-benefit paradigm to be tested in nature. The lifetime seed production of MeJA-treated *N. attenuata* plants grown in natural populations was lower than that of untreated plants if herbivores were absent, however, when plants were exposed to moderate or high levels of herbivory, fitness benefits exceeded the costs of JA elicitation (Baldwin, 1998). In wild radish, Agrawal et al. (1999) detected fitness costs of JA induction not in terms of lower number or mass of seeds, but rather the time to first flowering and amount of pollen produced. However, JA applications do not faithfully mimic the responses to herbivore attack as this phytohormone functions substantially downstream of the herbivore-specific elicitors which activate JA signaling in *N. attenuata* (Wu et al., 2007) and is known to interact with other herbivore-

elicited signaling molecules in complex ways (Thomma et al., 1998; Mur et al., 2006). Moreover, exogenous treatments are not likely to realistically imitate the frequently highly tissue-specific and transient changes in endogenous pools of signal molecules that are elicited by herbivore attack.

The identification of herbivore-specific elicitors, such as the fatty acid-amino acid conjugates (FACs) in the oral secretions of lepidopteran larvae (Alborn et al., 1997; Halitschke et al., 2001), has made it possible to elicit those plant responses that are most similar to the responses elicited by actual herbivore attack (Halitschke et al., 2003). But elicitation studies are usually unable to demonstrate the fitness costs of any particular biochemically characterized induced defense because many of the responses include physiological and morphological changes, which although unrelated to resistance, nevertheless affect fitness parameters. For example, as well as mediating resistance to herbivores, JA is involved in numerous processes such as fruit ripening, pollen fertility, root growth, tendril coiling, and resisting pathogens (Creelman and Mullet, 1997). Treatment with MeJA inhibits the gene transcription of proteins essential for growth, for example, RuBPCase and chlorophyll a/b binding proteins (Reinbothe et al., 1994; Halitschke et al., 2001); and as a consequence photosynthesis is reduced (Metodiev et al., 1996). This suppression can decrease fitness when plants are grown under resource-limited conditions. The down-regulation of growth and the degradation of photosynthetic proteins may be necessary to free up the resources required for the *de novo* production of resistance traits but may otherwise not be related to resistance traits. To date we know very little about the traits that are necessary and sufficient for establishing resistance. These pleiotropic effects may cause the fitness costs of resistance to be overestimated in elicitation studies, or they may accurately represent the true fitness costs of defense elicitation by including the metabolic readjustments required for defense activation.

4.2.3 The quantitative genetics approach

Elicitation studies make use of the phenotypic plasticity of inducible plants to compare the fitness of plants with and without activated defenses; another approach uses the genetic variation in resistance. Early attempts to estimate the genetic tradeoff involved in allocating resources to defense compared the fitness parameters of offspring from crosses of species, cultivars, or populations that differ in the degree of their defense investment (Krischik and Denno, 1983). Because genetic differences within populations encompass many traits in

addition to those that control resistance, sib analysis and artificial selection experiments have been used to examine negative correlations between the genetic variation in resistance and in fitness. In a pioneering study using half-sib families of *Pastinaca sativa*, Berenbaum et al. (1986) provided strong evidence for such a genetic tradeoff, showing that the genetically controlled ability to produce an anti-herbivore defense is negatively associated with fitness parameters. The production of furanocoumarins, which explains 75% of the variation in resistance to a specialist herbivore, correlated negatively with seed production in the absence of herbivores. Others studies have also found negative correlations between inherited defense traits and fitness parameters; for example, higher glucusinolate production in *Brassica rapa* is associated with lower female fitness (Mitchell-Olds et al., 1996; Stowe, 1998) and fewer pollinator visits (Strauss et al., 1999), but many studies have also failed to detect any significant correlations between the level of resistance and reproductive success (for example Simms and Rausher, 1987, 1989; Agren and Schemske, 1993; Vrieling et al., 1996; Juenger and Bergelson, 2000; Agrawal et al., 2002). A meta-analysis conducted in 1996, examining conditions under which costs were detected, revealed that only about one-third of the studies found significant costs and that greater control over the genetic background increased the probability that costs of resistance traits would be detected (Bergelson and Purrington, 1996). That the fraction of studies that were able to detect costs subsequently more than doubled can be attributed to increased experimental control in both elicitation studies and genetic correlations (Heil and Baldwin, 2002; Strauss et al., 2002; Cipollini et al., 2003). However, without control over the genetic background, the presence or absence of fitness costs cannot be ascribed to the expression of resistance (Bergelson et al., 1996).

4.2.4 Mutants and transformant studies

Advances in molecular techniques during the past decade have enabled specific defense genes to be altered and therefore obviated the problems of confounding factors that vary with defense expression. The use of mutants and transformed plants that constitutively express resistance genes or that are hindered in the expression of such genes allows the fitness consequences to be examined against a common genetic background. Initial support for the hypothesis that the constitutive expression of resistance lowers fitness came from the phenotypes of mutants that constitutively express genes of the signaling cascades, which mediate pathogen resistance; these mutants typically have stunted growth and development (references in Heil and Baldwin, 2002). Subsequent studies actually measured the fitness

consequences of such mutations. Constitutive activation of systemic required resistance (SAR) by the mutations *cpr1*, *cpr5*, and *cpr6* decrease seed production in *Arabidopsis thaliana* (Heidel et al., 2004). An elegant study that rigorously controlled for potential differences in genetic background unambiguously revealed the fitness costs of a particular R gene conferring resistance to the pathogen *Pseudomonas syringae* (Tian et al., 2003). Sense-expression of this R gene, *rpm1*, encoding a peripheral plasma membrane protein that functions as a receptor for the pathogen elicitors, decreased reproductive output in *A. thaliana* by 9%. However, the specific responses elicited by this pathogen recognition system which are responsible for the decrease in reproductive output are unknown.

Only a few studies have investigated the fitness consequences of mutations or transformations in the signal transduction of herbivore defense. In *Solanum tuberosum* antisense-silencing a biosynthetic gene of the jasmonate signaling cascade (*lox*) increased tuber yield (Royo et al., 1999). Surprisingly, a study that used the *jar1* mutant in *A. thaliana*, which is deficient in JA signaling and in the expression of protease inhibitors (PIs), found greater reductions in seed production after elicitation with JA in the mutant line than in the wild type (WT) lines (Cipollini, 2002). However, transgenic *A. thaliana* that overexpress carboxyl methyltransferase (*jmt*), and thereby increase MeJA production, have decreased seed production (Cipollini, 2007).

As in elicitation studies, the interpretation of signaling mutants or plants transformed to constitutively express signaling molecules is confounded by the various roles the signaling molecules play, especially with regard to controlling growth and photosynthesis. Therefore, the fitness costs of specific defenses are best determined in isogenic lines that differ in a biosynthetic gene of a given defense trait. Furthermore, if plants are transformed to over-express a gene, the chemical and also the ecological (if the gene is heterologously expressed) context in which the trait evolved are altered. Expression of a defense trait in a particular tissue or cell compartment may be essential for its defensive function as well as minimization of its fitness costs. Hence ectopic over-expression of a defense in all cells may provide spurious results. Primary metabolites and the chemical milieu are known to affect the function of secondary metabolites (Broadway and Duffey, 1988; Govenor et al., 1997; Green et al., 2001). Additionally, secondary metabolites can affect each other (Felton et al., 1989; Steppuhn and Baldwin, 2007) and in response to herbivore attack, most plants increase their concentrations of a diverse bouquet of secondary metabolites. The potentially confounding effects of this chemical diversity can be minimized by silencing endogenous genes that only influence the production of a particular defense metabolite. This approach allows the

ecological function as well as costs of a particular trait to be studied in its natural context, as has been shown for the case of inducibly produced PIs in *N. attenuata* (Zavala et al., 2004a; Zavala et al., 2004b).

To be able to attribute altered fitness or resistance to the targeted gene, the genetic consequences of the transformation or mutation procedure need to be characterized. Transgenic lines should harbor a single insertion of the transgene and be homozygous; the success of the silencing or sense-expression should be verified by measuring transcript levels of the targeted gene; and the observed phenotypes should not be due to either the effect of interrupting the genetic code at the site of insertion or the vector used for transformation. The likelihood that an insertion site is the basis of an observed fitness effect can be excluded by backcrossing (for example Tian et al., 2003), who used site-specific recombination to excise the *rpm1* transgene), or by using multiple lines of independent transformation events (Zavala et al., 2004b). Similarly, it should be verified that the phenotypes of a certain mutants is not caused by other mutations that occurred during its creation. To control for the transformation vector and the procedure, plants transformed with empty vectors should be included in the analysis.

4.2.5 Protease inhibitors – a case study

A variety of plants produce JA-elicited PIs as a direct defense. PIs are antidigestive proteins that can decrease the performance of many herbivores by suppressing gut protease activity (Birk, 2003). To determine whether *N. attenuata*'s production of trypsin PIs incurs a fitness cost, Zavala et al. (2004b) transformed two natural genotypes: in one, endogenous PI production was silenced by introducing a fragment of the *pi* gene in antisense orientation; in the other, a genotype that had a nonsense mutation in the *pi* gene, PIs were constitutively expressed. Plants deficient in PI activity grew faster and taller, flowered earlier, and produced more seed capsules (25–53%) than did the isogenic PI-producing genotypes against which they were competing. Results were similar regardless of whether PI activity was suppressed in two independent lines or restored. The difference in seed capsule production between two competing neighbors correlated with the difference in PI activity. Clearly, PI production exacts a large fitness cost when plants are grown under herbivore-free competitive conditions, which is consistent with the hypothesis that inducibility evolved as a cost-saving mechanism.

A subsequent study with the same isogenic lines, some of which were unattacked and on others of which *Manduca sexta* larvae were allowed to feed freely, demonstrated that the

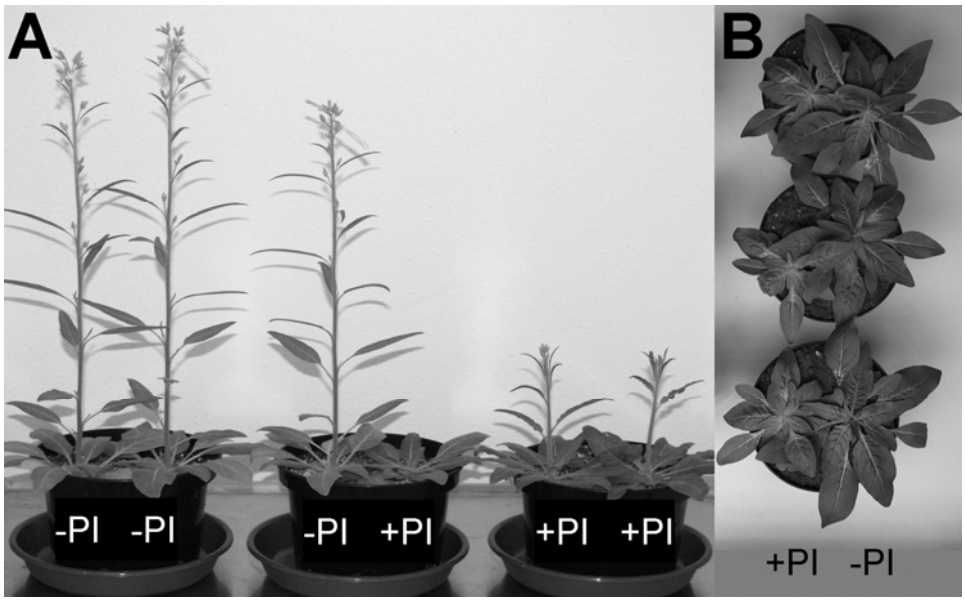

Figure 1: Competitive growth reveals the costs of protease inhibitor (PI) production in *Nicotiana attenuata*. When isogenic pairs of plants that differ only in their expression of PIs are grown in the same pots, PI-producing plants are out-competed by PI-free plants and produce significantly fewer seeds. (A) Natural genotypes harboring a mutation resulting in a premature stop codon in the PI gene; (B) isogenic genotypes transformed to silence PI production by RNAi. Photograph: (A) G. Glawe (B) H. Wünsche

PI-mediated decreases in *M. sexta* performance translate into a fitness benefit for the plant that outweighs the costs of PI production under greenhouse conditions (Zavala and Baldwin, 2004). Again, PI production of unattacked plants was negatively correlated with seed capsule production and caterpillar attack not only reduced seed capsule production but also reversed this pattern of seed capsule production among genotypes; high-PI-producing genotypes had higher fitness than those which produced few or no PIs. The use of isogenic plants that are altered in the expression of a particular defense genes enable the cost-benefit-paradigm to be examined under various natural conditions: field studies with transgenic *N. attenuata* plants silenced for genes involved in the biosynthesis of the alkaloid nicotine or in the JA cascade that elicits it revealed their function as anti-herbivore defenses in nature (Kessler et al., 2004; Steppuhn et al., 2004).

4.3 The impact of linkage

4.3.1 Types of linkage

The expression of defense traits can be linked to other traits by different mechanisms. Genes mediating resistance are linked to other genes located on the same chromosome. Even

though this linkage is disrupted by crossing over during sexual reproduction, genes that are close to each other may not segregate independently; this dependency can be quantified as linkage disequilibrium. In *A. thaliana* polymorphisms are typically independent if separated by more than fifty kilo bases, whereas below this distance linkage disequilibrium is significant (Plagnol et al., 2005). However these are only rough estimates, as a gene's location in particular chromosomal regions can dramatically influence its recombination rate. The advances in minimizing the genetic linkage to a manipulated gene has decreased many of the potential confounding effects that have plagued tests of the cost-benefit paradigm, but none of the previously described approaches excludes another confounding effect, which is the linkage of defense traits to the plant's metabolism.

Eliminating one component of the metabolic machinery is bound to cause changes in metabolite flow, which can in turn regulate metabolism through complicated feedback and feed-forward controls. The extent to which metabolism is altered depends on how the trait is regulated and how deeply it is involved in the metabolic machinery. The large extent to which the various signaling pathways that regulate resistance genes also profoundly influence metabolism is clear (Halitschke and Baldwin, 2003; Reymond et al., 2004), but changes at the end branches of metabolic pathways can also have consequences due to the accumulating precursors or side products. Hence a comparison of two plants that differ genetically only in the expression of a single resistance gene may include the consequences of significantly altered metabolisms. And these alterations may be the cause or consequence of defense costs.

In addition to genomic and metabolic linkage, functional linkage also influences the costs and benefits of defense traits. How a defense trait functions can depend on the presence or absence of other metabolites. The ecological costs of a broadly toxic direct defense that interferes with indirect defenses when tolerant herbivores co-opt the plant's resistance trait for their own defense is one example already mentioned. Moreover, the functions of different defense traits might be directly linked: synergistic interactions, for example, increase the resistance above the sum of the resistance provided by each defense alone (Berenbaum and Neal, 1985; Nelson and Kursar, 1999). By increasing the benefits more than the costs of defenses, functional linkage may be a very efficient cost-saving mechanism.

4.3.2 Metabolic linkage

The difficulties of disentangling the fitness costs of allocating resources to a specific defense trait from fitness consequences of shifts in allocation resulting from other processes

activated during elicitation have already been mentioned. Furthermore, it is not clear whether in isogenic genotypes, disabled in the expression of a specific trait, the resources that are not used for defenses are redirected to be available for growth and reproduction. The resource allocation shifts required for a defense may occur far up-stream of the portion of biosynthetic pathway committed to the actual metabolite production, especially if a defense is produced by a long, multi-step pathway. A physiologically well-studied example of such a defense is nicotine accumulation in leaves of attacked *Nicotiana* species. This accumulation involves a large spatial separation between the site of synthesis and the site of herbivore attack and a long-distance signaling system is required to bridge the gap.

The induced increases of nicotine in the shoots of *N. sylvestris* and *N. attenuata* is mediated by JA-signaling and results from increased *de novo* nicotine biosynthesis, which takes place in the roots (Baldwin et al., 1994; Baldwin et al., 1998). In both species, the elicitation of leaves with MeJA increases the root transcripts of putrescine N-methyl transferase (PMT), which is the enzyme that regulates nicotine biosynthesis (Voelckel et al., 2001; Winz and Baldwin, 2001). The signal that moves from the site of attack to the roots to induce nicotine biosynthesis is transported via the phloem (Baldwin, 1989), whereas the nicotine is transported into the shoot via the xylem (Baldwin, 1991). Pulse-chase experiments with ^{15}N revealed that nitrogen reduction and assimilation are unaffected after induction but the proportion of reduced nitrogen that is allocated to nicotine doubles (Baldwin et al., 1993; Baldwin et al., 1994). This suggests large allocation costs of nicotine production may be due to a shift in nitrogen allocation, especially because nicotine is not sufficiently catabolized to recycle its nitrogen for use in reproductive processes (Baldwin et al., 1994; Baldwin et al., 1998). Eliciting *N. attenuata* plants with MeJA applied to the roots in order to specifically stimulate nicotine synthesis reduced seed production by 43-71% in glasshouse experiments and by 17-26% in field plantations and native populations of plants that had not been attacked by herbivores (Baldwin, 1998; Baldwin et al., 1998). MeJA elicitation of roots will elicit processes in addition to those required for nicotine biosynthesis. To circumvent these confounding secondary effects of MeJA elicitation, the *pmt* gene was silenced by RNAi, and isogenic lines of *N. attenuata* were generated which accumulated less than 10% of the nicotine of WT plants. However, these lines accumulated levels of an alkaloid not detected in WT plants, anatabine, that were about a quarter of the nicotine levels in WT plants (Chintapakorn and Hamill, 2003; Steppuhn et al., 2004). Silencing PMT disrupts nicotine biosynthesis at the formation of the five-membered pyrrolidine ring. Pulse-chase experiments with deuterated nicotinic acid, the precursor of the six-membered pyridine ring of nicotine,

revealed that the excess of nicotinic acid dimerizes to form anatabine. Though nicotine biosynthesis is specifically addressed by silencing the regulatory enzyme, the elicited increase in nitrogen-containing metabolites in the nicotine biosynthetic pathway was not completely interrupted, as evidence by the accumulation of a novel side product. No evidence was found that the precursor of the five-membered ring (putrescine) accumulates. The derivatives of putrescine that are known to accumulate after MeJA elicitation (such as caffeoylputrescine) showed no changes as a consequence of PMT silencing. However, a quarter of the nitrogen normally allocated to nicotine is still allocated to an alkaloid (anatabine) even when PMT is silenced. This example underscores the importance of a careful characterization of the metabolism of lines disabled in defense production and more generally of the importance of metabolic linkage in studying allocation costs.

Metabolic linkage may also occur if biosynthetic enzymes for secondary metabolite production have an additional catalytic function in primary metabolism or if the products themselves regulate other processes. Though the production of PIs in *N. attenuata* functions defensively when they are ingested by herbivores, they may also regulate proteases in the plant (Laing and McManus, 2002). Whether the fitness costs of PI production result from resource-based allocation costs or from alternative physiological functions such as the regulation of an endogenous protease, remains to be determined (Zavala et al., 2004b). To determine whether the fitness costs of a defense are due to a resource allocation shift from growth and reproduction to secondary metabolism, the flow of fitness limiting resources into these traits warrants following. This is possible in pulse-chase experiments with isotopically labeled fitness-limiting resources. Metabolic linkage via PIs' secondary function in *N. attenuata* may explain why the lack of PIs in the natural genotype with the nonsense mutation is coupled with a lack of the predator-attracting release of cis-α-bergamotene (Glawe et al., 2003), alternatively these traits may also be functionally linked.

4.3.3 Functional linkage

Because plants produce a cocktail of chemically and functionally diverse defenses, it has long been debated whether these mixtures of secondary metabolites have adaptive value. The selective advantage of producing combinations of different allelochemicals may be a broader range of enemies against which defense is provided, an increased toxicity of mixtures, or a delayed evolution of herbivores resistant to plant defenses. Yet, the defensive functions of allelochemicals are usually examined separately, regardless of evidence for synergistic

interactions among secondary metabolites (Berenbaum and Neal, 1985; Dyer et al., 2003). Functional linkage may allow plants to shift the fitness consequences of defense metabolites towards the benefits within the cost-benefit tradeoff.

The existence of a functional linkage between *N. attenuata*'s PI production and VOC release, which interestingly share the same elicitor and are therefore coordinately expressed (Halitschke et al., 2001), can be assumed, if their defensive functions depend on each other's presence. Because PI production decreases herbivore growth rates by inhibiting insects' digestive processes (Birk, 2003), the fitness benefits of PI expression may result from extending the period during which larvae can be successfully attacked by natural enemies. The predators in *N. attenuata*'s native habitat are small predatory bugs that prey only on early instars of large lepidopteran herbivores such as *Manduca* species (Fig. 2). Hence, the defensive benefit of PI expression should be considerably enhanced when the plants can also attract a herbivore's natural enemies (Zavala et al., 2004b). However, under glasshouse conditions, PI production increases plant fitness when plants are attacked by *M. sexta* even in the absence of predators (Zavala and Baldwin, 2004), but the fitness benefits of PIs have yet to be determined in natural environments with the native herbivore community.

Herbivore attack elicits a JA-mediated and coordinated increase in both leaf PI and nicotine levels. When both of these defenses are individually silenced in *N. attenuata,* each can be shown to function effectively as defense against one of this species's major generalist herbivores, *Spodoptera exigua* (Steppuhn and Baldwin, 2007). However, silencing PI and

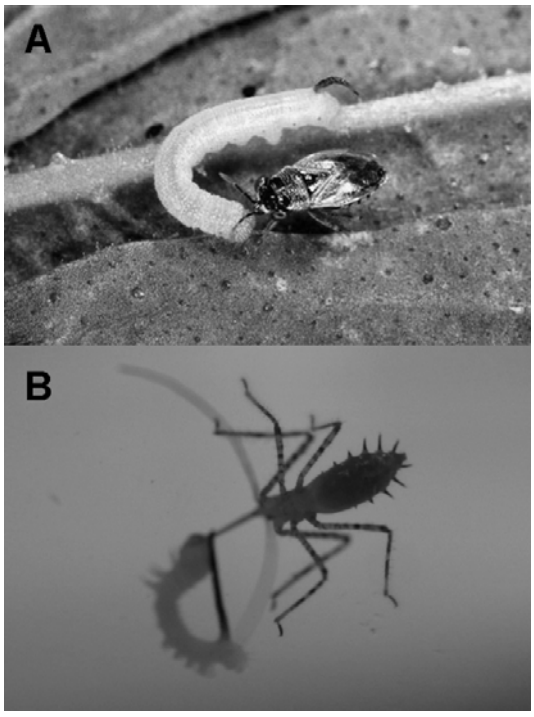

Figure 2: Native predators preying on first instar larvae of *Manduca sexta*, which increase their body mass 10,000 fold from eclosion through 5 instars to pupation. *Geocoris pallens* (**A**) is the dominant predator in *Nicotiana attenuata*'s native habitat throughout the growing season and Reduvidae nymphs (**B**) are abundant predators during flowering and seed set; by the time *M. sexta* larvae reach the third instar, both predators are substantially smaller than their prey. Induced direct defenses that delay growth and development of the herbivores likely increase the probability of predation by prolonging the period during which larvae can be successfully attacked by these small predators.

Photographs: (A) A. Kessler, (B) A. Steppuhn

nicotine simultaneously demonstrates that the defensive function of PIs is strongly dependent on the presence of nicotine: *S. exigua* larvae performed better and consumed more on PI-producing but nicotine-deficient plants than they did on plants silenced in both defenses. The reason for the synergism lies in the compensatory responses of the larvae feeding PI containing diets. Nicotine prevented *S. exigua* from overcompensating for PI's antinutritive activity by increasing consumption. Therefore, deploying both defenses provides a defensive synergism. Synergistic interactions of defenses are rarely studied but might well be the rule rather than the exception (Berenbaum, 1985; Dyer et al., 2003).

4.3.4 Dynamic view of costs and benefits of induced responses

In summary, though many theories about the ecology and evolution of plant defenses are based on tradeoffs between costs and benefits, the evidence for costs is scarce. This is due to the difficulties of separating the fitness costs of a specific defense trait from genetically, metabolically, and functionally linked traits. Methodological advances allow the genetic background and genetic linkage to be controlled for, but to elucidate the full spectrum of metabolic and ecological roles of induced defenses, detailed analyses of the transcriptional and metabolic consequences of their expression are required. Elicitation with herbivore-specific elicitors is known to result in large changes of the transcriptome (Halitschke et al., 2003) and metabolomes of plants (Giri et al., 2006). The massive change in primary metabolism (for example the down-regulation of photosynthesis) that commonly accompanies defense elicitation may be required to re-allocate resources to fuel secondary metabolism. Alternatively, this metabolic shift may itself function as a defense. The herbivore-induced response in *N. attenuata* includes a re-configuration of source-sink relationships in the plant so that recently fixed assimilates are transported to roots rather than young leaves. This shift is mediated by the rapid down-regulation of a subunit (GAL83) of a SNF1-related kinase (SnRK1) in the attacked leaves (Schwachtje et al., 2006). This carbon diversion response leads to increased root reserves, which delays senescence and prolongs flowering and seed production. The SnRK1-mediated bunkering of assimilates to the roots allows plants to better tolerate herbivory, but may also function as a "passive" defense by decreasing the nutritional value of the remaining above-ground parts to above-ground herbivores.

The simplistic view of induced defenses – that an initially constitutively expressed defense evolves to become inducible so as to minimize the defense costs when the defenses are not needed – is difficult to harmonize with the complex and flexible nature of plant

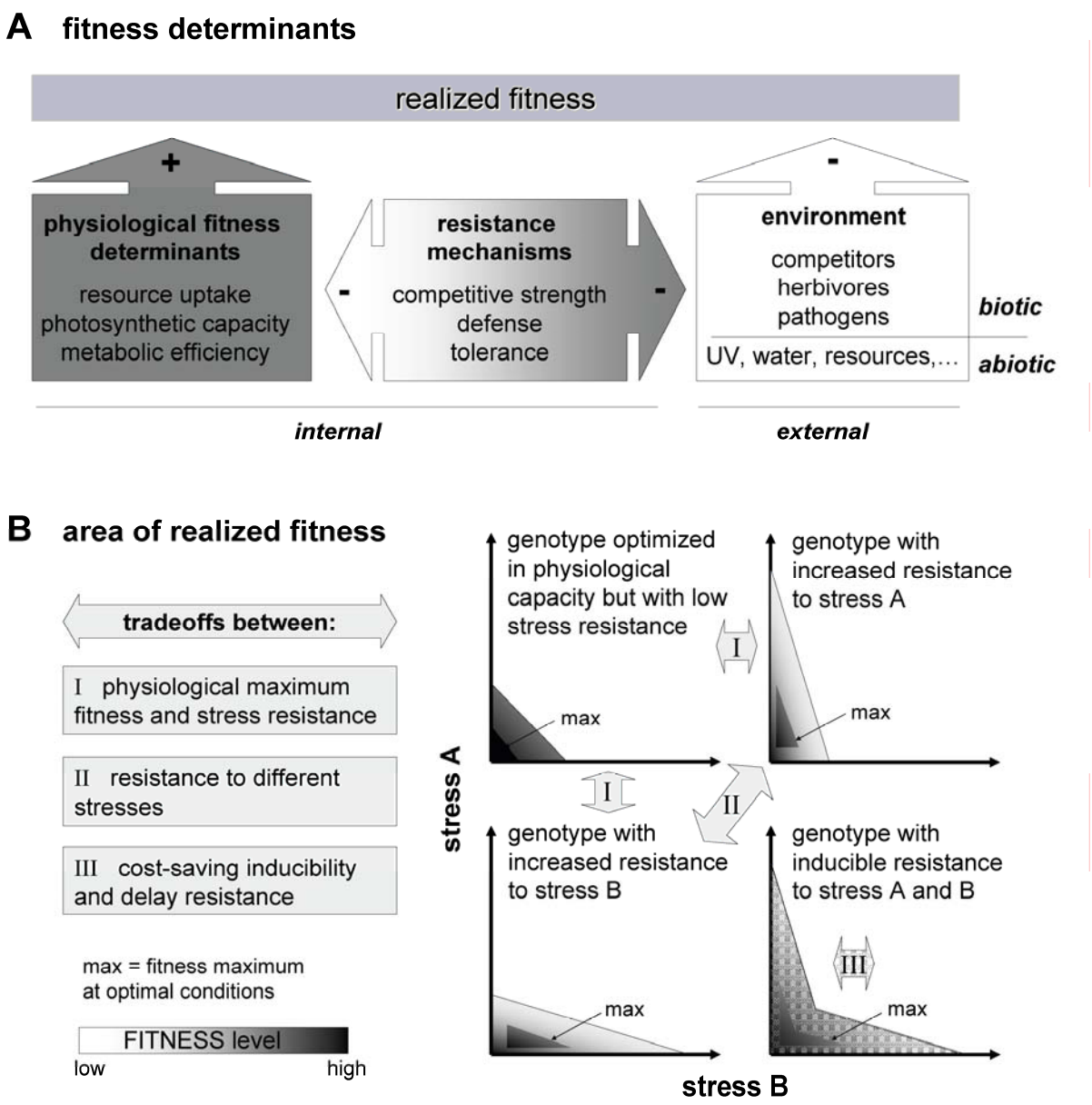

Figure 3: (**A**) Conceptual framework for how internal and external factors determine the fitness a plant can achieve. Environmental conditions vary with respect to many biotic and abiotic stresses which influence a plant's realized fitness. Resistance mechanisms activated by these stresses adjusts plant metabolism so as to optimize physiological capacities in the diversity of environments in which a plant lives. (**B**) The resistance mechanisms increase the area of realized fitness when plants grow in environments with particular stresses and alter the conditions under which maximum fitness can be achieved.. Increasing resistance to a certain stress results in different tradeoffs with respect to physiological capacities or resistance to other stresses and these costs can be minimized when resistance is deployed inducibly, which in turn, produces a cost resulting from the delay in the activation of resistance traits.

metabolism. The diverse linkages among various responses to herbivory suggests that a more dynamic view of the costs and benefits of induced defense would be more appropriate. The fitness of a plant depends on internal and external factors (Fig. 3A). The latter are the environmental conditions including resource, light, and water availability as well as biotic and abiotic stresses. The internal factors are first the physiological capacities such as resource uptake rates, photosynthetic rates, and metabolic efficiency, and second, the ability of metabolism to maintain these capacities under the environmental stresses which the plant is likely to experience. The resistance mechanisms of a plant are the means by which plant metabolism is adjusted to maximize reproductive output in the face of diverse suite of stresses that plants face. The costs of the resistance mechanisms result from the tradeoffs between maximizing physiological capacity and I) increasing the stress level and II) expanding range of conditions under which this capacity is optimized, and III) the tradeoff between the advantage of phenotypic plasticity, i.e. inducible resistance, and their drawbacks, such as the delays inherent in inducible activation (Fig. 3B). If a genotype produces more of a defense against herbivores it may be that this production results in decreased fitness under conditions at which a less defended genotype has its fitness maximum. However, small environmental changes might reverse this fitness outcome. The conditions under which the better defended genotype realizes its maximum fitness are likely altered due to metabolic and functional linkages. By deploying defenses inducibly, plants may be able to increase their fitness opportunities with respect to many stresses as the tradeoffs with other stress resistances occur only when required. The increasing evidence of crosstalk between the signaling cascades of various induced responses suggests that plants are able to "fine-tune" their metabolism to maximize fitness under various stress combinations. One example for such crosstalk is the concentration-dependent interaction between the pathogen-related salicylate (SA) and the herbivore-related JA signaling in *N. tabacum* (Mur et al., 2006). JA and SA signaling synergize each other at low concentration but at high concentrations they antagonize each other's biosynthesis and the subsequent signaling cascades. Thus, the induced reconfiguration of metabolism to increase herbivore resistance changes the plant's physiological capacities and its ability to maintain these capacities under other environmental stresses. This view of induced defenses does not presume the existence of intrinsically costly defenses, which can be calculated in a currency of resources or energy, but rather emphasizes the environmental contingency of defense costs.

4.3 Conclusions

The wide-spread acceptance of the assumption that plant defenses are intrinsically costly is more likely a reflection of our shared experience (or beliefs) that what benefits us is expensive, than the weight of the experimental evidence. Photosynthetic rate may not necessarily be the most important determinant of plant growth, and therefore the quantity of carbon diverted from growth into defense production may not be the best currency with which to measure costs, particularly when resources other than carbon are more limiting for growth. The divisions of metabolism into primary or secondary, into herbivore defense or pathogen resistance, and so forth, are useful in parsing the complexity of metabolic changes in more manageable parts, but such parsing may not be helpful for questions that can only be answered in the currency of Darwinian fitness. The various metabolic pathways are linked in complex ways so that – as in the game of pick-up-sticks – changing a specific trait invariably has ramifications throughout metabolism. Perhaps induced responses to herbivory do not automatically incur fitness cost but reduce the ability of the plant to optimize its fitness as the environment changes. Induced responses can be seen as responses that not only alter the physiological maximum fitness but also the size, shape, and position of the plant's opportunity to realize fitness in the multi-dimensional space of environmental conditions that each plant must successfully occupy during its life. This view on costs and benefits is depicted in Figure 4 showing two genotypes, one of which evolved a greater defense against herbivores. This genotype (II) is still able to reproduce under high levels of herbivory; however these benefits are constrained by a lower resistance to a pathogen, drought, and a competitor. The latter are those conditions which benefits the other genotype (I) but at a cost of reduced herbivore resistance. Yet, under many conditions both genotypes can achieve comparable fitness, underscoring the main point: defense costs are not absolute but highly environmentally contingent.

If the production of plant defenses is not intrinsically costly, cost minimization may not be the raison d'être of induced defenses. A model which does not assume that defense incurs costs is the moving-target model stating that inducibility evolved to present a changing metabolic phenotype to the plant's herbivores (Karban et al., 1997). Because nutritional variability decreases herbivore performance, inducibility may itself represent a defense strategy. Another hypothesis that does not rely on costs supposes that the adaptive value of inducibility arises from increasing the level of defense to highly toxic levels at a time when the herbivore is at its most voracious stage, thereby stimulating the herbivore to move to an

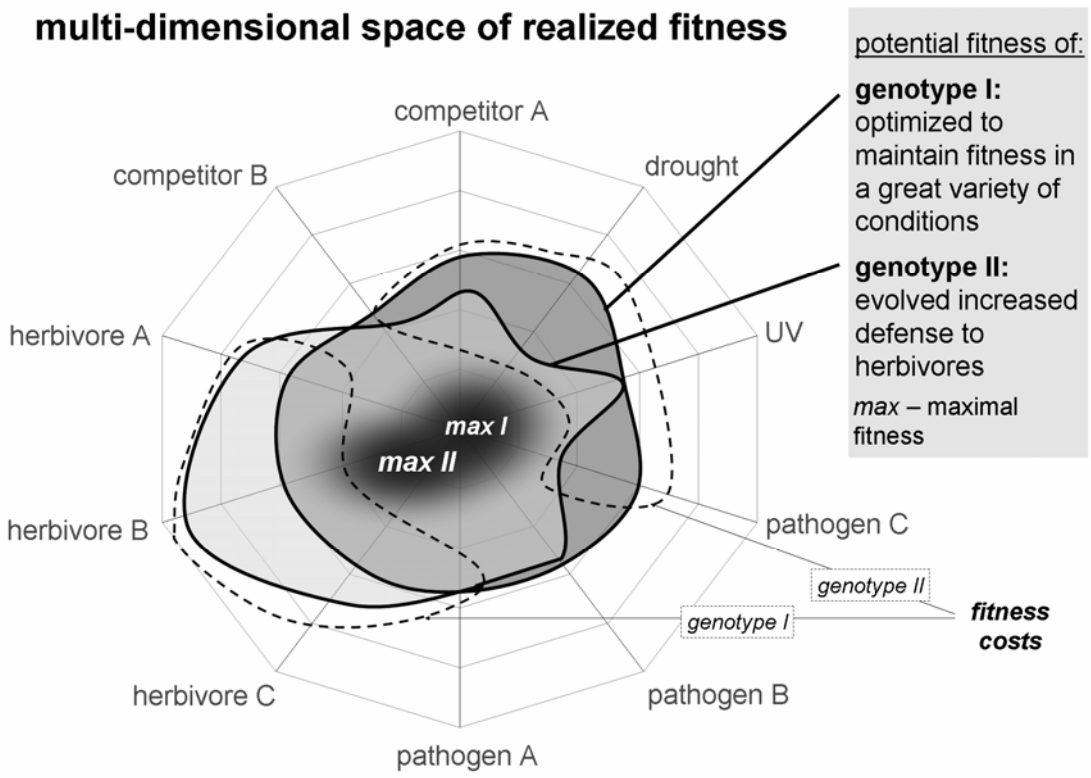

Figure 4: Model of costs and benefits of induced defense in the multi-dimensional space of environmental conditions. Within this space, each plant genotype has a limited opportunity to realize a certain fitness level. The realized fitness of a genotype with increased resistance to one or more environmental stresses can be restricted under a variety of conditions. Fitness of genotype II (light gray) with increased anti-herbivores defense is lower than that of the less-defended genotype I (dark gray) in environments with high levels of competition, drought, or attack by pathogen C (right dotted line). However in environments with high levels of herbivore attack (left dotted line), the realized fitness of genotype II is greater than that of genotype I. In intermediate stress environments, (in the centre of the overlap area) the two genotypes have similar realized fitness levels.

adjacent competitor (van Dam et al., 2001). All these hypotheses, including the cost-benefit paradigm, assume that inducibility is a derived trait and constitutive expression of defense the pleisiomorphic condition. However, defenses may have originally all been inducibly expressed, and evolved constitutive expression under particular conditions (Karban and Baldwin, 1997). If costs are not the driving force behind the evolution of induced plant defenses, they can also evolve under conditions of predictable and high herbivory, even if permanently expressed. This would be consistent with the observations that induced defense are common, despite the fact that herbivores are everywhere and their attack on plants, relentless.

On the other hand inducibility per se may be a costly trait, which is difficult to examine because such costs need to be separated from the costs of defense production and

other induced traits (Cipollini et al., 2003). The costs of maintaining the required signaling systems, which are likely co-opted from developmental processes, are generally assumed to be small. Some studies suggest that genetically inherited phenotypic plasticity for defense expression is costly (such as Agrawal et al., 2002), but the underlying mechanism remain unclear. The obvious drawback of inducibility is the delayed activation of defenses, and plants may optimize the benefits of inducibility by increasing their ability to predict attack from natural enemies. Potential mechanisms for this may be "eavesdropping" the neighboring plants for signals emitted by attacked plants, (such as MeJA or VOC), or a "memory" of previous attacks. For example, plants respond to pathogen attack with increased resistance to future pathogen attack, which is attributed to a faster and stronger response of specific defense genes. This priming uses specific signaling components and is less costly than activated induced defense although providing similar resistance (van Hulten et al., 2006). Similarly, plants that experienced herbivore attack can be vaccinated by attack from a less damaging herbivore against attack from a more damaging herbivore species (Kessler and Baldwin, 2004; Voelckel and Baldwin, 2004b). We still have much to learn about the mechanisms by which plants rapidly adjust their phenotypes to the environments in which they live.

Outlook

The empirical evidence for costs of induced defense remains underwhelming, and more comprehensive research is required to test the different concepts of costs and benefits of induced defense. Our understanding of the eliciting compounds, the signaling cascades, and the metabolic pathways involved has increased dramatically, and advances in molecular methods enable individual traits to be isolated and their consequences for an organism's Darwinian fitness to be determined. The simple model of intrinsically costly defenses does not jibe with the complexity with which plants adjust their metabolism in response to herbivore attack as well as to other environmental stresses. Hence, simply seeking evidence for negative correlations between defense level and realized fitness is not sufficient to adequately test the existence of defense costs. The analysis needs to proceed at many levels, examining the underlying metabolic mechanisms and their consequences for whole-organism performance in the range of environments that a plant likely experiences in nature. Advanced molecular and analytical tools in the areas of transcriptomics and metabolomics provide insights to the metabolic adjustments; together with manipulative approaches such as gene silencing, it is possible to examine the connections in the metabolic grid that is responsible for

the phenotype. In addition, the methodological advances will allow for refinements of hypotheses about tradeoffs occurring at a whole-organismic level. The transgenic approach of altering specific defense traits, allows their function as well as the tradeoffs among traits to be determined under different natural conditions. Quantitative genetic studies elucidate the mechanisms for the underlying evolution of induced responses (Mitchell-Olds and Schmitt, 2006). Extending the cost-benefit paradigm from a purely resource-based model to a dynamic view of tradeoffs among different metabolic and functional traits may improve the falsifiability of the hypotheses and broaden the experimental approaches to test them. The field of research that Clarence Ryan started more than three decades ago when he described increases in PIs in potato leaves when they were attacked by Colorado potato beetles (Green and Ryan, 1972) has blossomed into a field which is shaping our understanding of how metabolism is regulated and continues to hold the promise of developing crop plants that are as sophisticated as their wild ancestors in dodging environmental stresses.

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Manuscript II

Nicotine's Defensive Function in Nature

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6 Supplemental Figures (in Appendices)

1 Supplemental Protocol

Abstract

Plants produce metabolites that directly decrease herbivore performance, and as a consequence, herbivores are selected for resistance to these metabolites. To determine whether these metabolites actually function as defenses requires measuring the performance of plants that are altered only in the production of a certain metabolite. To date, the defensive value of most plant resistance traits has not been demonstrated in nature. We transformed native tobacco (*Nicotiana attenuata*) with a consensus fragment of its two *putrescine N-methyl transferase* (*pmt*) genes in either antisense or inverted-repeat (IR*pmt*) orientations. Only the latter reduced (by greater than 95%) constitutive and inducible nicotine. With D_4-nicotinic acid (NA), we demonstrate that silencing *pmt* inhibits nicotine production, while the excess NA dimerizes to form anatabine. Larvae of the nicotine-adapted herbivore *Manduca sexta* (tobacco hornworm) grew faster and, like the beetle *Diabrotica undecimpunctata*, preferred IR*pmt* plants in choice tests. When planted in their native habitat, IR*pmt* plants were attacked more frequently and, compared to wild-type plants, lost 3-fold more leaf area from a variety of native herbivores, of which the beet armyworm, *Spodoptera exigua*, and *Trimerotropis* spp. grasshoppers caused the most damage. These results provide strong evidence that nicotine functions as an efficient defense in nature and highlights the value of transgenic techniques for ecological research.

Abbreviations: IR*pmt*, inverted-repeat *putrescine N-methyl transferase*; MeJA, jasmonic acid methyl ester; NA, nicotinic acid; *pdk*, *pyruvate orthophosphate dikinase*; *PMT*, putrescine N-methyl transferase; SE, standard error; T-DNA, transferred DNA; TPI, trypsin protease inhibitor; WT, wild-type

Introduction

Plants produce many secondary metabolites, of which some are thought to function as direct defenses against pathogens and herbivores by reducing their performance, survival, and reproduction. Numerous plant allelochemicals with antiherbivore properties are classified according to their mode of action (e.g., toxins, antifeedants, antidigestive proteins, etc.) (Bennett and Wallsgrove 1994) and have been used in agriculture to control insect pests (Hedin 1991). The fact that a secondary metabolite reduces herbivore performance does not by itself demonstrate that the endogenously expressed metabolite functions defensively in the plant's natural environment (Bell 1987), because the evolutionary interaction between

herbivores and their host plants may have reduced the defensive efficacy of the metabolite. Phytophagous insects have evolved various strategies to cope with allelochemicals (Karban and Agrawal 2002) and tend to tolerate, or even co-opt, plant defenses for their own defenses (Wink and Theile 2002).

Pharmacological studies demonstrating a resistance effect of metabolites applied to plants or artificial diets (Yamamoto et al. 1968; Bowers and Puttick 1988), and studies using heterologously expressed genes in agricultural systems (Carozzi and Koziel 1997; Hilder and Boulter 1999), represent a first step in evaluating the defensive function of a secondary metabolite. The interpretation of these studies is confounded by both the altered ecological context in which the resistance is measured and the altered chemical milieu, which is also known to influence the defensive function of a metabolite. Stronger evidence for resistance effects of allelochemicals arises from studies establishing correlations between plant resistance against herbivores and the genetically variable accumulation of secondary metabolites (Berenbaum et al. 1986; Shonle and Bergelson 2000) or from studies demonstrating the defensive role played by a suite of elicited metabolites (Orozco-Cardenas et al. 1993; Baldwin 1998; Halitschke and Baldwin 2003). Ideally, the benefits of a putative defense trait should be determined in plants differing only in a single gene that controls the expression of a resistance trait and are otherwise identical (Bergelson and Purrington 1996). To date, studies measuring resistance of ''near isogenic'' lines with altered metabolite accumulations (Jackson et al. 2002) provide the strongest evidence for their resistance, but these lines, which are created by repetitive backcrossing, are likely to differ in many loci linked to the target locus, which may also affect resistance. Such problems of genetic linkage have been overcome through the use of genetic transformation to explore the fitness effects of herbicide resistance (Bergelson et al. 1996; Purrington and Bergelson 1997) and pathogen resistance (Tian et al. 2003) in field populations of *Arabidopsis*. In this study, we use transgenic silencing to alter a single putative resistance trait—the production of nicotine—and thereby establish its contribution to plant resistance in the field.

The pyridine alkaloid nicotine is one of the best-studied putative plant resistance traits. Because it can interact with the acetylcholine receptors in the nervous systems of animals, nicotine is extremely toxic to most herbivores and, consequently, was one of the first insecticides used to control pests in agriculture (Schmeltz 1971). Evidence for the resistance value of nicotine arises from the agricultural practice of using nicotine sprays and genotypes of cultivated tobacco differing in nicotine levels (Jackson et al. 2002). Although nicotine is widely toxic, insects adapted to nicotine-producing plants have evolved resistance to this

alkaloid (Glendinning 2002). The tobacco specialist *Manduca sexta* (tobacco hornworm) tolerates doses of nicotine that are fatal to unadapted herbivores but grows more slowly on high-nicotine diets (Appel and Martin 1992; Wink and Theile 2002). Other studies suggest that *M. sexta* might even be better defended by dietary nicotine against its parasitoid, *Cotesia congregata*, which suffers higher mortality when parasitizing larvae fed on high- rather than low-nicotine diets (Barbosa et al. 1986; Thorpe and Barbosa 1986). Thus, the coevolutionary arms race between nicotine-producing plants and their adapted herbivores may have reduced the defensive value of nicotine.

In the native tobacco species *Nicotiana attenuata* and *N. sylvestris*, nicotine is the most abundant alkaloid. Elicitation of *N. attenuata* with jasmonic acid methyl ester (MeJA) in its native habitat increases nicotine content, which is correlated with enhanced plant fitness when plants are attacked (Baldwin 1998). However, herbivore attack and MeJA elicitation (as well as the plant's endogenous jasmonic acid cascade [Halitschke and Baldwin 2003]) regulate many resistance traits, including trypsin protease inhibitors (TPIs), diterpene glycosides, and volatile emissions involved in indirect defense. Hence, nicotine is only one of a suite of putative defense traits elicited by herbivore attack, and its specific role remains to be determined.

In laboratory trials, resistance benefits of nicotine production against *M. sexta* larvae were established using transgenic *N. sylvestris* plants silenced in their nicotine biosynthesis by antisense expression of *putrescine N-methyl transferase* (*pmt*). Plant consumption and the performance of *M. sexta* larvae were negatively correlated with constitutive nicotine levels in laboratory feeding trials (Voelckel et al. 2001); whether this result applies to plants in their natural habitat is unclear. To examine the resistance effect of nicotine, we transformed *N. attenuata* with inverted-repeat *pmt* (IR*pmt*) and antisense *pmt* constructs and found that only IR*pmt* plants had strongly reduced nicotine content. We characterized the defense and growth phenotypes of two independently transformed homozygous IR*pmt* lines and found that measured direct and indirect defenses did not differ from those of the wild-type (WT) plants, except for a dramatic reduction (greater than 95%) of MeJA-elicited and constitutive nicotine production and an increase in anatabine content. In pulsechase experiments with D_4-nicotinic acid (NA) ethyl ester, we demonstrated that the increased anatabine likely results from a dimerization of the NA that would normally have been used in nicotine biosynthesis. In feeding trials, *M. sexta* larvae preferred and grew faster on IR*pmt* than WT leaves. We transplanted WT and IR*pmt* plants into *N. attenuata*'s native habitat in southwestern Utah and elicited a subset with MeJA. Several naturally occurring herbivore species attacked and

damaged unelicited IR*pmt* plants more than unelicited or elicited WT and elicited IR*pmt* plants. These results demonstrate that nicotine functions as an effective resistance trait under natural conditions.

Results/Discussion

IR*pmt* Constructs Silence Nicotine Production

Nicotine accumulation was not reduced in most of the independent lines transformed with antisense *pmt* constructs (25 lines of pNATPMT1 and six lines of pCAMPMT1) compared to WT (Figure 1A). None of the five lines with lower nicotine accumulation in the T1 screen had nicotine levels lower than those of WT in the homozygous T2 generation. In contrast, 29 of 34 independently transformed lines with the IR*pmt* construct pRESC5*PMT* had dramatically reduced constitutive and MeJA-induced nicotine accumulations (Figure 1B). The suppression of nicotine accumulation was stable during plant development and when plants were grown in the glasshouse or in the field in Utah. Clearly, inverted-repeat constructs are more efficient at silencing the expression of endogenous genes, as has been previously described (Wesley et al. 2001).

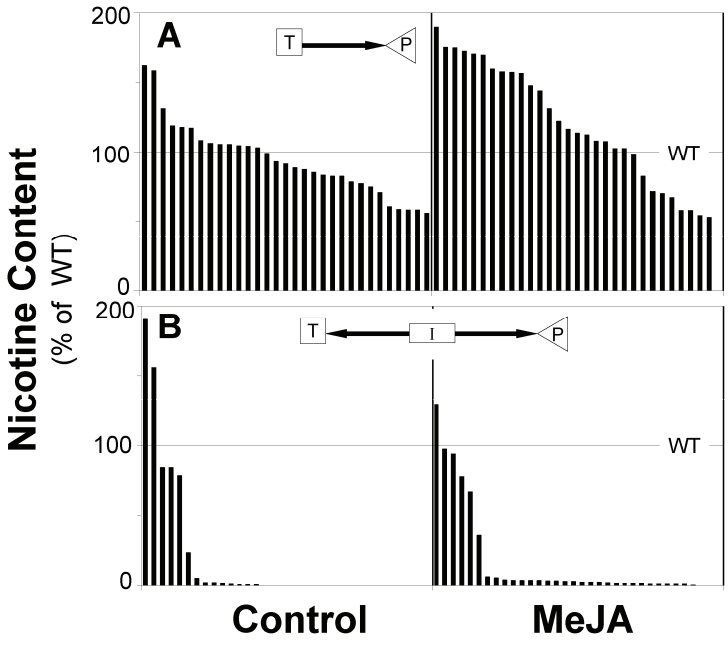

Figure 1. Comparison of Antisense and Inverted-Repeat Silencing of *pmt*

Nicotine content (mean of 5–6 plants/line) normalized to mean of WT of unelicited (control) *N. attenuata* plants and plants 5 d after elicitation with 150 μg of MeJA per plant from independent lines transformed with (A) antisense *pmt* constructs and (B) an IR*pmt* construct. In contrast to the 31 lines transformed with the antisense *pmt* construct, 29 of the 34 IR*pmt* lines had dramatically reduced constitutive and MeJA-induced nicotine levels.

T, terminator; P, promoter; I, spliceable intron; arrow, 950-bp consensus fragment of pmt1 and pmt2. For details of transformation constructs see Protocol S1.

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Genomic and Transcriptional Characterization

Two homozygous T2 IR*pmt* lines (108 and 145) with reduced nicotine levels were further characterized. Southern blot analysis using a probe hybridizing to the selective marker in the IR*pmt* construct demonstrated that both lines contained a single insertion (Figure S1). Transformation with a pRESC transformation vector allowed the transferred DNA (T-DNA) and flanking DNA at the insertion site to be recovered from the plant genomic DNA. These experiments demonstrated that the T-DNA integrated into the *N. attenuata* genome at a single site in each line, since all sequenced clones from a line (108, $n = 4$; 145, $n = 5$) contained the same flanking sequence (see Figure S1 and Protocol S1).

Transcripts of the *pmt* genes in the two lines were significantly reduced to approximately 10% of the constitutive and MeJA-induced WT mRNA levels (Figure 2A), demonstrating that the targeted genes were successfully silenced.

Metabolic Consequences of *pmt* Silencing in *N. attenuata*

Consistent with the observed silencing of *pmt* transcripts, the constitutive and induced nicotine levels in transformed plants of both lines were dramatically reduced to 3%–4% of the levels found in WT plants (Figure 2B). All 29 IR*pmt* lines with reduced nicotine levels accumulated the alkaloid anatabine, which was not detected in WT plants. Constitutive and MeJA-induced total (nicotine, anabasine, and anatabine) alkaloid contents of the two IR*pmt* lines were about one-half and one-third of the WT levels, respectively, of which anatabine comprised 30% and 23% (Figure 2C). Levels of anabasine representing 20% of the constitutive and 8% of the MeJA-elicited total alkaloid contents in WT plants were unchanged in IR*pmt* plants (Figure S2). Elevated anatabine levels were also found in recently published studies with antisense *pmt* transformation of *N. tabacum*; elevated anatabine levels did not affect transcript levels of other genes encoding enzymes involved in alkaloid metabolism (Chintapakorn and Hamill 2003).

Anatabine consists of a pyridine and a piperideine ring. Both are likely derived from NA, which is also the precursor of the pyridine ring of nicotine (Leete and Slattery 1976). Disrupting nicotine biosynthesis at the formation of the pyrrolidine ring by silencing PMT activity might cause an oversupply of the NA used in the biosynthesis of anatabine. Feeding the roots of hydroponically grown MeJA-elicited WT plants with NA ethyl ester resulted in formation of anatabine at levels of about a third of the total alkaloids (nicotine and anatabine) (Figure 3); in the IR*pmt* lines, anatabine constitutes 98% of the total alkaloids. Feeding plants

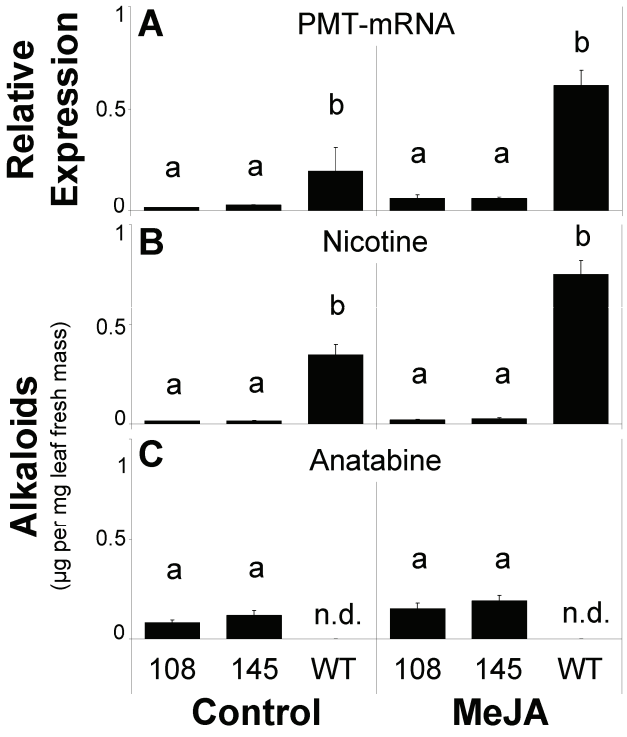

Figure 2. PMT Transcript and Alkaloid Levels of IR*pmt* Lines

Mean (± SE) relative PMT mRNA transcript levels in the roots (A), and leaf levels of (B) nicotine and (C) anatabine, in two independent lines of IR*pmt*-transformed (108 and 145) and WT *N. attenuata* plants. Elicited (150 µg of MeJA) and unelicited (control) plants were harvested at 10 h for transcript (A) and at 4 d for alkaloid (B and C) quantification. Both IRpmt lines had significantly reduced PMT transcript and nicotine but featured anatabine not present in WT plants. Lowercase letters signify differences at $p < 0.01$, Bonferroni corrected ([A] $n = 3$, ANOVA: $F_{2,12} = 12.55$; [B] $n = 8–10$, ANOVA: $F_{2,50} = 135.4$; [C] $n = 8–10$, ANOVA: $F_{2,50} = 39.611$]. n.d., not detected.

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with D_4-NA ethyl ester results in the formation not only of D_4-nicotine and D_4-anatabine but also of D_8-anatabine, demonstrating that the last integrates two D_4-NA units. When these experiments are conducted with WT plants, about half of the anatabine is labeled, suggesting that the unlabeled half was formed from endogenous unlabeled NA. In addition, about one-fourth of the WT nicotine was D_4-nicotine. In IR*pmt* plants, in contrast, only traces of D_4-nicotine were found, but one-third of the anatabine was either D_4- or D_8-labeled. In summary, exogenously supplied NA is taken up by the roots of *N. attenuata* plants and used in alkaloid biosynthesis, and an oversupply of NA results in the formation of anatabine. These results support the hypothesis that the silencing of *pmt* disrupts nicotine biosynthesis, causing an oversupply of NA and the subsequent formation of anatabine.

IR*pmt* plants did not differ from WT plants in any other measured secondary metabolite or growth parameter. Constitutive or MeJA-induced levels of caffeoylputrescine, chlorogenic aid, rutin (Figure S2), TPI activity, or the release of cis-a-bergamotene (Figure S3) in IR*pmt*-transformed plants did not differ from those of WT plants. Rosette-stage and elongation-stage growth in individual pots in both the glasshouse and the field (Figure S4) did not differ between WT and IR*pmt* lines, and transformed lines were not visually or morphologically distinguishable from WT plants. Hence, the IR*pmt* plants represent an ideal construct with which to examine the ecological consequences of nicotine production.

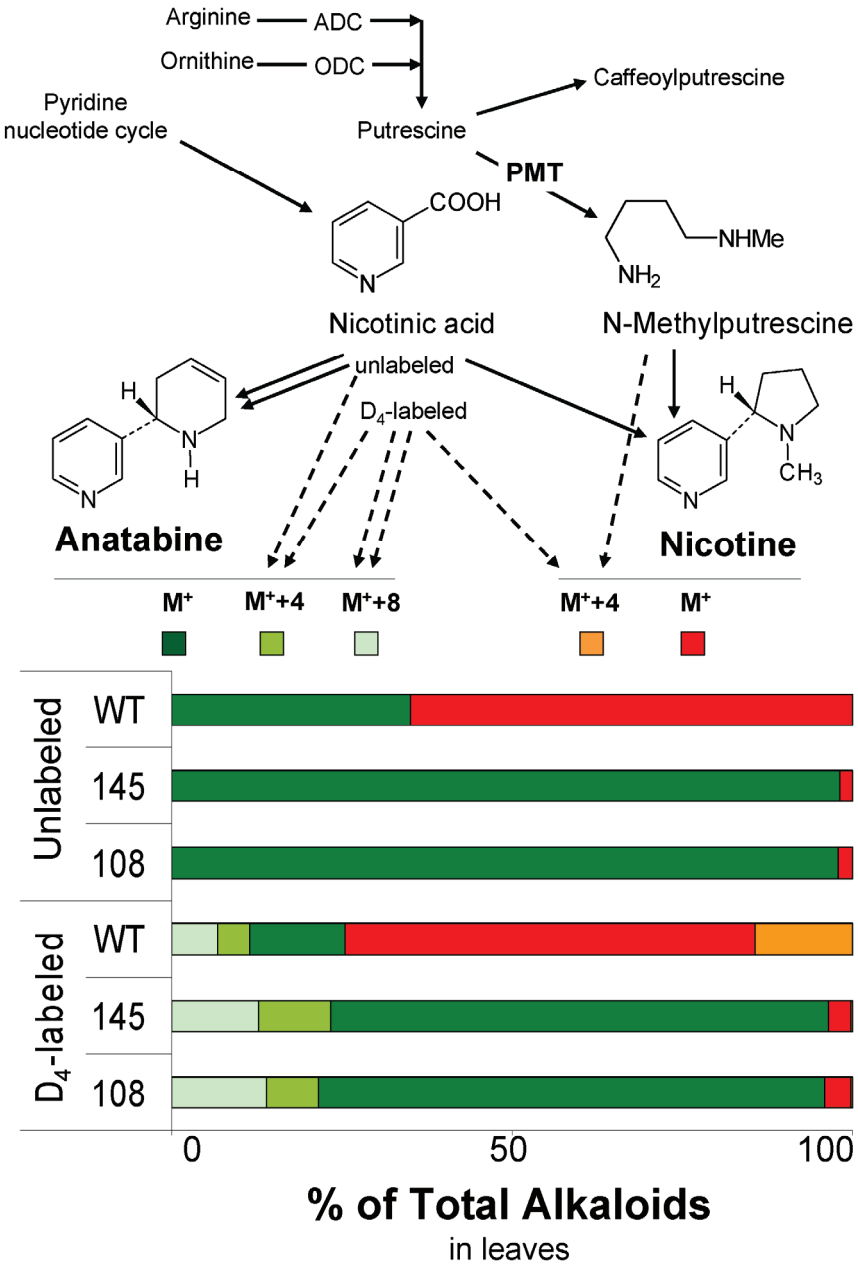

Figure 3. Alkaloid Biosynthesis and the Consequences of a NA Oversupply

Biosynthesis scheme and proportion of unlabeled (M^+) and labeled (M^++4, M^++8) nicotine and anatabine in the leaves of two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) and WT plants 5 d after elicitation with 150 µg of MeJA per plant. Plants were grown in hydroponic solutions and supplied with either unlabeled or D_4-ring-labeled NA ethyl ester (1 mM) 24 h after elicitation (n = 3 or 4). The oversupply of NA resulted in the formation of anatabine even in WT plants from both labeled exogenous and unlabeled endogenous NA pools.

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Effects of Nicotine Silencing on *N. attenuata* Herbivores

M. sexta larvae reared on IR*pmt* plants in the glasshouse gained significantly more mass and changed instars faster than larvae reared on WT plants (n = 17–20; ANOVA: $p < 0.01$, $p_{\text{WT-}PMT108} < 0.02$, $p_{\text{WT-}PMT145} < 0.01$). The differences were comparable to those observed for *M. sexta* larvae reared on nicotine-enriched artificial diets (Parr and Thurston 1972; Appel and Martin 1992) or on nicotine-enhanced WT (Baldwin 1988) or antisense-*pmt*-transformed *N. sylvestris* plants (Voelckel et al. 2001). Two-thirds of freshly eclosed *M. sexta* larvae, given the choice between leaf material from WT or IR*pmt* (108) plants, preferred to initiate feeding on the latter (n = 43; $\text{Chi}^2 = 6.7$, $p < 0.01$). Such behavior suggests that nicotine plays an important role in determining feeding sites of *M. sexta* larvae, as has been suggested in a study with cultivated tobacco (Kester et al. 2002). While the relative toxic effects of anatabine and nicotine remain unstudied, these results are likely to underestimate the influence of nicotine on *M. sexta* choice and performance, because IR*pmt* plants had enhanced levels of anatabine.

Since secondary metabolism is known to be sensitive to environmental parameters that differ between glasshouse and field conditions (e.g., UV-B influence; Caldwell et al. 1983), nicotine, anatabine, and TPI levels of WT and IR*pmt* plants grown in the field plantation were analyzed: they were found not to differ from plants grown under laboratory conditions (Figure 4A). A *M. sexta* feeding choice test evaluating the larvae's choice between field-grown WT and IR*pmt* plants (n = 57; $\text{Chi}^2 = 7.74$, $p < 0.01$) verified the results described above for the same experiment conducted with glasshouse grown plants. Thus, the phenotype of glasshouse-grown IR*pmt* plants was not altered by growth under field conditions. In addition, choice tests with field-collected *D. undecimpunctata*, which was observed colonizing only IR*pmt* plants in the field plantation, revealed that 77% of these beetles preferred the nicotine-deficient IR*pmt* leaf material over WT (n = 35; $\text{Chi}^2 = 10.31$, $p < 0.001$). Another beetle species observed occasionally on WT plants, *Trichobarus mucorea*, does not distinguish between WT and IR*pmt* leaf material in choice tests (n = 19; $\text{Chi}^2 = 0.05$, $p = 0.8$).

In the field plantation, IR*pmt* plants lost significantly more leaf area to herbivores than did WT plants (Figure 4B), demonstrating that nicotine indeed functions as a direct resistance trait of *N. attenuata* in its native habitat. Over a period of 16 d, IR*pmt* plants exposed to naturally occurring herbivores lost 16% of their total leaf area to herbivores, an amount that is more than double the amount of damage incurred by WT plants. In order to meet compliance requirements described in the Code of Federal Regulations (7CFR340.3c) for the introduction

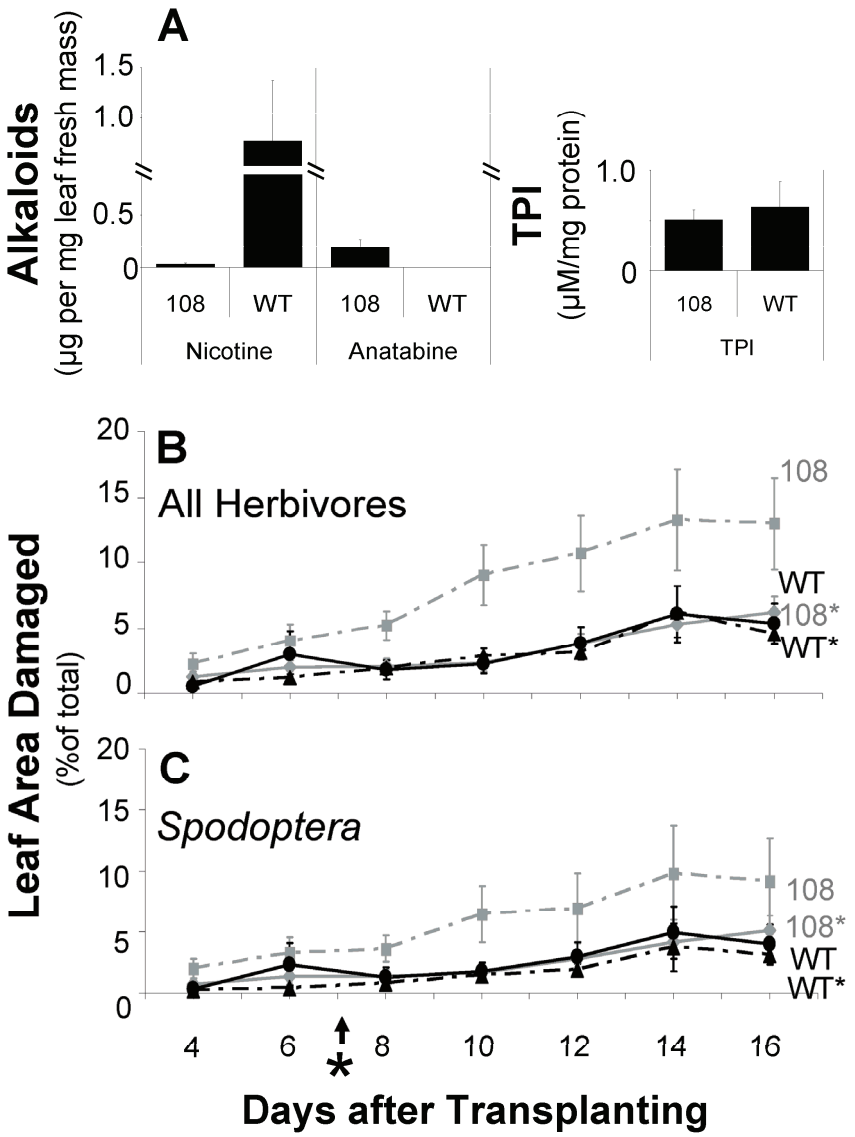

Figure 4. Herbivore Damage to IR*pmt* and WT *N. attenuata* Plants in Nature

(A) Leaf alkaloids (nicotine and anatabine) and TPIs 7 wk after transplantation ($n = 6$). Mean (± SE) percentage total leaf area damaged by (B) all herbivores and (C) only by *Spodoptera exigua* on WT *N. attenuata* plants and plants transformed with an IR*pmt* construct (108) that were either untreated (dotted lines) or elicited (solid lines; asterisk) with MeJA 7 d after plants were transplanted into a field plot in a native habitat. Differences between 108 and WT, 108*, and WT* are significant at $p < 0.05$ ($n_{PMT} = 36$, $n_{WT} = 50$, $n_{PMT}* = 28$, $n_{WT}* = 27$; [B] ANOVA: $F_{3,822} = 5.73$, $p = 0.001$; [C] ANOVA: $F_{3,822} = 4.6$, $p = 0.004$). Plants of the nicotine-deficient transformed line 108 suffered significantly higher leaf area damage than did WT plants, but when line 108 was elicited, leaf damage by all herbivores was reduced to WT levels.

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of organisms altered through genetic engineering, flowers were removed as they matured, and therefore we could not directly measure the fitness consequences of this greater herbivore load. However, in other experiments with *N. attenuata* plants grown in natural populations, leaf area damage is negatively correlated with capsule number (Baldwin 1998; Kessler and Baldwin 2004), suggesting that the strongly enhanced herbivore damage of the nicotine-deficient IR*pmt* plants translates into a fitness loss.

IR*pmt* plants were attacked by a variety of insect herbivores. About half of the total herbivore damage resulted from *S. exigua* feeding (Figure 4C). One-third of the total herbivore damage was damage from grasshoppers of the genus *Trimerotropis*, which followed the same general pattern of distribution as *S. exigua* damage, but the differences between unelicited IR*pmt* and WT plants were not significant. The damage caused by *Epetrix hirtipennis* was variable but significantly higher for unelicited IR*pmt* compared to WT plants (ANOVA: $F = 2.81$, $df = 3$, $p = 0.04$, $p_{PMT\text{-}WT} < 0.05$).

MeJA elicitation significantly reduced the damage of IR*pmt* plants to levels found on WT plants, suggesting that MeJA treatment elicits defense traits that are as efficient as the constitutive levels of nicotine in protecting plants. MeJA elicitation of *N. attenuata* plants is known to induce a diverse suite of transcriptional responses and secondary metabolites including TPIs, phenolics, flavonoids, phenolic putrescine conjugates, diterpene sugar esters, and volatile organic compounds (Halitschke and Baldwin 2003; Roda and Baldwin 2003), some of which apparently function as resistance traits. Which component of this complex suite of elicited metabolites is as effective as nicotine remains to be determined. It should be noted that the overall amounts of leaf area lost to herbivores was relatively low during the field experiments. Only 5% of the canopy area was lost from control and MeJA-elicited WT plants. In previous experiments (Baldwin 1998), fitness differences were observed between control and MeJA-elicited WT plants in populations that had lost approximately 40% of their canopy area to herbivores.

Altogether, these results provide direct evidence for the defensive value of nicotine. In a field trial, we established that a native tobacco, which produces large amounts of nicotine, is better defended against its natural herbivores than are nicotine-deficient transformants of the same genetic background. This is likely mediated by the reduction of herbivore performance and by the fact that these phytophagous insects prefer low-nicotine diets. In contrast to studies demonstrating genetic correlations between the production of secondary metabolites and herbivore resistance (Berenbaum et al. 1986; Shonle and Bergelson 2000), the resistance effects established in this study can be directly attributed to the altered traits. The fact that the

silencing of one enzyme in the nicotine biosynthetic pathway redirects metabolite flux, resulting in the accumulation of an apparently less toxic alkaloid, anatabine, underscores the importance of characterizing single-gene transformants for secondary effects.

Conclusion

Plant secondary metabolites are widely accepted as essential components of a plant's direct defenses against its natural enemies, but unambiguous proof has been lacking, mainly because of the difficulty of altering the expression of single traits in plants and testing the consequences of these manipulations under natural conditions. Transformation technology has provided biologists with the ability to manipulate and study the ecological consequences of single-gene manipulations. To date, the technology has largely been used for the heterologous expression of resistance genes (e.g., *Bacillus thuringiensis* d-endotoxin) in agricultural systems (see Tian et al. [2003] for an elegant exception), and therefore has provided little evidence for the defensive value of endogenously expressed traits against a plant's native herbivore community. The scientific value of transgenically silencing endogenous genes in native plants to understand the ecological function of particular genes has been undermined by the polarized attitudes towards the use of genetically modified organisms in agriculture. Transgenic down-regulation of nicotine demonstrates that *N. attenuata* is under relentless herbivore pressure. Disabling this resistance trait, even in a year of low herbivore abundance, results in a large increase in opportunistic herbivory and supports the conclusion that secondary metabolites play an important role in explaining why the earth is largely green (Hairston et al. 1960).

Materials and Methods

Plant material and transformation. *N. attenuata* Torr. ex Watson (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae) grown from field-collected seeds (Baldwin 1998) and inbred 11 or 14 generations were used for transformation and all experiments. Seed germination and the *Agrobacterium tumifaciens* (strain LBA 4404)–mediated transformation procedure are described in Krügel et al. (2002). In order to silence the expression of the two *N. attenuata pmt* genes, plants were transformed with pCAMPMT1 and pNATPMT1 vectors, which contain a gene fragment of *pmt*1 (which has 95% identity to *pmt*2) in an antisense orientation, and pRESC5PMT, which contains the *pmt* gene fragment twice in an inverted

orientation separated by intron 3 of the *Flaveria trinervia* gene pyruvate *orthophosphate dikinase* (*pdk*) (for vector construction and plasmids see Figure S5 and Protocol S1). T1 plants were screened for hygromycin resistance (hygromycin phosphotransferase II gene of the vector pCAMBIA-1301) and constitutive and induced nicotine accumulation; homozygosity was determined by resistance screening of the T_2 plants. Two independently transformed homozygous IR*pmt* lines (108 and 145) were further characterized by Southern blot analysis and by the rescuing of the transformation vector from genomic DNA into *Escherichia coli* to identify copy number and insertion site of the TDNA (see Figure S1 and Protocol S1).

PMT mRNA accumulation and secondary metabolites. Transformed lines (108 and 145) and WT plants were grown in 1-l hydroponic vessels in a climate chamber as described in Hermsmeier et al. (2001), and 4-wk-old rosette-stage plants were treated (elicited) on the first two fully expanded (source) leaves with 150 µg of MeJA per plant applied in 20 µl of lanolin paste, or left untreated. Approximately 200 mg of young roots was harvested and frozen in liquid nitrogen 10 h after elicitation, and RNA was extracted with Tri Reagent (Sigma, Taufkirchen, Germany) according to the manufacturer's instructions (n = 3/line/treatment). PMT transcript accumulation was analyzed by real-time PCR (ABI PRISM 7000; Applied Biosystems, Darmstadt, Germany). cDNA was generated from 20 ng of RNA with MultiScribe reverse transcriptase (Applied Biosystems), and amplified using the qPCR core reagent kit (Eurogentec, Searing, Belgium) and a probe and primers that were gene-specific (for sequences see Figure S6). For analysis of secondary metabolites, leaves growing one node above the sink-source transition leaf and young root tissue were harvested 4 d after elicitation (n = 8–10/line/ treatment). Samples were analyzed by HPLC for alkaloids, caffeoylputrescine, chlorogenic acid, and rutin (Keinänen et al. 2001; Halitschke and Baldwin 2003). A peak occurring in IR*pmt* alkaloid extracts but not in extracts of WT *N. attenuata* was collected and identified by nuclear magnetic resonance imaging as anatabine (for spectra and method, see Protocol S1).

To determine whether a NA oversupply was responsible for the formation of anatabine in the transformed lines, we supplied 4-wk-old plants with either unlabeled or D4-NA ethyl ester (1 mM) in their hydroponic solution 24 h after MeJA elicitation (n=4/line/treatment). After 4 d, the treated leaf was harvested and extracted as above, but analyzed by LC/MS/MS to detect incorporation of the deuterium into nicotine and anatabine (for instrument conditions, see Protocol S1).

To examine the release of cis-a-bergamotene in the transformed lines compared to WT, volatiles from hydroponically grown plants (n = 3–5/line/treatment) enclosed in open-top

volatile collection chambers were collected for an 8 h period starting 24 h after MeJA elicitation of the first two source leaves, and analyzed by GC/MS (Halitschke et al. 2000). TPI activity in the MeJA-treated leaf 3 d after elicitation was analyzed in plants (n = 5/line/treatment) by radial diffusion activity assay (van Dam et al. 2001).

***M. sexta* performance and feeding choice.** In the glasshouse, 2-wkold seedlings were planted individually into 2-l pots with potting soil (C 410; Stender, Schermbeck, Germany) at 26–28 8C under 16-h supplemental light from Philips Sun-T Agro 400- or 600-W Na lights. For analysis of performance, newly eclosed *M. sexta* larvae (North Carolina State University, Raleigh, North Carolina, United States) were placed on the first-stem leaf of 8-wk-old WT and IR*pmt* (108 and 145) plants and allowed to feed for 14 d. Larval mass was recorded at 8, 10, 12, and 14 d.

The first feeding choice of *M. sexta* was determined by placing newly eclosed larvae in the center of a 3-cm–diameter cup containing, on opposite sides, 1.5-cm2 WT and IR*pmt* (108) leaf pieces and recording the leaf on which larvae started feeding (n = 44).

Resistance of WT and IR*pmt* plants to herbivores in the natural habitat. In a field plantation (15 m318 m; GPS: lat 3780894599N, long 11480191299) in *N. attenuata*'s natural habitat in southwest Utah, transformed IR*pmt* (108) and WT plants were exposed to naturally occurring herbivores dispersing from adjacent populations. To allow for spatial heterogeneity, plants were transplanted in a paired design (with 0.3 m and 1.5 m between plants of a pair and between pairs, respectively) in which plants were matched for equal rosette diameters. Plants were grown in soil (Potting Mix; Miracle-Gro, Marysville, Ohio, United States) for 5 wk after germination (Krügel et al. 2002), and were transplanted into the field plot (10 columns by 15 lines) in their 3.8-l pots. Seven days after transplantation, 30 WT and IR*pmt* plants were elicited with 150 μg of MeJA per plant applied in 20 μl of lanolin paste to the two youngest rosette leaves. Starting 4 d after transplantation, each plant was examined for damage and insects (including predators and eggs) every other day for 14 d. Damage amount was estimated as a percentage of the total leaf area, and the characteristic damage caused by caterpillars, beetles, grasshoppers, myrids, and leafhoppers was noted separately. The most abundant herbivores observed in the field plantation during the release were *S. exigua*, *Trimerotropis* spp., *E. hirtipennis*, and *D. undecimpunctata*. *M. sexta* and *M. quinquemaculata* occurred in the season only rarely, and no eggs were laid in the plantation during the 14 d. As plants began to elongate and produce flowers, they were examined daily, and all flowers were removed before opening and anthesis to meet the performance standards

in the Code of Federal Regulations (7CFR340.3c). Consequently, direct fitness measures were unobtainable in this experiment.

For analysis of alkaloids and TPIs under field conditions, leaf samples of WT and IR*pmt* plants in the plot (n = 6) were taken 7 wk after transplantation and frozen (dry ice). To determine if the herbivore phenotype of IR*pmt* plants observed in glasshouse-grown plants was retained in plants grown under natural light conditions, the *M. sexta* choice experiment was repeated. The first feeding choices of freshly eclosed *M. sexta* larvae (North Carolina State University) and of adults of field-collected *D. undecimpunctata* and *Trichobarus mucorea* (Chrysomelidae and Curculionidae) found on *N. attenuata* were determined as described above.

Supporting Information

Figure S1. Copy Number of T-DNA in the Two Studied IR*pmt* Lines

(A) Southern blot analysis of two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) and WT plants. Genomic DNA (15 μg) from individual plants of the three genotypes and the plasmid used for transformation pRESC5PMT (4 ng) were digested with EcoRV and blotted onto nylon membranes (Winz and Baldwin 2001). The blot was hybridized with a PCR fragment of the *hygromycin phosphotransferase II* gene from pCAMBIA-1301, which is specific for the selective marker on the T-DNA and signifies one insertion in each of the two lines.

(B) Ethidium bromide staining of the DNA revealed an overload of the DNA of the IR*pmt* lines and therefore loading of the WT was controlled by stripping and rehybridization with a PMT probe, which clearly revealed the endogenous *pmt*1 and *pmt* 2 genes described (Winz and Baldwin 2001) (unpublished data).

Found at DOI: 10.1371/journal.pbio.0020217.sg001 (6.3 MB TIF) and in the Appendices.

Figure S2. Secondary Metabolite Levels in the Studied IR*pmt* Lines

Inverted-repeat silencing of *pmt* did not change the levels of (A) anabasine, (B) caffeoylputrescine, (C) chlorogenic acid, and (D) rutin (mean ± standard error [SE]) in two independently transformed *N. attenuata* lines (108 and 145) compared to WT plants. Plants were harvested 4 d after receiving one of four treatments: untreated control (Con), wounding (W), wounding and regurgitate application (W+R), and application of 150 μg of MeJA per plant applied in a lanolin paste. Plants were treated at the first two fully expanded (source) leaves and wounding was performed by generating three rows of puncture wounds on each leaf side using a pattern wheel. Subsequently, 10 μl per leaf of either water or *M. sexta* regurgitate diluted 1:1 (v:v) was dispersed over the puncture wounds (n = 8–10).

Found at DOI: 10.1371/journal.pbio.0020217.sg002 (179 KB PPT) and in the Appendices.

Figure S3. Proteinase Inhibitor and Volatile Emission of the Studied IR*pmt* Lines
Levels of (A) TPI and (B) cis-a-bergamotene emission (mean ± SE) in two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) did not differ from WT plants 4 d (for TPI) and 10 h (for cis-abergamotene) after receiving one of four treatments (as described for S2): untreated control (Con), wounding (W), wounding with additional regurgitate application (W+R), and MeJA elicitation. IS, internal standard.
Found at DOI: 10.1371/journal.pbio.0020217.sg003 (73 KB PPT) and in the Appendices.

Figure S4. Growth Parameters Under Glasshouse and Field Conditions of the Studied IR*pmt* Lines
N. attenuata plants transformed with an IR*pmt* construct (108 or 145) did not differ in (A) stalk length [n_{PMT} = 43, n_{WT} = 57, n_{PMT}* and n_{WT}* = 28] and (B) rosette diameter [n = 8] from WT grown under either field (A) or glasshouse (B) conditions. Plants in (A) were untreated or elicited (*) with MeJA 7 d after plants were transplanted into a field plot in a native habitat.
Found at DOI: 10.1371/journal.pbio.0020217.sg004 (98 KB PPT) and in the Appendices.

Figure S5. Transformation Vectors
This figure shows plasmids used for the generation of *N. attenuata* lines with reduced levels of two PMTs due to posttranscriptional gene silencing. Both (A) pCAMPMT1 (10.7 kb) and (B) pNATPMT1 (9.7 kb) allow the synthesis of *pmt* antisense RNA. (C) pRESC5PMT (12.4 kb) was used for the synthesis of *pmt* RNA capable of forming an inverted repeat. Functional elements: bla, *beta-lactamase* gene from plasmid pUC19; *hptII*, gene for hygromycin resistance from pCAMBIA-1301; LB and RB, left and right border of T-DNA; nptIII, aminoglycoside phosphotransferase of type III from *Streptococcus faecalis*; ori ColE1, origin of replication from pUC19; ori pVS1, origin of replication from plasmid pVS1; PCaMV and TCaMV, 35S promoter and terminator of cauliflower mosaic virus; pdk i3, intron 3 of pdk; *pmt*1, gene fragment of *pmt*1 (95% identical with *N. attenuata pmt*2); PNOS and TNOS, promoter and terminator of the nopaline synthase gene; repA pVS1, replication protein gene from pVS1; *sat-1*, nourseothricin resistance gene; staA pVS1, partitioning protein gene from pVS1. Displayed restriction sites mark the borders of functional elements, which are displayed in gray if on the T-DNA and in black if outside the T-DNA.
Found at DOI: 10.1371/journal.pbio.0020217.sg005 (56 KB PPT) and in the Appendices.

Figure S6. PMT Sequences and TaqMan Probe
Nucleotide sequences of *N. attenuata pmt*1 and *pmt*2 mRNA (Winz and Baldwin 2001) aligned with ClustalW. Primers and probe (underlined) used for real-time PCR of *pmt* mRNA are highlighted and bold.
Found at DOI: 10.1371/journal.pbio.0020217.sg006 (396 KB TIF) and in the Appendices.

Protocol S1. Molecular and Analytical Methods
Found at DOI: 10.1371/journal.pbio.0020217.sd001 (58 KB DOC) and in the Appendices.

Accession Numbers. GenBank accession numbers for the genes discussed in this paper are bla from puc19 (L09137), hygromycin phosphotransferase II from pCAMBIA-1301 (AF234297), pdk (X79095), *pmt*1 (AF280402), and *pmt*2 (AF280403).

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Plant biology

The benefits of nicotine

F. HABEGGER/GRANT HELLMAN PHOTOGRAPHY

Gardeners and farmers know that nicotine has its uses: a quick squirt of a nicotine solution keeps pests at bay. But do the plants that make this compound use it to deter invaders? This isn't as obvious as one might think; evolution might have rendered the insects that eat these particular plants completely resistant to nicotine. Anke Steppuhn *et al.* aimed to find out (*PLoS Biol.* **2,** e217; 2004).

They produced transgenic tobacco plants (*Nicotiana attenuata*) in which a key enzyme in nicotine synthesis was silenced. The nicotine concentration in these plants dropped to 3–4% of normal levels. Experiments with greenhouse-grown tobacco showed that the larvae of a key pest — the tobacco hornworm *Manduca sexta* (pictured) — much preferred leaves from the transgenic plants than from wild-type plants. The larvae also grew much more quickly when reared on the transgenic plants, implying that although these bugs can tolerate nicotine, they fare better without it.

Experiments with field-grown tobacco gave similar results, and showed that the transgenic plants also lost significantly more leaf area to pests than did wild-type plants. The inescapable conclusion is that secondary metabolites such as nicotine, although not essential for normal plant growth and reproduction, nonetheless make significant contributions to ecology.

Amanda Tromans

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Nicotine Keeps Leaf-Loving Herbivores at Bay

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Sooner or later, a gardener looking for "nontoxic" ways to control the inevitable attack on a favorite plant will discover the nicotine remedy. Steep a cup of loose tobacco in a gallon of water, let it sit overnight, strain, and spray away. Caterpillars, aphids, and a diverse array of insects predisposed to devouring plants will soon abandon your vegetables and flowers in search of less disagreeable forage.

The ultimate sitting duck, plants rely on an arsenal of chemical metabolites to fend off predators. Many of these chemicals harbor anti-herbivore properties, which have been exploited for commercial use. Nicotine, it turns out, is so toxic that it was one of the first chemicals used in agricultural insecticides. It's not clear, though, whether these toxic metabolites are really defending plants against hungry herbivores in their natural environment, especially since many insects can tolerate various plant chemicals and sometimes even incorporate them into their own defenses. Though scientists have cataloged a long list of these presumed resistance traits, there's no evidence that they offer plants a competitive advantage against their leaf-covetous foes in nature.

With plant and plant-eater engaged in an ever-escalating battle of evolutionary one-upmanship and with plants capable of producing an array of defensive responses, teasing out the predator-resistant effects of individual plant metabolites has proved challenging. Theoretically, one could track down a resistance gene by breeding plants that are genetically identical save for the gene that controls expression of a particular resistance trait. In practice, however, traditional breeding techniques aren't that precise and tend to generate additional variations in genomic regions that are linked to the target gene and that might affect resistance as well.

The tools of genetic engineering have largely overcome such limitations, allowing scientists far greater control and specificity. Following this approach, Ian Baldwin and colleagues use transgenic silencing (which introduces gene "constructs" into an organism to inactivate a gene of interest) to investigate a single resistance trait, nicotine production. Even though nicotine is one of the best-studied putative resistance traits, its specific role has been unclear.

To isolate the resistance effects of nicotine from possible confounding factors, Baldwin and colleagues blocked nicotine production in the *Nicotiana attenuata* tobacco plant. Focusing on an enzyme, called putrescine methyl transferase (PMT), central to nicotine biosynthesis, the authors used two techniques that interfere with PMT production by silencing the gene, *pmt*, that encodes the enzyme. One of the techniques (which adds genetic sequences called "inverted repeats" to gene fragments) proved far more effective at silencing *pmt*, producing 29 out of 34 plant lines with only 3%–4% of normal nicotine levels.

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Spodoptera exigua* larva feeding on *Nicotiana attenuata

With suitably nicotine-deprived plants, Baldwin and colleagues could directly test nicotine's role in tobacco fitness. They transplanted the transgenic plants, along with nonmutant cultivated plants, in southwestern Utah, *N. attenuata*'s native habitat. A subset of the plants was also treated with a chemical known to increase both nicotine content and resistance to herbivore attack. Predictably, several of the plant's natural insect enemies made an appearance. Untreated nicotine-deficient transgenic plants fared the worst, losing twice as much foliage to herbivores as nonmutant plants. Transgenic plants treated with the chemical boost performed much better, showing about the same amount of damage as the nonmutants. Interestingly, tobacco hornworms—which, as their name implies, feed primarily on tobacco—preferred nicotine-free plants when given the choice. Though the worms have evolved strategies for coping with nicotine's deleterious effects, these adaptations come at a price: worms feeding on nicotine-deficient tobacco grew bigger and faster than those feeding on plants with normal nicotine levels.

These results clearly show that nicotine protects tobacco plants in their native habitat, the authors conclude, and that tobacco-chewing insects "prefer low nicotine diets." Removing nicotine from the equation reveals the relentless pressure that plants face from herbivores. Without such defenses, plants would be unceremoniously eliminated posthaste, leaving a world without greenery, not to mention oxygen. But these experiments also demonstrate the unprecedented power of transgenic tools to peel back the obfuscating layers inherent in ecological interactions to reveal the fundamental properties of those interactions. And that scientists working to unravel the tangled web of ecological interactions would do well to take advantage of the longest running experiment on earth—the natural environment.

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. DOI: 10.1371/journal.pbio.0020217

Manuscript III

Resistance Management in a Native Plant: Nicotine Prevents Herbivores from Compensating for Plant Protease Inhibitors

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Abstract

Plants deploy chemical defenses in complex mixtures which are thought to be adaptive, but experimental tests have used artificial diets rather than plants. Herbivore attack to *Nicotiana attenuata* rapidly increases the production and accumulation of trypsin proteinase inhibitors (TPIs) and the toxic alkaloid nicotine. By transgenically silencing their respective biosynthetic genes, we were able to abolish TPI activity and reduce inducible nicotine by 85%. Nicotine production was not affected by silencing *pi* or vice versa, and transformation did not alter levels of other metabolites examined. *Spodoptera exigua*, a native generalist herbivore that can compensate for heterologous TPI expression, performed better on TPI- or nicotine-deficient plants compared to wild-type. Due to a compensatory feeding response to TPIs when nicotine is absent, larvae performed better on nicotine-deficient plants than they did on plants silenced in both defenses. The antifeedant toxin, nicotine, prevents this compensatory response. We conclude that *N. attenuata* counters an insect adaptation with a defensive synergism.

Keywords: plant defense, synergistic interaction, secondary metabolites, trypsin proteinase inhibitors, *Nicotiana attenuata*, *Spodoptera exigua*, tobacco

Introduction

Plants defend themselves against herbivores with secondary metabolites, which assume forms as diverse (alkaloids, glycosides, proteins) as their functions (toxins, antifeedants, antidigestive proteins). Most plants produce cocktails of these metabolites (Bennett & Wallsgrove 1994). Ecologists have long debated whether mixtures of secondary metabolites represent metabolic waste (Waterman & Mole 1989) or evolutionarily sculpted mixtures of adaptive value (Jones 1983; Berenbaum 1985). The ability to produce mixtures of allelochemicals may broaden a plant's resistance to a range of herbivores, increase the toxicity of defense compounds, and delay herbivores' ability to evolve resistance to those defenses. Yet, the defensive functions of allelochemicals are usually examined separately, despite evidence for synergistic interactions among secondary metabolites enhancing the resistance provided by each metabolite alone (Berenbaum & Neal 1985; Dyer *et al.* 2003). Similarly, agronomists typically introduce single resistance genes into high-yielding lines, but combining two genes can increase plant resistance synergistically and simultaneously delay the evolution of herbivore resistance (Cao *et al.* 2002; Zhao *et al.* 2003). Whether multiple

defenses help the plant to resist herbivores has not been systemically addressed in a native plant.

Producing protease inhibitors (PIs) that target insect digestive proteins can decrease insect growth, survival, and fertility by suppressing gut protease activity and thereby reducing the availability of essential amino acids (Birk 2003). However, several insects are known to counter the effects of ingested PIs by overexpressing and desensitizing existing proteases, producing PI-insensitive proteases, proteolytically inactivating PIs, or increasing consumption (Jongsma & Bolter 1997; Winterer & Bergelson 2001; Zhu-Salzman *et al.* 2003b). PI production can even become a liability for the plant, if the compensatory response requires the herbivore to eat more leaf material to complete development. The ubiquity of PIs in plants raises the question of how plants prevent compensatory responses during the evolutionary arms race with their herbivores.

The resistance provided by PIs and subsequent compensatory insect responses have been investigated either by supplementing artificial diets with PIs or heterologously expressing *pi* genes in plants. Both types of studies alter the ecological and chemical context in which endogenous PIs are expressed. But primary metabolites and the chemical milieu of a leaf-- for example, pH, protein quality, or phytic acid --can affect insects' ability to detoxify plants' secondary metabolites (Broadway & Duffey 1988; Govenor *et al.* 1997; Green *et al.* 2001). Additionally, secondary metabolites can affect each other: for example wound-induced quinones may deactivate PIs (Felton *et al.* 1989). Many plants increase PI production after herbivore attack in concert with the production of numerous secondary metabolites. Few studies have addressed how defense compounds in plants interact (Berenbaum & Zangerl 1993; Zhu-Salzman *et al.* 2003a) or have pharmacologically determined the dosage-dependent effects of mixing two secondary metabolites (Berenbaum & Neal 1985; Nelson & Kursar 1999). Transferring PIs to other plant species or artificial diets changes the chemical environment in a way that may confound its function. Recent advances in molecular techniques have enabled genes to be individually silenced; by altering the expression of a putative resistance trait, the ecological function of a trait can be studied in the context of the normal background of other traits.

Nicotiana attenuata Torr. ex Watson produces many secondary metabolites, most of which are elicited by the jasmonate (JA)-signaling pathway. Silencing the genes responsible for the biosynthesis of trypsin PIs (TPIs), the alkaloid nicotine, or the JA cascade produces plants with diminished resistance to herbivores (Halitschke & Baldwin 2003; Steppuhn *et al.* 2004; Zavala *et al.* 2004a) and demonstrated their function as direct defenses. Whereas TPIs

are anti-digestive, nicotine acts as a neurotoxin on all animals with nervous systems that have acetylcholine receptors. Both defenses are synchronously elicited by JA-signaling (Pohlon & Baldwin 2001). Depending on how they work, plant defenses can diminish herbivore growth and development by reducing the consumption, digestibility, or conversion of the consumed material into body tissue. PI production can act as an antifeedant, e.g. in the case of peptidyl PIs in corn pollen against a coleopteran herbivore (Kim & Mullin 2003), but compensatory feeding has frequently been reported for lepidopteran and coleopteran herbivores. Overexpressing potato TPIs (PIN2) in cabbage or tobacco caused several lepidopteran larvae to consume more leaf material (De Leo *et al.* 1998; Winterer & Bergelson 2001; Abdeen *et al.* 2005). Nicotine generally reduces consumption by herbivores; even the nicotine-tolerant specialist *Manduca sexta* ingested less diet that contained more supplemented or endogenous nicotine than diet that didn't (Parr & Thurston 1972; Appel & Martin 1992; Voelckel *et al.* 2001). Hence these two defenses may complement each other functionally, restraining consumption of plant tissue (via nicotine) and reducing its digestibility (via TPIs).

To study why plants deploy chemical defenses in mixtures, we examined the function of two direct defenses in a native plant both with and without the other. The generalist herbivore *Spodoptera exigua* Hübner, occasionally responsible for half of the canopy lost to herbivores in *N. attenuata* plants growing in its native populations (Steppuhn *et al.* 2004), is known to compensate for heterologously expressed potato TPIs (Jongsma *et al.* 1995). We aimed to determine the effects of endogenous nicotine and TPI production on the performance of this herbivore and whether one of the metabolites alters the effect of the other. We transformed *N. attenuata* to silence the genes for *pi* and *putrescine N-methyl transferase* (*pmt*), which is the regulatory enzyme for nicotine biosynthesis separately and concomitantly (Hibi *et al.* 1994; Winz & Baldwin 2001). Two independently transformed lines of each genotype were genetically and metabolically characterized and shown to differ from wild-type plants (WT) only in the silenced defense trait. Using nutritional indices (Waldbauer 1968), we measured the performance of *S. exigua* when feeding on material from plants that produce either both defenses or nicotine alone or TPIs alone, or from plants that do not produce either of the two. The results demonstrate that TPIs alone have no detrimental effect for the generalist and even enhance its performance, but in combination with nicotine TPIs contribute to a strong defensive synergism. In other words, when it comes to deploying defenses, the whole is greater than the sum of its parts.

Material & Methods

Plant material & transformation. We used an inbred genotype of *N. attenuata* (synonymous with *N. torreyana* Nelson and Macbr.) derived from field-collected seeds (Baldwin 1998) for transformation and all experiments. Seed germination and the *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure are described elsewhere (Krügel *et al.* 2002).

To silence the expression of the *N. attenuata pmt* and *pi* genes, inverted repeat (IR) constructs were used, which contained either a consensus fragment for the two *pmt* genes (GenBank AF280402 & AF280403) or a fragment of the *pi* gene (GenBank DQ158200) twice in inverted orientation; these were separated by intron 3 of the *Flaveria trinervia pdk* gene for pyruvate, orthophosphate dikinase (GenBank X79095). Vector construction and plasmid of pRESC5PMT to generate IR*pmt* transformants are described in Steppuhn *et al.* (2004). IR*pi* plants were transformed with a pSOL3PI construct, which harbored a fragment of the *N. attenuata pi* gene (for PCR fragment see Zavala *et al.* 2004b) in the pSOL3 plasmid described in Bubner *et al.* (2006). In order to produce plants silenced for both genes, we transformed homozygous T2 IR*pmt* plants (line A03-108-3) with a pSOL4PI plasmid, which is based on the pSOL3 but its selective marker gene was exchanged for the *streptothricin-acetyl-transferase* (*sat-1*) gene (GenBank X15995). T1 plants were screened for resistance to hygromycin (*hygromycin phosphotransferase II gene* from pCAMBIA-1301; GenBank AF234297 contained in the pRESC5 and pSOL3 vectors) or nourseothricin (*sat-1*gene contained in the pSOL4 vector), constitutive and induced nicotine and TPI accumulation; homozygosity was determined by screening the T2 plants for resistance.

Southern blot and flow cytometric analysis. Several independently transformed homozygous lines for each vector were further characterized by Southern blot analysis. Genomic DNA (15 µg) from WT, IR*pi*, and IR*pmt/pi* plants was extracted and digested with EcoRV. After gel electrophoresis, the digested plant DNA, including 4 ng plasmid-DNA (from the constructs used for transformations--pSOL3PI, pSOL4PI, pRESC5PMT—which had been digested with EcoRV and XhoI), the DNA was blotted onto nylon membranes (Winz & Baldwin 2001; Bubner *et al.* 2004). The blot was hybridized with a PCR fragment of the respective selective marker gene (*hygromycin phosphotransferaseII* or *sat-1,*) to identify lines harboring only one insertion from each transformation (Supplemental Fig. 1; for IR*pmt* see Steppuhn *et al.* 2004). Flow cytometric analysis (Bubner *et al.* 2006) revealed that all the lines were diploids.

Characterization of transformants. WT *N. attenuata* and two homozygous lines of each genotype IR*pmt*, IR*pi*, and IR*pmt/pi* (IR*pmt*: line 1: A04-145-1-1, line 2: A03-108-3-1, IR*pi*: line 1: A04-186, line 2: A04-169, IR*pmt/pi*: line 1: A04-103, line 2: A04-226) were grown in 1 L hydroponic vessels in a climate chamber (16/8 h photoperiod at 200-300 µmol m^{-2} s^{-1}, 32/28°C, and 65 % r.h.) as described in Hermsmeier *et al.* (2001); four-week-old rosette-stage plants were elicited on the first fully expanded source leaf (+1) with 20 µl of 1/1 dilution of *M. sexta* regurgitate, which is known to elicit the full response to attack (Halitschke *et al.* 2001). The +1 leaves (200-300 mg) of elicited and of untreated control plants were harvested after 30 minutes for phytohormone analysis. Subsequently, the second fully developed source leaf (+2) was treated with 150 µg of methyl jasmonate (MeJA) applied in 10 µl of lanolin paste. After 10 h, elicited and untreated control plants were harvested for the + 2 leaf (200-300 mg) and young root tissue (400-500 mg) to analyze *pi* and *pmt* transcripts respectively. All samples were immediately frozen in liquid nitrogen. To analyze secondary metabolites, leaves growing one node above the treated leaves were harvested 4 days after elicitation.

For hormone analysis, 200 to 300 mg of the +1 leaf (N = 3) were homogenized and extracted twice in 1 ml ethyl acetate containing labeled internal standards for JA and SA, and the supernatant was then dried completely. The residue was re-suspended in 70 % (v/v) methanol before 15 µl were analyzed on Varian 1200L Triple-Quadrupole-MS (Palo Alto, CA, USA) in negative electrospray ionization (ESI) mode according to Schmidt & Baldwin (2006). The amount of JA (parent-ion / daughter-ion: *m/z* = 209 / *m/z* = 59) and SA (*m/z* = 137 / *m/z* = 93) were calculated relative to the corresponding internal standard [2-^{13}C]-JA (*m/z* = 211 / *m/z* = 61) and [4-^{2}H]-SA (*m/z* = 141 / *m/z* = 97) and per g fresh leaf mass. The amounts of JA conjugated to isoleucine or leucine (JA-Ile/-Leu; parent-ion / daughter-ion: *m/z* = 322 / *m/z* = 130) were calculated relative [2-^{13}C]-JA and g fresh leaf mass and represent therefore relative quantities.

RNA from the +2 leaves and roots (N = 3) was extracted with Tri Reagent (Sigma, Taufkirchen, Germany) according to the manufacturer's instructions, and cDNA was generated from 20 ng of RNA with MultiScribe reverse transcriptase (Applied Biosystems). Levels of transcript accumulation of *pmt* and *pi* genes were analyzed by real-time PCR (ABI PRISM 7000; Applied Biosystems, Darmstadt, Germany), during which the cDNA was amplified using the qPCR core reagent kit (Eurogentec, Searing, Belgium) and the probes and primers that were gene-specific for *pmt* and *pi* genes, respectively [for sequences see Steppuhn *et al.* (2004) and Zavala *et al.* (2004b)]. Relative expression levels were calculated

according to plasmid (pRESC5PMT and pSOL3PI, respectively) dilution series, which were performed for each 96-well PCR plate.

To analyze alkaloids and phenolics (N = 5–8) 100 mg leaf were extracted and analyzed with an Agilent HPLC system according to Keinänen *et al.* (2001). Nicotine, caffeoylputrescine, and chlorogenic acid were quantified based on external standard curves (in mg g^{-1} fresh weight). Rutin and the most abundant diterpene glycoside (DTG) were relatively quantified in area per fresh mass. TPI activity was examined in protein extracts of another 100 mg with a radial diffusion assay relative to protein content, which was determined with a Bradford assay (N= 4-7; described in van Dam *et al.* 2001).

Profiles of the secondary metabolite in transformed lines in comparison to WT lines were additionally determined under growth conditions similar to the *S. exigua* trials (see below). The first fully expanded source leaf (+1) was harvested 4 days after elicitation with 200 µg JA-Ile on two pre-wounded leaves per plant and on unelicited control plants. JA-Ile is an active form of JA on the octadecanoid pathway (Kang *et al.* 2006). Leaf samples were extracted and analyzed by HPLC as mentioned above (N = 5–10) above for the alkaloids nicotine, anatabine, and anabasine as well as for the phenolics caffeoylputrescine, chlorogenic acid, and rutin.

***Spodoptera exigua* assay.** We chose *S. exigua* because after *M. sexta,* it is the most damaging lepidopteran folivore of the *N. attenuata* in nature (Steppuhn *et al.* 2004); unlike *M. sexta*, however, it is not highly adapted to nicotine. Initial experiments revealed large differences among larvae maintained on the four different genotypes with respect to their development and the amount of tissue they consumed. Because differential tissue damage would have caused dissimilar elicitation levels among genotypes, the bioassay was carried out with excised leaf material from constitutive and MeJA-elicited plants, thus standardizing the degree of elicitation. Plants were grown in the glasshouse (16/8 h photoperiod at 200-300 µmol m^{-2} s^{-1}, 25/21°C, and 45-55 % r.h.) in 1 L pots with soil. For induction 150 µg MeJA in 20 µL lanolin was applied to the petioles of the first two source leaves (10 µL on each leaf). Plants were induced 5 days after caterpillars started feeding to allow the very small and sensitive first instars of this generalist to establish themselves on *N. attenuata*.

Spodoptera exigua neonates hatched from eggs supplied by the Plant Protection Centre of Bayer AG (Monheim, Germany) were kept communally in boxes with leaves from uninduced plants (either WT or the other genotypes) until separated into individual containers (ø12 mm) with leaf disks (ø10 mm) in three different setups. Three leaf disks were taken from a leaf of the same plant. One day after emergence, the first set of larvae was separated and

placed on material from uninduced plants to compare larval performance on two lines of each genotype. For each line, 21 larvae were placed on leaf disks of 7 plants of each genotype. Twice as many were established on WT (42 larvae on 14 plants), because we expected that mortality would be higher on WT plants than on the less-defended transformants. The mass of the surviving larvae (N = 5 - 13 see survival in Fig. 3) was measured on day six, which is when individual larvae reached a size that was readily weighable with a balance with 0.1 mg resolution.

Two additional repetitions of the experiment were separated in individual containers with leaf disks of line 1 of each genotype to measure larval performance and consumption. Two days after larvae emerged, 39 larvae per 13 plants of each genotype were placed on leaf disks of unelicited plants (in the case of IR*pi* additionally 24 larvae were placed on line 2). Four days after larvae emerged, 21 larvae (7 plants per genotype) were placed on leaf disks of plants that were MeJA-elicited on day five. All insect boxes contained a moist filter paper and leaf material that was refreshed at least every 24 h; boxes were maintained in a climate chamber (16/8 h light to dark 26/24 °C, and 65 % r.h.). After day eight, larger containers (ø 40 mm) were used and the leaf amount provided was increased. Caterpillar mass was determined daily (days 6, 7, 8, 9, and 10). Consumption was determined for a 24-h period (days 7-8), which is when 21-28 larvae per genotype survived on the experiment using unelicited plant material and 10-15 larvae survived on elicited plant material. The leaf area eaten was determined using SIGMA SCAN PRO 5 on scans of photocopies of the leaf disks after larvae had fed on them. The leaf mass consumed was estimated from the area, and the fresh-weight-to-area ratio that was determined for the same lines under similar growth conditions. The efficiency of conversion of the ingested food (ECI) was calculated from the mass gain per consumed mass over the 24-h period (days 7-8).

Data analysis & statistics. The production of secondary metabolites in the transformed lines relative to WT was evaluated by unpaired *t*-tests (performed with Statview) with adjusted α according to the sequential Bonferroni procedure to control for family-wise type I error.

Univariate ANOVA (genotype as factor) was used to test for differences between larval mass of the *S. exigua* that fed on the two lines of each genotype at day 6, which was followed by a Ryan-Einot-Gabriel-Welsch-F post hoc test. The effects of nicotine and TPIs on *S. exigua* growth either with (WT vs. IR*pmt* and WT vs. IR*pi*,) or without (IR*pi* vs. IR*pmt/pi* and IR*pmt* vs. IR*pmt/pi*) the expression of the other defense were determined separately by two-way repeated measures ANOVAs (factors: genotype, MeJA-treatment). To detect

interactions of endogenous nicotine and TPI production, as well as the effect of MeJA elicitation on caterpillar growth, consumption and efficiency of utilization of the consumed material, analyses with three factors: MeJA-treatment, nicotine, TPIs were used. Larval mass (from day 6-10) was analysed with a repeated measures ANOVA, 24 h consumption data from day 7-8 with an ANCOVA using larval mass of day 7 as the covariate (to account for caterpillar size), and larval mass gain from day 7-8 with an ANCOVA with consumption as the covariate. Because the effect of TPIs on consumption changed sign depending on the presence or absence of nicotine, 2-way-ANCOVAs (WT vs. IR*pi* and IR*pmt* vs. IR*pmt/pi* with genotype and MeJA as factors) were used to examine its effect. Mortality for the first repetition of larvae on both lines of each genotype was compared with a χ^2 test for differences of the survivorship between lines and a binary logistic regression model was used to test for differences caused by nicotine, TPIs, and their interaction. Survivorship of the larvae feeding continuously on uninduced and MeJA-elicited leaves of line 1 were analyzed with a binary logistic regression model for effects of nicotine, TPIs, MeJA elicitation, and the interaction of nicotine and TPIs.

All data (except mortality data) were graphically inspected for normality and variance homogeneity was determined with the Levene's test and most metabolite data and all insect performance data were log-transformed to meet the requirements of equal variance and normality. For the ANCOVAs, we graphically checked for homogeneity of the residuals plotted against the predicted values of a linear regression model of the dependent variable with the covariate. SPSS was used to perform ANOVAs, ANCOVAs, and regression models.

Results

Characterization of the transformed lines. Transcript levels of the *pmt* gene in IR*pmt* and IR*pmt/pi* lines were reduced to 18% of constitutive and 6% of MeJA-induced WT mRNA levels (Fig. 1A). Levels of *pi* transcript accumulation in IR*pi* lines and IR*pmt/pi* lines were reduced to below 1% of constitutive and MeJA-elicited WT mRNA levels (Fig. 1C). Consistent with transcript analysis, constitutive and MeJA-induced nicotine levels were significantly reduced to 15 - 20% of levels in WT plants (Fig. 1B; Supplemental Fig. 2A, t-tests: $P < 0.01$) in lines transformed to silence *pmt*. TPI activity was not detectable in any of the *pi*-silenced lines (Fig. 1D).

Transgenic lines were not morphologically distinguishable from WT plants and only differed in the traits silenced (t-tests: $P > 0.05$ for all other metabolites measured,

Supplemental Tables 1 and 2), with the exception of anatabine, which accumulated in all *pmt*-silenced lines to levels that were about 25% of nicotine levels in WT plants (Supplemental Fig. 2B) and was slightly higher in MeJA-elicited in IR*pi* line 2 (3.6% of nicotine level in WT), but anatabine levels in WT and IR*pi* plants are at the detection limit. TPI activity was

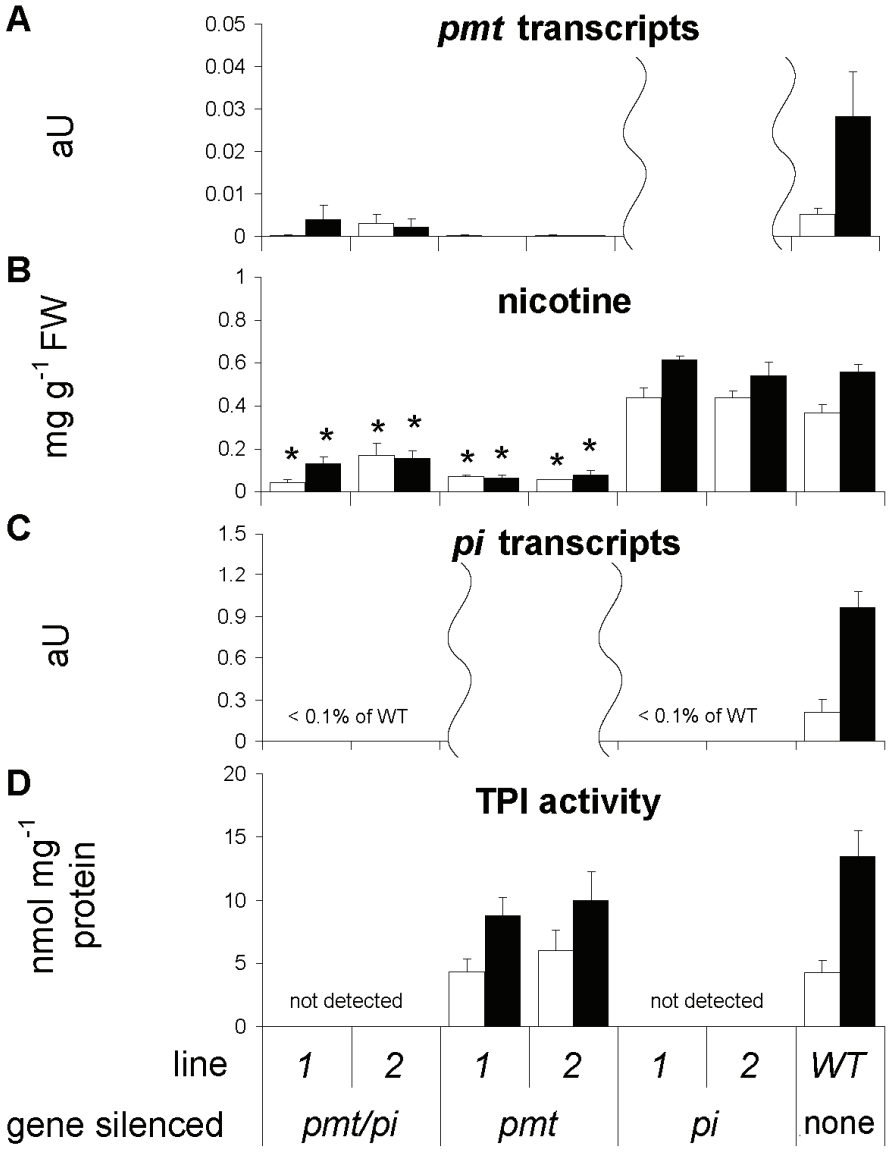

Figure 1: Mean (± SE) (**A**) *putrescine N-methyl transferase* (*pmt*) transcript levels (**B**) nicotine, (**C**) *protease inhibitor* (*pi*) transcript levels, and (**D**) trypsin PI (TPI) activity in wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) of each genotype silenced for *pmt*, or *pi*, or both. Plants were either unelicited (white bars) or MeJA elicited (black bars) and transcript levels (N = 3) were quantified after 10 h relative to standard curves obtained from *pmt* or *pi* plasmid dilution rows (N = 3; aU = arbitrary units). Nicotine and TPI levels were determined after 4 days (N = 4-8) and tested for significant differences between each transformed line and WT by t-tests. Significant differences to WT at $P < 0.05$ are indicated by asterisks.

not affected by silencing *pmt.* Constitutive or *M. sexta* oral secretion-inducible levels of JA, its conjugates with isoleucine (Ile) and leucine (Leu), and salicylic acid (SA) were not significantly affected in any of the transformed lines (Fig. 2A; Supplemental Fig. 3A, B) as well as constitutive and JA-Ile-elicited levels of anabasine, and the phenolics caffeoylputrescine, chlorogenic acid, and rutin (Supplemental Fig. 2C-F). Levels of constitutive and MeJA-induced total protein, level of the phenolics and an abundant DTG were unchanged in transformed lines compared to WT (Fig. 2B, Supplemental Fig. 3C).

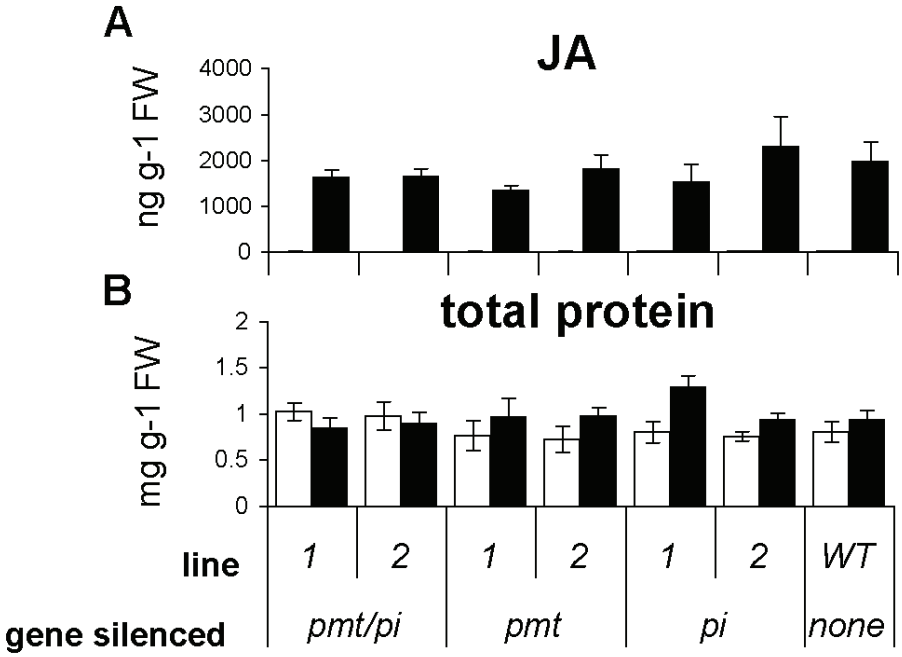

Figure 2: Mean (± SE) of (**A**) jasmonic acid levels (JA) and (**B**) total protein content in leaves of wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) of each genotype silenced for *putrescine N-methyl transferase* (*pmt*), or *protease inhibitor* (*pi*), or both. JA (N = 3) and total protein concentration (N = 4-7) of unelicited plants (white bars) and elicited plants (black bars) 30 min after treatment with *M. sexta* oral secretion or 4 days after MeJA-treatment, respectively. T-tests did not detect significant differences between each transformed line and WT ($P < 0.05$).

Effects of silencing nicotine and TPIs on *S. exigua* performance. Larval mass of *S. exigua* did not differ between the two lines of each genotype on day six, at which time larvae that fed on nicotine-deficient *N. attenuata* genotypes (IR*pmt* and IR*pmt/pi*) had already gained twice the mass of larvae that fed on nicotine-producing genotypes (IR*pi*, WT). Although mortality did not differ significantly among the lines ($\chi^2 = 8.79$, $P = 0.186$) only five replicates (of 21 initial) remained for each IR*pi* line due to high mortality (50-75%) of larvae that fed on the nicotine-producing genotypes (logistic regression model without the

non-significant interaction between nicotine and TPIs: $B_{nicotine}$ = -1.09 , P = 0.037, B_{TPI} = -0.28 , P = 0.958). By day 6, their mass did not differ from those feeding on WT (Fig. 3A). However, mass did not differ between larvae that fed continuously on the two uninduced IR*pi* lines for the duration of the experiment, even when they gained significantly more mass than larvae on WT plants (Fig. 3B).

Silencing nicotine had significantly increased larval mass by day six (Fig 3A), which is when the larvae were large enough to be weighed. This effect was visually evident by day two and lasted for the duration of the experiment (Fig 4A: repeated measures 2-way-ANOVA between WT and IR*pmt*: $F_{genotype}$ = 31.944, P < 0.001). Silencing TPI production affected larval growth much later and less substantially (repeated measures 2-way-ANOVA between

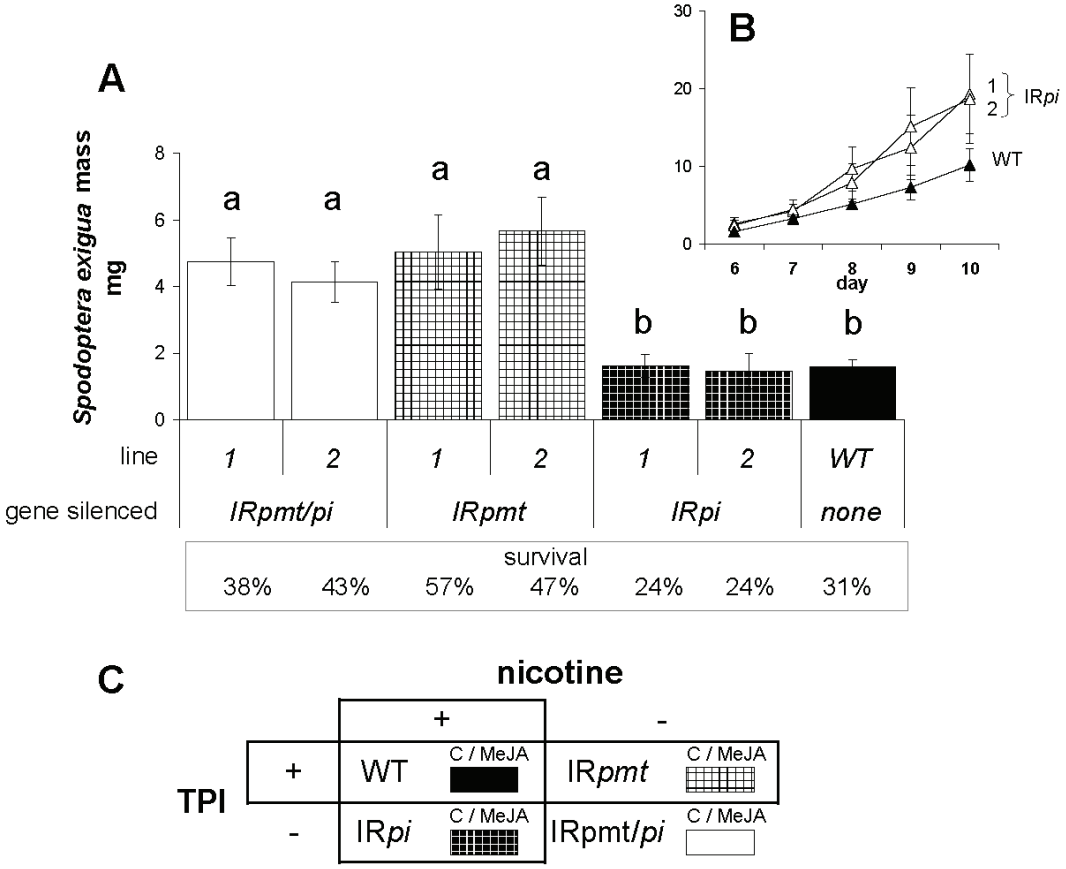

Figure 3: *Spodoptera exigua* mass (mean ± SE) (**A**) 6 days after larvae hatched and fed on either wild-type (WT) *Nicotiana attenuata* (black bars) or 2 independently transformed lines (1 & 2) silenced for *putrescine N-methyl transferase* (*pmt*; light squared bars), *protease inhibitor* (*pi*; dark squared bars), or both (white bars). Letters indicate significant differences (P < 0.05). The number of larvae surviving until day 6 is given below the graph in % of initially 21 larvae per line (42 in case of the WT). (**B**) *S. exigua* mass of another experimental repetition with larvae feeding continuously on 2 lines inverted repeat silenced (IR) of *pi*-silenced (white triangles; on day 10 N = 11 & 12) or on WT (black triangles; on day 10 N = 17) plants. Larvae grow significantly larger when fed on IR*pi* plants. (**C**) Overview of the genotypes used to study interactions between nicotine and TPIs and their elicitation by MeJA as defenses against *S. exigua*.

WT and IR*pi*: $F_{genotype} = 1.58$, $P = 0.21$), but larval growth over the experimental period increased significantly (within subject effects: $F_{genotype*time} = 2.36$, $P = 0.05$). By the end of the experimental period IR*pi*-fed larvae had twice the mass of WT-fed larvae, whereas IR*pmt*-fed larvae were four-fold heavier than WT-fed larvae. There were no significant differences in mortality due to nicotine, TPIs, or MeJA elicitation ($P > 0.14$ for all effects in logistic regression models of day 7 and 10 with and without the interaction of nicotine and TPIs).

Interactions of nicotine and TPI mediated resistance to *S. exigua*. The crossed design of the feeding trials with four genotypes differing in nicotine and TPI levels enabled us to examine interactions of these two direct defenses and their elicitation with MeJA (Fig. 3C). In addition to larval mass gain, we measured the Waldbauer indices, consumption (CI) and the efficiency of conversion of ingested food into body mass (ECI), which enable behavioral and physiological counter responses of insects to plant defenses to be detected (Rayapuram & Baldwin 2006).

Repeated measures three-way ANOVA (with nicotine, TPI, and MeJA-elicitation as the 3 factors) for larval mass across the experimental period revealed a significant effect of nicotine but not of TPIs; instead there was a significant interaction between nicotine and TPIs on caterpillar growth (Table 1A). This interaction was due to the fact that although TPIs decreased larval mass gain in the nicotine-producing WT, larvae that fed on TPI-producing plants that are nicotine deficient grew significantly better than larvae that fed on plants lacking both metabolites (repeated measures 2-way-ANOVA between IR*pmt* and IR*pmt/pi*: within subject effects: $F_{genotype*time} = 5.06$, $P = 0.001$; Fig. 4A). Nicotine significantly decreased larval mass even in the absence of TPI production (repeated measures 2-way-ANOVA between IR*pmt* and IR*pmt/pi*: $F_{genotype} = 14.48$, $P < 0.001$), but the growth reduction was greater when TPIs were present.

The amount of tissue consumed by one-week-old larvae was determined for a 24-h period; during this time larval mass gain mirrored the pattern observed over the entire experiment (Fig. 4A). Because the extent of consumption depends on caterpillar size (Table 1B), consumed amounts are depicted relative to larval mass on day 7, which was used as a covariate in the statistical analysis (Fig. 4B). Consumption was significantly decreased by nicotine, which was dependent on the presence of TPIs and of the MeJA treatment. In the presence of nicotine, TPIs had no effect on consumption and MeJA treatment significantly reduced consumption (2-way-ANCOVAs WT vs. IR*pi*: $F_{genotype} = 1.35$, $P = 0.25$; $F_{treatment} = 5.45$, $P = 0.023$), but in the absence of nicotine TPIs significantly increased consumption and

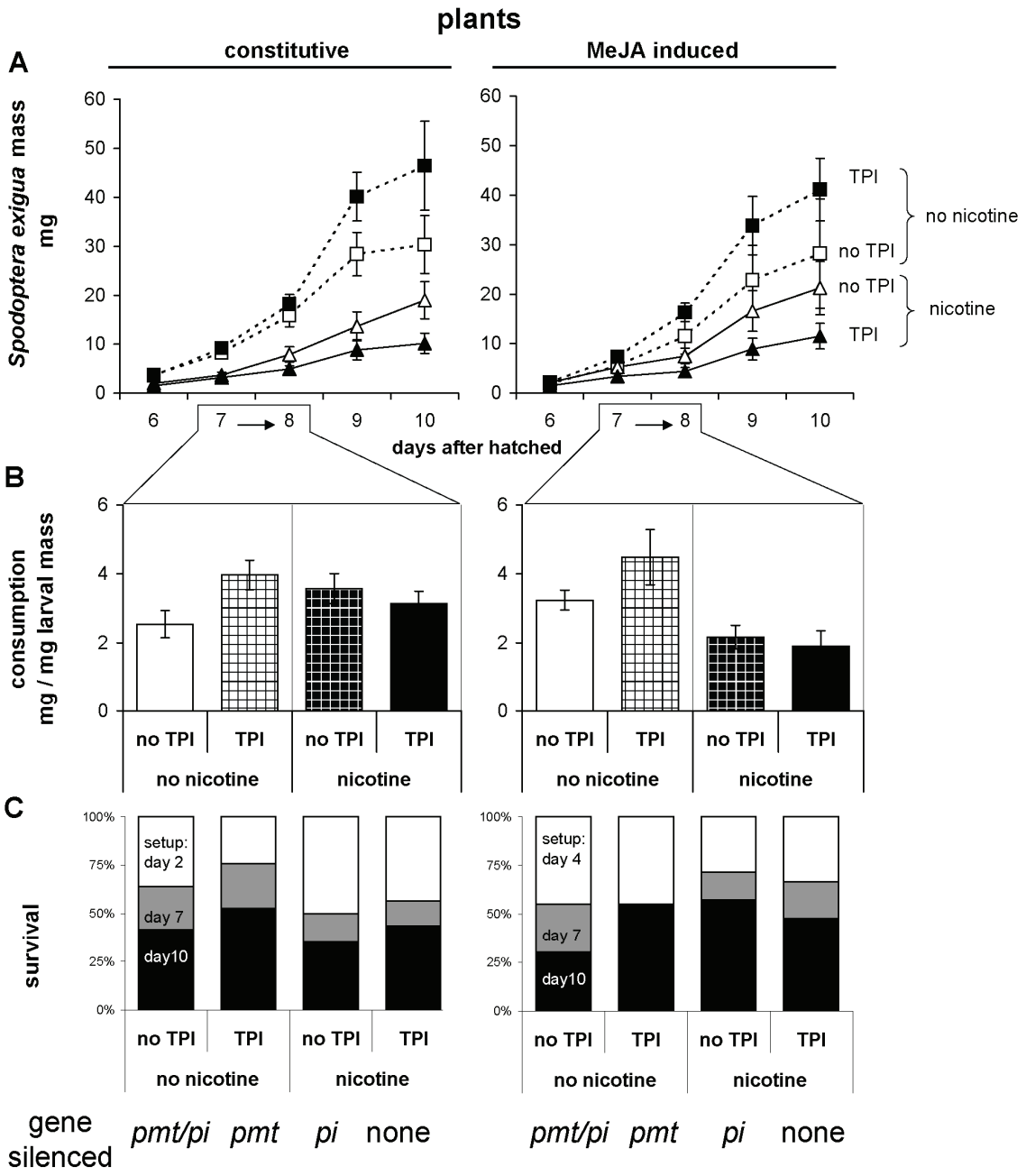

Figure 4: Mean (± SE) (**A**) larval mass and (**B**) consumption relative to larval mass (during 24 h from days 7 - 8) of *Spodoptera exigua* feeding on wild-type (WT) *Nicotiana attenuata* (black triangles and bars) and isogenic lines transformed to silence nicotine (black squares / light squared bars), trypsin protease inhibitors (TPIs; white triangles / dark squared bars), or both simultaneously (white squares and bars) due to inverted repeat silencing of *putrescine N-methyl transferase* (*pmt*) and *protease inhibitor* (*pi*). Larvae were reared on leaf material of unelicited plants (left side) or MeJA-elicited plants (right side). For statistical analyses see Table 1. (**C**) Number of larvae surviving until day 7 and day 10 as percentage of a cohort of 27 and 21 larvae that started on each unelicited (on day 2; both IR*pi* lines were used) and MeJA elicited (day 4) genotypes of plants, respectively.

MeJA treatment had no significant effect (2-way-ANCOVA IR*pmt* vs. IR*pmt/pi*: $F_{genotype}$ = 9,92, $P = 0.003$; $F_{treatment} = 2.49$, $P = 0.12$).

The mass gain relative to the consumed mass was significantly affected by nicotine, MeJA elicitation, marginally by TPIs and significantly by the interaction among these three factors (Table 1C). In the utilization plot (Fig. 5A) depicting mass gain over consumption, the regression of larvae feeding on MeJA-elicited WT (bold black line) is located below all other linear regression lines, even though it has the steepest slope, which results from the very small mass gain of the smallest individuals. MeJA-elicitation decreased the slopes of regressions of larvae feeding on IR*pmt* and IR*pi* plants to values similar to that of WT-fed larvae. The Utilization plot also revealed the antifeedant effect of nicotine; larvae on nicotine-containing genotypes (black and orange lines) appear at lower consumption values whereas larvae on nicotine-deficient genotypes (blue and green lines) consumed generally more. The regression of larvae feeding on unelicited IR*pmt/pi* plants (light green line) appears above all other regressions, but this effect is more apparent when calculating the mean for the efficiency of conversion in ingested food (ECI = mass gain / consumption; Fig. 5B). ECI was reduced by each of the factors TPI, nicotine, and MeJA elicitation relative to larvae feeding on plants that are minimally defended but the combination of all three factors had the strongest effect on the ECI.

Discussion

Because interactions among different secondary metabolites might contribute to plant defense, this study was designed to determine whether and how *N. attenuata*'s endogenous nicotine and TPI production influence each other in the resistance they provide against *S. exigua* larvae. We transformed plants to silence nicotine and TPI production separately or simultaneously. We found that nicotine and TPIs act synergistically and that nicotine production was required to prevent *S. exigua* from overcompensating for TPIs by increasing consumption.

Transforming *N. attenuata* with inverted repeat constructs of the *pmt* and *pi* genes successfully silenced the production of nicotine and TPIs without affecting other plant characteristics except for the elevated levels of the alkaloid anatabine in plants silenced for nicotine (Supplemental Fig. 2B) which results from diverting metabolite flow after the loss of *pmt* function (Chintapakorn & Hamill 2003; Steppuhn *et al.* 2004). The *pmt* gene encodes the enzyme catalyzing the first step in the biosynthesis of nicotine's five-membered ring from putrescine, and its removal results in an accumulation and dimerization of nicotinic acid

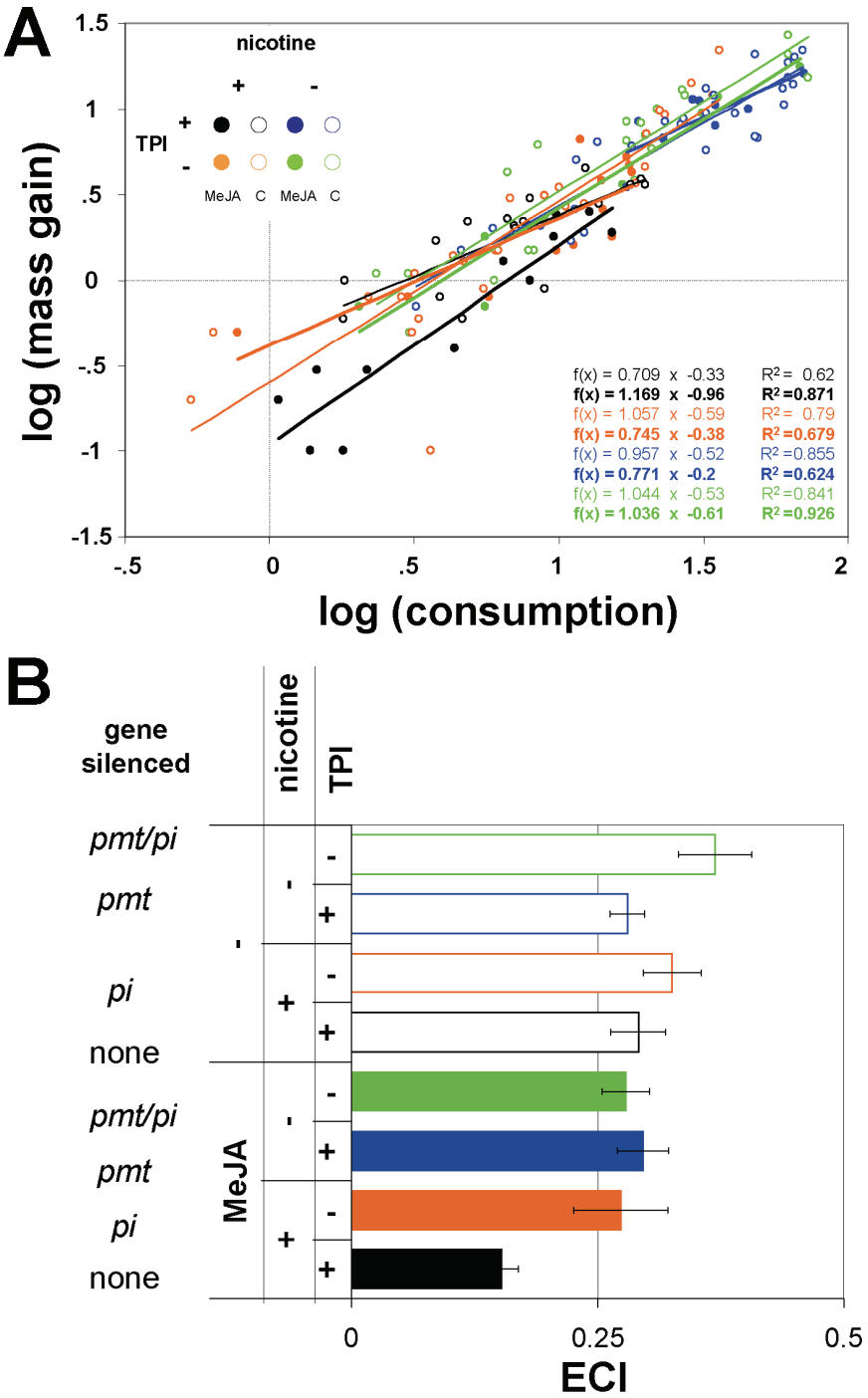

Figure 5: (**A**) Utilization plot showing the mass gain relative to consumption for a 24 h-period of each individual *Spodoptera exigua* larvae feeding either on MeJA-elicited (closed points) or unelicited (open points) wild-type (WT) *N. attenuata* (black), or plants deficient for nicotine (blue), trypsin protease inhibitors (TPIs; orange), or both (green) due to inverted repeat silencing of *putrescine N-methyl transferase* (*pmt*) and *protease inhibitor* (*pi*). The lines represent the linear regression for the mean of each treatment genotype combination. (**B**) Mean (± SE) of the efficiency of conversion of ingested food (ECI = mass gain per mg consumed) of these *S. exigua* larvae.

(Steppuhn *et al.* 2004), which is the precursor of the nicotine's six-membered ring. Other secondary metabolites (Supplemental Fig. 2) -- the phytohormones (SA, JA, JA-Ile/-Leu) that elicit them, the total protein content, which can considerably influence insect performance, and plant morphology -- were not affected by *pmt* and/or *pi* silencing (Fig. 2, Supplemental Fig. 3). Hence, these isogenic transgenic plants, which differ only in the production of the targeted resistance traits, allowed us to examine the effects of nicotine and TPI production and their interaction on larval performance.

Plants are attacked by adapted specialist herbivores as well as by generalists; the latter are thought to be more susceptible to taxon-specific (qualitative) plant defenses but not to commonly employed (typically quantitative) plant defenses (Feeny 1976; Rhoades & Cates 1976). In its natural habitat, *N. attenuata* is attacked by the tobacco specialist *M. sexta* and the generalist herbivore *S. exigua*. In the *S. exigua* feeding trials, larvae gained less mass on WT plants than on isogenic lines silenced for either TPIs (IR*pi*) or nicotine (IR*pmt*; Fig. 4A). The 2-fold greater mass of *S. exigua* larvae feeding on leaves of TPI-deficient plants is in the range of that observed for the specialist herbivore *M. sexta* feeding on TPI-deficient plants (Zavala *et al*. 2004a). In contrast, when *S. exigua* fed on nicotine-deficient plants, they increased their mass four-fold compared to WT-fed larvae; *M. sexta*, on the other hand, which has evolved a strong resistance to this genus's specific alkaloid (Glendinning 2002), increased mass only 1.5-fold when feeding on the same isogenic lines (Steppuhn *et al.* 2004). In field experiments, IR*pmt* plants received twice as much leaf damage as WT plants and about half of that damage resulted from *S. exigua* attack (Steppuhn *et al.* 2004). *S. exigua* suffered high mortality rates on *N. attenuata*, which was significantly reduced by the silencing nicotine (Fig. 3A). Thus, although both secondary metabolites function defensively against specialist and generalist herbivores, the latter is clearly more susceptible to the alkaloid.

Whereas nicotine by itself strongly affects *S. exigua* larvae, TPIs alone are ineffective, which is consistent with the ability of larvae to adapt to TPIs [e.g. by producing PI-insensitive protease activity in their midguts (Jongsma et al. 1995; Lara et al. 2000)]. However, when TPIs are expressed in concert with nicotine, *N. attenuata*'s endogenous TPIs clearly functioned defensively against this herbivore. Thus, the resistance effect of TPIs depends on the production of nicotine and this interaction is also evident from the amount of leaf material larvae consumed: more leaf mass was consumed on TPI-containing plants but only if nicotine was not present (Fig. 4B).

Table 1: Analysis to detect the effects of nicotine, TPIs, MeJA-elicitation and their interactions on *Spodoptera exigua* feeding on *Nicotiana attenuata* (A) 3-way repeated measures ANOVA of larval mass (from day 6-10), (B) 3-way ANCOVA of consumption during 24h from day 7-8 with larval mass of day 7 covariate (to account for caterpillar size), and (C) 3-way ANCOVA of the larval mass gain from day 7-8 with consumption as covariate. The 3 factors are nicotine (wild-type level or silenced), trypsin protease inhibitors (TPIs; wild-type level or silenced), and treatment (constitutive or induced with 150µg MeJA). Displayed are the degree of freedom (DF), the sum of squares (SS – adjusted (type I) or not (type III)), mean squares (MS), F- and *P*-values and significant differences are indicated by a bold "s".

	DF	**SS**	**MS**	**F**	***P-value***	
(a) Repeated measures ANOVA for larval mass (day 6-10)		(type I)				
Within-subjects effects						
Day	4	58.54	14.64	660.23	0.000	**s**
Day x nicotine	4	1.08	0.27	12.20	0.000	**s**
Day x TPI	4	0.70	0.02	0.79	0.534	
Day x treatment	4	0.14	0.03	1.54	0.190	
Day x nicotine x TPI	4	0.63	0.16	7.12	0.000	**s**
Day x nicotine x treatment	4	0.08	0.02	0.93	0.444	
Day x TPI x treatment	4	0.02	0.00	0.19	0.944	
Day x nicotine x TPI x treatment	4	0.02	0.01	0.25	0.910	
Error (day)	424	9.40	0.02			
Between-subjects effects						
Intercept	1	384.61	384.61	609.08	0.000	
Nicotine	1	20.48	20.48	32.43	0.000	**s**
TPI	1	0.15	0.15	0.24	0.626	
Treatment	1	0.38	0.38	0.60	0.442	
Nicotine x TPI	1	1.15	1.15	1.82	0.180	
Nicotine x treatment	1	0.29	0.29	0.46	0.500	
TPI x treatment	1	0.08	0.08	0.13	0.719	
Nicotine x TPI x treatment	1	0.37	0.37	0.58	0.448	
Error	106	66.936				
(b) *ANCOVA for mg consumption day 7-8*		(type III)				
Between-subjects effects						
Intercept	1	7.60	7.60	28.65	0.000	
TPI	1	0.10	0.10	88.94	0.284	
Nicotine	1	1.06	1.06	1.16	0.001	**s**
Treatment	1	0.04	0.44	12.35	0.473	
Larval mass on day 7	1	10.17	10.17	0.52	0.000	**s**
TPI x nicotine	1	0.59	0.59	119.03	0.010	**s**
TPI x treatment	1	0.10	0.10	6.93	0.276	
Nicotine x treatment	1	0.69	0.69	1.20	0.005	**s**
TPI x nicotine x treatment	1	0.00	0.00	8.10	0.953	
Error	125	10.68	0.09			

Table 1 continued

	DF	SS	MS	F	*P-value*	
(c) ANCOVA for larval mass gain day 7-8		(type I)				
Between-subjects effects						
intercept	1	30.55	30.55	760.45	0.000	
TPI	1	0.12	0.12	2.92	0.090	
nicotine	1	10.00	10.00	24.78	0.000	**s**
treatment	1	0.48	0.48	1185.00	0.001	**s**
consumption day 7-8	1	23.12	23.12	575.54	0.000	**s**
TPI x nicotine	1	0.01	0.01	0.26	0.612	
TPI x treatment	1	0.01	0.01	0.21	0.650	
TPI x consumption day 7-8	1	0.00	0.00	0.21	0.886	
nicotine x treatment	1	0.17	0.17	4.27	0.410	
nicotine x consumption day 7-8	1	0.00	0.00	0.27	0.869	
treatment x consumption day 7-8	1	0.02	0.02	0.47	0.490	
TPI x nicotine x treatment	1	0.20	0.20	4.91	0.029	**s**
TPI x treatmentx consumption day 7-8	1	0.18	0.18	4.57	0.035	**s**
Error	121	4.86	0.40			

Several studies have documented increased consumption by herbivores in response to the heterologous expression of PIs in different plants (De Leo *et al.* 1998; Cloutier *et al.* 2000; Winterer & Bergelson 2001; Abdeen *et al.* 2005). Compensatory feeding is a well-described response in larvae that feed on diets low in nutritional protein (Simpson & Simpson 1990, and references therein). Since TPIs are anti-digestive and reduce gut protease activity, they can also elicit compensatory consumption. *S. exigua* larvae consumed significantly less leaf material in the presence of nicotine and its elicitation by MeJA amplified this repellent effect (Fig. 4B). Nicotine is a potent antifeedant, which causes even nicotine-adapted herbivores to curtail consumption (Parr & Thurston 1972; Appel & Martin 1992; Voelckel *et al.* 2001). That it repels herbivores from feeding on *N. attenuata* is clear because silencing nicotine leads to attack from herbivores not normally found on this plant (Steppuhn *et al.* 2004). Moreover, nicotine in the nectar of *N. attenuata* flowers also functions as an antifeedant to floral visitors (Kessler & Baldwin 2007). *S. exigua* larvae on nicotine-containing plants did not increase their consumption in response to TPIs. In short, these results demonstrate that by limiting consumption, nicotine prevents *S. exigua* from compensating for TPIs by increasing consumption.

In response to herbivore attack, *N. attenuata* regulates various JA-mediated traits in addition to nicotine and TPI production (Halitschke & Baldwin 2003). This study genetically separated the production of nicotine from that of TPIs to examine both their individual and combined effects and how they interact with JA elicitation. Growth of *S. exigua* was not significantly reduced by the MeJA treatment. The treatment might have been too late (day 5)

to affect larval mass, because large increases in nicotine and TPI levels occur 3 to 4 days after MeJA elicitation (Pohlon & Baldwin 2001), which corresponds to days 8 and 9 in the feeding trials. Plants were not treated with MeJA on the first day of the experiment to avoid exacerbating the high mortality that this generalist herbivore normally suffers on *N. attenuata*. However, MeJA-elicited effects were clearly discernable in the generally lower larval masses on treated plants at the end of the experimental period (Fig. 4A), and in the reduced consumption by larvae feeding on nicotine containing leaf material (WT and IR*pi*; Fig. 4B). The mass gain was dependent on the amount consumed but additionally, it was influenced by the overlapping effects of nicotine, TPIs, and MeJA treatments (Table 1C). ECI increased when plants were minimally defended (no JA-mediated defenses present) and decreased if all JA-mediated defenses were present (Fig. 5B). That increasing nicotine or TPIs by MeJA elicitation in IR*pi* and IR*pmt* lines did not decrease ECI but their individual constitutive production did (because ECI was higher on constitutive IR*pmt/pi* plants) demonstrates that simply producing more of one defense is not as effective as eliciting a mixture of defenses. Generalist insects are confronted with nutritionally different foods and have evolved compensatory responses which allow them to adapt to anti-digestive plant defenses and nutritionally variable foods (Simpson *et al.* 1995). Adding antifeedants with different modes of action can help plants to reduce herbivores' ability to compensate.

The results from this study are consistent with the hypothesis that the ability to produce many different metabolites is adaptive due to synergistic interactions among defenses. These synergistic interactions can result from incompatibilities in the compensatory responses elicited in the herbivores as they adjust their physiologies in an attempt to adapt to individual defenses. Synergistic interactions are rarely studied but might well be the rule rather than the exception (Dyer *et al.* 2003; Berenbaum 1985). The fact that secondary metabolites interact, regardless of whether they are synergistic or antagonistic, underscores the difficulty of inferring a defensive function of a trait as it is transferred from one particular chemical environment to another. For example, heterologously expressing a TPI gene from tomato in tobacco to examine its defensive properties may not accurately reflect the defensive value of the TPI gene in tomato.

This study demonstrates that two of the most important secondary metabolites of *N. attenuata* are functionally linked by the compensatory responses of its herbivore and highlights the importance of studying plant defenses in native systems with an intact chemical background and the plant’s natural enemies.

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Supplemental Material

Supplemental Figure 1: Southern blot analysis demonstrates that each of the 2 independently transformed homozygous lines per genotype harbored a single insertion of the transgene. Both genotypes are inverted repeat silenced (IR) for the *pi* gene in either WT *Nicotiana attenuata* using the construct pSOL3PI or with pSOL4PI in a line previously silenced for nicotine (A03-108). Genomic DNA and the plasmids used for transformation were EcoRV digested (plasmids additionally with XhoI), separated by electrophoresis and blotted onto nylon membranes. The blot was hybridized with a PCR fragment specific for the selective marker genes on the respective T-DNA, namely *hygromycin phosphotransferaseII* (pSOL3PI) and *sat-1* gene (pSOL4PI).
Found in the Appendices.

Supplemental Figure 2: Levels (mean ± SE) of different secondary metabolites in wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) silenced for *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. (**A**) nicotine (**B**) anatabine, (**C**) anabasine, (**D**) caffeoylputrescine, (**E**) chlorogenic acid, and (**F**) rutin. Plants were grown in the glasshouse (same conditions as in the feeding trials) and were either unelicited (white bars) or elicited with jasmonic acid isoleucine conjugate (black bars) and harvested after 4 days (N = 5-7). T-tests were used to test for significant differences between each transformed line and WT. Significant differences to WT at $P < 0.05$ are indicated by asterisks.
Found in the Appendices.

Supplemental Figure 3: Constitutive (white bars) and induced (black bars) levels (mean ± SE) of different secondary metabolites in wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) silenced for *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. Levels of (**A**) jasmonic acid conjugates to isoleucine and leucine JA-Ile + JA-Leu and (**B**) SA (N = 3) were determined in leaves of untreated control plants and plants elicited with *M. sexta* oral secretion 30 min prior to being harvested for analysis. (**C**) Levels of an abundant diterpene glycoside in unelicited and methyl jasmonate (MeJA)-elicited plants. Plants were hydroponically grown in the climate chamber. T-tests did not detect significant differences between each transformed line and WT ($P < 0.05$).
Found in the Appendices.

Supplemental Table 1: Comparisons (unpaired *t*-tests; α adjusted according to sequential Bonferroni procedure) of levels of secondary metabolites between wild type (WT) *Nicotiana attenuata* and each of 2 independently transformed lines (1 & 2) silenced for either *putrescine N-methyl transferase* (*pmt*), *protease*

inhibitor (*pi*), or both. Plants were hydroponically grown in the climate chamber and phytohormones (jasmonic acid – JA, salicylic acid – SA, and JA conjugates) were analyzed in unelicited plants and plants that were elicited with *Manduca sexta* oral secretions 30 min prior to analysis. All other metabolites were analyzed in control plants and plants that were treated with methyl jasmonate 4 days prior to analysis.
Found in the Appendices.

Supplemental Table 2: Comparisons (unpaired *t*-tests; α adjusted according to sequential Bonferroni procedure) of levels of secondary metabolites between wild type (WT) *N. attenuata* and each of 2 independently transformed lines (1 & 2) silenced for either *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. Plants were grown in the glasshouse under same conditions as the *Spodoptera exigua* assay and secondary metabolites were analyzed in control plants and plants that were treated with jasmonic acid conjugated to isoleucine 4 days before.
Found in the Appendices.

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Manuscript IV

Silencing Jasmonate (JA) Signaling and JA-Mediated Defenses Reveals Different Survival Strategies Between Two Ecotypes of *Nicotiana attenuata*

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Abstract

To determine the role of ecotypic variation in plant defenses, we transgenically silenced the biosynthetic genes for nicotine, trypsin proteinase inhibitors (TPI), and jasmonate (JA) production in two ecotypes of *Nicotiana attenuata*: Utah (UTE) with JA-induced nicotine and TPI production and Arizona (AZE), which is TPI-deficient. Transient JA-silencing of both wild-types increased *Manduca sexta* larval growth, but not on TPI-deficient UTEs or nicotine-deficient AZEs, demonstrating that JA-mediated resistance is TPI-dependent in UTE and nicotine-dependent in AZE. When transplanted into a native Utah population, AZEs and UTEs, rendered equally able or unable to produce nicotine and TPIs by transformation, received ecotype-specific herbivory. Resistance to rare voracious herbivores was higher in AZEs than UTEs, and nicotine-dependent, while resistance to small abundant herbivores was higher in UTEs and TPI-dependent. AZEs were fitter than UTEs, independently of TPI production costs. These two ecotypes have developed different defenses which differ in the cost-benefit functions.

Keywords: plant defense, synergistic interaction, secondary metabolites, trypsin proteinase inhibitors, nicotine, costs of defenses, genotypic plasticity

Introduction

Native plants persist in a world full of herbivores, relying on survival strategies which include toxic or antidigestive direct defenses, indirect defenses that attract predators of herbivores, and tolerance mechanisms. Variation in survival strategies may itself be adaptive and comprises both plasticity of a single genotype and polymorphisms among individuals and populations. Plasticity, exemplified by inducibility of many plant defenses, allows individual plants to adapt to changing environments (Agrawal 2001). Polymorphisms in antiherbivore resistance traits may result from varying selection pressures among populations or from diversifying selection, which may constrain the herbivores' ability to adapt to plant defenses (Meyers *et al.* 2005). The native tobacco *Nicotiana attenuata* Torr. Ex Wats. produces diverse secondary metabolites, most of which are inducibly elicited via the jasmonate (JA)-signaling pathway (Halitschke & Baldwin 2003; Roda & Baldwin 2003). Several *N. attenuata* populations across the southwestern USA vary markedly in their production of anti-herbivore defenses (Glawe *et al.* 2003). Because the adaptive value of particular traits is best determined

in isogenic lines differing only in the gene(s) coding for a given trait (Bergelson *et al.* 1996), we used gene silencing to determine the contribution to anti-herbivore resistance and plant fitness of different JA-mediated defense traits that vary among ecotypes of *N. attenuata*.

Evidence for the defensive value of JA-mediated traits comes from the increased resistance to native herbivores of *N. attenuata* in wild populations in Utah when elicited with the methyl ester of JA (MeJA), and the diminished resistance of plants genetically silenced for the expression of the *13-lipoxygenase* gene (*lox*) of JA biosynthesis, and of *Coronatine insensitive1* (*Coi-1*), an F-box protein essential for JA perception (Baldwin 1998; Kessler *et al.* 2004; Paschold *et al.* 2007). Much of the JA-mediated resistance in the ecotype from UT (UTE) is attributable to the elicitation of the toxic alkaloid nicotine and antidigestive trypsin proteinase inhibitors (TPIs), as demonstrated by the independent silencing of their biosynthetic genes: *putrescine N-methyltransferase* (*pmt*), and *pi* (Steppuhn *et al.* 2004; Zavala & Baldwin 2004; Zavala *et al.* 2004a). However, an ecotype of *N. attenuata* collected from a population in Arizona (AZE) does not produce TPIs due to a nonsense mutation in the *pi* gene (Glawe *et al.* 2003; Wu *et al.* 2006). That TPIs can also function defensively in the AZE is evident from increased resistance to *Manduca sexta* when TPI activity is restored in AZEs by expressing *pi* in a sense orientation (Zavala *et al.* 2004a; Zavala *et al.* 2004b). In addition to this direct defense, the AZE does not emit the sesquiterpene *cis*-α-bergamotene in response to herbivore attack. *Cis*-α-bergamotene is among the most prominent volatile compounds produced by herbivore-attacked UT plants and can reduce herbivore load by over 90% through the attraction of predatory insects (Halitschke *et al.* 2000; Kessler & Baldwin 2001). This raises the questions: how does the AZE survive without these potent direct (TPI) and indirect (*cis*-α-bergamotene) defenses, and does it employ other adaptive strategies to offset these deficiencies?

A defense trait is adaptive only if its benefits exceed its costs (Strauss *et al.* 2002). Physiological costs of defenses arise from allocating resources and energy normally used for growth and reproduction to plant resistance traits. TPI production is costly in the absence of herbivores for both ecotypes: silencing *pi* in the UTE increased plant fitness, measured in seed production, whereas restoring TPI activity by sense-expressing *pi* in the AZE reduced fitness (Zavala *et al.* 2004b; Zavala & Baldwin 2006). That the AZE realized a higher fitness when competing with the UTE for resources is consistent with a high allocational cost of TPI production (Glawe *et al.* 2003). If the benefits of TPI production do not outweigh its costs in the AZ population, perhaps due to an altered herbivore community, loss of TPI function might

be adaptive. The AZE may employ other defense traits that are less costly than those in the UTE, but effective against herbivores present in its own environment.

The release of volatiles likely exacts only moderate allocation costs (Halitschke *et al.* 2000; Kessler & Baldwin 2001), but other costs may select for the AZE's inability to elicit *cis*-α-bergamotene release, for example if herbivores co-opt such volatile signals when foraging for hosts (Takabayashi & Dicke 1996; Halitschke *et al.* 2007). The loss of TPI and *cis*-α-bergamotene production could be also functionally linked in the AZE, e.g. if the resistance value of TPIs in nature depends on attracting predators (Glawe *et al.* 2003). TPIs retard herbivore growth, thereby prolonging insects' smaller stages and making them easier prey; but if they remain on a TPI-producing plant, many lepidopteran herbivores compensate for dietary PIs by increasing consumption, as is known for one native herbivore of *N. attenuata* (Steppuhn & Baldwin 2007). The major predator in *N. attenuata*'s habitat, *Geocoris pallens,* can kill only the eggs and the first two instars of *M. sexta* (Kessler & Baldwin 2001) and thus the AZE's inability to elicit *cis*-α-bergamotene might have reduced the benefits of TPI production. However, the AZE may also have evolved other traits to attract predators. In this study we investigate whether the AZE is less-defended than the UTE, whether the cost-benefit-relation of TPI production is altered to be more costly in the AZ ecotype, and whether AZ evolved alternative defensive traits to compensate for its lack of TPI and *cis*-α-bergamotene.

To determine whether the AZE employs JA-mediated direct defenses different from those in UTE, we transiently silenced *lox* while also stably silencing *pi* and/or *pmt* in both ecotypes and examined *M. sexta* larval performance on *lox*-silenced plants versus empty vector (ev)-treated control plants in a controlled laboratory setting. To examine ecotypic differences in resistance that are independent of TPI and nicotine production, we planted pairs of AZ and UT plants which had been equalized for TPI and nicotine production by stable transformation with either sense (S) overexpression or inverted-repeat (IR) silencing constructs into a native population in Utah and documented levels of herbivory, herbivore predation, and reproductive performance in a double-blind study. Our results show that the two direct defenses contribute differently to JA-mediated resistance to *M. sexta* in both ecotypes, which also differed in their resistance to native herbivores in the field, even when equalized for nicotine and TPI production. Predation rates on *M. sexta* eggs and larvae did not differ between ecotypes, indicating that AZE has other signals that offset the lack of *cis*-α-bergamotene. The AZE was significantly fitter than the UTE independent of the costs of TPI

production, even within the native Utah population. The two ecotypes have thus developed alternative defense strategies against herbivores.

Materials and Methods

Plant material & stable transformation. We used inbred lines of UT and AZ wild-type (WT) *N. attenuata* (synonymous with *N. torreyana* Nelson and Macbr.; Solanaceae) derived from field-collected seeds (Baldwin 1998; Glawe *et al.* 2003) for experiments and transformation. Seed germination, glasshouse growth conditions, and the *Agrobacterium tumefaciens* (strain LBA 4404)-mediated transformation procedure are described in Krügel *et al.* (2002). For the field experiment, seedlings were transferred to 50 mm peat pellets (Jiffy, Shippegan, New Brunswick, Canada) 15 days after germination and gradually hardened to environmental conditions (high sun exposure and low relative humidity) over 2 weeks. Small, adapted, rosette-stage plants of equal size were transplanted into a Utah population and watered every other day for two weeks until roots were established in the native soil. We used plants stably transformed to silence expression of the *N. attenuata pmt* genes in the AZE and UTE or the *pi* gene alone and together with *pmt* in the UTE; additionally, we used AZE transformed to express *pi* (for details see Protocol S1).

Virus-induced gene silencing (VIGS) of lox. In climate chambers (for conditions see Protocol S1), early rosette-stage plants of either WT AZE, WT UTE, and IR*pi* UTE (experiments 1 & 2) or IR*pmt* AZE and IR*pmt/pi* UTE (experiment 3) were pressure-injected with *A. tumefaciens* (strain GV3101) harboring either the *lox*-silencing vector pTVLOX3 (Lou & Baldwin 2006) or the empty vector (ev) pTV00. A *phytoene desaturase* (*pds*)-silencing construct (pTVPDS5) that causes photobleaching was used as a visible positive control for the VIGS process. Inoculation and constructs are described in Saedler & Baldwin (2004).

Successful *lox* silencing by VIGS was verified (N = 5) by determining *lox* transcripts (experiments 1 and 2) and/or JA levels (experiments 2 and 3) in unelicited and elicited leaves 1 h after wounding with a pattern wheel and the addition of either distilled water or *M. sexta* oral secretion (OS) diluted 1:4 with distilled water as described in Halitschke *et al.* (2001). Either the second fully-expanded source leaves (+2) were harvested from extra plants at the start of the *M. sexta* performance assay (experiments 1 and 3) or the youngest unattacked rosette leaves were harvested 1h after wounding or OS treatment from the same plants used for the performance assay on day 3 following the removal of caterpillars (experiment 2).

Control samples were taken prior to elicitation (+3 leaves) and to caterpillar feeding (+ 2 leaves for phytohormone and + 1 leaves or RNA quantification). TPI activity and nicotine content were analyzed (experiments 2 and 3) in +1 leaves harvested prior to (control) and at the end of the feeding assay (elicited) in leaves that had been recently fed on (for TPI activity) or unattacked stem leaves (for root-produced nicotine). All samples were immediately frozen in liquid nitrogen and stored at -80°C until extraction.

***M. sexta* performance assay on VIGS plants.** Approximately 2 weeks after inoculation, when VIGS*pds* plants had thoroughly bleached, *M. sexta* neonates were placed on +1 leaves (N = 20-30 per genotype-VIGS vector combination, see Fig. 1A). Larval mass was determined on days 3, 9, and 12. Due to larval movement off the plants and mortality, replicate number decreased during the assay: by the end of the feeding assay (day 12), 7 to 19 larvae remained within each treatment group in all experiments. Plants were randomized spatially in the growth chamber and temporally in placement and weighing of larvae.

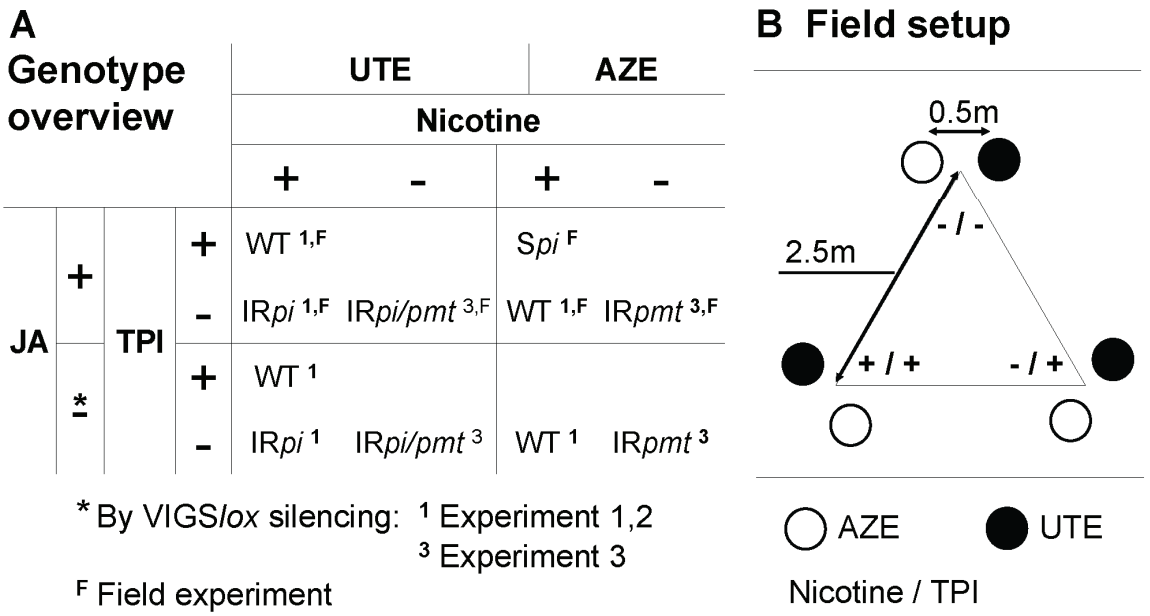

Figure 1: (A) Overview of the genotypes used to investigate the role of nicotine and trypsin protease inhibitors (TPI) in two ecotypes of *Nicotiana attenuata* from Utah (UTE) and Arizona (AZE) that differ in their jasmonic acid (JA)-mediated defenses. In three experiments the regulatory enzyme of JA biosynthesis (*lox*) was transiently silenced by virus-induced gene silencing (VIGS) in wild-type (WT) AZEs and UTEs and lines stably transformed to silence nicotine and TPI production by inverted repeated (IR) silencing of their biosynthetic genes (*pmt* and *pi*) or to express TPIs in AZEs by using a sense (S) construct. (B) The same genotypes were examined for differences between ecotypes in herbivory, predation, and fitness levels in a field experiment, in which AZ and UT plants were transplanted in pairs that produced either nicotine and TPIs, only nicotine, or neither nicotine nor TPIs.

Northern blot and metabolite analysis. RNA was extracted with Tri Reagent (Chomczynski 1993) and 15 μg of total RNA per sample were separated by gel electrophoresis and blotted onto nylon membranes as described in Wu *et al.* (2006). Levels of *lox* transcripts were detected by hybridizing the blot with a α-^{32}P-dCTP-labeled PCR fragment of *N. attenuata lox3* (Protocol S1).

Phytohormones were extracted from 300 mg frozen tissue with 1 mL ethyl acetate containing isotope-labeled internal standards for JA and SA (200 $ng^{-1}mL^{-1}$) and analyzed by LC-MS as previously reported (Schmidt & Baldwin 2006). TPI activity relative to protein content as determined by a microwell Bradford assay was examined in protein extracts of 100 mg tissue using a radial diffusion assay, described in van Dam *et al.* (2001). Nicotine, chlorogenic acid, rutin, and two diterpene glycosides (DTGs) were analyzed from extracts of 150 mg tissue with an Agilent HPLC system according to Keinänen *et al.* (2001). Nicotine, chlorogenic acid, and rutin were quantified based on external standard curves (in mg g^{-1} fresh leaf mass) and DTGs were quantified by peak area relative to fresh leaf mass, as synthetic standards are unavailable.

Field experiments. Twenty replicates consisting of three UTE-AZE pairs (WT UTE vs. S*pi* AZE, IR*pi* UTE vs. WT AZE, and IR*pmt/pi* UTE vs. IR*pmt* AZE) were transplanted (5/6/2005) into a natural *N. attenuata* population near Santa Clara (Utah) on a wildfire burn from 2004 (Fig. 1B). Plants of a pair were matched for size. The positions of pairs in the triangular planting formation and of plants in a pair were randomized by a coin toss. Plant labels were hidden in soil to allow blind data acquisition. Relative leaf area damage by herbivores was examined by a researcher not involved in the planting as percentage of canopy on May 9, 21, 25 and 31, as well as on June 6 and 12. Measurements from the latter two days included damage from *M. sexta* larvae which had been applied to examine predation rates. Characteristic damage caused by lepidopteran larvae (present in this season were *Spodoptera exigua* Hübner, *M. sexta* and *Manduca quinquemaculata* Haworth, *Agrotis* spp.), flea beetles (*Epitrix hirtipennis* Melsheimer and *Epitrix subcrinita* Le Conte), grasshoppers (*Trimerotropis* spp), and mammals (rabbits or ground squirrels) was recorded separately. Damage from sucking herbivores including leafhoppers (among others *Empoasca spp.*), mirids (*Tupiocoris notatus* Distant), and thrips (Thysanoptera) was estimated ranging from 0 (no attack) to 3 (heavily attacked). Because newly transplanted plants were heavily damaged by grasshoppers, they were protected with metal cages (ø = 10 cm, 20 cm high, 1 cm mesh size) from May 9 to 21. On June 6, abundant herbivores and predators (spiders and predatory bugs such as *Geocoris pallens* Stäl, and Reduvidae) present on the plants were counted.

Predation rates on *M. sexta* first-instar larvae and eggs were determined in 2 and 3 repetitions, respectively. On June 2, one larva per plant and on June 6, two larvae were applied to the first stem leaf of each plant and their survival was recorded after 24, 48, and 72 h. On three subsequent evenings (June 16-18) 4 eggs were glued to the underside of the first stem leaf and 24 h later the number of predated eggs was examined (Kessler & Baldwin 2001).

On May 21, rosette diameter and stalk elongation, and on May 25 and 29 and June 21 stalk elongation was measured. Because plant health differed, health condition was recorded on June 12 on a scale ranging from 0 (healthy: green, straight leaves) to 5 (sick: yellowish, wilting leaves). On June 21 all plants were examined for the number and length of basal side branches, and number of flower buds, flowers, and capsules.

Data analysis. Data were graphically inspected for normality and variance homogeneity, transformed if required, and analyzed with Statview 5.0 (SAS Institute, Cary, NC, USA). Square root transformed levels of phytohormones, and untransformed TPI and nicotine levels of VIGS-treated plants were analyzed by ANOVAs and untransformed *M. sexta* growth by repeated-measures ANOVAs. Square root transformed herbivory and fitness data and untransformed growth data were analyzed by paired *t*-tests between UTE and AZE for each pair type and between ecotypes and pair types with 2-way ANOVAs. Untransformed survival rates of *M. sexta* larvae and eggs applied to UTE and AZE plants were compared by paired *t*-tests.

Results

Characterization of transformants. Two independent IR*pmt* AZE lines were successfully silenced for *pmt* transcript accumulation, resulting in >85 %-reduced nicotine levels compared to WT; levels of other secondary metabolites measured were not altered (Protocol S1, Fig. S1). Successful silencing of *pi* and *pmt* transcripts in IR*pi* UTE and IRp*mt/pi* UTE plants and levels of phytohormone levels, total protein content, and secondary metabolites similar to those of WT plants has been shown in Steppuhn & Baldwin (2007). (The only secondary effect was the accumulation of the alkaloid anatabine in all *pmt*-silenced lines.) Stable silencing of nicotine and TPI was not affected by VIGSev treatment: there was no TPI activity in VIGSev IR*pi* UTE and, additionally, no increase of nicotine in VIGSev IR*pmt* AZE and IR*pmt/pi* UTE plants in experiments 2 and 3.

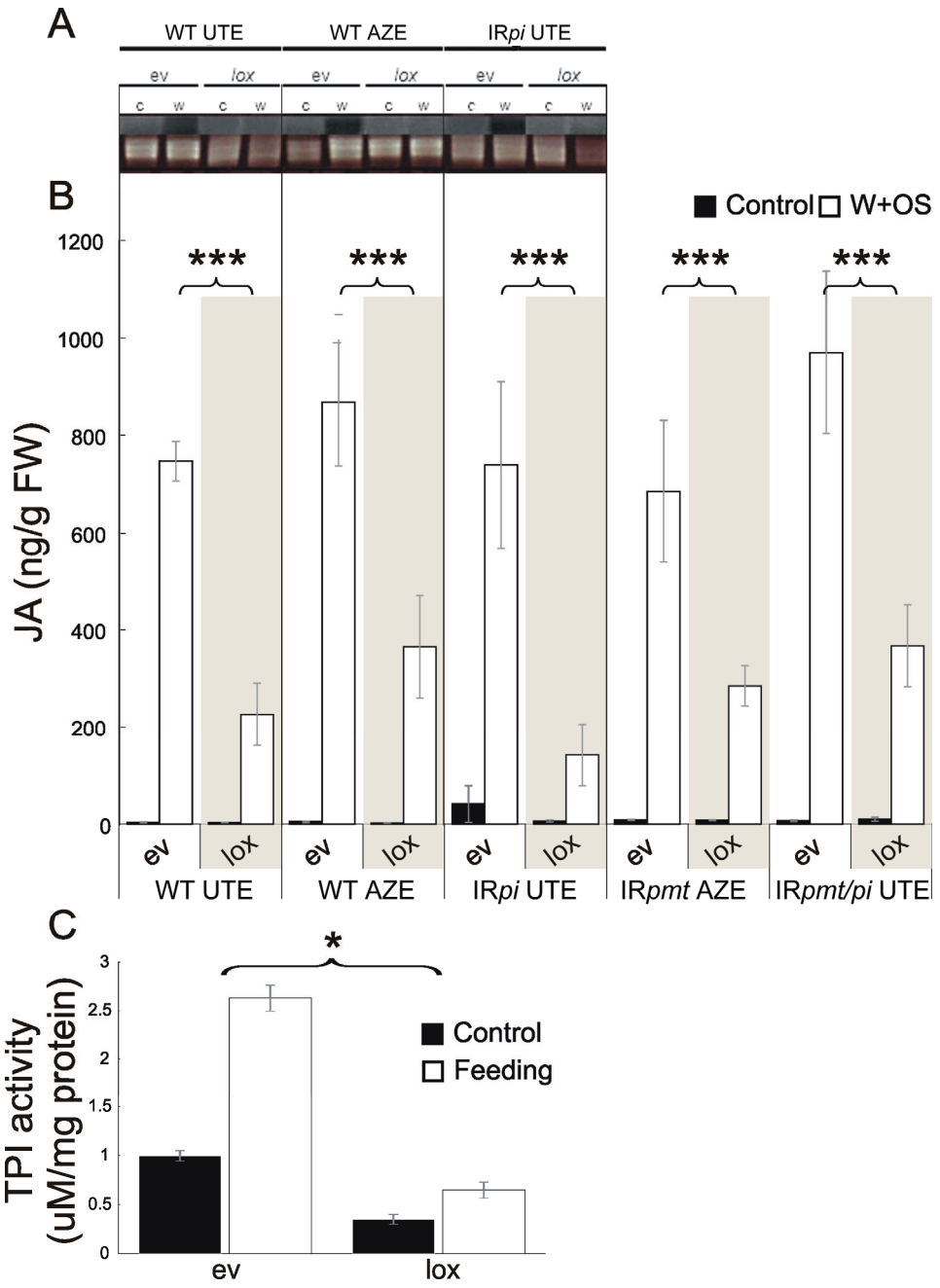

Figure 2: Silencing *13-lipoxygenase* (*lox*) of the jasmonate-signaling cascade by VIGS. Empty vector control plants (ev) and *lox*-silenced plants were left untreated (c/Control), or treated with wounding and the addition of either water (W; for transcript analysis, because *lox* expression is not amplified by oral secretions) or *Manduca sexta* oral secretions (W + OS) and harvested after 1 h. (A) Northern blot analysis shows no detectable *lox* transcripts (top) in VIGS*lox* treated plants (bottom: ethidium bromide-stained total RNA as loading control). (B) W + OS-induced JA levels (mean ± SE) were reduced in *lox*-silenced plants. (**C**) Activity of trypsin protease inhibitors (TPI, mean ± SE) in VIGS-treated WT UTEs either prior to or following 12 days of feeding by *M. sexta* (N = 3-5). Asterisks signify significant differences between the VIGS vectors (ev or *lox*) of ANOVAs on JA levels at $P < 0.001$ (***, separate 3-way ANOVAs with the factors vector, treatment, and genotype: for Experiment 2: vector $F_{1, 59} = 54.43$, vector * treatment $F_{2, 59} = 12.84$; for Experiment 3: $F_{1, 48} = 21.75$, vector * treatment $F_{2, 48} = 10.65$; no significant effect of genotype) and of unpaired *t*-tests on TPI activity at $P = 0.002$ (*, effect of vector after feeding: $t_{6\ df} = 5.21$; before feeding $P = 0.06$: $t_{8\ df} = 2.18$).

Transcripts of *lox* were markedly reduced in VIGS*lox* compared to VIGSev plants (Fig. 2A), as were wound- and OS-elicited levels of JA (Fig. 2B). Silencing was maintained throughout feeding because *lox* transcripts and JA were reduced irrespective of whether samples were taken at the beginning or 3 days after the end of the caterpillar feeding assay. JA conjugates to isoleucine and leucine were also reduced by silencing *lox* (Protocol S1).

Diminished JA levels in VIGS*lox* WT UTE plants significantly reduced TPI activity induced by *M. sexta* feeding and marginally reduced constitutive levels of TPI in plants at the beginning of the feeding assay (Fig. 2C). In post-feeding samples of VIGS*lox* plants, nicotine levels of all nicotine-producing genotypes tended to be reduced (2-way ANOVA for experiment 2: vector $F_{1, 18} = 2.26$, $P = 0.15$; genotype not significant).

Effects of silencing JA-mediated defenses in AZEs and UTEs on *M. sexta* performance. Silencing *lox* by VIGS significantly increased growth of *M. sexta* larvae that fed on WT UTEs and WT AZEs, but not on IR*pi* UTEs (Table 1A, Fig. 3A-B). Whereas experiments 1 and 2 show the effects of ecotype and TPIs on JA-mediated resistance to *M. sexta*, experiment 3 adds the impact of nicotine (in the absence of TPIs). Silencing nicotine eliminated the effect of VIGS*lox* on *M. sexta* performance in the AZE (Table 1B, Fig. 3C). Caterpillar growth varied by experiment, but the effect of silencing *lox* was independent of experimental conditions as the only significant interaction term with experiment was larval growth (day), not vector (Table 1A).

Field herbivory on UTEs and AZEs equalized for nicotine and TPI production. Seasonal average herbivory levels were low, with only 4-6 % canopy loss to herbivores, and differed significantly between UTEs and AZEs producing nicotine and TPIs (Fig. 4A). Relative leaf area loss varied throughout the season as did the herbivore group responsible for the majority of the damage. Three days after plants were transplanted, damage by *Trimerotropis* grasshoppers was very high. To prevent loss of replicates during establishment, plants were protected with cages until May 21, decreasing damage to basal levels. Damage then increased through the end of May due to browsing mammals and in June due to flea beetles (mainly *E. subcrinita*) and lepidopteran larvae (*M. sexta* and *Agrotis* ssp.), reaching a maximum of 12% canopy loss on June 12 (Fig. 4B).

Herbivores responded differently to the two ecotypes and their defense traits. Grasshoppers tended to prefer UTE over AZE plants; this preference was significant in the pair producing nicotine and TPIs, which received the greatest attack from this herbivore, and the same tendency was observed in the pair producing only nicotine (Fig. 4C). Because only a few plants were attacked by mammals, which resulted in very high leaf area loss, this damage

Table 1: ANOVA tables for *Manduca sexta* growth on *Nicotiana attenuata* silenced for jasmonic acid production by virus-induced gene silencing (VIGS) of its *13-lipoxygenase* (*lox*) and on plants treated with empty vector control (ev) in (a) experiments 1 and 2 and (b) experiment 3. The factors are genotype and VIGS vector and additionally experiment in (a), which used the same genotypes in both experiments: an ecotype from Utah (UTE), one from Arizona (AZE), and a UTE stably silenced (via inverted repeat construct, IR) for its protease inhibitor gene (*pi*). Experiment 3 used UTEs additionally silenced for the nicotine biosynthetic genes, *putrescine N-methyl transferase* (*pmt*) and AZEs silenced for *pmt*. (c) ANOVA tables for plant growth and fitness measures of the field experiment with pairs of AZEs and UTEs that were equally able to produce nicotine and TPIs (+/+), only nicotine (+/-), or neither of the two (-/-). The factors were ecotype and pair type and interactions are only displayed if significant. Displayed are the degree of freedom (DF), the sum of squares (SS), mean squares (MS), F- and *P*-values and significant differences are bold and indicated by "**s**" or "**m**" (marginally significant).

	DF	SS	MS	F	*P – value*	
(a) ***repeated measures ANOVA for VIGS experiment 1 and 2***						
Between-subject effects						
Genotype (UTE, AZE, or IR*pi* UTE)	2	5146	2573	0.49	0.62	
Vector (ev or *lox*)	1	45484	45484	8.64	**<0.01**	**s**
Experiment (1 or 2)	1	46558	46558	8.84	**<0.01**	**s**
Genotype x vector	2	32864	16432	3.12	**<0.05**	**s**
Genotype x experiment	2	5227	2613	0.50	0.61	
Vector x experiment	1	433	433	0.08	0.77	
Genotype x vector x experiment	2	16039	8020	1.52	0.22	
Residual	161	847945	5267			
Within-subject effects						
Day (3, 9, 12)	2	1552750	776375	263.94	**< 0.01**	**s**
Day x genotype	4	6042	1511	0.51	0.73	
Day x vector	2	42709	21354	7.26	**<0.01**	**s**
Day x experiment	2	28328	14164	4.82	**<0.01**	**s**
Day x genotype x vector	4	26602	6650	2.26	**0.06**	**m**
Day x genotype x experiment	4	6819	1705	0.58	0.68	
Day x vector x experiment	2	2105	1053	0.36	0.70	
Day x genotype x vector x experiment	4	13035	3259	1.11	0.35	
Residual (day)	322	947171	2942			
(b) ***repeated measures ANOVA for VIGS experiment 3***						
Between-subject effects						
Genotype (IR*pi/pmt* UTE or IR*pmt* AZE)	1	436	436	0.17	0.68	
Vector (ev or *lox*)	1	5877	5877	2.26	0.14	
Genotype x vector	1	18	18	0.01	0.94	
Residual	52	135401	2604			
Within-subject effects						
Day (3, 9, 12)	2	400335	200167	82.06	**< 0.01**	**s**
Day x genotype	2	558	279	0.11	0.89	
Day x vector	2	7418	3709	1.52	0.22	
Day x genotype x vector	2	1073	537	0.22	0.80	
Residual (day)	104	253690	2439			
(c) ***ANOVAs for plant fitness in the field experiment***						
Rosette diameter (May 21)						
Ecotype (UTE or AZE)	1	7363.3	7363.3	14.65	**<0.01**	**s**
Pair type (+/+, +/-, or -/-)	2	1958.6	979.3	1.95	0.15	
Residual	114					
Stalk length (May 21 - 29)						
Ecotype (UTE or AZE)	1	8817.7	8817.7	1.31	0.26	
Pair type (+/+, +/-, or -/-)	2	409151.8	204575.9	30.31	**<0.01**	**s**
Ecotype x Pair type	2	44877.5	22438.8	3.32	**0.04**	**s**
Residual	100					

Table 1 continued

	DF	SS	MS	F	*P – value*	
Plant health (June 12)						
Ecotype (UTE or AZE)	1	0.41	0.41	3.44	**0.07**	**m**
Pair type (+/+, +/-, or -/-)	2	0.78	0.39	3.25	**0.42**	**s**
Residual	114					
Side branch length (June 12)						
Ecotype (UTE or AZE)	1	18.8	18.8	1.75	0.19	
Pair type (+/+, +/-, or -/-)	2	52.2	26.1	2.43	**0.09**	**m**
Residual	114					
Number of flowers (June 12)						
Ecotype (UTE or AZE)	1	5.1	5.1	1.80	0.37	
Pair type (+/+, +/-, or -/-)	2	5.7	2.9	1.01	0.18	
Residual	114					
Number of flower buds (June 12)						
Ecotype (UTE or AZE)	1	7.6	7.6	1.57	0.21	
Pair type (+/+, +/-, or -/-)	2	10.0	5.0	1.0	0.35	
Residual	114					
Number of capsules (June 12)						
Ecotype (UTE or AZE)	1	16.9	16.9	3.46	**0.06**	**m**
Pair type (+/+, +/-, or -/-)	2	29.5	14.7	3.02	**0.05**	**s**
Residual	114					

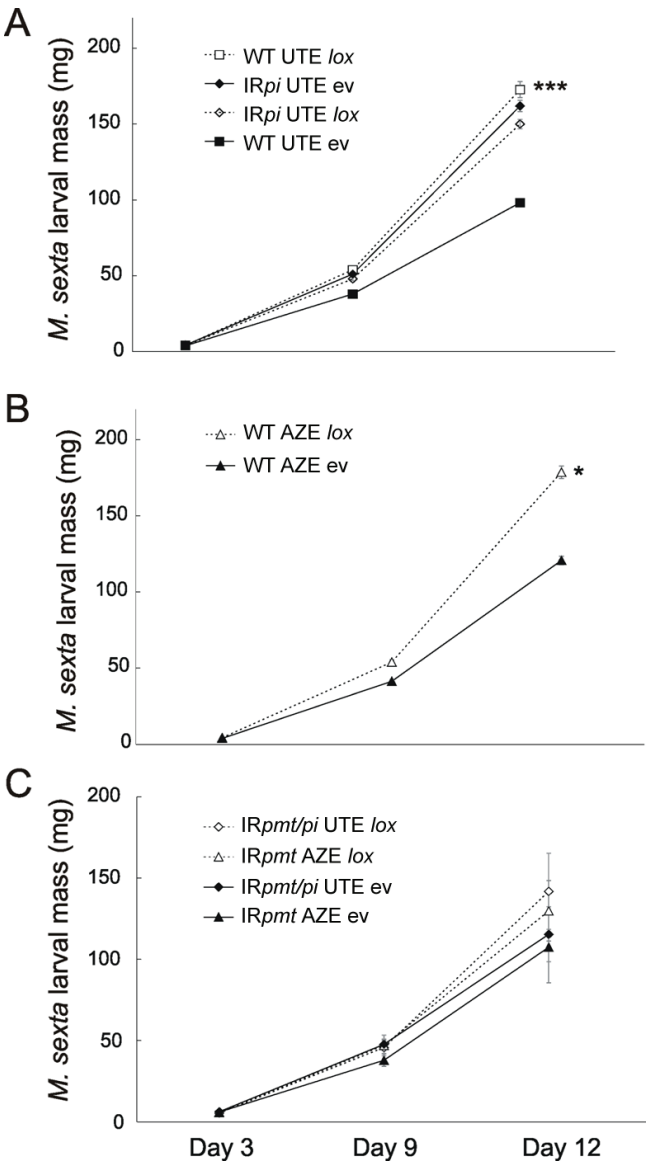

Figure 3: Larval mass (mean ± SE) of *Manduca sexta* feeding on different genotypes of *N. attenuata* ecotypes from Arizona (AZE) and Utah (UTE) that were transiently silenced for *13-lipoxygenase* (*lox*) of the jasmonate-signaling cascade by VIGS or treated with an empty vector construct (ev): (A) wild-type (WT) UTEs and UTEs stably transformed to silence the *protease inhibitor* (*pi*) gene, (B) AZE WT plants, and (**C**) nicotine-deficient AZEs and UTEs due to silencing the biosynthetic *putrscine-N methyl transferase* (*pmt*) genes (UTEs were additionally silenced for *pi*). Larval masses in (A) and (B) represent the average of two independent experiments with similar outcomes: larval mass was increased by *lox*-silencing in WT UTE and WT AZE but not in IR*pi* UTE relative to empty vector controls ($t_{58\ df} = 0.34$, $P = 0.73$). Asterisks signify significant differences between VIGS vectors (ev or *lox*) in separate unpaired t-tests at $P < 0.001$ (**; WT UT: $t_{61\ df} = 3.53$) and at $P = 0.021$ (*; WT AZ: $t_{56\ df} = 2.38$). Silencing *lox* did not significantly affect larval growth in genotypes producing neither TPIs nor nicotine.

was not statistically analyzed (data were not normally distributed nor were variances homogeneous). However, the preferences of mammals were clear: 8 of 11 attacked plants belonged to the UTE, 9 did not produce TPIs, and plants producing neither nicotine nor TPIs were damaged the most (Fig. 4D). Damage from flea beetles was significantly different between ecotypes and among the three defense genotypes (Fig. 4E). Unlike grasshoppers and mammals, flea beetles preferred AZEs over UTEs and attacked TPI-deficient but nicotine-producing plants significantly more than other pair types. Damage caused by lepidopteran larvae was not different between ecotypes or among pairs (Fig. 4F). *M. sexta* larvae applied to plants for predation assays caused most of this damage but wild *Agrotis* cutworms, which attacked only few plants (15), caused on average twice the damage on UTE compared to AZE plants in the pairs producing nicotine and TPIs or only nicotine.

Most damage caused by leaf-sucking herbivores was due to thrips, which significantly differentiated between ecotypes and among the three defense genotypes (Fig. S2). Thrips attacked primarily nicotine- and TPI-deficient plants and caused the most damage to the AZE. Only two UTE plants were among 8 plants attacked by leafhoppers and 6 by mirids; but damage levels on all plants were too low to be analyzed. There were no differences among all genotypes in the number of sucking herbivores and flea beetles observed on the plants on June 6.

Field predation on *M. sexta* applied to UTEs and AZEs. On June 6, an average of 2.7 *Geocoris pallens* individuals per plant was observed. Within 24 h 40%-60% of applied *M. sexta* larvae disappeared and within 72 h >80%. Plants were elicited by the applied larvae and the high native herbivory (Fig. 4B). However, larval survival did not differ at any time (24, 48, 72 h) between the two ecotypes within each pair in two experimental setups (all $t_{19df} < 1.6$, $P > 0.1$). The percentage of eggs remaining 24h after 4 eggs were applied per plant ranged from 14 to 43% during the three-day replication of this experiment. There were no significant differences between ecotypes in all three pairs and in three repetitions of the egg assay with the single exception of the pair producing nicotine and TPIs in the second repetition ($t_{19df} = 2.1$, $P = 0.05$). There were no significant differences in the number of predators counted on the plants on June 6.

Fitness of UTEs and AZEs in the field. AZEs had developed significantly larger rosettes than UTEs by May 21 and stalk elongation was significantly affected by pair type and its interaction with ecotype (Table 1C). Stalks elongated faster the more defenses the plants had, independent of ecotype (nicotine + TPI > nicotine alone > neither nicotine nor TPI, Fishers PLSD for all three comparisons $P < 0.01$), but in the pair producing nicotine only,

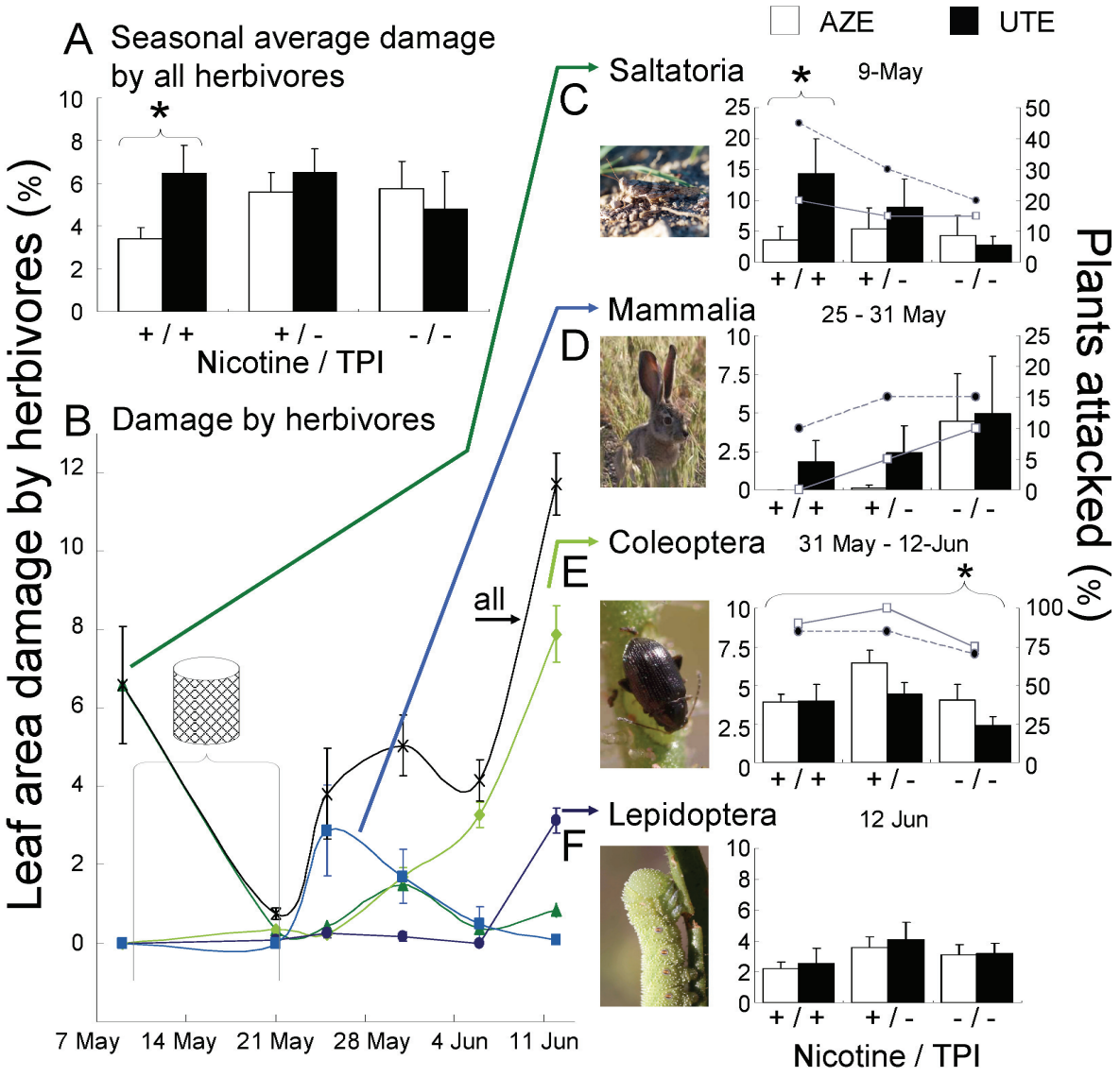

Figure 4: Herbivory rates on ecotypes of *N. attenuata* from Arizona (AZE) and Utah (UTE) planted into a wild Utah population in 2005. (A) Average leaf area lost to herbivores (mean ± SE) over the growing season in AZE-UTE pairs that were matched to produce trypsin protease inhibitors (TPI) and nicotine, only nicotine, or neither nicotine nor TPIs (via stable transformation; N = 20). (B) Leaf area lost (mean ± SE) to attack from different herbivore taxa over the growing season. (Plants were caged from May 9-21.) (C) Leaf area lost (bars and primary Y-axis) and percentage of AZEs and UTEs attacked (line charts and secondary Y-axis) by the different herbivore taxa at a time when the attack was greatest by these herbivores. (Note that scales change for better visualization.). Asterisks on brackets signify significant differences between the members of a pair analyzed by paired *t*-tests (A: $t_{19df} = 2.2$, $P = 0.039$; C: $t_{19df} = 2.6$, $P = 0.016$) and by repeated measures 2-way ANOVA in the case of coleopteran-caused damage (for ecotype $F_{1df,111} = 5.44$, $P = 0.02$, for pair type $F_{2df,111} = 4.87$, $P < 0.01$). The 2-way ANOVAs for damage caused by grasshoppers on May 9 (for ecotype $F_{1df,114} = 2.72$, $P = 0.10$, for pair type $F_{2df,114} = 1.25$, $P = 0.29$) revealed only tendencies and for damage caused by Lepidotera on June 12 (for ecotype $F_{1df,114} = 0.12$, $P = 0.73$, pair type $F_{2df,114} = 1.31$, $P = 0.27$) were not significant. (Photographs: A. Steppuhn)

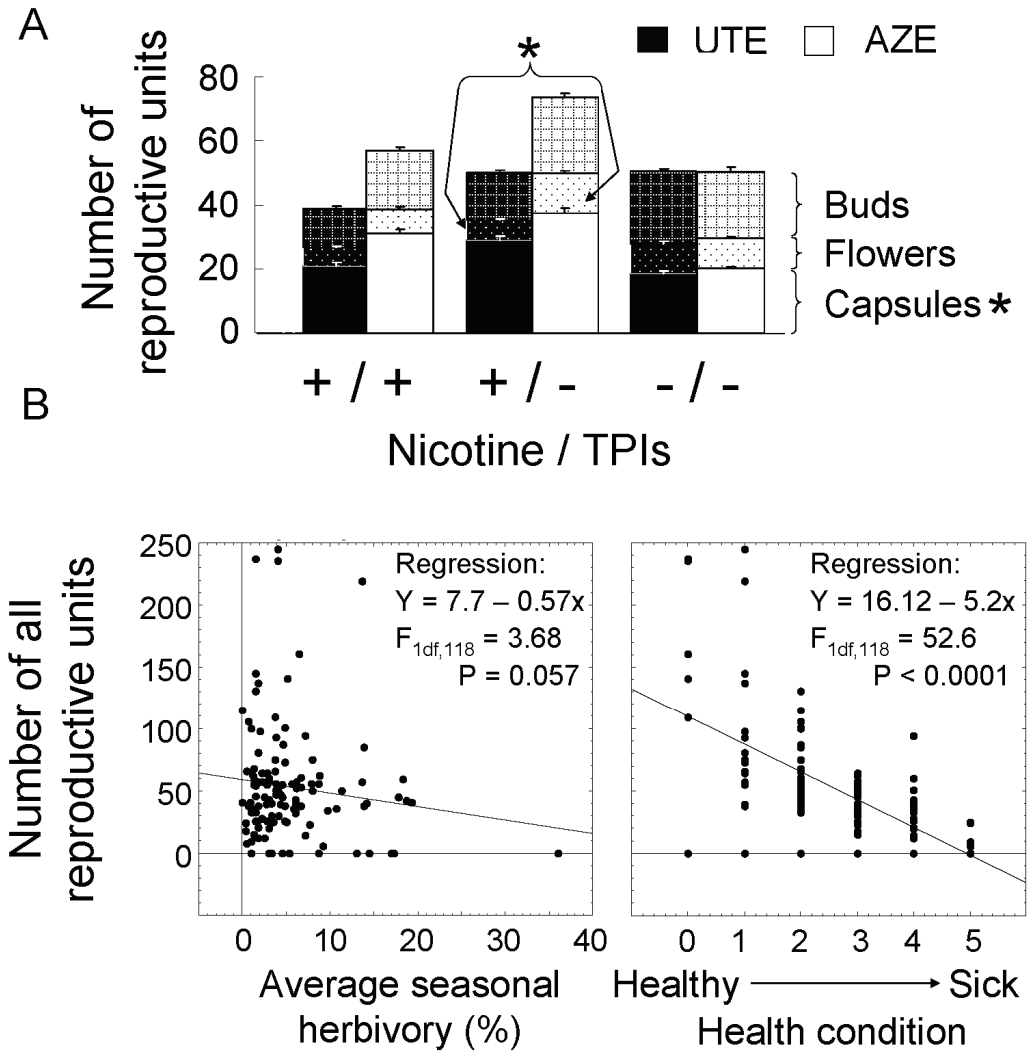

Figure 5: Mean ± SE of (**A**) reproductive units of 2 ecotypes of *N. attenuata* from Arizona (AZE) and Utah (UTE) planted in pairs that were matched by their ability to produce trypsin protease inhibitors (TPI) and nicotine, only nicotine, or neither nicotine nor TPIs (via stable transformation; N = 20) into a native Utah population. Asterisks for flowers signify a significant differences between AZEs and UTEs in the TPI-deficient pair (paired *t*-tests: $t_{19df} = 2.45$, $P = 0.05$) and, for capsules, significant main effects of ecotype and pair type (2-way ANOVA: ecotype $F_{1df,114} = 3.4$, $P = 0.06$, pair type $F_{2df,114} = 3.0$, $P = 0.05$). (**B**) Linear regression of the number of reproductive units with average seasonal herbivory (left) and plant condition (right).

UTEs grew faster than AZEs. Plant health on June 12 differed significantly between ecotypes and among the three pair types (Table 1C): AZEs appeared healthier than UTEs in all pairs and the plants producing only nicotine appeared healthier than the plants also producing TPIs. Total side branch length was greater in AZEs than in UTEs in the pair producing only nicotine ($t_{19df} = 2.45$, $P = 0.02$); that pair tended to have longer side branches than other pairs (Table 1C). AZEs produced more seed capsules than UTEs and the lowest-defended pair type had fewer capsules than the others (Fig. 5A). Total number of flowers did not differ among pairs,

but did differ between ecotypes in the pair producing nicotine but no TPIs. No significant differences were detected for the number of flower buds, but the pattern was similar to that for flower number.

Discussion

This study examined whether the AZE of *N. attenuata,* which lacks two herbivore-induced traits that function as major defenses in the UTE, is either less defended and benefits from not incurring the costs associated with the production of these defenses, or has evolved other defenses. Using transient and stable gene silencing of the defense metabolites in ecotypes that produce them and restoring TPI production in the AZE, we demonstrate differences in resistance between the ecotypes that are both dependent and independent of two JA-mediated defenses in *N. attenuata*: nicotine and TPIs. In laboratory experiments, silencing JA-signaling revealed that both ecotypes exhibit JA-mediated resistance to *M. sexta*, which was TPI-dependent in the UTE but nicotine-dependent in the AZE. When planted into a native Utah population, AZEs lost less canopy area than UTEs as a result of attack from voracious herbivores (grasshoppers and mammals) but only when both ecotypes produced nicotine. In TPI-deficient pairs, small herbivores such as flea beetles and thrips preferred to feed on AZEs rather than UTEs. Both ecotypes were equally able to attract predators, though AZEs lack the sesquiterpene cis-α-bergamotene, which attracts *G. pallens* predators to attacked UTEs. AZEs were generally fitter than UTEs, but not in the nicotine- and TPI-deficient pair. We conclude that AZEs, rather than being relatively defenseless in comparison to UTEs, have other traits that function as both direct and indirect defenses and that either these defenses are less costly to produce or AZEs have additional fitness-enhancing traits which result in greater reproductive performance when planted into the UTE's habitat.

Silencing *lox* by VIGS reduced JA levels by 60-75 % compared to levels in VIGSev-treated plants in all genotypes (Fig. 2) and, as a consequence, *M. sexta* growth increased in the WT of the UTEs and AZEs (Fig. 3). Increased caterpillar growth on VIGS*lox* UTEs was similar to that previously shown in antisense *lox* stable UTE transformants (Halitschke & Baldwin 2003) and was more pronounced than in the AZE, perhaps due to that ecotype's natural TPI deficiency. TPIs function as an effective direct defense against *M. sexta* (Zavala & Baldwin 2004; Zavala *et al.* 2004a) and silencing JA-signaling in UT plants reduced TPI activity by 75% (Fig. 2). Larval performance on TPI-deficient VIGSev IR*pi* UTEs was as high as on VIGS*lox* WT UTEs, and silencing *lox* had no additional effect on larvae feeding on

IR*pi* UTEs or on nicotine- and TPI-deficient IR*pmt/pi* UTEs (Fig. 3). Silencing *lox* in nicotine-deficient isogenic IR*pmt* AZE transformants which otherwise resemble WT AZE plants (Fig. S1) also did not increase larval growth (Fig. 3C). Therefore, JA-mediated resistance to *M. sexta* depends on TPI production in UTEs, and AZEs must have evolved defensive mechanisms independent of TPIs but dependent on nicotine, which is an effective defense against *M. sexta* although this herbivore is nicotine-tolerant (Steppuhn *et al.* 2004).

While JA-mediated resistance depends on only a few compounds, many may contribute, because the function of plant defenses can change according to the metabolites with which they are produced (Green *et al.* 2001). In *N. attenuata*, nicotine and TPI production interact synergistically against *S. exigua* larvae (Steppuhn & Baldwin 2007): the defensive function of TPIs depends on the concomitant production of nicotine, which prevents compensatory feeding by this TPI-adapted generalist. The tobacco specialist *M. sexta* can tolerate doses of nicotine fatal for non-adapted herbivores (Glendinning 2002; Wink & Theile 2002), but its capacity may be compromised by other secondary metabolites. The AZE may have evolved such mechanisms to increase the effectiveness of nicotine against its adapted specialist.

In their native habitat, WT UTEs suffered more herbivore damage than AZEs transformed to produce comparable amounts of nicotine and TPIs (Fig. 4), which is consistent with the hypothesis that the AZE has evolved additional defenses, due primarily to damage by voracious native folivores, including grasshoppers and mammals. At the beginning of the season, *Trimerotropis* grasshoppers attacked twice as many UTE (19) as AZE plants (10) and caused more damage on UTE. While this difference was significant only for the pair producing nicotine and TPIs, which received the most damage from *Trimerotropis* ssp., the effect of pair type was not significant, and in a previous field season *Trimerotropis* grasshoppers caused more damage to nicotine-silenced than to WT UTEs (Steppuhn *et al.* 2004). Though only a few plants were attacked by mammals, 75% were UTEs, but in the nicotine- and TPI-deficient pair, which received most damage, both ecotypes suffered equally. Lepidopteran damage was primarily caused by *M. sexta* larvae applied to measure predation rates (and did not differ between ecotypes or among pair types) but occasionally by native *Agrotis* larvae, which caused twice as much damage to UTEs as to AZEs in pairs producing nicotine. These results indicate that AZEs produce additional defenses effective against voracious native herbivores and which require the presence of nicotine, since herbivores preferred to attack UTEs over AZEs only in nicotine-producing pairs. Thus, as shown for JA-mediated resistance to *M. sexta* in the laboratory, the AZE relies on nicotine production for

resistance to herbivores in a native environment. The major herbivores later in the growing season were small flea beetles of the genus *Epitrix*, which unlike grasshoppers and mammals, damaged AZEs more than UTEs, and TPI-deficient more than TPI-producing plants. Although the AZE is less resistant to this abundant herbivore than is the UTE, resistance of AZEs transformed to produce TPIs was similar to that of UTEs. The AZE was also less resistant to thrips, which particularly preferred this ecotype over the UTE in nicotine- and TPI-deficient pair type, which suffered 3-4 fold greater damage, indicating high sensitivity to nicotine (Fig. S2). Thus, most herbivores, except the adapted specialist *M. sexta,* clearly distinguished between ecotypes, but only in the presence of nicotine in the case of herbivores preferring the UTE and in the absence of TPIs in the case of herbivores preferring the AZE. Thus, although VIGS*lox* assays showed that JA-mediated resistance to *M. sexta* is TPI-dependent in the UTE, some defenses must act independently of TPI production. Restoring TPI production in the AZE equalized it to the UTE in the resistance to flea beetles and thrips, confirming the defensive of TPIs in the AZE, as previously shown for resistance to *M. sexta* and *T. notatus* (Zavala & Baldwin 2004; Zavala *et al.* 2004a).

Because herbivore-attacked AZEs do not emit *cis*-α-bergamotene (Glawe *et al.* 2003), which functions as an efficient indirect defense in the UTE, we expected lower predation rates for *M. sexta* larvae and eggs applied to the AZE. Although *G. pallens* was abundant during the field experiment and survival rates of applied *M. sexta* were low, there was no difference between UTEs and AZEs in 5 experimental repetitions. This suggests that the AZE uses other, yet-to-be identified VOCs produced after herbivore elicitation to attract predators. If there is a functional linkage between indirect defenses and growth-retarding TPI as suggested by Glawe *et al.* (2003), it occurs only in the UT ecotype .

The AZE grew larger rosettes and more and longer side branches, and, as a consequence, produced more reproductive units in plants of both nicotine-producing pair types, especially in the pair producing only nicotine (Fig. 5A). Contrary to arguments in previous TPI-focused studies (Glawe *et al.* 2003; Zavala *et al.* 2004b), the AZE's fitness advantage was independent of TPIs, which therefore contribute to but do not cause the higher reproductive performance in AZEs. Fitness was partly explained by average seasonal herbivory: plants that suffered very high levels of herbivory had less reproductive output (Fig. 5B). Because voracious herbivores preferred the UTE, the fitness benefit for the AZE can be partially attributed to its resistance to these herbivores. Herbivore pressure can not fully account for the fitness differences, and because the fitness of TPI-deficient genotypes exceeded that of nicotine- and TPI-producing genotypes, costs of TPI production likely

trumped benefits during this season of low herbivory. However, plants investing the least in defense – those producing neither nicotine nor TPIs – did not incur more herbivory but had a lower fitness than plants producing nicotine only, and the fitness advantage for the AZE was aboished. Only damage patterns from thrips matched this fitness outcome (Supplemental Fig. S2), but thrips attack is unlikely to have been sufficiently intense to influence plant fitness. Overall, fitness was only weekly correlated with herbivory to above-ground tissue but much better predicted by estimates of plant health (Fig. 5B), which probably mirrors the nourishment it has received, and the diseases and root-herbivory it has suffered. Whereas nutrient allocation costs of TPIs could be responsible for the poor performance of nicotine- and TPI-producing plants, below-ground herbivores or pathogens could account for the poor performance of nicotine-deficient plants: nicotine is produced in the roots and is an effective defense against nematodes in cultivated tobacco (Hanounik & Osborne 1977; Davis & Rich 1987).

Together, results from the laboratory and the field demonstrate that the AZE is not just a less-defended variant of the UTE that survives by avoiding the fitness costs of TPI production; instead it has evolved other direct and indirect anti-herbivore defenses as well as fitness-enhancing traits independent of defense metabolite production. The genetic variation between these ecotypes is not greater than that among individuals within Utah populations (Bahulikar *et al.* 2004) and implies that great variety in defense strategies may be ubiquitous in populations of *N. attenuata*. This study highlights the value of transgenic approaches for untangling the roles of individual defense traits in different ecotypes and demonstrating how differently a herbivore community responds to the varying defenses produced by a plant.

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Supplemental Material

Figure S1: Characterization of the 2 independently-transformed homozygous lines in which the *putrescine N-methyl transferase* (*pmt*) genes were silenced in *N. attenuata* wild-type (WT) from a population in Arizona (AZE). (**A**) Southern blot analysis reveals that each transformed line harbors only a single insertion (DNA was EcoRV digested and the plasmid used for transformation was additionally digested with XhoI; probe was specific for the selective marker gene *hygromycin phosphotransferaseII*). Levels (mean ± SE) of constitutive (C) and methyl jasmonate (MeJA)-elicited (**B**) *pmt*-transcripts, (**C**) nicotine, (**D**) a diterpene glycoside (DTG), (**E**) chlorogenic acid, and (**F**) rutin (N = 5). Asterisks signify significant differences between transformed lines and WT at $P < 0.05$ determined by unpaired *t*-tests with log-transformed data. see Appendices

Figure S2: Damage levels caused by thrips (mean ± SE) on ecotypes of *N. attenuata* from Arizona (AZE) and Utah (UTE) planted into a native Utah population in 2005. AZEs and UTEs were arranged in pairs which were matched to produce trypsin protease inhibitors (TPI) and nicotine, only nicotine, or neither nicotine nor TPIs (via stable transformation; N = 20). Percentage of AZEs and UTEs attacked is indicated by the line graphs and secondary Y-axis. Asterisks on brackets signify significant differences in damage levels between the partners of a pair (paired *t*-tests) and different letters signify significant differences for the pair types as analyzed by repeated measures 2-way ANOVA (May 31–June 6, for ecotype $F_{1df,112} = 4.5$, $P = 0.035$, for pair type $F_{2df,112} = 7.7$, $P < 0.001$). see Appendices

Supplemental Protocol S1: see Appendices

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4. Discussion

The aim of this thesis was to examine different aspects of the cost-benefit-paradigm for induced plant defenses, which has long been hypothesized by ecologists, using recent molecular tools. This general discussion will evaluate experience gained with gene silencing to address ecological questions (4.1), summarize experimental support for the benefits of nicotine and trypsin protease inhibitors (TPIs) (4.2), review ecotypic variation of their cost-benefit functions (4.3), and discuss findings of interactions among them (4.4). Further, ongoing research originating from results achieved during the thesis will be presented (4.5).

4.1 Gene silencing: An elegant tool for dissecting ecological questions

Interdisciplinary approaches have opened new fields in ecology and allowed researchers to examine hypotheses about costs and benefits of putative defense compounds, which was not possible with classical ecological methods. Pharmaceutical methods, in which secondary metabolites were added to artificial diets and plants to explore potential negative effects on herbivores, provided only a weak indication for the function as anti-herbivore defense (Yamamoto *et al.*, 1968; Bowers & Puttick, 1988), because toxicity to herbivores may be just a side effect of metabolites that serve other ecological and physiological functions (Rausher, 1992). The book chapter (manuscript I) reviewed and structured the available studies according to the methodology used and showed how technical advances allowed confounding factors arising from complexity of a plant's metabolism and ecology to be to overcome. The use of improved analytical chemistry in methods of investigating interactions among plants and animals meant the identification of not only an increasing number of secondary metabolites, which are specifically produced in response to herbivore attack, but also of elicitors that activate such plant responses. Elicitation studies, which compare resistance to herbivores and the fitness of plants that are activated in their defense responses with these characteristics in unelicited plants, presented the first conclusive evidence for costs and benefits of induced defenses (Baldwin, 1998; Agrawal *et al.*, 1999). However, the contribution of specific substances could not be separated from various co-regulated factors within primary and secondary plant metabolism. The introduction of genetic tools in ecological research allowed specific factors to be correlated with changes in plant resistance and fitness among different genotypes (Berenbaum *et al.*, 1986; Shonle & Bergelson, 2000). However, different genotypes differ in various genetic factors and defense-related traits can

be linked to other traits, e.g. if their genes are close to each on the same chromosome. The confounding effects of genetic linkage can be circumvented by using isogenic genotypes, which differ only in the trait of interest (Bergelson & Purrington, 1996). Therefore, studies with mutants and transformed plants provide the strongest evidence for costs and benefits of defense traits (Tian *et al.*, 2003; Zavala *et al.*, 2004b). In the past decade the gene-silencing technique became available to precisely manipulate specific traits. However, only a small – but currently increasing – number studies of have used it in ecological research.

The aim of this thesis was to establish gene silencing as a method to test the cost-benefit-paradigm. The first study presented in this thesis (manuscript II) compared the efficiency of two different silencing constructs, the anti-sense (AS)- and the inverted repeat (IR)-construct for their ability to silence *putrescine-N-methyl transferase* (*pmt*), the regulatory enzyme of nicotine biosynthesis. Whereas the silencing process with AS-constructs starts only after the mRNA of the target gene is gathered, the two inverted sequences of IR-constructs immediately hybridize and form dsRNA after splicing the resulting mRNA. Consistent with the theoretical principles and results from a previous study (Wesley *et al.* 2001) we found that IR-constructs are clearly more efficient than AS-constructs at silencing the expression of *Nicotiana attenuata*'s endogenous nicotine production. Whereas nicotine accumulation was not reduced in most of the 31 independently transformed AS*pmt* lines compared to WT, constitutive and induced nicotine accumulations in 29 of 34 independently transformed IR*pmt* lines was dramatically reduced. Similarly, *N. attenuata*'s endogenous *protease inhibitor* (*pi*) gene expression was more efficiently silenced in IR*pi* transformants, whose PI activity was not detectable (manuscript III), than in AS*pi* lines, whose PI was activity reduced by 50-77% (Zavala *et al.*, 2004b). However, the silencing success is also gene dependent. AS-silencing of *N. attenuata*'s *13-liopoxygenase* (*lox*) of the jasmonate (JA)-signaling cascade reduced wound-inducible JA levels and JA levels inducible by the oral secretions of *Manduca sexta* between 33-65% (Halitschke & Baldwin, 2003) but JA levels were not further suppressed in IR*lox* lines (Halitschke, personal communication).

All transformed lines used in this thesis were carefully characterized for their genetic modification and for the specificity of the metabolic consequences of gene silencing (manuscripts II-IV). The genetic characterization included verification that the transformed line integrated a single insertion of T-DNA, did not change ploidity, and was suppressed for the targeted transcripts; in addition it was screened for those T1 plants that are homozygous for T-DNA. Only every sixth line screened during this thesis integrated more than a single insertion. Fifty-one independently transformed lines in the UT ecotype of *N. attenuata* that

were created in the Department for Molecular Ecology were diploid as the WT plants are. However, 55% of 33 lines of the transformed AZ ecotype became tetraploid during the transformation procedure (Bubner *et al.*, 2006). As previously mentioned, the silencing success with the IR-construct was very high (85% of 34 IR*pmt* lines were successfully suppressed). The suppression of nicotine accumulation and of TPI activity was stable during plant development in *pmt*- and/or *pi*-silenced lines, respectively, and when plants were grown in the glasshouse or in the field in Utah. After identification of homozygous, diploid single-insertion lines, two lines for each silencing construct were more thoroughly investigated for differences in their metabolite profile and in their response to herbivore attack. Transgenic lines were not morphologically distinguishable from WT plants and only differed in the traits silenced, with one exception (see below). We examined those metabolites that are known to play role in determining herbivore performance on *N. attenuata*, among them 3 alkaloids, 3 phenolics, 3 phytohormones, TPI activity, total protein content, and the most abundant DTG (in addition one sesquiterpene in case of IR*pmt* lines). Some of these metabolites were determined twice under different conditions. The very few and minor variations in certain metabolite levels from WT plants were not consistent for both independently transformed lines of each genotype or between experiments. Therefore isogenity for all lines of one ecotype can be assumed. The only exception was anatabine, which accumulated in all *pmt*-silenced lines to levels up to 25% of nicotine levels in WT plants. Elevated anatabine levels were also published for AS*pmt N. tabacum* lines, but transcript levels of other genes encoding enzymes involved in alkaloid metabolism were not affected (Chintapakorn & Hamill, 2003).

Anatabine consists of a pyridine and a piperideine ring. Both are likely derived from nicotinic acid (NA), which is also the precursor of the pyridine ring of nicotine (Leete & Slattery, 1976). Silencing *pmt* disrupts nicotine biosynthesis at the formation of the pyrrolidine ring and might therefore cause an oversupply of the NA used in the biosynthesis of anatabine. Feeding the roots of elicited WT plants with NA ethyl ester resulted in the formation of anatabine at levels of about one third of the total alkaloid levels (manuscript II). Feeding IR*pmt* plants with D_4-NA ethyl ester resulted in the formation not only of D_4-labeled nicotine and anatabine but also of D_8-anatabine, demonstrating that the last integrates two D_4-NA units. About half of the anatabine in WT plants was labeled, suggesting that the unlabeled half was formed from endogenous unlabeled NA. In addition one-fourth of the WT nicotine was D_4-labeled and in IR*pmt* plants, one-third of the anatabine was either D_4- or D_8-labeled. These results demonstrate that exogenously supplied NA is taken up by the roots of *N. attenuata* and used in alkaloid biosynthesis and that an oversupply of NA results in the

formation of anatabine. Thus, silencing *pmt* to disrupt nicotine biosynthesis causes an oversupply of NA and subsequently the formation of anatabine, which demonstrates a metabolic linkage between both alkaloids and underlines the importance of a careful metabolic characterization of transformants.

Altogether the studies presented in this thesis illustrate that gene silencing is an elegant tool for dissecting different questions about the functions and costs of a defense trait as well as to discover the linkages among different traits that may occur at several levels. Together with another study from our department (Kessler *et al.*, 2004), manuscript II represents the first use of gene silencing in field ecology. Manuscript III demonstrates how interactions among different secondary metabolites can be determined; and manuscript IV how different ecotypes can be investigated for the contribution of defense compounds to differences in resistance and fitness.

4.2 Anti-herbivore defense in *N. attenuata*: The contribution of nicotine & TPIs

In manuscripts II, III and IV it has been explored, what the contribution of *N. attenuata*'s nicotine and TPI to plant defense is and against which herbivores they provide resistance. Plants are attacked by adapted specialist herbivores as well as by generalists; the latter are thought to be more susceptible to taxon-specific (qualitative) plant defenses but not to commonly employed (typically quantitative) plant defenses (Feeny, 1976; Rhoades & Cates, 1976). In its natural habitat, *N. attenuata* is attacked by the tobacco specialist *M. sexta* and the generalist herbivore *S. exigua*. That nicotine provides resistance even against the nicotine-adapted specialist *M. sexta* is evident from its increased mass gain and faster development between instars when reared on IR*pmt* rather than on WT plants (manuscript II, Fig. 1A). The 50% body mass increase of larvae feeding on IR*pmt* plants is in a similar range as the difference between *M. sexta* larvae reared on nicotine-free or nicotine-enriched artificial diets (Appel & Martin, 1992), or reared on AS*pmt* and WT *N. sylvestris* plants (Voelckel *et al.*, 2001). *M. sexta* neonates also preferred to initiate feeding on IR*pmt* rather than on WT plants (both under glasshouse- and field-conditions; Fig. 1B), which suggests that nicotine plays an important role in determining the feeding sites of *M. sexta* larvae, as has been suggested in a study with cultivated tobacco (Kester *et al.*, 2002). Larvae of the generalist *Spodoptra exigua* feeding on the same IR*pmt* lines increased their mass four-fold relative to WT-fed larvae (manuscript III), indicating they are more sensitive to nicotine than are larvae of *M. sexta*, which has evolved a strong resistance to this genus's specific alkaloid

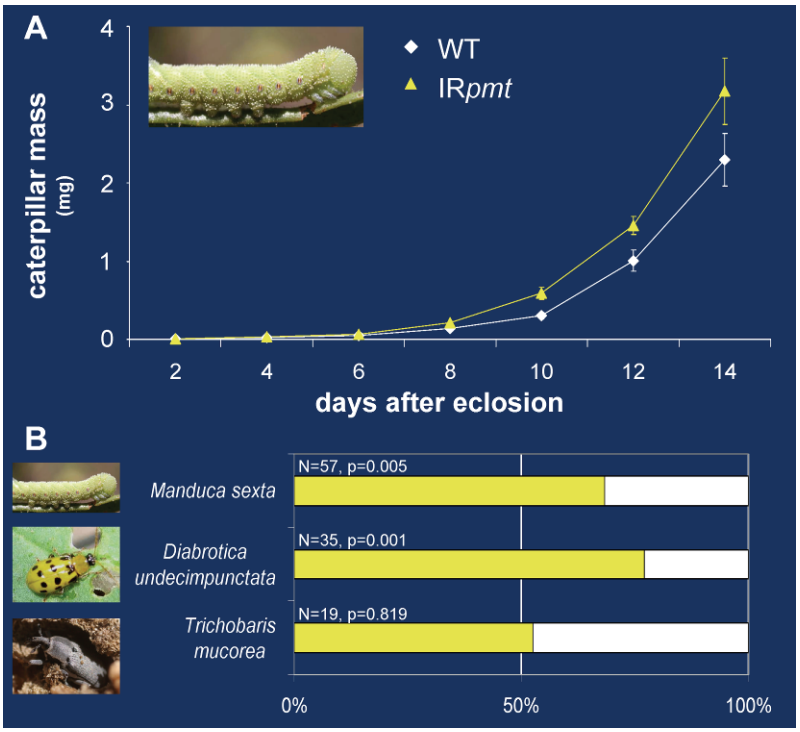

Figure 1: A Body mass (mean ± SE) in mg of *Manduca sexta* larvae reared on WT (white) and IR*pmt* (yellow) *N. attenuata*. **B** Feeding choice in % of *M. sexta* neonates, and adult beetles of *Diabrotica undecimpunctata* and *Trichobarus mucorea* between IR*pmt* and WT leaves. P-values refer to paired t-tests.

Photographs: *D. undecimpunctata* by R. Halitschke, others by A. Steppuhn

(Glendinning, 2002). In addition, *S. exigua* suffered very high mortality rates on *N. attenuata* (not observed for *M. sexta*), which were significantly reduced by silencing nicotine. Another generalist herbivore, the coleopteran *Diabrotica undecimpunctata*, did not colonize WT *N. attenuata* but rather IR*pmt* plants in the field (manuscript II; and also nicotine-deficient IR*lox* plants, see Kessler *et al.*, 2004). Choice tests with field-collected *D. undecimpunctata* revealed that 77% of these beetles preferred the nicotine-deficient IR*pmt* leaves over WT, but another beetle species observed occasionally on WT plants, *Trichobarus mucorea*, does not distinguish between WT and IR*pmt* plants (Fig. 1B). Altogether, these results signify that many generalist herbivores are very susceptible to nicotine.

Whereas nicotine's effectiveness varies according to whether the attacking herbivore is a specialist or generalist, the contribution of TPIs to the resistance against *M. sexta* and *S. exigua* is similar. In *S. exigua* feeding trials, larvae gained 2-fold greater mass on isogenic lines IR*pi* lines than on WT plants (manuscript III), which is similar to the mass gain observed for specialist *M. sexta* feeding on AS*pi* lines (Zavala *et al.*, 2004a). Thus, although both secondary metabolites function defensively against specialist and generalist herbivores, the latter is clearly more susceptible to the alkaloid. The fact that the silencing of one enzyme in the nicotine biosynthetic pathway redirects metabolite flux, resulting in the accumulation of anatabine, an apparently less toxic alkaloid, indicates that the influence of nicotine on herbivore choice and performance is likely underestimated.

That nicotine indeed functions as a direct defense of *N. attenuata* in its native habitat has been shown in field plantations: IR*pmt* plants suffered more than double the canopy loss to herbivores than did WT plants (manuscript II). About half of the total herbivore damage resulted from *S. exigua* feeding and about one-third was damage from *Trimerotropis* grasshoppers, which tended to attack IR*pmt* more than WT plants, and damage caused by *Epetrix hirtipennis* was significantly higher for IR*pmt* compared to WT plants. Because leaf area damage was negatively correlated with capsule number in other field experiments with *N. attenuata* (Baldwin, 1998; Kessler *et al.*, 2004), the strongly enhanced herbivore damage of the nicotine-deficient IR*pmt* plants likely translates into a fitness loss.

Elicitation with the methyl ester of JA (MeJA) significantly reduced the damage of IR*pmt* plants to levels found in WT plants. This result suggests that other JA-mediated defense traits are as efficient as the constitutive levels of nicotine in protecting plants. However, overall damage by herbivores during that field season might have been too low (5% canopy loss on WT plants) to detect differences between MeJA-elicited IR*pmt* and WT plants and between unelicited and MeJA-elicited WT; in previous experiments, fitness differences were observed only between unelicited and MeJA-elicited *N. attenuata* of populations that had lost approximately 40% of their canopy area to herbivores (Baldwin, 1998). MeJA elicitation of *N. attenuata* plants induces diverse transcriptional responses and secondary metabolites including TPIs, phenolics, flavonoids, phenolic putrescine conjugates, diterpene sugar esters, and volatile organic compounds (Halitschke & Baldwin, 2003; Roda & Baldwin, 2003). Which component of this complex suite of elicited metabolites was as effective as constitutive nicotine is unclear.

4.3 Ecotypic variation the cost-benefit functions of nicotine & TPIs in *N. attenuata*

In manuscript IV the contribution of nicotine and TPIs for JA-mediated resistance was explored in two ecotypes of *N. attenuata*, the TPI-producing ecotype from Utah (UTE) and the naturally TPI-deficient ecotype from Arizona (AZE). Virus-induced gene silencing (VIGS) of *lox* reduced JA levels and as a consequence *M. sexta* feeding on VIGS*lox* plants gained more mass than did those on empty vector control (VIGSev) plants of both ecotypes. The increase in caterpillar growth on VIGS*lox* UTEs was similar to that previously shown for AS*lox* lines in the UTE (Halitschke & Baldwin, 2003) and was more pronounced than in the AZE, perhaps because of the lack of TPI production in the latter. Silencing *lox* in the UTEs reduced TPI activity by 75%. The particular role of TPIs amongst the JA-mediated resistance

traits was examined using IR*pi* UTEts. Larval performance on VIGSev-treated IR*pi* UTEs was similarly increased as on VIGS*lox*-treated UTE WT plants, and silencing *lox* had no additional effect on larvae feeding on IR*pi* UTE or on IR*pmt/pi* UTEs. Therefore, the JA-mediated resistance of the UTE to *M. sexta* depends on TPI production and the AZE must have evolved defensive mechanisms that are independent of TPIs. Silencing *lox* in the nicotine-deficient IR*pmt* AZEs also did not increase larval growth and thus, JA-mediated resistance in the AZE must depend on nicotine production. Though the JA-mediated defenses of both ecotypes depend for their effectiveness on TPIs or nicotine, respectively, it cannot be assumed that these are the only active defense compounds, because the function of plant defenses can change according to the metabolites with which they are produced (Green *et al.*, 2001). In *N. attenuata* nicotine and TPI production have been shown to interact synergistically to promote resistance to *S. exigua* larvae (manuscript III), as will be discussed in the next section (4.3). The AZE may have evolved mechanisms to increase the effectiveness of nicotine against its adapted specialist, which could explain the nicotine dependence of its JA-mediated resistance to *M. sexta*.

In their native habitat, UTE WT plants were more heavily damaged by herbivores than were AZEs transformed to produce comparable amounts of nicotine and TPIs, indicating that the AZE has evolved additional defenses. Higher herbivore damage to UTEs resulted from voracious herbivores such as grasshoppers and mammals, but this effect was nicotine-dependent, because AZEs and UTEs silenced for nicotine and TPI production received similar damage by these herbivores. However, small insect herbivores such as flea beetles and thrips preferred to feed on AZEs rather than UTEs but only if the plants were deficient in TPI production. When TPI production is restored in the AZE, its resistance to flea beetles and thrips was similar to that of the UTE , indicating that TPIs also act defensively in the AZE, which has been previously shown for the resistance to *M. sexta* and *Tupiocoris notatus* (Zavala & Baldwin, 2004; Zavala *et al.*, 2004a).

Besides the effects on resistance, manuscript IV also investigated differences between the plant fitness of the UT and AZ ecotypes in relation to TPI and nicotine production. The data reflect both costs and benefits under the conditions of the respective field season. Divergent the conclusion drawn in previous TPI-focused studies (Glawe *et al.*, 2003; Zavala *et al.*, 2004b), AZEs had a higher fitness than UTEs even if equalized for TPI production, which therefore only contributes to but does not cause the higher reproductive performance of the AZE. However, AZEs only produced more reproductive units if the plants produced nicotine and the effect was more pronounced when plants were TPI-deficient, which may

have been due to herbivory, because the fitness of those plants that had suffered from attack by voracious herbivores, which preferred UTEs, was reduced. But the correlation between plant and herbivory levels was very weak, and the fitness of TPI-deficient genotypes exceeded that of nicotine- and TPI-producing genotypes, indicating that the costs of TPI production were greater than its benefits during this season of rather low herbivory rates.

Plant fitness correlated better with plant health than with herbivory levels, which probably mirrors the nourishment the plant received as well as the diseases and root herbivory that it suffered. Whereas the nutrient allocation costs of TPIs could have been responsible for the poor condition of nicotine- and TPI-producing plants, below-ground herbivores or pathogens might have reduced the health and fitness of the nicotine-deficient plants. Root-produced nicotine is an effective defense against nematodes in cultivated tobacco (Hanounik & Osborne, 1977; Davis & Rich, 1987). Thus, the AZE is not just a less defended variant of the UTE that survives by avoiding the fitness costs of TPI production; instead it has evolved other anti-herbivore defenses that may either be less costly or it has evolved other fitness-enhancing traits, for example, tolerance traits.

4.4 The plant defense network: Secondary metabolites interact

The expression of defense traits can be linked to other traits by different mechanisms. First, they can be genetically linked to nearby genes on the same chromosome, as previously mentioned. The technical advances to control the genetic background have decreased many of the potential confounding effects of genetic linkage that have plagued tests of the cost-benefit paradigm. Second, defense traits are linked to the plant's metabolism, and eliminating one component of the metabolic machinery is bound to cause changes in metabolite flow, which can in turn regulate metabolism through complicated feedback and 'feed-forward' controls. Even changes at the end branches of metabolic pathways can also have consequences due to the accumulation of precursors or byproducts, as indicated by the accumulation of anatabine due to *pmt* silencing (manuscript II). Hence, a comparison of two plants that differ genetically only in the expression of a single resistance gene may include the consequences of significantly altered metabolisms. And these alterations may be either the cause or the consequence of defense costs. Third, the function of a defense trait can depend on the presence or absence of other metabolites, what can be termed functional linkage. For example, broadly toxic direct defenses can interfere with indirect defenses, e.g. by poisoning the attracted predators, and adapted herbivores even co-opt such direct plant defenses for their

own defense (Karban & Agrawal, 2002). Moreover, the functions of different defense traits might be directly linked: synergistic interactions, for example, amplify resistance to more than the sum of the resistance provided by each defense alone (Berenbaum & Neal, 1985; Nelson & Kursar, 1999). Because most plants produce a cocktail of chemically and functionally diverse defenses, it has long been debated whether these mixtures of secondary metabolites have adaptive value.

To discover whether nicotine and TPI production interact, manuscript III examined both these compounds' defensive function against *S. exigua* with and without the background of the other. Whereas nicotine by itself strongly affects *S. exigua* larvae, TPIs alone are ineffective, which is consistent with the ability of larvae to adapt to TPIs (Jongsma *et al.*, 1995; Lara *et al.*, 2000). However, when TPIs are expressed in concert with nicotine, *N. attenuata*'s endogenous TPIs clearly functioned defensively against this herbivore. Thus, the resistance effect of TPIs depends on the production of nicotine, an interaction which is also evident from the amount of leaf material larvae consumed: more leaf mass was consumed on TPI-containing plants but only if nicotine was not present. Increased consumption in response to heterologous PI expression is known from different plants (De Leo *et al.*, 1998; Cloutier *et al.*, 2000; Winterer & Bergelson, 2001; Abdeen *et al.*, 2005) and compensatory feeding is a well-described response in larvae that feed on diets low in nutritional protein (Simpson & Simpson, 1990). Because TPIs reduce gut protease activity, they can also elicit compensatory consumption. *S. exigua* larvae consumed significantly less leaf material in the presence of nicotine, and its elicitation by MeJA amplified this repellent effect. Nicotine is a potent antifeedant even for nicotine-adapted *M. sexta* larvae (Parr & Thurston, 1972; Appel & Martin, 1992; Voelckel *et al.*, 2001). *S. exigua* larvae on nicotine-containing plants did not increase their consumption in response to TPIs. Thus, it can be concluded that by limiting consumption, nicotine prevents *S. exigua* from compensating for TPIs by increasing consumption. Generalist herbivores feeding have evolved compensatory responses which allow them to adapt to anti-digestive plant defenses and nutritionally variable foods (Simpson *et al.*, 1995). Adding antifeedants with different modes of action can help plants to reduce herbivores' ability to compensate.

4.5 Testing the cost-benefit-paradigm: to be continued …

In addition to the questions explored in the manuscript that are part of this thesis, further aspects of the cost-benefit-paradigm were addressed. Three more studies are in

progress: one on physiological costs, one on ecological costs and one on the costs and benefits of nicotine and TPI production under an array of different conditions in the natural environment of the UTE. In the following the questions addressed and the data acquired will be briefly summarized.

In a further study in preparation the central questions are: Does *N. attenuata*'s nicotine and TPI production exhibit fitness costs in the absence of herbivores? And if so can these be attributed to nitrogen related resource allocation costs? This study reveals that *N. attenuata*'s nicotine and TPI production are bound to fitness costs, as in the absence of herbivores IR*pmt*, IR*pi* and IR*pmt/pi* realize an increased fitness than WT plants they are competing with (Fig. 2). Similarily do nicotine- and TPI-deficient IR*pmt/pi* have a higher fitness than competing single transformants that are only deficient for one defense. To examine the role of nitrogen in the increased fitness of plants silenced for the biosynthetic genes, *pmt* or *pi*, when competing with WT plants, ^{15}N-nitrate was fed to plants shortly after they were elicited with MeJA to produce secondary metabolites. The levels of ^{15}N that were incorporated into the seeds were analyzed and revealed, that whereas IR*pmt* plants incorporated more ^{15}N than a competing WT, IR*pi* plants incorporated similar ^{15}N amounts as the WT, indicating that fitness costs of nicotine are N-based resource costs, but fitness costs of TPIs are N-independent.

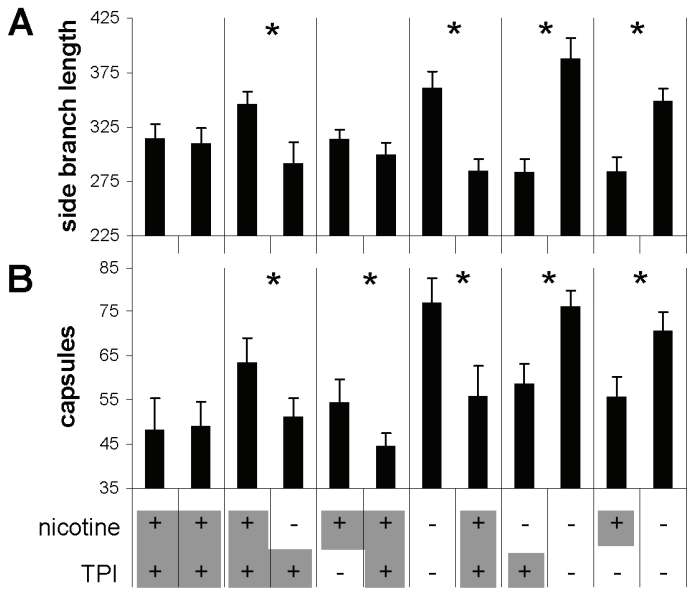

Figure 2: Mean ± SE of **A** total side branch length and **B** capsule number of *N. attenuata* plants in 6 different competition combinations: 2 wild-type (WT) competing with each other, WT vs. nicotine-deficient IR*pmt* plants, WT vs. TPI-deficient IR*pi* plants, WT vs. nicotine- and TPI-deficient IR*pmt/pi* plants, IR*pmt/pi* vs. IR*pmt* plants, and IR*pmt/pi* vs. IR*pi* plants. Asteriks signify significant ($P < 0.05$) paired *t*-tests.

A second study is evaluating whether *N. attenuata*'s nicotine and TPI production incurs ecological costs. Predation rate and predator survival on *M. sexta* larvae that fed either on WT plants or on nicotine and/or TPI-deficient transformants are examined. Predation experiments in a field plantation revealed no differences between predation on IR*pmt*- and WT-reared larvae when both larvae were placed on separate plants or leaves. However, when both larvae were placed on the same leaf, predators preferred IR*pmt*- over WT-reared larvae.

Field-collected *G. pallens*, the major predator in *N. attenuata*'s native habitat (Kessler & Baldwin, 2001) survived longer on IR*pmt*-reared larvae than on those reared on isogenic WT plants or on wild *N. attenuata* individuals (Fig. 3A). This suggests that nicotine may indeed interfere with *N. attenuata*'s indirect defense system of attracting predators. The parasitoid *Cotesia congregata* suffers higher mortality in response to nicotine in the diet of its host *M. sexta* (Barbosa *et al.*, 1991). Parasitoids are exposed to plant toxins in the host hemolymph throughout their larval development. Our results indicate that even predatory bugs that feed on their prey's hemolymph are negatively affected by nicotine. This may explain why *N. attenuata* suppresses *pmt*-transcription via an ethylene burst, which is in turn elicited by fatty acid amino acid conjugates in the oral secretions of *M. sexta* (Halitschke *et al.*, 2001; Winz & Baldwin, 2001). In glasshouse experiments, similar results were obtained with *G. punctipes* and Reduvidae nymphs (the latter reared from field-collected individuals; Fig. 3B, C). However, in addition both predators survived longer on IR*pi*-reared larvae than on WT-reared larvae, indicating that producing TPI may also have ecological costs, reducing the performance of the natural enemies of *N. attenuata*'s herbivores. That also PIs can

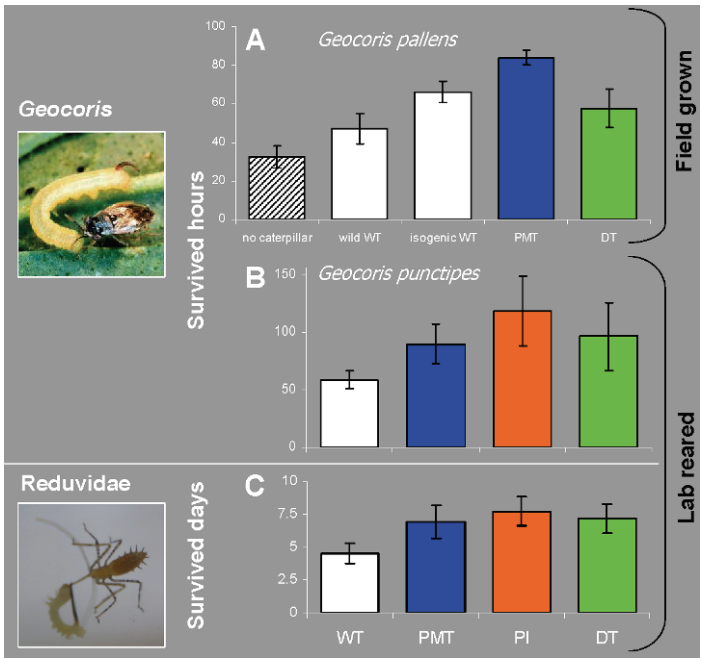

Figure 3: Mean ± SE of survival time of **A** field-collected *Geocoris pallens*, **B** lab-reared *G. punctipes*, and **C** Reduvidae nymphs when fed with *M. sexta* larvae that were reared on either wild-type (WT) *N. attenuata* or lines silenced for either nicotine (PMT), protease inhibitors (PI), or both (DT-double transformed). Plants in **A** are field-grown and in addition to the WT used for transformation, includes individuals from a wild population.

Photographs: *G. pallens* by A. Kessler, Reduvidae nymph by A. Steppuhn

negatively affect the third trophic organisms has been previously described for potato oryzacystatin I, which decreases the female fertility of the two-spotted stinkbug, *Perillus bioculatus* a predator of the Colorado potato beetles, *Leptinotarsa decemlineata* (Ashouri *et al.*, 1998; Bouchard *et al.*, 2003).

In another field study, pairs of WT *N. attenuata* with IR*pmt*, IR*pi* and IR*pmt/pi* lines as well as pairs of the latter with IR*pmt*, and IR*pi* lines were transplanted into a wild population to directly compare the costs and benefits of nicotine and TPI production and investigate the conditions under which they occur in the natural environment. The data revealed that the defensive benefits of these direct defenses outweigh their costs only if herbivory was high enough and under specific abiotic conditions. IR*pmt* and IR*pi* plants suffered higher herbivore damage than did WT plants on field sites with poor growth conditions, which resulted in a decreased biomass of nicotine and/or TPI-deficient lines (Fig. 4). However, field sites with a high ash content promoted plant growth, and relative canopy damage by herbivores was lower than on sites with lower ash content and the fitness outcome discovered costs of nicotine and TPI production. Under high ash conditions, only the extremely disarmed IR*pmt/pi* lines still received higher levels of herbivory than WT plants

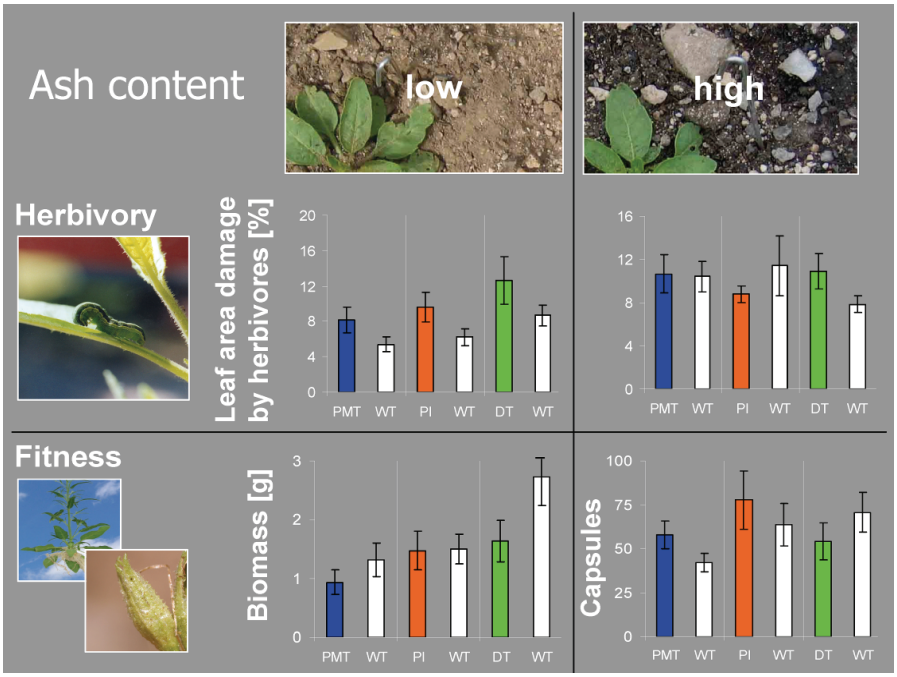

Figure 4: Mean ± SE of seasonal average herbivory and the fitness parameters biomass and capsule number of wild type (WT) plants paired with lines silenced for either nicotine (PMT), protease inhibitors (PI) or both (DT-double transformed) on side of low or high ash content in a wild *N. attenuata* population.
Photographs: plant in front of sky by J. Schwachtje, others by A. Steppuhn

and were consequently less fit. In addition to confirming costs and benefits of *N. attenuata*'s nicotine and TPI production in nature, the data demonstrate the large effect of other factors, such as nutrient availability, on the cost-benefit ratio of secondary metabolite production. High ash content corresponds to high nutrient availability, which severely affects the plant's primary and secondary metabolism. The effects of nutrient availability on costs and benefits of the two direct defenses are will be established under standardized conditions in the glasshouse to support the conclusions from the field data.

5.1 Summary

Many secondary metabolites are assumed to be defensive but costly in terms of resource allocation. In a book chapter (**I**) I reviewed studies on costs and benefits of plant defenses and the advantages and challenges of the approaches used. The literature survey pointed to a lack of evidence due to the methodological problems of dissecting the effects of a certain trait from the effects of genetically, metabolically, or functionally linked traits.

In four studies, the costs and benefits of *Nicotiana attenuata*'s nicotine and trypsin protease inhibitor (TPI) production were investigated by silencing their biosynthetic genes *putrescine-N-methyltransferase* (*pmt*) and *pi* using stable transformation with inverted repeat (IR)-constructs. IR*pmt* lines had a more than 90% reduction in nicotine levels and IR*pi* lines had no detectable TPI activity. Otherwise IR-lines and untransformed WT plants were similar in metabolic and growth traits, with the exception of IR*pmt* lines, which accumulated the alkaloid anatabine as a result of excess nicotinic acid, the precursor of the six-membered ring of nicotine, when the formation of the five-membered ring was silenced. Herbivores preferred and performed better on IR*pmt* than on WT plants, which received more leaf area damage from herbivores in field plantations. Thus, gene silencing enabled the defensive function of nicotine production in nature to be confirmed (**II**).

Both nicotine and TPIs are jasmonic acid (JA) mediated and synchronically induced. A functional linkage between both was examined using plants silenced for *pmt* and *pi* separately or together. The effects of nicotine and TPIs on the growth and consumption of the generalist herbivore *Spodoptera exigua*, a native in *N. attenuata*'s habitat, were established with and without the background of the other. Both metabolites functioned defensively in WT plants, but whereas nicotine alone reduced larval performance, TPIs alone did not. A compensatory feeding response to TPIs was prevented by nicotine in the WT plants, signifying a defensive synergism between both defenses (**III**).

Two ecotypes of *N. attenuata*, one from Utah (UT), which was the subject of all studies, and one additional one from Arizona (AZ), which is naturally TPI-deficient, were compared with respect to the role of JA-mediated traits for their herbivore resistance and plant fitness. Both ecotypes were stably transformed so that they either produce nicotine and TPIs, only nicotine, or neither of the two. In laboratory experiments, in which these transformants were in addition transiently silenced for JA biosynthesis, the contribution of nicotine and TPIs to the JA-mediated resistance against *Manduca sexta* was examined and found to be TPI-dependent in the UT ecotype, but nicotine-dependent in the AZ ecotype. In field experiments,

AZ and UT plants were compared for differences in herbivore resistance and fitness, when equally able to produce nicotine and/or TPI or not. UT-AZ plant pairs of all genotypes – those that produce nicotine and TPIs, those only producing nicotine, or those that produced neither – were transplanted into a wild UT population. The two ecotypes differed in their resistance to different herbivores – in a TPI-dependent manner for the UT ecotype and in a nicotine-dependent manner for the AZ ecotype. AZ plants were fitter than UT plants independent of their ability to produce TPIs. Thus both ecotypes evolved different defense and survival strategies (**IV**).

5.2 Zusammenfassung

Pflanzen müssen eine Vielzahl verschiedener Fraßfeinde abwehren. Viele pflanzliche Sekundärmetabolite sind für bestimmte Tiergruppen und Krankheitserreger giftig und deswegen wird eine Funktion für die Verteidigung gegen Herbivoren vermutet. Viele dieser Substanzen werden durch Herbivorenbefall induzierbar produziert. Dass Pflanzen den Nachteil der daraus resultierenden Zeitverzögerung eingehen, wird vielfach dadurch begründet, dass die Produktion solcher Abwehrsubstanzen auch Kosten verursacht. So werden etwa Ressourcen und Energie in die Biosynthese dieser Verbindungen investiert, die dann nicht mehr für Reproduktion zur Verfügung stehen. Die durch Induzierbarkeit erreichte örtliche und zeitliche Beschränkung der Produktion solcher Substanzen auf Befallssituationen würde demnach eine Optimierung der Kosten-Nutzen-Relation darstellen. Die vielfältigen ökologischen Wechselbeziehungen zwischen Pflanze und abiotischer und biotischer Umwelt sowie physiologische Wechselwirkungen zwischen verschiedenen Metaboliten erschweren es, die Konzepte von Kosten und Nutzen potenzieller Abwehrsubstanzen experimentell zu überprüfen. Die rasante Entwicklung der letzten Jahrzehnte auf dem Feld der Molekularbiologie hat neue Methoden hervorgebracht, die es erleichtern können, komplexe ökologische Theorien zu testen.

Zielsetzung meiner Arbeit war die Etablierung von gene silencing zur Untersuchung ökologischer Fragestellungen, die Verifizierung oder Falsifizierung von Nutzen und Kosten zweier potenzieller Abwehrsubstanzen einer Wildpflanze, sowie die Untersuchung weiterer ökologischer Hypothesen zur Pflanzenabwehr (z.B. Synergismus als Abwehrstrategie).

Der experimentelle Nachweis von Nutzen und Kosten potenzieller Abwehrsubstanzen ist unzureichend – erst moderne Methoden erlauben eindeutige Schlußfolgerungen.

Den experimentellen Studien geht ein Review (Manuskript I) voraus, welcher einen konzeptionellen Überblick über die bereits vor Jahrzehnten formulierten Hypothesen zu Kosten und Nutzen pflanzlicher Abweher gibt. Wesentliche Studien, welche diese Hypothesen testeten, werden nach der verwendeten Methodologie zusammengefasst sowie die Vor- und Nachteile der verschiedenen experimentellen Herangehensweisen evaluiert. Aus dieser Literaturrecherche ließen sich folgende Schlussfolgerungen ziehen:

- Drei generelle Typen von Studien zu Kosten und Funktion pflanzlicher Sekundär-metabolite können unterschieden werden. Resistenz und Fitness der Pflanze werden entweder 1) nach dem Auslösen einer Abwehrreaktion durch Herbivoren oder Elicitoren

untersucht, 2) mit gentischer Variabilität korreliert oder 3) mit Mutanten oder Transformanten verglichen, die genetisch identisch sind, sich aber in spezifischen Faktoren unterscheiden.

- Obwohl das Kosten-Nutzen-Model von Pflanzenabwehr relativ alt ist, ist die experimentelle Überprüfung noch sehr lückenhaft, da die ersten beiden Methodiken keine eindeutigen Rückschlüsse auf Kosten und Nutzen spezifischer Abwehrfaktoren zulassen und letztere Methodik erst in jüngerer Zeit in zunehmendem Maße angewendet wird.
- Verkettungen von Abwehrmechanismen mit anderen Faktoren auf genetischer, stoffwechselphysiologischer sowie funktioneller Ebene erschweren die Deutung einfacher Kosten-Nutzen-Relationen und weisen vielmehr auf ein kompliziertes Netzwerk von Zielkonflikten hin.

Gene silencing ermöglicht den Nachweis der Abwehrfunktion von Nikotin in einer wilden Pflanze in ihrem natürlichen Habitat.

In den folgenden Studien wurden Kosten und Nutzen der induzierbaren Produktion des Toxins Nikotin sowie verdauungshemmender Protease-Inhibitoren (PI) durch *Nicotiana attenuata* untersucht. Dieser wilde Tabak ist heimisch in den noch relativ natürlichen Ökosystemen der Great Basin Wüste Nordamerikas, aber nah verwandt zu den genetisch sehr gut untersuchten Kulturpflanzen Tomate, Kartoffel und Tabak, und damit besonders geeignet für die Anwendung molekularer Methoden zur Untersuchung ökologischer Fragestellungen. In Manuskript II wurde gene silencing durch stabile Transformation auf seine Anwendbarkeit in der Feldökologie evaluiert und am Beispiel der Nikotinproduktion von *N. attenuata*, die durch gene silencing des die Biosynthese regulierenden Enzyms Putrescin-N-Methyltransferase (PMT) „stillgelegt“ wurde, konnte folgendes gezeigt werden:

- Die Transformation mit inverted repeat-Konstrukten (enthalten eine Sequenz des Zielgens zweifach in entgegengesezter Orientierung) ist effizienter als die mit antisense-Konstrukten (enthalten lediglich eine umgekehrte Sequenz des Zielgens).
- Primär- und Sekundärstoffwechsel sowie Pflanzenwachstum entsprechen weitgehend dem des untransformierten Wildtyps (WT), jedoch mit der Ausnahme erhöhter Produktion das Alkaloids Anatabin. Dies ließ sich auf einen Überschuß an Nikotinsäure, dem Ausgangsstoff für den im Nikotin enthaltenen Sechsring, in den Transformanten zurückführen, in welchen lediglich die Zufuhr des Fünfrings zur Nikotinsynthese blockiert wurde. Ein Nikotinsäureüberschuß führt zur Dimerisierung und damit zur Bildung von Anatabin (Nachweis durch von den Wurzeln aufgenommene ^{15}N-markierte Nikotinsäure).

- Die Produktion von Nikotin führt zur Abschreckung und Verlangsamung der Entwicklung von verschieden Herbivoren.
- Der Nutzen von Nikotin im natürlichen Habitat einer wilden Pflanze konnte erstmals nachgewiesen werden. Die PMT-Transformanten erlitten einen zweifach höheren Blattflächenverlust durch Herbivoren als der WT.

Die gleichzeitige Produktion von Nikotin und Protease-Inhibitoren wirkt als Abwehr-Synergismus gegen einen generalistischen Fraßfeind von *N. attenuata*.

Nikotin- und PI-Produktion nach Herbivorenbefall wird durch das Phytohormon Jasmonsäure (JA) vermittelt, was zu einer synchronen Induktion beider Substanzen führt. In Manuskript III wurde untersucht, ob sich die beiden Substanzen in ihrer Wirkung als Abwehr beeinflussen. Die Entwicklung und Fraßmenge eines an *N. attenuata* häufig vorkommenden Generalisten, *Spodoptera exigua*, wurde quantifiziert. Die Raupen fraßen an Blättern des WT und von Transformanten, die durch gene silencing unabhängig voneinander oder gleichzeitig in ihrer Nikotin- und PI-Biosynthese blockiert waren. Folgende Schlussfolgerungen ließen sich ziehen:

- Sowohl die Produktion von Nikotin als auch die von PI stellen im WT effektive Abwehrstrategien gegen *S. exigua* dar. Raupen, die Transformanten fraßen, welche entweder kein Nikotin oder kein PI produzierten, entwickelten sich besser als WT-fessende Raupen.
- Nikotin wirkt auch unabhängig von PI-Produktion als Abwehr, denn fraßen die Raupen nikotinproduzierende PI-Transformanten, entwickelten sie sich schlechter als an Transformanten, die weder Nikotin noch PI produzierten.
- Die Abwehrfunktion von PIs ist abhängig von Nikotin, denn Raupen auf PI-produzierenden PMT-Transformanten entwickelten sich besser und fraßen mehr als Raupen auf PI- und nikotinfreien Transformanten. PIs können kompensatorisches Fraßverhalten auslösen und *N. attenuata*s Nikotinproduktion unterbindet dieses Verhalten der PI- adaptierten *S. exigua* Larven.
- Die Produktion beider Substanzen stellt einen Abwehr-Synergismus gegen *S. exigua* dar, da beide zusammen eine größere Resistenz verursachten, als die Einzelsubstanzen.

Verschiedene Ökotypen von *N. attenuata* unterscheiden sich in ihren Abwehrstrategien.

Neben dem bisher beschriebenen Ökotyp aus Utah (UT) wurde ein weiterer aus Arizona (AZ) untersucht, welcher im Gegensatz zum erstgenannten keine PI produziert. In

Manuskript IV wurden beide Ökotypen hinsichtlich der Rolle JA-vermittelter Abwehr für Resistenz und Fitness verglichen, wobei zusätzlich zu den bereits erwähnten UT- auch AZ-Transformanten verwendet wurden, die kein Nikotin produzierten oder in denen die PI-Produktion wiederhergestellt wurde. Laborexperiemte, in denen durch Virus-induziertes gene silencing (VIGS) die JA-Biosynthese ausgeschaltet wurde, und Feldexperimente mit Paaren von UT und AZ Pflanzen die durch Transformation in ihren Fähigkeit Nikotin bzw. PI zu produzieren oder nicht angeglichen wurde, ergaben folgendes:

- Während die durch JA vermittelte Resistenz gegen *M. sexta* im UT Ökotyp von dessen PI-produktion abhängt, ist diese Resistenz im AZ Ökotyp Nikotin-abhängig.
- Der AZ Ökotyp ist resistenter gegen gefräßige Herbivoren wie Grashüpfer und Säuger als der UT Ökotyp, aber dieser Effekt ist abhängig von Nikotinproduktion.
- Der UT Ökotyp ist resistenter gegen abundante Kleinherbivoren wie Flohkäfer und Thripsen, jedoch nicht, wenn beide Ökotypen PIs produzieren können.
- Der AZ Ökotyp hat eine höhere Fitness als der UT Ökotyp, unabhängig von ihren unterschiedlichen Fähigkeiten, PI zu produzieren.

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8. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Univeristät Jena erkläre ich, dass ich die vorliegende Promotion eigenständig angefertigt und alle von mir benutzen Hilfsmittel angegeben habe. Personen, die mich bei der Erhebung und Auswahl des Materials sowie bei der Erstellung der Manuskripte unterstützt haben, sind in der Auflistung der Manuskripte genannt. Ich habe weder die Hilfe eines Promotionsberaters in Anspruch genommen noch haben Dritte für Arbeiten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Dienstleistungen erhalten. Die vorgelegte Dissertation wurde weder als Prüfungsarbeit für eine Staatliche oder andere Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Anke Steppuhn
Jena, den 11. Juli 2007

9. Curriculum vitae

Personal data

Name:	Anke Steppuhn
Date of birth:	26.04.1977
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Home address:	Magdelstieg 30, 07745 Jena, Germany
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Education

09\83 – 08\91	Allgemeinbildende Politechnische Oberschule
09\91 – 06\96	Max-Reinhardt-Gymnasium Hellersdorf, Berlin Abitur in June 1996

Study

10\96 – 03\02	Free University of Berlin (FU Berlin), Diploma in Biology Major in ecology; minors in botany, zoology, and paleontology
04\01 – 01\02	Diploma thesis at the Netherlands Institute of Ecology (NIOO) in the Multitrophic interaction department (head: Dr. Wim van der Putten) Title: "Body sugar levels of the parasitoids *Cotesia glomerata* and *Microplitis mediator* as an indicator for feeding and the effect of agro ecosystem diversity on their nutrient state in the field" Supervisor: Dr. Felix L. Wäckers

Practical training

07\97 – 08/97	Employment as student assistant at the Siemens factory for Installation
02\98 – 03/98	Technology (Werkstudent im Werk für Antriebs-, Schalt- und
08\98 – 10/98	Installationstechnik, Siemensstadt, Nonnendammallee 72, 13629, Berlin)

12/99 – 06/00	Employment as health nurse (as student assistant) at the mobile health nursing service in Berlin (Mobiler Pflegedienst, Elsenstr. 6, 12435, Berlin, Inhaberin: Petra Schaumann)
02/00 – 02/00	Internship at the Institute of Biology - Department of Applied Zoology / Animal Ecology of the FU Berlin; under supervision of Dr. Johannes L.M. Steidle
03/00 – 04/00	Undergraduate academic for field work on mate finding of the maybeetles *Melolontha melolontha* and *Melolontha hippocastani* under supervision of Dr. Joachim Ruther (Institute of Biology of the FU Berlin)
05/00 – 10/00	Undergraduate academic assistant at the Institute of Biology - Department of Applied Zoology / Animal Ecology of the FU Berlin; under supervision of Dr. Johannes L.M. Steidle I worked on the olfactory host recognition of the parasitoid *Lariophagus distinguendus*

Scientific career

04/02 – 09/02	Research associate at the Max-Plank-Institute for Chemical Ecology (MPI-CE) in Jena; research topic: in collaboration with Dr. Amy L. Roda I worked on the role of variability in herbivore-specific elicitors for plant defense
10/02 – now	Ph.D. student at the MPI-CE and Friedrich-Schiller-University in Jena

10. Scientific publications & talks

Publications:

Steppuhn, A. & Baldwin, I.T. (2007) Resistance management in a native plant: Nicotine prevents herbivores from compensating for plant protease inhibitors. *Ecology Letters*, **10**, 499-511.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R., & Baldwin, I.T. (2004) Nicotine's defensive function in nature. *PloS Biology*, **2**, 1074-1080.

Roda, A., Halitschke, R., Steppuhn, A., & Baldwin, I.T. (2004) Individual variability in herbivore-specific elicitors from the plant's perspective. *Molecular Ecology*, **13**, 2421-2433.

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Wäckers, F.L. & Steppuhn, A. (2003) Characterizing nutritional state and food source use of parasitoids collected in fields with high and low nectar availability. *IOBC WPRS Bulletin*, **26**, 203-208.

Steidle, J.L.M., Steppuhn, A., & Reinhard, J. (2001) Volatile cues from different host complexes used for host location by the generalist parasitoid *Lariophagus distinguendus* (Hymenoptera : Pteromalidae). *Basic and Applied Ecology*, **2**, 45-51.

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Steppuhn, A. & Baldwin, I.T. (in press). Induced Defenses and the Cost-Benefit Paradigm. In *Induced defenses to herbivory* (ed A. Schaller). Springer, Berlin

Talks

2007-03-02 Steppuhn, A. Freunde und Feinde des Wüstentabaks *Nicotiana attenuata*. 10. Treffen der Arbeitsgruppe "Biologischer Pflanzenschutz" / Verband Botanischer Gärten, Westphälische Wilhelms-Universität Münster, Botanischer Garten, Münster, Germany

2006-11-11 Steppuhn, A. The benefits and the costs of *N. attenuata's* nicotine & TPI production. 5th Biannual IMPRS Symposium / MPI for Chemical Ecology, Jena, Germany

2006-06-30 Steppuhn, A. Preis-Leistungs-Verhältniss von pflanzlichen Abwehrmechanismen. ICE Symposium / MPI for Chemical Ecology, Jena, Germany

2006-06-22 Steppuhn, A., Rayapuram, C. Interactions among plant defenses – The impact on herbivore consumption & digestion: I) LOX & NPR1, II) LOX & aDOX, III) nicotine & trypsin protease inhibitors. Institute seminar / MPI for Chemical Ecology, Jena, Germany

2006-01-27 Steppuhn, A. Using gene-silencing to study ecological interactions in the field. BIORHIZ Workshop: Rhizosphere processes and induced defense, Jena, Germany

2004-08-06 Steppuhn A., Baldwin I.T., Gase K., Halitschke R., Krock B. Nicotine's defensive function in nature. ESA Annual Meeting / The Ecological Society of America, Portland, United States of America

2004-01-31 Steppuhn, A. The role of nicotine in tritrophic plant-insect interactions in nature. ICE Symposium / MPI for Chemical Ecology, Jena, Germany

2003-11-20 Steppuhn A. Nicotine – ecological costs and benefits. Institute seminar, Institute of Biology - Department of Applied Zoology / Animal Ecology of the FU Berlin, Germany

2003-01-31 Steppuhn A. Ecological benefits of nicotine & the role of α-DOX1 after herbivore attack. MPI for Molecular Plant Physiology, Golm, Germany

2002-04-11 Steppuhn A. Einfluss von Nektarpflanzen auf den Ernährungszustand von Parasitoiden im Feld. multitrophic interaction - meeting, Göttingen, Germany

2001-06-05 Steidle, J.L.M., Steppuhn, A., & Ruther, J.: Specific and general foraging cues used by the generalist parasitoid *Lariophagus distinguendus* Förster (Hymenoptera: Pteromalidae). 94. Annual Meeting of the German Society for Zoology (DZG), Osnabrück, Germany

11. Appendices

Manuscript II Supplemental Material

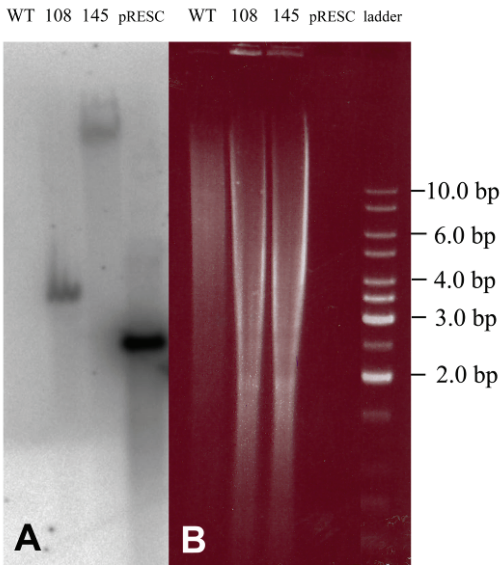

Figure S1. Copy Number of T-DNA in the Two Studied IR*pmt* Lines

(A) Southern blot analysis of two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) and WT plants. Genomic DNA (15 µg) from individual plants of the three genotypes and the plasmid used for transformation pRESC5PMT (4 ng) were digested with EcoRV and blotted onto nylon membranes (Winz and Baldwin 2001). The blot was hybridized with a PCR fragment of the *hygromycin phosphotransferase II* gene from pCAMBIA-1301, which is specific for the selective marker on the T-DNA and signifies one insertion in each of the two lines. (B) Ethidium bromide staining of the DNA revealed an overload of the DNA of the IR*pmt* lines and therefore loading of the WT was controlled by stripping and rehybridization with a PMT probe, which clearly revealed the endogenous *pmt*1 and *pmt* 2 genes described (Winz and Baldwin 2001) (unpublished data).

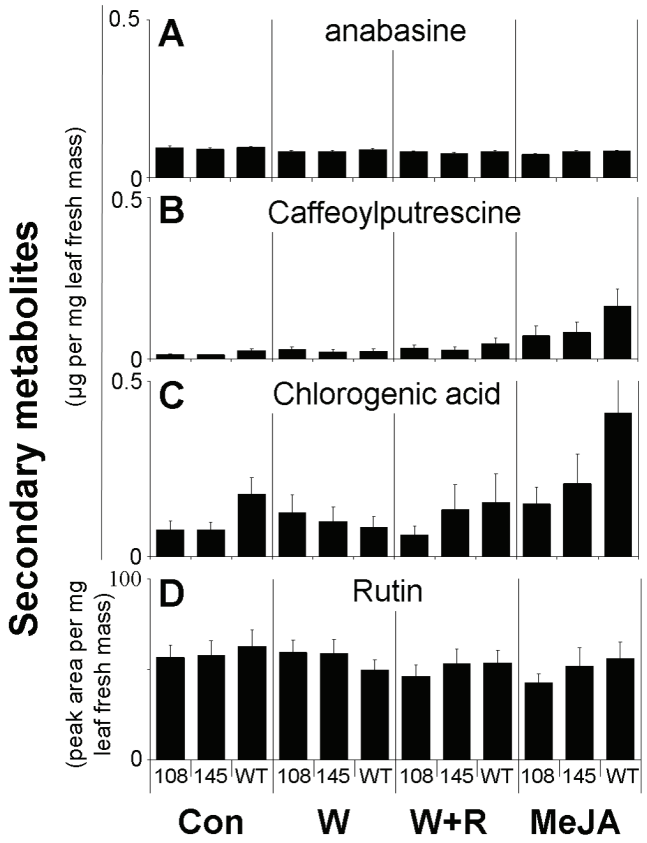

Figure S2. Secondary Metabolite Levels in the Studied IR*pmt* Lines

Inverted-repeat silencing of *pmt* did not change the levels of (A) anabasine, (B) caffeoylputrescine, (C) chlorogenic acid, and (D) rutin (mean ± standard error [SE]) in two independently transformed *N. attenuata* lines (108 and 145) compared to WT plants. Plants were harvested 4 d after receiving one of four treatments: untreated control (Con), wounding (W), wounding and regurgitate application (W+R), and application of 150 µg of MeJA per plant applied in a lanolin paste. Plants were treated at the first two fully expanded (source) leaves and wounding was performed by generating three rows of puncture wounds on each leaf side using a pattern wheel. Subsequently, 10 µl per leaf of either water or *M. sexta* regurgitate diluted 1:1 (v:v) was dispersed over the puncture wounds (n = 8–10).

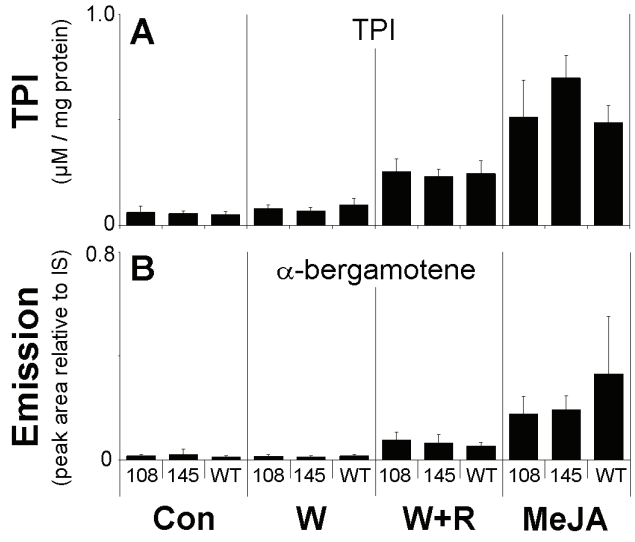

Figure S3. Proteinase Inhibitor and Volatile Emission of the Studied IR*pmt* Lines

Levels of (A) TPI and (B) cis-a-bergamotene emission (mean ± SE) in two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) did not differ from WT plants 4 d (for TPI) and 10 h (for cis-abergamotene) after receiving one of four treatments (as described for S2): untreated control (Con), wounding (W), wounding with additional regurgitate application (W+R), and MeJA elicitation. IS, internal standard.

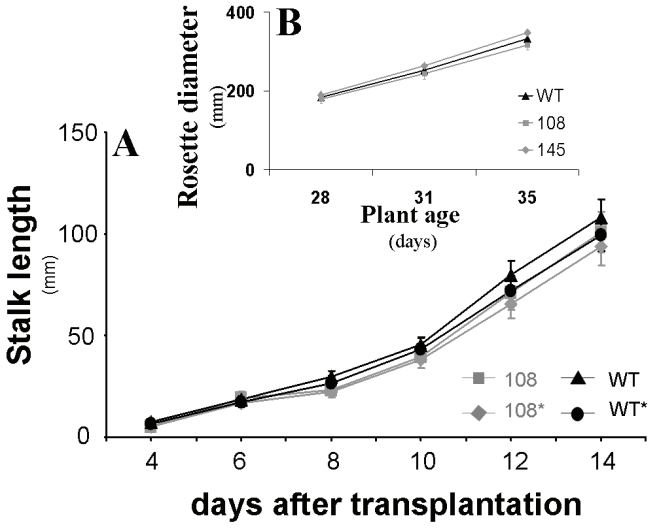

Figure S4. Growth Parameters Under Glasshouse and Field Conditions of the Studied IR*pmt* Lines

N. attenuata plants transformed with an IR*pmt* construct (108 or 145) did not differ in (A) stalk length [n_{PMT} = 43, n_{WT} = 57, n_{PMT}* and n_{WT}* = 28] and (B) rosette diameter [n = 8] from WT grown under either field (A) or glasshouse (B) conditions. Plants in (A) were untreated or elicited (*) with MeJA 7 d after plants were transplanted into a field plot in a native habitat.

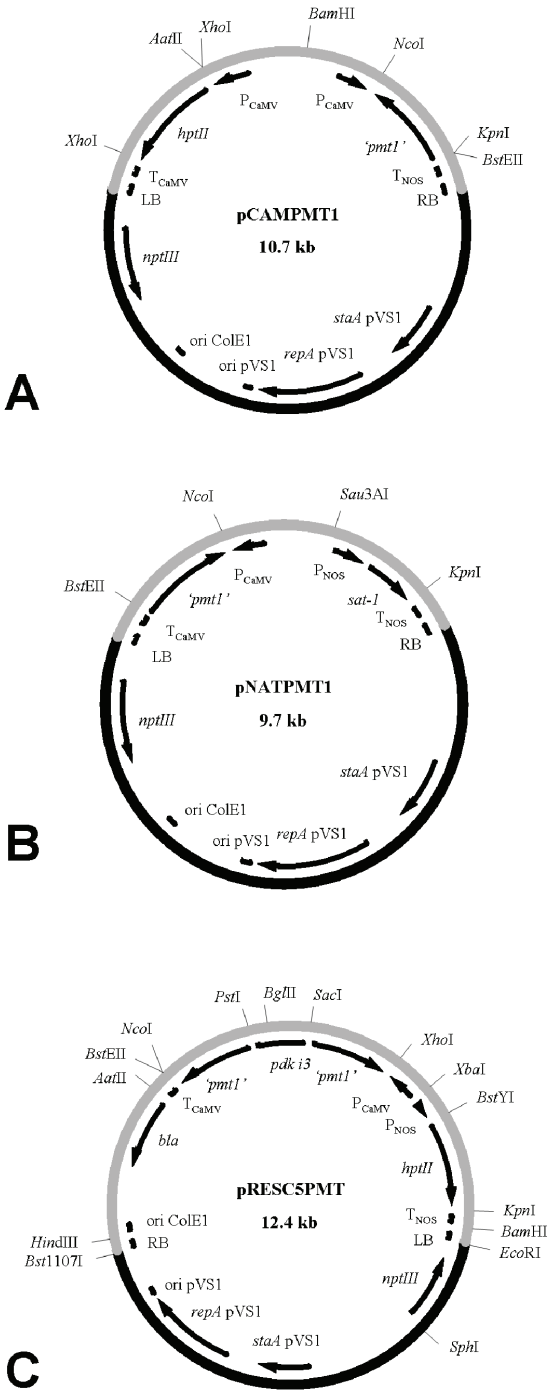

Figure S5. Transformation Vectors

This figure shows plasmids used for the generation of *N. attenuata* lines with reduced levels of two PMTs due to posttranscriptional gene silencing. Both (A) pCAMPMT1 (10.7 kb) and (B) pNATPMT1 (9.7 kb) allow the synthesis of *pmt* antisense RNA. (C) pRESC5PMT (12.4 kb) was used for the synthesis of *pmt* RNA capable of forming an inverted repeat. Functional elements: bla, *beta-lactamase* gene from plasmid pUC19; *hptII*, gene for hygromycin resistance from pCAMBIA-1301; LB and RB, left and right border of T-DNA; nptIII, aminoglycoside phosphotransferase of type III from *Streptococcus faecalis*; ori ColE1, origin of replication from pUC19; ori pVS1, origin of replication from plasmid pVS1; PCaMV and TCaMV, 35S promoter and terminator of cauliflower mosaic virus; pdk i3, intron 3 of pdk; *pmt*1, gene fragment of *pmt*1 (95% identical with *N. attenuata pmt*2); PNOS and TNOS, promoter and terminator of the nopaline synthase gene; repA pVS1, replication protein gene from pVS1; *sat-1*, nourseothricin resistance gene; staA pVS1, partitioning protein gene from pVS1. Displayed restriction sites mark the borders of functional elements, which are displayed in gray if on the T-DNA and in black if outside the T-DNA.

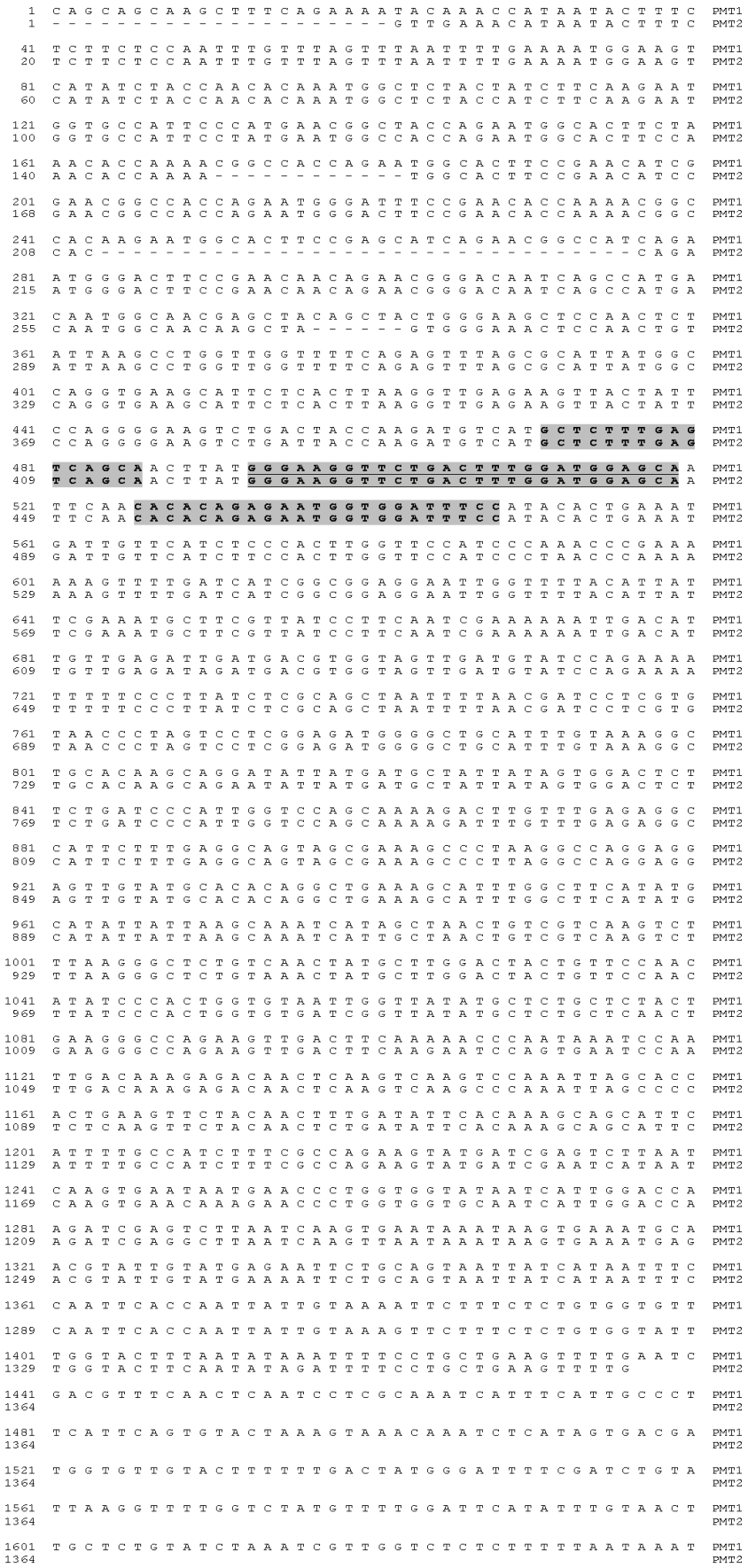

Figure S6. PMT Sequences and TaqMan Probe Nucleotide sequences of *N. attenuata pmt*1 and *pmt*2 mRNA (Winz and Baldwin 2001) aligned with ClustalW. Primers and probe (underlined) used for real-time PCR of *pmt* mRNA are highlighted and bold.

Protocol S1. Molecular and Analytical Methods

I. Vector construction: The construction of vectors pCAMPMT1 (10.7 kb; Fig. 9A) and pNATPMT1 (9.7 kb; Fig. 9B), both containing the same 0.9 kb cDNA portion of the *Nicotiana attenuata* putrescine N-methyltransferase 1 gene (*pmt1*; GenBank AF280402, Fig. 10) expressed in antisense orientation, were described elsewhere (Krügel et al. 2002 and Hui et al. 2003, respectively).

In order to obtain a basic cloning vector with two inverted copies of a gene fragment separated by an intron functional in *Nicotiana attenuata*, cDNA of the *N. benthamiana* phytoene desaturase gene (*pds*; GenBank AJ571700) resident on a plasmid kindly provided by D. Baulcombe, JIC Norwich, United Kingdom, was PCR amplified using primer pairs PDS13-40 (5´-GCGGCGAAGCTTGAGCTCAGGCACTCAACTTTATAAACCC-3´), pDS14-40 (5´-GCGGCGGAATTCCTCGAGTCAGCTCGATCTTTTTTATTCG-3´) and PDS15-40 (5´-GCGGCGAAGCTTCTGCAGAGGCACTCAACTTTATAAACCC-3´), pDS16-40 (5´-GCGGCGGAATTCCCATGGTCAGCTCGATCTTTTTTATTCG-3´). After digestion with *Eco*RI and *Hin*dIII both 0.2 kb PCR fragments were cloned in pUC18 (Norrander et al. 1983); GenBank L08752) cut with the same enzymes, yielding pUCPDS4 (2.9 kb) and pUCPDS5 (2.9 kb), respectively. After treatment with *Pst*I, T4 DNA Polymerase and *Eco*RI the 4.5 kb fragment of pHYB34 (Gase et al. 1998), served as cloning vector for the 0.2 kb *pds* fragment obtained by treating pUCPDS4 with *Hin*dIII, T4 DNA Polymerase and *Eco*RI, resulting in pUCPDS6 (4.2 kb). pUCPDS7 (4.5 kb) was created by fusing the 2.4 kb *Aat*II-*Hin*dIII vector fragment of pUCPDS5 to the 2.1 kb *´pds´* containing fragment of pUCPDS6. To prepare an appropriate plant transformation vector the 9.8 kb *Bst*EII- *Nco*I fragment of pRESC20 (Zavala et al. 2004) was blunt ended with T4 DNA Polymerase and circularized. The resulting plasmid pRESC201 (9.8 kb), cut with *Nco*I and *Xho*I, served as cloning vector for the 1.8 kb *Nco*I-*Xho*I *´pds´* inverted repeat fragment from pUCPDS7, yielding pRESC500 (11.5 kb). Intron 3 of the *Flaveria trinervia* pyruvate, orthophosphate dikinase gene (GenBank X79095) resident on a plasmid kindly provided by P. Westhoff, Düsseldorf, Germany, was PCR amplified with primers PDK1-29 (5´-GCGGCGGAGCTCC TTGGTAAGGAAATAAT-3´) and PDK2-30 (5´-GCGGCGCTGCAGTCCCAACTGTAAT CAATC-3´). After digestion with *Pst*I and *Sac*I the resulting 0.8 kb PCR fragment was used to replace the 1.4 kb *Pst*I - *Sac*I spacer between both *´pds´* copies on pRESC500, yielding pRESC501 (10.9 kb) which allowed efficient silencing of the *pds* gene in *N. attenuata* (data not shown), thus proving the function of the inverted repeat structure. The 0.2 kb *Nco*I-*Pst*I *pds* fragment of pRESC501 was replaced with a 0.9 kb *Nco*I-*Pst*I fragment containing the *pmt1* cDNA portion resident on pCAMPMT1, PCR synthesized with Primers PMT7-31 (5´-GCGGCGCCATGGAGCCCTTAAAGACTTGACG-3´) and PMT8-33 (5´-GCGGCGCTGC AGTACCAACACAAATGGCTCTAC-3´). In the resulting plasmid pRESC503 the 0.2 kb *Sac*I-*Xho*I *pds* fragment was replaced with a 0.9 kb *Sac*I-*Xho*I fragment carrying the *pmt1* cDNA portion of pCAMPMT1, PCR amplified with primers PMT9-33 (5´-GCGGCGCTC GAGCGAGCCCTTAAAGACTTGACG-3´) and PMT10-33 (5´-GCGGCGGAGCTCTAC CAACACAAATGGCTCTAC-3´), thus yielding plant transformation vector pRESC5PMT (12.3 kb; Fig. 9C).

II. Plasmid Rescuing: We took advantage of the presence of the pUC19 (Yanischperron et al. 1985; GenBank L09137) origin of replication and the beta-lactamase gene as selectable marker, which are both functional in *E. coli,* to recover the plant transformation vector pRESC5PMT T-DNA integrated into the *N.*

attenuata chromosome. This rescuing was performed as follows: chromosomal DNA of the two independently transformed homozygous *N. attenuata* lines, 108 and 145, was isolated from 2 week-old plants as described previously (Krügel et al. 2002). A mixture of 250 µl A1, 250 µl A2 and 300 µl A3 from the Macherey & Nagel (Düren, Germany) Nucleospin Kit was prepared and added to 50 µl of DNA solution. Subsequently the chromosomal DNA was purified with the kit as recommended for pDNA. Each 2 µg of the obtained DNA were cut with *Bcl*I, *Eco*RI or *Hin*dIII. After a precipitation step, the pellets were washed with 70% ethanol. Circularization of the fragments was done with T4 DNA ligase for 16 hr in a final volume of 14 µl. *E. coli* TOP10 (Grant et al. 1990; F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC} ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ (*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G) was transformed with the circularized chromosomal DNA fragments. After selection on 100 µg/ml Ampicillin 10 *E. coli* clones with rescued plasmids per digestion were isolated if rescuing was successful.

Nine rescued plasmids obtained from the different digestions were sequenced with primers binding close to and reading over the borders of pRESC5PMT T-DNA. This allowed us to identify the integration site of the T-DNA into the *N. attenuata* chromosome and to determine if the expected pRESC5PMT region was integrated. In line 108, the exact T-DNA fragment was fully integrated, whereas in line 145, a 227 bp deletion occurred: the integrated T-DNA comprised the region from right border to the middle of the NOS-terminator; the 3´ region of the NOS-terminator and the left border sequences were deleted. However, the integrated T-DNA is still functional, since the plants of this transformed line are hygromycin resistant and nicotine deficient. The analysis of the sequenced clones for line 108 revealed the T-DNA integration occurred into a gene homologous to GenBank CB214928 (SAM Demethylmenaquinone methyltransferase like gene from *Lycopersion esculentum*).The integration in Line 145 was identified to be in a region homologous to GenBank BI933931 (cDNA from the anthesis of *L. esculentum* flower).

III. ^{1}H-NMR of anatabine (D_4-MeOH). NMR measurement was performed on a Bruker DRX 500 NMR spectrometer, operating at 500,13 MHz for ^{1}H. The sample was measured in D_4-MeOH. Chemical shifts are given in δ values referring to TMS as internal standard and coupling constants in Hz. Literature data are given in square brackets (Yang and Tanner 1997): 2.62 (d) 3´H; 2.73 (m) 3´H [2.18, m]; 3.79 (d 16.0) 6´H [3.40, dm 17.0, 4.0]; 3.95 (d 16.0) 6´H [3.53 dm, 17.0, 2.0]; 4.59 (dd 11.6, 4.7) 2´H [3.80, t 7.0]; 5.90 (d 10.0) 4´H [5.78, d 10.0]; 6.10 (d 10.0) 5´H [5.72, ddd 10.0, 4.0, 2.0]; 7.60 5H [7.20, ddd 8.0, 5.0, 0.8]; 8.03 (d 8.0) 4H [7.67, dt 8.0, 1.8, 2.0]; 8.67 6H [8.42, dd 5.0, 1.8]; 8.72 2H [8.54, d 2.0].

IV. Analysis of nicotine and anatabine and their deuterated forms by LC/MS-MS. The samples were measured by an Agilent LC1100 HPLC system (Agilent, Waldbronn, Germany) with degasser, binary pump, auto injector, column thermostat, and DAD coupled with a LCQ DECA XP mass spectrometer (Thermo-Finnigan, Egelsbach, Germany). Mobile phase A consisted of 0.5% NH_4OH in water and mobile phase B of 0.5% NH_4OH in acetonitrile. The mobile phase gradient was linearly increased from 20 % B (initial value) to 100 % B at 18 min. The mobile phase flow was 1.0 mL/min and the injection volume 15 µL. Stationary phase was a Luna 5u C18 column (250 x 4.60 mm, 5 µm particle size, Phenomenex, Aschaffenburg, Germany). Under these conditions anatabine eluted at 6.9 min and nicotine at 8.6 min. The MS conditions were as follows: APCI Ion source, positive polarity; 500°C vaporizer temperature; 175°C capillary temperature; 10 µA discharge current. 6 scan events (MS/MS experiments) were programmed: 1. m/z 161 @ 27 collision energy (arbitrary units) for anatabine; 2. m/z 163 @ 27 for nicotine and d_2-anatabine; 3. m/z 165 @ 27 for d_2-nicotine and d_4-

anatabine; 4. m/z 167 @ 27 for d_4-nicotine and d_6-anatabine; 5. m/z 169 @ 27 for d_6-nicotine and d_8-anatabine; 6. m/z 171 @ 27 for d_8-nicotine.

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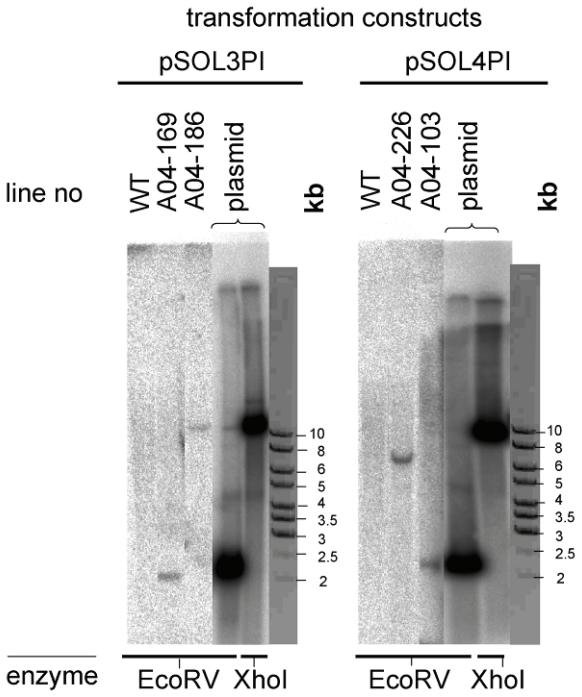
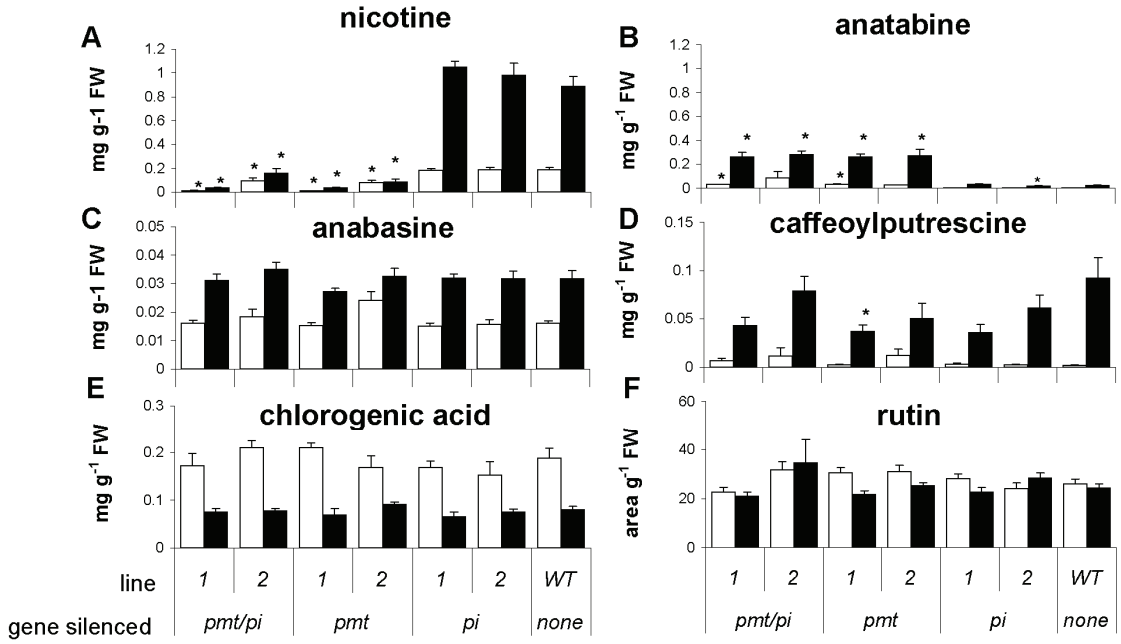
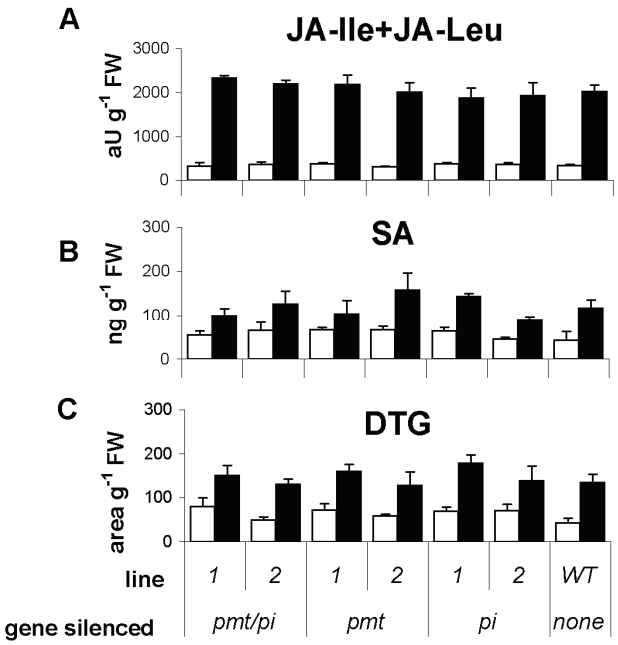
Manuscript III Supplemental Material



Supplemental Figure 1: Southern blot analysis demonstrates that each of the 2 independently transformed homozygous lines per genotype harbored a single insertion of the transgene. Both genotypes are inverted repeat silenced (IR) for the *pi* gene in either WT *Nicotiana attenuata* using the construct pSOL3PI or with pSOL4PI in a line previously silenced for nicotine (A03-108). Genomic DNA and the plasmids used for transformation were EcoRV digested (plasmids additionally with XhoI), separated by electrophoresis and blotted onto nylon membranes. The blot was hybridized with a PCR fragment specific for the selective marker genes on the respective T-DNA, namely *hygromycin phosphotransferaseII* (pSOL3PI) and *sat-1* gene (pSOL4PI).



Supplemental Figure 2: Levels (mean ± SE) of different secondary metabolites in wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) silenced for *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. (**A**) nicotine (**B**) anatabine, (**C**) anabasine, (**D**) caffeoylputrescine, (**E**) chlorogenic acid, and (**F**) rutin. Plants were grown in the glasshouse (same conditions as in the feeding trials) and were either unelicited (white bars) or elicited with jasmonic acid isoleucine conjugate (black bars) and harvested after 4 days (N = 5-7). T-tests were used to test for significant differences between each transformed line and WT. Significant differences to WT at $P < 0.05$ are indicated by asterisks.



Supplemental Figure 3: Constitutive (white bars) and induced (black bars) levels (mean ± SE) of different secondary metabolites in wild-type (WT) *Nicotiana attenuata* and 2 independently transformed lines (1 & 2) silenced for *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. Levels of (**A**) jasmonic acid conjugates to isoleucine and leucine JA-Ile + JA-Leu and (**B**) SA (N = 3) were determined in leaves of untreated control plants and plants elicited with *M. sexta* oral secretion 30 min prior to being harvested for analysis. (**C**) Levels of an abundant diterpene glycoside in unelicited and methyl jasmonate (MeJA)-elicited plants. Plants were hydroponically grown in the climate chamber. T-tests did not detect significant differences between each transformed line and WT ($P < 0.05$).

Supplemental Table 1: Comparisons (unpaired *t*-tests; α adjusted according to sequential Bonferroni procedure) of levels of secondary metabolites between wild type (WT) *Nicotiana attenuata* and each of 2 independently transformed lines (1 & 2) silenced for either *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. Plants were hydroponically grown in the climate chamber and phytohormones (jasmonic acid – JA, salicylic acid – SA, and JA conjugates) were analyzed in unelicited plants and plants that were elicited with *Manduca sexta* oral secretions 30 min prior to analysis. All other metabolites were analyzed in control plants and plants that were treated with methyl jasmonate 4 days prior to analysis.

climate chamber		constitutive							induced						
substance	comparison: WT vs.	mean diff.	df	*t*	*P*	rank	adj.α		mean diff.	df	*t*	*P*	rank	adj.α	
caffeoyl-putrescine	IR*pi/pmt* 1	0.001	11	0.027	0.98	1	0.050	ns	-0.092	11	-0.906	0.38	5	0.010	ns
	IR*pi/pmt* 2	-0.007	11	-0.267	0.79	2	0.025	ns	-0.1	11	-1.093	0.30	6	0.008	ns
	IR*pmt* 1	0.051	11	0.97	0.35	6	0.008	ns	0.066	11	0.677	0.51	4	0.013	ns
	IR*pmt* 2	0.011	11	0.399	0.70	4	0.013	ns	0.001	11	0.01	0.99	1	0.050	ns
	IR*pi* 1	0.015	11	0.614	0.55	5	0.010	ns	0.06	11	0.625	0.55	3	0.017	ns
	IR*pi* 2	0.007	11	0.286	0.78	3	0.017	ns	-0.022	11	-0.201	0.84	2	0.025	ns
DTG	IR*pi/pmt* 1	36.979	11	1.748	0.11	6	0.008	ns	15.844	11	0.537	0.60	4	0.013	ns
	IR*pi/pmt* 2	6.508	11	0.431	0.68	1	0.050	ns	-3.507	11	-0.139	0.89	2	0.025	ns
	IR*pmt* 1	29.626	11	1.636	0.13	4	0.013	ns	25.901	11	0.985	0.35	5	0.010	ns
	IR*pmt* 2	16.61	11	1.166	0.27	2	0.025	ns	-6.727	11	-0.203	0.84	3	0.017	ns
	IR*pi* 1	26.761	11	1.696	0.12	5	0.010	ns	44.58	11	1.625	0.13	6	0.008	ns
	IR*pi* 2	27.94	11	1.572	0.14	3	0.017	ns	4.322	11	0.126	0.90	1	0.050	ns
JA	IR*pi/pmt* 1	-5.465	4	-1.899	0.13	5	0.010	ns	-354.34	4	-0.79	0.47	5	0.010	ns
	IR*pi/pmt* 2	-8.12	4	-4.015	0.02	6	0.008	ns	-326.25	4	-0.732	0.51	3	0.017	ns
	IR*pmt* 1	-6.539	4	-1.856	0.14	4	0.013	ns	-638.96	4	-1.49	0.21	6	0.008	ns
	IR*pmt* 2	-6.11	4	-1.551	0.20	3	0.017	ns	-164.3	4	-0.321	0.76	1	0.050	ns
	IR*pi* 1	-2.071	4	-1.195	0.30	2	0.025	ns	-446.2	4	-0.79	0.47	4	0.013	ns
	IR*pi* 2	-0.757	4	-0.162	0.88	1	0.050	ns	304.19	4	0.387	0.72	2	0.025	ns
JA-ILE	IR*pi/pmt* 1	-11.878	4	-0.164	0.88	1	0.050	ns	302.66	4	1.837	0.14	6	0.008	ns
	IR*pi/pmt* 2	27.542	4	0.503	0.64	3	0.017	ns	177.03	4	0.995	0.38	5	0.010	ns
	IR*pmt* 1	33.777	4	0.893	0.42	5	0.010	ns	166.22	4	0.643	0.56	4	0.013	ns
	IR*pmt* 2	-30.818	4	-1.132	0.32	6	0.008	ns	-11.094	4	-0.043	0.97	1	0.050	ns
	IR*pi* 1	30.836	4	0.681	0.53	4	0.013	ns	-144.08	4	-0.529	0.63	3	0.017	ns
	IR*pi* 2	21.167	4	0.421	0.70	2	0.025	ns	-95.007	4	-0.278	0.79	2	0.025	ns
nicotine	IR*pi/pmt* 1	-0.324	11	-6.324	0.00	5	0.010	*	-0.43	11	-8.627	0.00	5	0.010	*
	IR*pi/pmt* 2	-0.197	11	-2.98	0.01	3	0.017	*	-0.406	11	-7.874	0.00	6	0.008	*
	IR*pmt* 1	-0.298	11	-5.837	0.00	4	0.013	*	-0.496	11	-11.357	0.00	4	0.013	*
	IR*pmt* 2	-0.312	11	-6.18	0.00	6	0.008	*	-0.482	11	-10.526	0.00	3	0.017	*
	IR*pi* 1	0.069	11	1.102	0.29	1	0.050	ns	0.053	11	1.19	0.26	2	0.025	ns
	IR*pi* 2	0.069	11	1.224	0.25	2	0.025	ns	-0.02	11	-0.315	0.76	1	0.050	ns
PI	IR*pmt* 1	0.025	10	0.197	0.85	1	0.050	ns	-0.158	10	-1.569	0.15	2	0.025	ns
	IR*pmt* 2	0.127	10	0.908	0.39	2	0.025	ns	-0.125	10	-1.123	0.29	1	0.050	ns
protein	IR*pi/pmt* 1	0.218	10	1.408	0.19	6	0.008	ns	-0.086	10	-0.571	0.58	5	0.010	ns
	IR*pi/pmt* 2	0.171	10	0.911	0.38	5	0.010	ns	-0.039	10	-0.247	0.81	3	0.017	ns
	IR*pmt* 1	-0.042	10	-0.225	0.83	2	0.025	ns	0.03	10	0.146	0.89	2	0.025	ns
	IR*pmt* 2	-0.082	10	-0.458	0.66	4	0.013	ns	0.039	10	0.271	0.79	4	0.013	ns
	IR*pi* 1	-0.003	10	-0.021	0.98	1	0.050	ns	0.352	10	2.171	0.06	6	0.008	ns
	IR*pi* 2	-0.049	10	-0.346	0.74	3	0.017	ns	0.001	9	0.004	1.00	1	0.050	ns
rutin	IR*pi/pmt* 1	0.085	11	0.67	0.52	4	0.013	ns	0.039	11	0.362	0.72	4	0.013	ns
	IR*pi/pmt* 2	0.002	11	0.016	0.99	1	0.050	ns	0.024	11	0.258	0.80	3	0.017	ns
	IR*pmt* 1	0.082	11	0.78	0.45	5	0.010	ns	0.24	11	2.447	0.03	6	0.008	ns
	IR*pmt* 2	-0.04	11	-0.31	0.76	2	0.025	ns	-0.019	11	-0.162	0.87	1	0.050	ns
	IR*pi* 1	0.094	11	0.885	0.40	6	0.008	ns	0.13	11	1.39	0.19	5	0.010	ns
	IR*pi* 2	-0.05	11	-0.477	0.64	3	0.017	ns	-0.018	11	-0.175	0.86	2	0.025	ns
SA	IR*pi/pmt* 1	11.519	4	0.532	0.62	2	0.025	ns	-16.686	4	-0.688	0.53	3	0.017	ns
	IR*pi/pmt* 2	22.529	4	0.831	0.45	3	0.017	ns	9.479	4	0.272	0.80	1	0.050	ns
	IR*pmt* 1	23.802	4	1.185	0.30	6	0.008	ns	-12.079	4	-0.348	0.75	2	0.025	ns
	IR*pmt* 2	24.736	4	1.177	0.30	5	0.010	ns	41.172	4	0.956	0.39	4	0.013	ns
	IR*pi* 1	21.352	4	1.01	0.37	4	0.013	ns	26.476	4	1.362	0.25	6	0.008	ns
	IR*pi* 2	3.088	4	0.156	0.88	1	0.050	ns	-25.561	4	-1.334	0.25	5	0.010	ns

Supplemental Table 2: Comparisons (unpaired *t*-tests; α adjusted according to sequential Bonferroni procedure) of levels of secondary metabolites between wild type (WT) *N. attenuata* and each of 2 independently transformed lines (1 & 2) silenced for either *putrescine N-methyl transferase* (*pmt*), *protease inhibitor* (*pi*), or both. Plants were grown in the glasshouse under same conditions as the *Spodoptera exigua* assay and secondary metabolites were analyzed in control plants and plants that were treated with jasmonic acid conjugated to isoleucine 4 days before.

glasshouse		constitutive							induced						
substance	comparison: WT vs.	mean diff.	df	*t*	*P*	rank	adj.α		mean diff.	df	*t*	*P*	rank	adj.α	
anabasine	IR*pi/pmt* 1	5E-05	17	0.002	1.00	1	0.050	ns	-0.002	15	-0.05	0.96	1	0.050	ns
	IR*pi/pmt* 2	0.036	15	0.647	0.53	3	0.017	ns	0.051	12	0.933	0.37	5	0.010	ns
	IR*pmt* 1	-0.026	17	-0.746	0.47	4	0.013	ns	-0.056	16	-1.359	0.19	6	0.008	ns
	IR*pmt* 2	0.16	14	2.99	0.01	6	0.008	ns	0.019	12	0.341	0.74	3	0.017	ns
	IR*pi* 1	-0.026	17	-0.496	0.63	2	0.025	ns	0.003	16	0.068	0.95	2	0.025	ns
	IR*pi* 2	-0.036	17	-0.957	0.35	5	0.010	ns	0.014	16	0.343	0.74	4	0.013	ns
anatabine	IR*pi/pmt* 1	0.012	9	4.76	0.00	6	0.008	*	0.089	13	6.302	0.00	6	0.008	*
	IR*pi/pmt* 2	0.031	5	0.904	0.41	3	0.017	ns	0.097	10	10.982	0.00	5	0.010	*
	IR*pmt* 1	0.013	9	4.421	0.00	5	0.010	*	0.091	14	10.223	0.00	4	0.013	*
	IR*pmt* 2	0.009	6	2.849	0.03	4	0.013	ns	0.093	10	6.169	0.00	3	0.017	*
	IR*pi* 1	0.0002	6	0.252	0.81	1	0.050	ns	-0.001	12	-0.687	0.51	1	0.050	ns
	IR*pi* 2	-0.0004	3	-0.808	0.48	2	0.025	ns	0.004	14	3.179	0.01	2	0.025	*
caffeoyl-putrescine	IR*pi/pmt* 1	0.141	17	0.679	0.51	2	0.025	ns	-0.36	15	-2.416	0.03	4	0.013	ns
	IR*pi/pmt* 2	0.197	15	0.88	0.39	3	0.017	ns	-0.036	12	-0.258	0.80	1	0.050	ns
	IR*pmt* 1	0.092	17	1.058	0.30	4	0.013	ns	-0.368	16	-3.253	0.01	6	0.008	*
	IR*pmt* 2	0.406	14	1.767	0.10	6	0.008	ns	-0.271	12	-1.788	0.10	3	0.017	ns
	IR*pi* 1	-0.053	17	-0.418	0.68	1	0.050	ns	-0.177	16	-1.412	0.18	2	0.025	ns
	IR*pi* 2	0.154	17	1.204	0.25	5	0.010	ns	-0.517	16	-2.715	0.02	5	0.010	ns
chlorogenic acid	IR*pi/pmt* 1	-0.059	17	-0.547	0.59	3	0.017	ns	-0.048	15	-0.637	0.53	3	0.017	ns
	IR*pi/pmt* 2	0.079	15	0.928	0.37	4	0.013	ns	-0.004	12	-0.068	0.95	1	0.050	ns
	IR*pmt* 1	0.084	17	1.156	0.26	5	0.010	ns	-0.176	16	-1.222	0.24	5	0.010	ns
	IR*pmt* 2	-0.034	14	-0.338	0.74	2	0.025	ns	0.064	12	1.014	0.33	4	0.013	ns
	IR*pi* 1	-0.172	17	-1.184	0.25	6	0.008	ns	-0.036	16	-0.568	0.58	2	0.025	ns
	IR*pi* 2	-0.028	17	-0.333	0.74	1	0.050	ns	-0.161	16	-1.313	0.21	6	0.008	ns
nicotine	IR*pi/pmt* 1	-1.136	17	-20.37	0.00	6	0.008	*	-1.409	15	-22.789	0.00	6	0.008	*
	IR*pi/pmt* 2	-0.337	15	-3.541	0.00	3	0.017	*	-0.836	12	-6.553	0.00	5	0.010	*
	IR*pmt* 1	-1.132	17	-22.03	0.00	5	0.010	*	-1.374	16	-23.912	0.00	3	0.017	*
	IR*pmt* 2	-0.386	14	-4.264	0.00	4	0.013	*	-1.06	12	-9.952	0.00	4	0.013	*
	IR*pi* 1	-0.017	17	-0.228	0.82	2	0.025	ns	0.04	16	0.673	0.51	1	0.050	ns
	IR*pi* 2	-0.011	17	-0.204	0.84	1	0.050	ns	0.085	16	1.835	0.09	2	0.025	ns
rutin	IR*pi/pmt* 1	-0.066	17	-1.338	0.20	3	0.017	ns	-0.065	15	-1.34	0.20	5	0.010	ns
	IR*pi/pmt* 2	0.083	15	1.646	0.12	5	0.010	ns	0.111	12	1.328	0.21	4	0.013	ns
	IR*pmt* 1	0.072	17	1.715	0.10	6	0.008	ns	-0.054	16	-1.02	0.32	3	0.017	ns
	IR*pmt* 2	0.077	14	1.642	0.12	4	0.013	ns	0.026	12	0.537	0.60	1	0.050	ns
	IR*pi* 1	-0.045	17	-0.805	0.43	2	0.025	ns	0.07	16	1.471	0.16	6	0.008	ns
	IR*pi* 2	0.031	17	0.688	0.50	1	0.050	ns	-0.032	16	-0.643	0.53	2	0.025	ns

Manuscript IV Supplemental Material

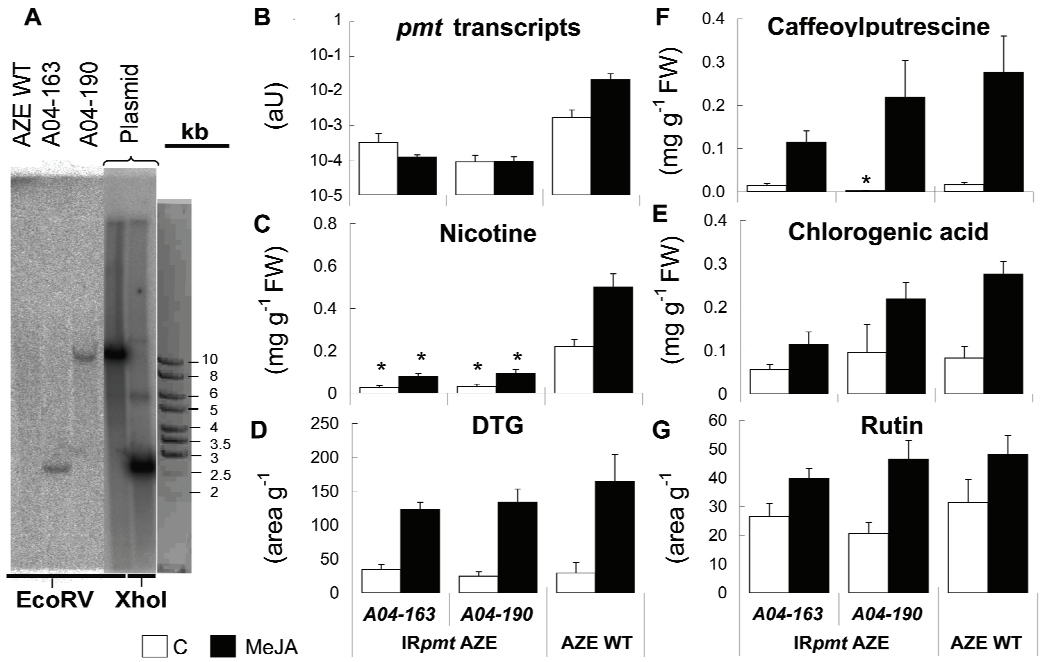


Figure S2: Characterization of the 2 independently-transformed homozygous lines in which the *putrescine N-methyl transferase* (*pmt*) genes were silenced in *N. attenuata* wild-type (WT) from a population in Arizona (AZE). (**A**) Southern blot analysis reveals that each transformed line harbors only a single insertion (DNA was EcoRV digested and the plasmid used for transformation was additionally digested with XhoI; probe was specific for the selective marker gene *hygromycin phosphotransferaseII*). Levels (mean ± SE) of constitutive (C) and methyl jasmonate (MeJA)-elicited (**B**) *pmt*-transcripts, (**C**) nicotine, (**D**) a diterpene glycoside (DTG), (**E**) chlorogenic acid, and (**F**) rutin (N = 5). Asterisks signify significant differences between transformed lines and WT at $P < 0.05$ determined by unpaired *t*-tests with log-transformed data.

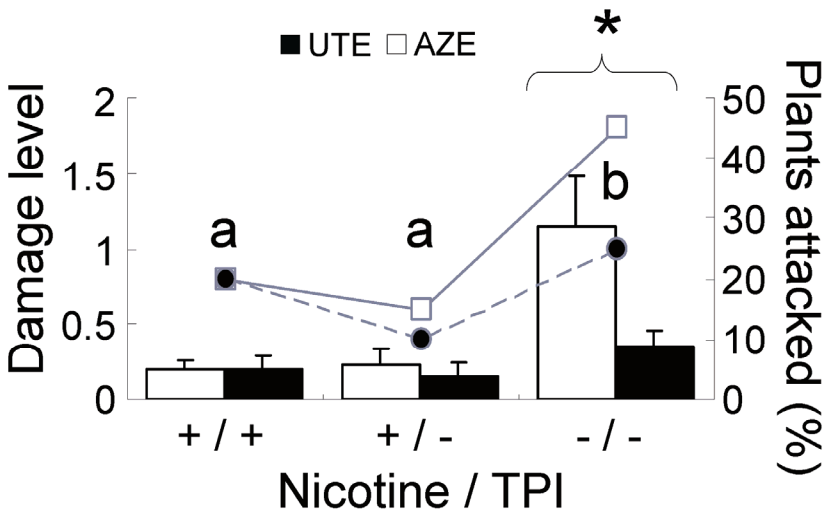


Figure S2: Damage levels caused by thrips (mean ± SE) on ecotypes of *N. attenuata* from Arizona (AZE) and Utah (UTE) planted into a native Utah population in 2005. AZEs and UTEs were arranged in pairs which were matched to produce trypsin protease inhibitors (TPI) and nicotine, only nicotine, or neither nicotine nor TPIs (via stable transformation; N = 20). Percentage of AZEs and UTEs attacked is indicated by the line graphs and secondary Y-axis. Asterisks on brackets signify significant differences in damage levels between the partners of a pair (paired *t*-tests) and different letters signify significant differences for the pair types as analyzed by repeated measures 2-way ANOVA (May 31–June 6, for ecotype $F_{1df,112} = 4.5$, $P = 0.035$, for pair type $F_{2df,112} = 7.7$, $P < 0.001$).

Supplemental Protocl S1:

Silencing constructs: For silencing we used IR constructs that contain either a consensus fragment for the two *N. attenuata pmt* genes (GenBank AF280402 & AF280403) or a fragment of the *pi* gene (GenBank DQ158200). Vector construction and plasmids are described elsewhere [see Steppuhn *et al.* (2004) for pRESC5 plasmid used to silence *pmt* and Bubner *et al.* (2006) for pSOL3 plasmid used to silence *pi*]. Homozygosity of T_2 plants was determined by screening for resistance to hygromycin (*hygromycin phosphotransferase II* gene from pCAMBIA-1301; GenBank AF234297 contained in the pRESC5 and pSOL3 vectors). To create plants silenced in both genes, we transformed homozygous T_2 IR*pmt* UTE plants with a pSOL4PI vector; this vector is based on pSOL3 but its selective marker gene has been exchanged for the *streptothricin-acetyl-transferase* (*sat-1*) gene (GenBank X15995), and thus T_1 plants from this second transformation were screened for resistance to nourseothicin. To express the full-length *pi* from the UTE in the AZE a pRESC2 plasmid was used and plants were screened for hygromycin resistance (Zavala *et al.* 2004b).

Characterization: Two independently-transformed homozygous lines for each vector were characterized by Southern blot analysis to reveal single insertions [see Steppuhn *et al.* (2004) for IR*pmt* UT, Steppuhn & Baldwin (2007) for IR*pi* UT and IR*pmt/pi* UT, Zavala *et al.* (2004b) for S*pi* AZ, and Fig. S1A for IR*pmt* AZ] and by flow cytometric analysis to confirm that all lines were diploid (Bubner *et al.* 2006). T_2 plants of both lines of each genotype were grown in 1L hydroponic vessels in a climate chamber (York Milwaukee,

WI, USA; 32/28 °C,16/8 h photoperiod, 65 % r.h., light intensity 200-300 µmol m^{-2} s^{-1}) and characterized for their constitutive and MeJA-elicited (150 µg in 10µL lanolin) expression of the target gene (10h after elicitation) as well as levels of nicotine, TPI, caffeoylputrescine, chlorogenic acid, rutin, and one DTG (4d after elicitation). Transformed lines were verified to differ from WT plants only in the targeted trait as previously described for all UTE transformants (Steppuhn & Baldwin 2007). Transcript levels of *pmt* in IR*pmt* AZE transformants were reduced to 12% of constitutive and less than 1% of MeJA-elicited WT mRNA levels (Fig. S1B) and nicotine levels were reduced to 13% of constitutive and 17% of MeJA-elicited levels of WT plants (Fig. S1C, t-tests: $P < 0.001$ for both lines). Transgenic lines were not morphologically distinguishable from WT plants and did not differ in other traits measured except that all *pmt*-silenced plants accumulated the alkaloid anatabine (Steppuhn *et al.* 2004). Constitutive and MeJA-elicited levels of other secondary metabolites such as caffeoylputrescine, chlorogenic acid, rutin, and one DTG were not significantly affected in IR*pmt* AZE (Fig. S1D-G: all t-tests, $P > 0.05$) with the exception of reduced constitutive caffeoylputrescine levels in one line (A04-190-1). Lines A04-186-1 (IR*pi* UTE), A03-108-3-2 (IR*pmt*/*pi* UTE), and A04-190-1 (IR*pmt* AZE) were used in VIGS and field experiments except that line A04-163-1 was used instead of A04-190-1 in the field.

VIGS-experiment: Plants were grown in 1 L pots with soil in a climate chamber (20/17 ºC, 16/8 h photoperiod, 60-75% r.h., light intensity in experiments 1 and 3: 405 µmol s^{-1} m^{-2} and in Experiment 2: 115 µmol s^{-1} m^{-2}) and only plants in experiment 2 were transferred to the glasshouse (20-22/18-20 ºC 16/8 h L/D, light intensity:165 µmol s^{-1} m^{-2} , 30-55% r.h.), 13 days after inoculation.

In addition to JA levels, its conjugates to isoleucine and leucine were also reduced in VIGS*lox* compared to VIGSev plants (separate 3-way ANOVAs with the factors vector, treatment, and genotype: for experiment 2: vector $F_{1, 59} = 20.70$, $P < 0.001$; for experiment 3: vector $F_{1, 48} = 4.68$, $P = 0.035$; no significances for genotype). Levels of *lox* transcripts were detected by hybridizing the blot with a α-^{32}P-dCTP-labeled PCR fragment (792 bp) amplified from a plasmid containing the full-length *N. attenuata lox3* gene (GenBank, AY254349) using primers binding at bp 1983-2002 (forward primer) and at bp 2755-2774 (reverse primer), which is outside of the region contained in the pTVLOX3 silencing vector (bp 509-947).

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