Nicotine’s Defensive Function in Nature

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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-methyltransferase (pmt) genes in either antisense or inverted-repeat (IRpmt) orientations. Only the latter reduced (by greater than 95%) constitutive and inducible nicotine. With Δ2-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 IRpmt plants in choice tests. When planted in their native habitat, IRpmt 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 beetle 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.

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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 plants in their natural habitat is unclear. To examine the resistance effect of nicotine, we transformed N. attenuata with inverted-repeat pmt (IRpmt) and antisense pmt constructs and found that only IRpmt plants had strongly reduced nicotine content. We characterized the defense and growth phenotypes of two independently transformed homozygous IRpmt 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 pulse-chase experiments with D2-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 IRpmt than WT leaves. We transplanted WT and IRpmt 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 IRpmt plants more than unelicited or elicited WT and elicited IRpmt plants. These results demonstrate that nicotine functions as an effective resistance trait under natural conditions.

Results/Discussion

IRpmt 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 pCAMPpmt1) 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 IRpmt construct pRESC5PMT 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).

Genomic and Transcriptional Characterization

Two homozygous T2 IRpmt lines (108 and 145) with reduced nicotine levels were further characterized. Southern blot analysis using a probe hybridizing to the selective marker in the IRpmt 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-elicted 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 with D4-NA ethyl ester results in the formation not only of D4-nicotine and D4-anatabine but also of D8-anatabine, demonstrating that the latter integrates two D4-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 D1-NA. In IR*pmt* plants, in contrast, only traces of D1-nicotine were found, but one-third of the anatabine was either D1- or D8-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.

IRpmt plants did not differ from WT plants in any other measured secondary metabolite or growth parameter. Constitutive or MeJA-induced levels of caffeoylputrescine, chlorogenic acid, rutin (Figure S2), TPI activity, or the release of cis-α-bergamotene (Figure S3) in IRpmt-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 IRpmt lines, and transformed lines were not visually or morphologically distinguishable from WT plants. Hence, the IRpmt plants represent an ideal construct with which to examine the ecological consequences of nicotine production.

**Effects of Nicotine Silencing on *N. attenuata* Herbivores**

*M. sexta* larvae reared on IRpmt 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*WT-PMt0108 < 0.02, *p*WT-PMt1145 < 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 1999) 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 IRpmt (108) plants, preferred to initiate feeding on the latter (*n* = 43; *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 IRpmt 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. 1985), nicotine, anatabine, and TPI levels of WT and IRpmt plants grown in the field plantation were analyzed: they were found not to differ from plants grown under laboratory conditions (*p* > 0.05; Figure 4A). A *M. sexta* feeding choice test evaluating the larval's choice between field-grown WT and IRpmt plants (*n* = 57; *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 IRpmt plants was not altered by growth under field conditions. In addition, choice tests with field-collected *D. undecimpunctata*, which was observed colonizing only IRpmt leaf material in the field plantation, revealed that 77% of these beetles preferred the nicotine-deficient IRpmt leaf material over WT (*n* = 35; *Chi*² = 10.31, *p* < 0.001). Another beetle species observed occasionally on WT plants, *Trichobarus mucorea*, does not distinguish between WT and IRpmt leaf material in choice tests (*n* = 19; *Chi*² = 0.05, *p* = 0.8).

In the field plantation, IRpmt 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, IRpmt 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 (7CFR340.5c) for the introduction 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 IRpmt plants translates into a fitness loss.

IRpmt 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 IRpmt and WT plants were not significant. The damage caused by Epitrix hirtipennis was variable but significantly higher for unelicited IRpmt compared to WT plants (ANOVA: F = 2.81, df = 3, p = 0.04, pMUT-WT < 0.05).

MeJA elicitation significantly reduced the damage of IRpmt 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 transfectants 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 (Berendse 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 transfectants 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 (Hastoin et al. 1960).

Materials and Methods

Plant material and transformation. N. attenuata Torr. ex Watson (synonym with N. toreyaya Nelson and Mabch.: 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 tumefaciens (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 pCAMBPT1 and pNATPMT1 vectors, which contain a gene fragment of pmt1 (which has 95% identity to pmt2) in an antisense orientation, and pRESCPMT, which contains the pmt1 gene fragment twice in an inverted orientation separated by intron 3 of the Flaveria trinervia gene pyruvate orthophosphate dikinase (poh) (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; homoygosity was determined by resistance screening of the T2 plants. Two independently transformed homozygous IRpmt 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 T-DNA (see Figure S1 and Protocol S1).

PMT mRNA accumulation and secondary metabolites. Transformed plants (108 and 145) and WT plants were grown in 1-L hydroponic vessels in a climate chamber as described in Hermensier 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 250 μl of lanolin paste, or left unelicited (control). 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 sequence see Figure S6). For analysis of the production 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 (Keinanen et al. 2001; Halitschke and Baldwin 2003). A peak occurring in 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 D2-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 pmt1 (which has 95% identity to pmt2) in an antisense orientation, and pRESCPMT, which contains the pmt1 gene fragment twice in an inverted orientation separated by intron 3 of the Flaveria trinervia gene pyruvate orthophosphate dikinase (poh) (for vector construction and plasmids see Figure S5 and Protocol S1). To examine the release of cis-α-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 5 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-wk-old seedlings were planted individually into 2-L pots with potting soil (1 C 4:1 at 20–28 °C, under (ger, Schermbeck, Germany) at 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 m × 18 m; GPS: lat 37°08′45″*N, long 114°01′12″) 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, Maryville, 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, and leafhoppers was noted separately. The most abundant herbivores observed in the field plantation during the release were *S. exigua*, *Trimerotropis* spp., *E. hispilennis*, 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 (CFR340.3c). Consequently, direct fitness measures were unobtainable in this experiment.

For analysis of alkaloids and TPs 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 choice of freshly eclosed *M. sexta* larvae (North Carolina State University) and of adults of field-collected *D. undecimpunctata* and *Trichoborus muscorea* (Chromyselidae and Curculionidiae) 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 pRcCambia1301 (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 pCambia1301, 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 with a PCR fragment of the hygromycin phosphotransferase II gene from pCambia1301, which is specific for the selective marker on the T-DNA and signifies one insertion in each of the two lines. Found at DOI: 10.1371/journal.pbio.0020217.sg001 (6.3 MB TIF).

**Figure S2.** Secondary Metabolite Levels in the Studied IR*pmt* Lines Inverted-repeat silencing of *pmt* did not change the levels of the following (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 (W), wounding and regurgitator 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* regurgitation diluted 1:1 (v:v) was dispersed over the puncture wounds (*n* = 8–10). Levels of (A) TPI and (B) cis-α-bergamotene emission (mean ± SE) in two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) did not differ from WT plants 4 d after transformation (for TPI) and 10 h (for cis-α-bergamotene) after receiving one of four treatments (as described for S2): untreated control (Con), wounding, (W), wounding with additional regurgitator application (W+R), and MeJA elicitiation. IS, internal standard. Found at DOI: 10.1371/journal.pbio.0020217.sg003 (73 KB PPT).

**Figure S3.** Proteinase Inhibitor and Volatile Emission of the Studied IR*pmt* Lines Levels of (A) TPI and (B) cis-α-bergamotene emission (mean ± SE) in two independently transformed *N. attenuata* IR*pmt* lines (108 and 145) did not differ from WT plants 4 d after transformation (for TPI) and 10 h (for cis-α-bergamotene) after receiving one of four treatments (as described for S2): untreated control (Con), wounding, (W), wounding with additional regurgitator application (W+R), and MeJA elicitiation. IS, internal standard. Found at DOI: 10.1371/journal.pbio.0020217.sg004 (98 KB PPT).

**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* = 43, WT = 57, IR = 57, *P* = 0.28) 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.sg008 (95 KB PPT).

**Figure S5.** Transformation Vectors This figure shows plasmids used for the transformation of *N. attenuata* lines with reduced levels of two PtsMs due to postranscriptional gene silencing. Both (A) pCAMPMT1 (10.7 kb) and (B) pNATPMT1 (9.7 kb) allow the synthesis of *pmt* antisense RNA. (C) pRES5CMPT (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; Tn5, *lacZ*, *gus*, and *phoA*; 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; PCAMP1, promoter and terminator of *Nicotiana platura* pmt1; pmtn1, fragment identical with *N. attenuata* pmt2; *sos*, *sox*, and *KdpD* promoter and terminator of the *S. enterica* synthase gene; repA* pVS1, replication protein gene from pVS1; sat-1, nourseothricin resistance gene; *sta*, *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).

**Figure S6.** PMT Sequences and TaqMan Probe Nucleotide sequences of *N. attenuata* pmt1 and pmt2 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).

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

**Accession Numbers**

GenBank accession numbers for the genes discussed in this paper are *bla* from puc19 (L09137), *hygromycin phosphotransferase I* from pCAMBIA-1301 (AF234297), *pmt1* (AF280402), and *pmt2* (AF280405).

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**Conflicts of interest** The authors have declared that no conflicts of interest exist.

**Author contributions** AS and ITB conceived and designed the experiments. AS, RH, and ITB performed the experiments. AS, BK, RH, and ITB analyzed the data. KG contributed reagents/materials/analysis tools. AS and ITB wrote the paper.
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Several studies have examined the role of nicotine in plant defenses against herbivory. For example, Kessler and Baldwin (2004) found that herbivore-induced plant vaccination, a process where plants produce increased levels of secondary metabolites in response to herbivory, can benefit plants. This is consistent with the idea that plants have evolved mechanisms to increase their resistance to herbivory when they are under attack.

Nicotine is a major alkaloid found in tobacco plants and is toxic to many insects. For example, van Dam et al. (2001) found that nicotine can reduce the survival of Manduca sexta larvae on tobacco plants. This suggests that nicotine can play a role in plant defense against herbivory.

Overall, these studies highlight the importance of understanding the role of nicotine and other tobacco alkaloids in plant defenses against herbivory. Further research is needed to understand how these compounds interact with other factors, such as natural enemies, to influence plant survival and fitness.