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Innovation in anti-herbivore defense systems during neopolypoloidy – the functional consequences of instantaneous speciation

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Summary

Allopolyploid hybridization instantly merges two differentially adapted genomes into one individual. Allopolyploids are often evolutionarily successful, undergoing adaptive radiations despite the genetic and physiological challenges of merging genomes. We examine a suite of induced herbivore resistance traits in three independent lines of the synthetic allopolyploid *Nicotiana* × *mierata* (*Nma*) and its parent species, *N. miersii* (*Nmi*) and *N. attenuata* (*Na*), to determine how a complex polygenetic adaptation fares during the early stages of neoallopolyploid formation. All species responded to *Manduca sexta* oral secretions (OS) with a temporally prolonged jasmonate (JA) burst. In one parent (*Na*), the JA burst was additionally amplified and associated with the elicitation of direct and indirect defenses. In the other parent (*Nmi*), OS neither amplified the JA burst nor elicited defense responses, although applied MeJA confirmed the inducibility of the defense responses. All lines of *Nma* retained enough aspects of *Na*'s JA signaling to recognize OS and to accumulate sufficient direct defenses to impair the growth of *Manduca* larvae. Most defense-related metabolites were retained in *Nma* even if inherited from only one parent; however, OS-elicited volatiles, trypsin protease inhibitors (TPIs) and chlorogenic acid were lost in some lines, even though MeJA treatment elicited similar responses in all lines. Herbivore defense systems are flexibly inherited in allopolyploids, causing individuals to diverge over only a few generations; for example, line 1 of *Nma* could not produce TPIs after OS elicitation, whereas lines 2 and 3 could. This flexible integration of defense signaling systems with a diversity of elicited responses may explain why adaptive radiations are commonly found in allopolyploid lineages.

Keywords: ***Nicotiana attenuata*, direct defenses, indirect defenses, allopolyploidization, variation, metabolite.**

Introduction

Polyploidy is pervasive in the evolutionary history of the plant kingdom (Masterson, 1994). Allopolyploidization, the combination of multiple intact genomes in a single offspring, occurs frequently in plants and instantly creates a unique species, one that is often incompatible with either one or both parent species (Jackson, 1976). Despite the problems inherent in the first polyploid generations, such as decreased fertility (Ramsey and Schemske, 2002) and genomic instability (Comai *et al.*, 2000), allopolyploidization events have often preceded adaptive radiations and yielded lineages or species that are highly adapted to their environments (Barrier *et al.*, 1999; Jackson and Tinsley, 2003; reviewed in Soltis *et al.*, 2003). This potentially rapid process is probably driven by a more heterogenous genome in polyploids than in parent species due to homologous chromosome pairing and non-Mendelian genomic changes, such as transposon activity, in polyploid genomes (Song *et al.*, 1995). Duplicated genes can evolve independently in polyploid individuals (Cronn *et al.*, 1999), potentially leading to functional differences among homologous copies of a gene (Adams *et al.*, 2003; Cedroni *et al.*, 2003).

Despite the apparent importance of allopolyploidy in plant evolution, little is known about how allopolyploids incorporate into one individual two differentiated physiological systems that have probably been sculpted by different selective pressures. Even if a plant exhibits sufficient plasticity in its genomic or nuclear machinery to flawlessly accommodate the fusion of two genomes,

unexpected or non-functional physiological systems would seem to be the likely result. Whether physiological systems rapidly evolve in independently breeding lines of polyploids or whether these systems diverge at the normal rate of a genetically isolated population – or even more slowly, as argued by Stebbins (1950) – remains unknown. Mounting evidence suggests that polyploidization is followed in many species by a 'genomic burst'. This burst is characterized by chromosomal breaks or homologous synapse formation (Pires *et al.*, 2004), increased transposon activity (Madlung *et al.*, 2005), and differential patterns of DNA methylation (Madlung *et al.*, 2005) and gene expression (Comai *et al.*, 2000), which contribute to differences in RFLP patterns (however, see Liu *et al.*, 2001). In addition to a rapid, genome-scale rearrangement following allopolyploid formation (Pontes *et al.*, 2004), more variation in quantitative phenotypic characteristics has recently been observed in neoallopolyploids than in their corresponding parent species (Schranz and Osborn, 2000), which could correlate with transcriptome differences between polyploid lines (Pires *et al.*, 2004).

In order to examine the consequences of allopolyploidy for a complex suite of anti-herbivore traits, we created the novel synthetic allopolyploid, *Nicotiana miersii* × *N. attenuata* (hereafter, *N.* × *mierata* or *Nma*). Allopolyploidy occurs frequently in the genus *Nicotiana* (Chase *et al.*, 2003; Goodspeed, 1954), with 35 of the 75 *Nicotiana* species recognized as allopolyploids (Clarkson *et al.*, 2004). *Nicotiana* allopolyploids display a low degree of homologous chromosome pairing as shown by a conservation of parental chromosomal geography using genomic *in situ* hybridization (GISH) chromosome staining; however, *Nicotiana* allopolyploids display a pattern of 18–5.8–26S rDNA repeats consistent with concerted evolution (Kovarik *et al.*, 2004). In addition, established *Nicotiana* allotetraploids retain maternal, paternal or mixed-inheritance internal transcribed spacer (ITS) regions (Chase *et al.*, 2003), suggesting little and potentially confounding selection pressure for cytoplasmic/nuclear compatibility, unlike in other allopolyploid systems (Soltis and Soltis, 1995). Ancestors of two North American tobacco species, *N. attenuata* and *N. obtusifolia* (previously *N. trigonophylla*), hybridized to form two polyploid species, *N. quadrivalvis* (previously *N. bigelovii*) and *N. clevelandii* (Chase *et al.*, 2003; Qu *et al.*, 2004). Recently, the role of polyploidy in the inheritance of anti-herbivore defense traits at the metabolic (Lou and Baldwin, 2003) and transcriptome (Qu *et al.*, 2004) levels was studied in these species. These studies examined traits within two polyploid species of the same parental origin that are probably >2 million years old (Clarkson *et al.*, 2004); traits that were inherited or altered directly following polyploidization cannot be distinguished from traits that were altered in subsequent generations (of both the polyploids and parent species). Here we examine changes in the polygenic defense complex that occur immediately after hybridization.

The *Nma* synthetic polyploids were created to simulate the natural *N. quadrivalvis N. clevelandii* allopolyploid system. *N. miersii* was chosen as the maternal parent as it approximates the defense responses of *N. obtusifolia* (Wu *et al.*, 2006). Unlike extant *N. obtusifolia, N. miersii* can be readily hybridized with *N. attenuata* (*Na*); information about the traits that are important for herbivore resistance in this species is plentiful.

Jasmonic acid (JA)-elicited defenses against the *Solanaceous*-specialist lepidopteran herbivore *Manduca sexta* (Figure 1b) have been extensively studied in *Na* in both greenhouse and native North American populations for more than a decade (Baldwin, 2001). A large part of the plant's transcriptome is involved (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003; Voeckel and Baldwin, 2004), and evidence is growing that many of these *Manduca*-induced responses increase plant fitness when plants are attacked, but are associated with fitness costs in herbivore-free environments (Baldwin, 1999; Halitschke *et al.*, 2000; reviewed in Kessler and Baldwin, 2002). Herbivore attack is recognized by *Na* when *M. sexta* oral secretions and regurgitants (OS) are introduced into wounds during feeding. This recognition response is clearly visualized by a burst of JA, whose effects in defense elicitation can be mimicked by exogenous application of methyl jasmonate (MeJA). The JA burst elicits both direct defenses, such as nicotine, trypsin protease inhibitors (TPIs), phenolic compounds and diterpene glycosides (DTGs), as well as indirect defenses, such as volatile organic compounds (VOCs). The ability of *Na* to recognize herbivore attack and induce appropriate defensive compounds confers a large fitness benefit on the plant (Baldwin, 1998; Halitschke and Baldwin, 2003). Nicotine, although a hallmark of the genus *Nicotiana*, is tolerated by *M. sexta* (Self *et al.*, 1964; however, see Steppuhn *et al.*, 2004), and its production exacts a high nutrient cost (Baldwin, 2001). Trypsin protease inhibitors slow larval growth on *Na*, but TPI production is also associated with fitness costs for the plant (Zavala *et al.*, 2004a,b). The direct anti-herbivore effect and resource costs of induced phenolic compounds and DTGs in the *Na M. sexta* system remain unknown, although DTGs have been shown to reduce larval mass in tobacco budworm (Snook *et al.*, 1997). In native North American *Na* populations, plant-released VOCs attract the generalist predator of *M. sexta, Geocoris pallens*, which reduces herbivory (Kessler and Baldwin, 2001). Although the physiological costs of VOCs have yet to be determined and are probably small (Halitschke *et al.*, 2000), such mutualistic relationships, whether pollinator- or predator-driven, may be transient, and are probably the source of the high diversity of volatile terpenoid compounds in *Nicotiana* (Raguso *et al.*, 2003).

This study introduces *Nma* as a synthetic polyploid system, and compares the anti-herbivore defense

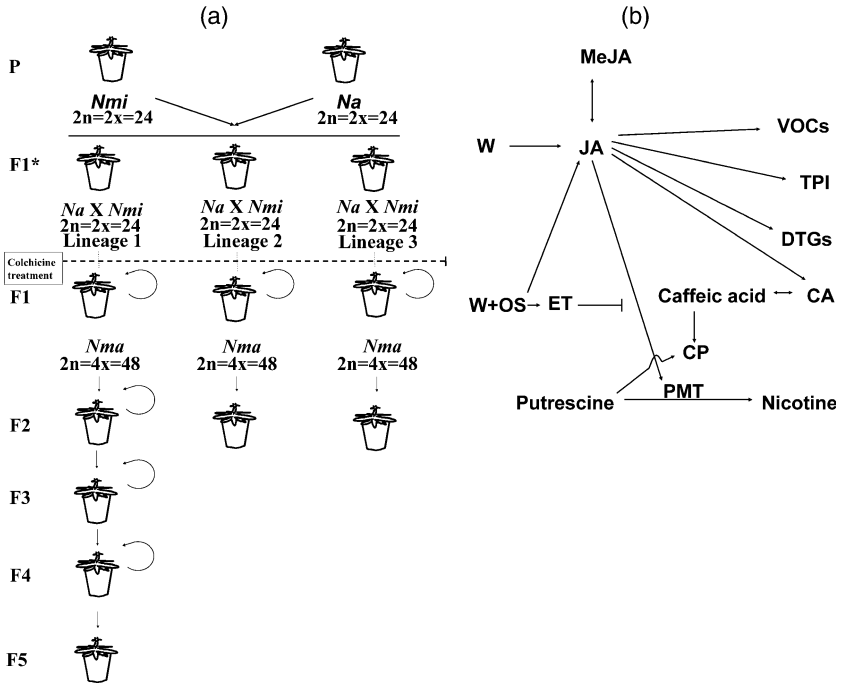

Figure 1. Breeding scheme and metabolic pathways.
(a) Breeding scheme for *N.* × *mierata* (*Nma*). An emasculated *N. miersii* (*Nmi*) flower was pollinated with an excised *N. attenuata* (*Na*) anther. Three seedlings of the primary hybrid were treated with colchicine to form the three lineages of *Nma.* Line 1 was inbred for five generations, while the F_2 generations of lines 2 and 3 were used. Functional ploidy level, ploidy level and chromosome counts are shown.
(b) Jasmonic-acid-dependent chemical defenses in *Na* (after Lou and Baldwin (2003)). Wounding (W) and wounding with applied *M. sexta* oral secretions (OS) elicit differential jasmonic acid (JA) 'bursts', which can be mimicked with exogenously applied methyl jasmonate (MeJA). This, in turn, elicits indirect defenses such as predator-attractant volatile organic compounds (VOCs) and direct defenses such as trypsin protease inhibitors (TPIs), diterpene glycosides (DTGs), caffeic-acid-derived phenolics such as chlorogenic acid (CA) and caffeoylputrescine (CP), and nicotine, which requires putrescine methyl transferase (PMT). Regulation of this system differs between *Na* and its sister species, *Nmi*, as well as from their allopolyploid, *Nma*.

phenotypes of independently formed lines of *Nma* as well as of its parent species, *Na* and *Nmi.*

Results

Botanical description of N. × mierata

Nicotiana × *mierata* is a synthetic hybrid between *Nicotiana miersii* J. Rèmy in C. Gay, *Fl. Chil.* 5: 56. 1849 and *N. torreyana* Nelson & J. F. Macbr. in *Bot. Gaz.* 61: 43, 1916 [Syn.: *N. attenuata* Torrey in Watson, *Botany* (Fortieth Parallel): 267. 1871; non-*N. attenuata* Steud., *Nomencl. Bot.* Ed. I:554, 1821]. *N.* × *mierata* differs from *N. miersii* in that it has oval to ovate rosette leaves larger than *N. miersii*'s lanceolate to broad-lanceolate rosette leaves, larger flowers, and a main inflorescence that is more dominant over lateral inflorescences than that which occurs in *N. miersii*. *N.* × *mierata* differs from *N. torreyana* in that it has larger, more undulated rosette leaves, a broader limbus with rounded petal tips, darker seed pigmentation, and a scent reminiscent of *N. miersii*. A Latin description of *N.* × *mierata* is found in the supplementary information online, as is an analysis of chromosome number ($2n = 48$; Figure S1).

Characterization of N. × mierata (Nma) *allopolyploids*

Reciprocal crosses of *N. miersii* (*Nmi*) and *N. attenuata* (*Na*) were attempted; however, only crosses with maternal *Nmi* produced viable seeds, perhaps due to large differences in stigma length between the two species. Viable hybrid seeds were reproducibly achieved from approximately 10 crossing events, although this study focuses on lines derived from a single flower. Of the seedlings treated with colchicine, 32% produced fertile plants with typical allopolyploid morphological characteristics, i.e. larger cell and stomatal size, a faster growth rate, and features that are intermediate between the parent species; the remaining putative polyploids produced no offspring. Primary hybrid seedlings, which were grown without colchicine treatment, produced completely sterile plants.

Nma seeds were larger than seeds from either parent and displayed the dark pigmentation typical of *Nmi*, but the flowers had the tightly woven lobes typical of *Na* (Figure 2a). The *Nma* rosette-stage leaf shape (Figure 2b) was intermediate between the lanceolate *Nmi* and ovate *Na* rosette-stage leaves. *Nma* bolted earlier and faster compared with both parent species (Figure 2c), and displayed intermediate numbers of auxiliary branches between the more bushy *Nmi* and the erect *Na*. Floral tube length was intermediate between *Nmi* and *Na*, whereas corolla limb shape and pigmentation were typical of *Nmi* (Figure 2d). Polyploids had larger stomata and *Nmi*-like trichome morphology but the intermediate trichome density and different floral characteristics typical of both parents, i.e. corolla length, pigmentation and corolla limb size (data not shown). One line of *Nma* was inbred for five generations, whereas the other lines were bred to F_2 (Figure 1a).

The polyploid state and genome organization were analyzed with flow cytometry and amplified fragment length polymorphism (AFLP), respectively. Flow cytometry indicated a stable total genome size for *Nma*, which was additive of the two parent species (Table 1), suggesting an absence

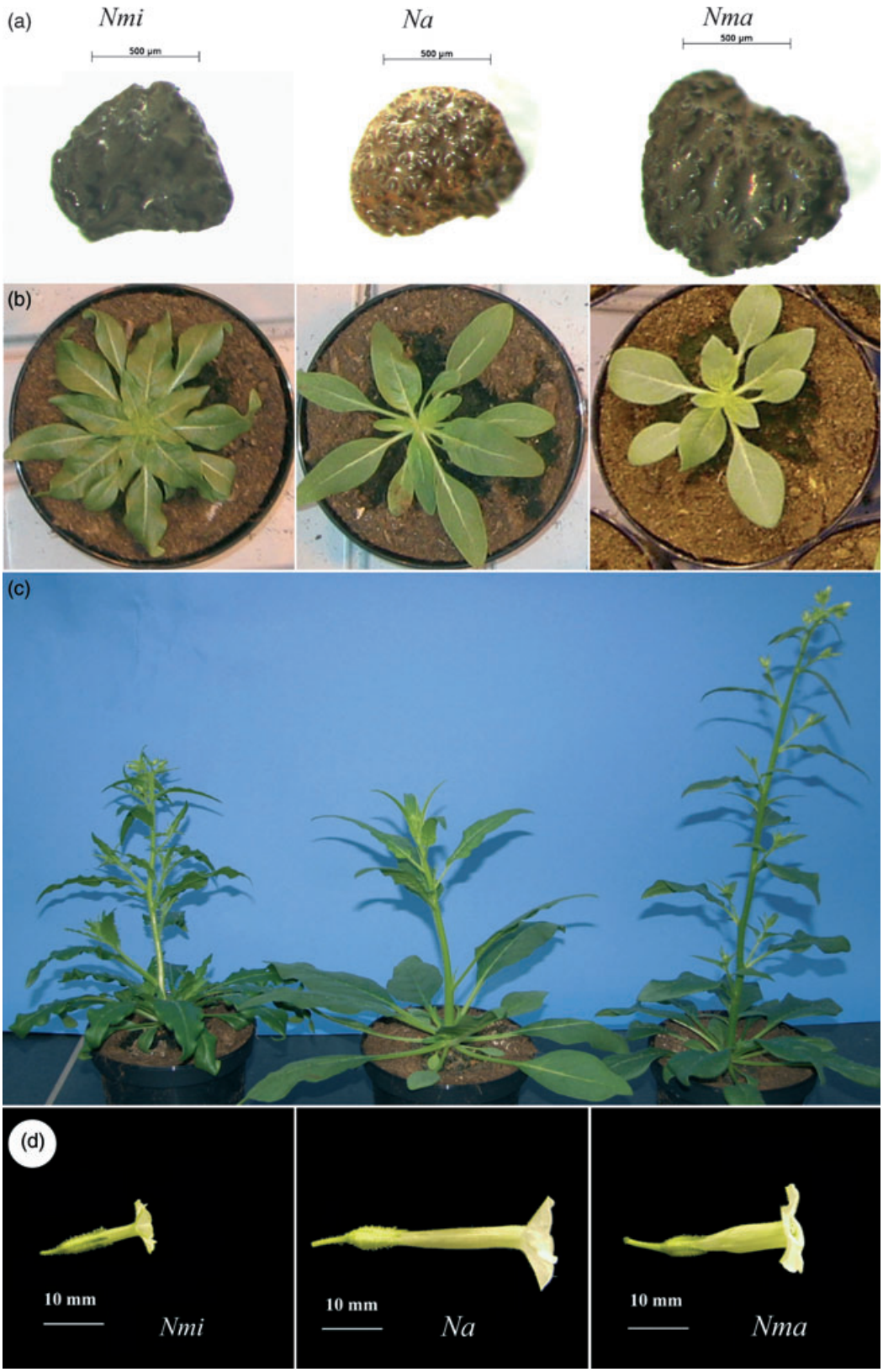

Table 1 Genome size (mean *C* value in pg DNA ± SE) was determined by flow cytometry for *N. attenuata* (*Na*), *N. miersii* (*Nmi*) and the allopolyploid *N.* × *mierata* (*Nma*) lines 1, 2 and 3

Species and lineage	*C* value (pg DNA) ± SE
Nmi	3.10 ± 0.06
Na	3.51 ± 0.02
Theoretical *Nma**	6.61 ± 0.11
Nma (line 1)	6.78 ± 0.01
Nma (line 2)	6.84 ± 0.05
Nma (line 3)	6.69 ± 0.08

*The theoretical genome size for *Nma* was calculated as the sum of *Na* and *Nmi*.

or low degree of aneuploidy, although the error in measurements (SD of approximately 0.1 pg) is larger than the size of any single chromosome [0.14 pg, i.e. the total genome size (6.7)/chromosome count (48) in the polyploids assuming homogenous chromosome lengths]. Analysis of 122 AFLPs (92 AFLPs unique to one or the other of the parents and 30 AFLPs common to both parents) revealed an entirely additive pattern of the two parental genomes in *Nma* (data not shown). Interestingly, no reproducible polymorphisms appeared between the polyploid and the sum of the two parental genetic fingerprints. This contrasts with the results of similar studies in wheat (Shaked *et al.*, 2001) and Arabidopsis (Madlung *et al.*, 2005), in which frequent (10% and 1% respectively) genomic changes accompanied polyploid formation. The genomic organization revealed by AFLP and flow cytometric analysis in *Nma* was more similar to that in synthetic tetraploid cotton, which did not yield any additions or deletions in an AFLP analysis (Liu *et al.*, 2001). It should be noted, however, that the total number of polymorphisms screened in this study was lower than in the studies with wheat and Arabidopsis.

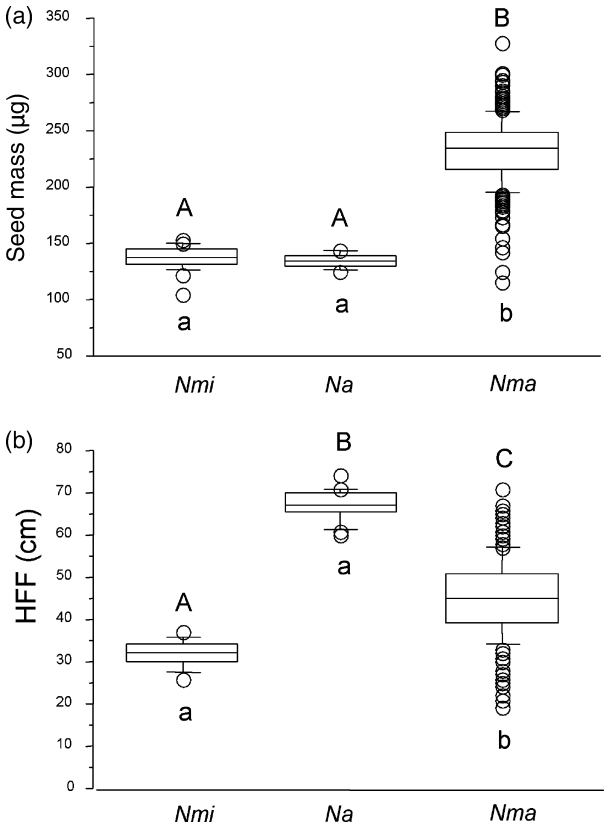

Figure 3. Seed mass and stalk height at first flower (HFF) were measured in *N. miersii* (*Nmi*), *N. attenuata* (*Na*) and *N.* × *mierata* (*Nma*) (line 1, F_3). Significant differences in means using a Games–Howell ANOVA *post hoc* test (to control for differences in sample size across species) are represented by unique capital letters. Unique lower-case letters indicate a significant difference in variation across species as determined by a Bonferroni coefficient-corrected *F* test. Center line, median; bars, flanking 25th percentiles; whiskers, flanking 45th percentiles; dots, all observations beyond the flanking 45th percentiles. Compared with diploids, polyploids commonly have drastically higher seed masses.

Variation in seed mass and stalk height

In order to determine whether novel phenotypic variation could arise in quantitative traits in the first generations of *Nicotiana* polyploids, seed mass and stalk height at first flower (HFF) were measured under controlled conditions in a population of *Nma* (seed mass: $n = 284$, HFF: $n = 334$) and populations of *Na* and *Nmi* (seed mass: $n = 24$, HFF: $n = 24$; Figure 3). Mean *Nma* seed mass exceeded the ranges of both parents, whereas *Nma* HFF was between the two parent species (Games–Howell ANOVA, *post hoc*). Interestingly, the variance within the *Nma* population exceeded that of either parent for both measured characteristics (Bonferroni corrected *F* test), although the opposite trend was expected due to uneven sample sizes.

JA burst

In *Na*, the transient JA increase elicited by wounding is amplified, attaining maximum concentrations of 1500 ng/g FW after 30 min and when *M. sexta* OS are added to

Figure 2. Seed, rosette-stage and bolting-stage morphologies of *N. miersii* (*Nmi*), *N. attenuata* (*Na*) and *N.* × *mierata* (*Nma*) (line 1, F_3).
(a) Polyploid seeds are about twice as large as either parent species and retain the dark pigmentation typical of *Nmi*.
(b) Rosette-stage plants of *Nma* develop approximately 3 days before either parent (photographs were taken to match stage). Polyploid leaf shape and pigmentation appear intermediate between the two parents.
(c) Bolting-stage plants. *Nma* displays a faster growth rate and a degree of branching between that of *Nmi* and *Na*.
(d) Flowers. *Nma* flowers are of intermediate size, and their floral limb resembles that of *Na* in size and that of *Nmi* in shape.

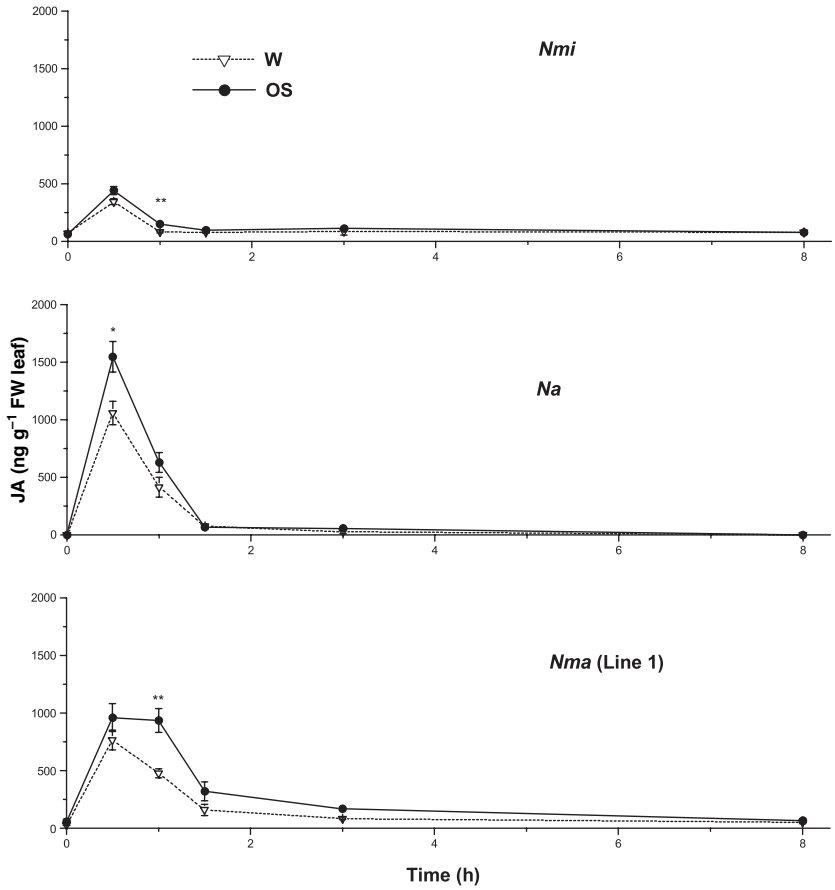

Figure 4. JA concentrations (mean ± SE) of five replicate plants over 8 h in *Nmi5 Na*, and *Nma* after wounding with applied *M. sexta* oral secretions (OS) or wounding followed by application of water (W). Significant differences between treatments at a given time point are indicated: $*P \leq 0.05$; $**P \leq 0.01$.

wounds, declining to constitutive levels within 60 min (Figure 4). *Nmi* reached a maximum JA concentration of only a third of the *Na* values, approximately 500 ng/g FW at 30 min, which was not amplified by OS treatment. This peak decayed quickly, although the decay was slightly delayed in OS treatments. JA concentrations in *Nma* reached a maximum at 30 min, attaining values that were between *Nmi* and *Na* (approximately 900 ng/g FW). This burst dissipated in water-treated plants, but remained at maximum levels until 1 h in OS-treated plants, returning to constitutive levels after about 3 h.

Defense compounds

TPIs. TPIs were harvested from the sink = source leaf 72 h after that leaf had been elicited by treating puncture wounds with either 20 μl of *M. sexta* OS or 20 μl water (W) or with 20 μl lanolin paste containing 150 μg methyl jasmonate (MeJA) or only lanolin (LC), or from a leaf from an untreated plant (Con). MeJA treatment resulted in an eightfold increase in TPI activity in *Na*, but constitutive levels were not detectable in *Nmi* or in any lines of *Nma*. Interestingly, OS treatment resulted in a sixfold induction of TPIs in *Na* and a 2.5-fold induction in *Nma* lines 2 and 3 compared with W levels, whereas *Nmi* and *Nma* line 1 plants showed no TPI elicitation after OS treatment. In *Nma* line 1 plants, this lack of induction results from relatively high TPI levels in W-treated plants (Figure 5). Although TPIs are present in both parents and all polyploid offspring, the regulation of TPI activity differed among species and even among polyploid lines.

Alkaloids. MeJA treatment elicited an approximately 1.5-fold increase in nicotine concentrations in *Na*, but not in *Nmi* or *Nma* lines 1 or 2 (Figure 6). Nicotine was induced in *Nma* line 3, but only 1.3-fold. OS treatment elicited a significant decrease in nicotine in *Nma* line 2, but not in *Nmi, Na* or *Nma* lines 1 or 3, despite a previously observed nicotine decrease after OS treatment in *Na* (Winz and Baldwin, 2001). However, this OS-mediated nicotine decrease was observed in *Nma* line 2. Nornicotine levels were not regulated by OS or MeJA, but constitutive levels were observed to be approximately 20-fold higher in *Nmi* and *Nma* compared with *Na* (Fisher's ANOVA *post hoc*, *P* values <0.0001; Figure 6), suggesting that nicotine is rapidly demethylated in these species.

Phenolics. The major extractable phenolics of *Nmi*, *Na* and *Nmi* (chlorogenic acid and caffeoylputrescine) were quantified in leaves 72 h following the five treatments (Figure 7).

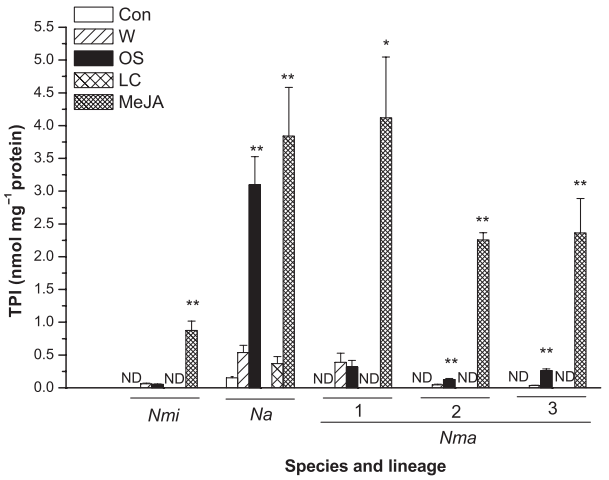

Figure 5. Trypsin protease inhibitor (TPI) concentrations (mean ± SE) in leaves of six or seven replicate plants normalized to total protein content across five elicitation treatments of *Na, Nmi* and three lineages of *Nma*. Leaves were induced with a lanolin paste containing 150 μg methyl jasmonate (MeJA) or with only lanolin as a control (LC); wounded with a fabric pattern wheel followed immediately by application of 20 μl *M. sexta* oral secretions (OS) or 20 μl water (W) to the puncture wounds; or left untreated (Con). Significant differences between treatment and control pairs (MeJA and LC; OS and W) are indicated: $*P \leq 0.05$; $**P \leq 0.005$. ND, not detected.

Chlorogenic acid was detected in all species. In *Na*, MeJA treatment elicited an approximately twofold increase in chlorogenic acid, whereas in *Nmi* and *Nma*, MeJA treatment decreased chlorogenic acid approximately twofold and three- to fivefold, respectively. MeJA treatment elicited caffeoylputrescine approximately eightfold in *Na* and likewise in *Nmi* (although both induced and constitutive caffeoylputrescine levels were lower in *Nmi* than in *Na*). A small MeJA-mediated increase of caffeoylputrescine was observed in *Nma* lines 1 and 2, although in *Nma* line 3, no caffeoylputrescine was detected with any treatment. Caffeoylputrescine showed a twofold increase after OS treatment in *Na* but not in any other species.

DTGs. No diterpene glycosides were observed in *Nmi*, but in *Na* and all lines of *Nma*, DTGs were elicited approximately 12-fold by OS (or an undetermined amount in treatments with no detectable constitutive levels of DTGs; Figure 8).

VOCs. VOCs (Figure 9) were trapped for 8 h from the headspace surrounding individual *Nmi, Na* and *Nma* plants 24 h after elicitation by either W, OS, LC or MeJA. Three sesquiterpenes (*cis*-α-bergamotene, germacrene A, and (–)-*trans*-caryophyllene) and two monoterpenes (linalool and an unknown monoterpene) were observably enhanced by MeJA or OS in one or more species. *Cis*-α-bergamotene was increased approximately threefold by MeJA in *Nmi* and *Na*, and also approximately threefold by OS treatment in *Na*. *Cis*-α-bergamotene levels were not regulated by any treatment in any lines of *Nma*. Germacrene A was only detected in *Na* plants and was upregulated significantly by OS and non-significantly by MeJA treatment. (–)-*trans*-caryophyllene was detected in *Nmi* and all lines of *Nma*, but not in *Na*. MeJA treatment increased caryophyllene levels approximately tenfold in *Nmi* and approximately threefold in *Nma* line 3, although no significant increase was found in the remaining *Nma* lines. Linalool was detected in *Nmi* and in all lines of *Nma*. MeJA upregulated linalool in *Nma* lines 1 and 2; a similar non-significant trend was observed in *Nmi* and *Nma* line 3. An identical trend in elicitation as that observed with

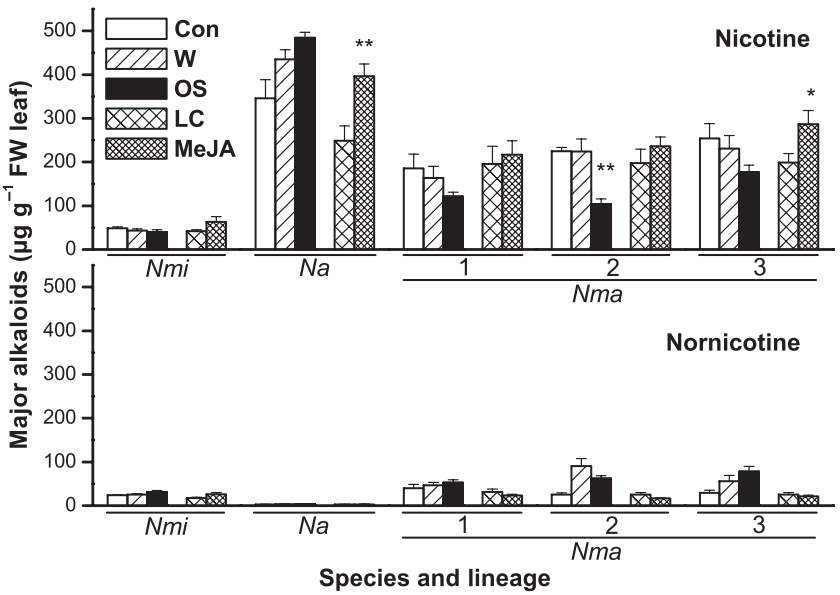

Figure 6. Concentrations (mean ± SE) of major alkaloids, nicotine and nornicotine, in *Nmi, Na* and three lineages of *Nma* across five elicitation treatments from nine or ten replicate plants per species and treatment. Leaves were induced with a lanolin paste containing 150 μg methyl jasmonate (MeJA) or only lanolin as a control (LC); wounded with a fabric pattern wheel followed immediately by application of 20 μl *M. sexta* oral secretions (OS) or 20 μl water (W) to the puncture wounds; or left untreated (Con). Significant differences between treatment and control pairs (MeJA and LC; OS and W) are indicated: $*P \leq 0.05$; $**P \leq 0.005$.

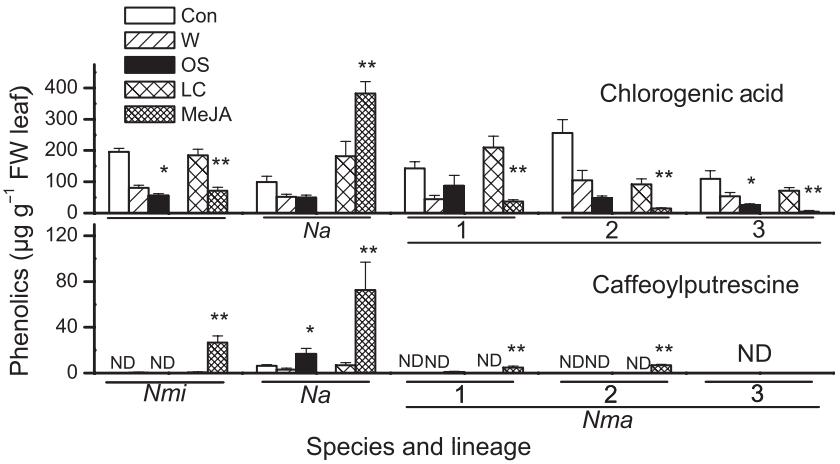

Figure 7. Concentrations (mean ± SE) of chlorogenic acid and caffeoylputrescine in *Nmi, Na* and three lineages of *Nma* across five elicitation treatments from nine or ten replicate plants per species and treatment.
Leaves were induced with lanolin paste containing 150 μg methyl jasmonate (MeJA) or only lanolin as a control (LC); wounded with a fabric pattern wheel followed immediately by application of 20 μl *M. sexta* oral secretions (OS) or 20 μl water (W) to the puncture wounds; or left untreated (Con). Significant differences between treatment and control pairs (MeJA and LC; OS and W) are indicated: $*P \leq 0.05$; $**P \leq 0.005$. ND, not detected.

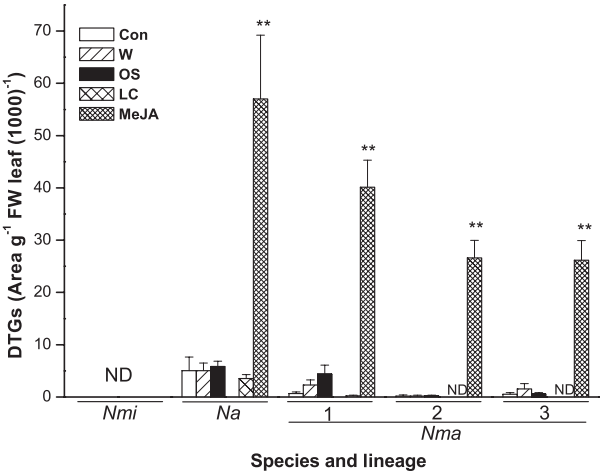

Figure 8. HPLC peak area (mean ± SE) of diterpene glycosides (DTGs) in *Nmi, Na* and three lineages of *Nma* across five elicitation treatments from nine or ten replicate plants per species and treatment.
Leaves were induced with lanolin paste containing 150 μg methyl jasmonate (MeJA) or only lanolin as a control (LC); wounded with a fabric pattern wheel followed immediately by application of 20 μl *M. sexta* oral secretions (OS) or 20 μl water (W) to the puncture wounds; or left untreated (Con). Significant differences between treatment and control pairs (MeJA and LC; OS and W) are indicated: $*P \leq 0.05$; $**P \leq 0.005$. ND, not detected.

linalool was observed for the other (unidentified) monoterpene (data not shown).

Larval performance. Freshly hatched first instar *M. sexta* larvae were reared on *Nmi, Na* and *Nma* (line 1, F_5; $n = 20$ per treatment) plants that had been treated 1 day earlier with either LC or MeJA. Caterpillars were weighed 4, 6, 8 and 10 days after being allowed to feed on the plants. MeJA elicitation did not affect caterpillar mass on *Nmi* but immediately decreased caterpillar weight in *Na*, ultimately resulting in caterpillars that on day 10 were almost three times smaller than their counterparts that fed on non-induced plants (Figure 10). MeJA also had a negative effect on caterpillar growth on *Nma* on days 8 and 10. Caterpillars that fed on non-induced *Nma* plants grew more slowly than did those that fed on the other two species (*Nma Nmi*, $P = 0.0051$; *Nma Na*, non-significant trend).

Discussion

Plants protect themselves from herbivore attack by activating combinations of direct defenses that interfere with insect feeding or with growth and indirect defenses that mediate mutualistic relationships with the natural enemies of the specialist herbivores (Kessler and Baldwin, 2002; Walling, 2000). Activated by elicitors from specialist insect herbivores, these polygenic defense responses are often highly specific. Given that the newly formed allopolyploid species will exist sympatrically with its parent species and the ecological interactions of the parents, the polyploids will probably benefit from retaining the chemical defense systems of the parent species. Conversely, by modifying these systems, a polyploid offspring may escape the ecological syndromes of the parent species. Lou and Baldwin (2003) demonstrated that two natural allopolyploid species, *N. quadrivalvis* and *N. clevelandii*, originating from hybridization events between *Na* and *N. obtusifolia* (Chase *et al.*, 2003; Wu *et al.*, 2006), retained a functional JA-mediated defense system; however, each species' response to *M. sexta* attack differed significantly. These findings suggest that polyploid speciation may be accompanied by a rapid differentiation of chemical defense systems while retaining the more generalized signaling pathways. A post-polyploidization genomic burst could be responsible (reviewed in Comai, 2000; Pikaard, 2001). However, how the differences in defense responses in *N. quadrivalvis* and *N. clevelandii* arose – either from heterogeneity in the parent populations that existed before the hybridization or from long-term evolutionary modifications that occurred after the

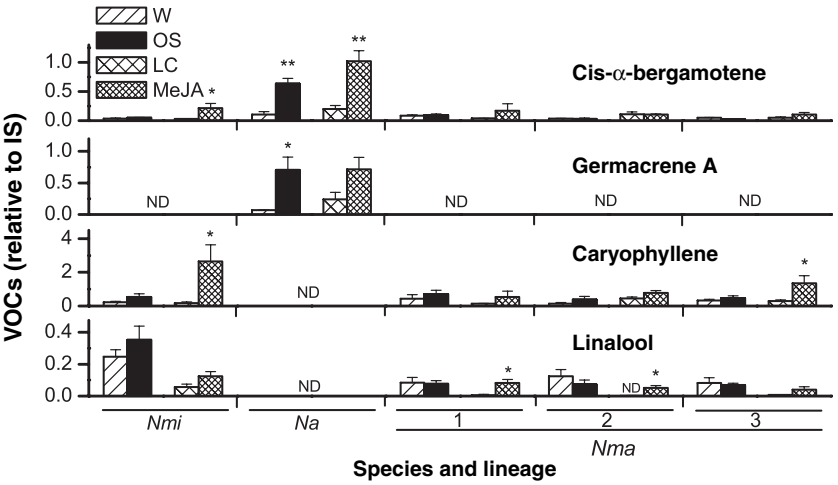

Figure 9. Level (mean ± SE) relative to an internal standard (IS) of selected headspace volatile organic compounds (VOCs) in *Nmi, Na* and three lineages of *Nma* across five elicitation treatments from four or five replicate plants per species and treatment.
One leaf per plant was induced with a lanolin paste containing 150 μg methyl jasmonate (MeJA) or only lanolin as a control (LC); wounded with a fabric pattern wheel followed immediately by application of 20 μl *M. sexta* oral secretions (OS) or 20 μl water (W) to the puncture wounds. Significant differences between treatment and control pairs (MeJA and LC; OS and W) are indicated: $*P \leq 0.05$; $**P \leq 0.005$. ND, not detected.

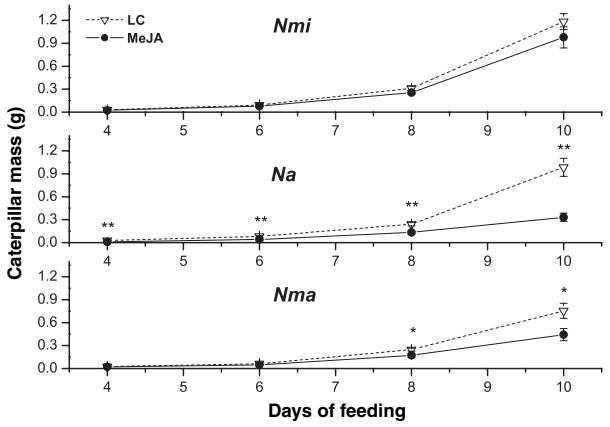

Figure 10. Mass (mean ± SE) of 20 replicate *M. sexta* larvae feeding on 20 *Nmi, Na* or *Nma* (line 1, F_5) plants that had been treated with 20 μl lanolin containing 150 μg methyl jasmonate (MeJA) or only 20 μl lanolin (LC) on two leaves the day prior to placing the neonate larvae on the plants. Larval masses were measured on days 4, 6, 8 and 10.

hybridization – cannot be uncovered in established polyploid species. Similarly, whether a functional JA defense system was retained in a few novel *N. quadrivalvis* and *N. clevelandii* individuals, and subsequently selected for in later generations cannot be resolved by studying established lines. Only comparisons between the defense systems among independent lines of newly formed *Nicotiana* allopolyploids and their parent species can resolve these questions.

Nma polyploids were created to mimic the natural *Nicotiana* polyploid systems. *Nma* primary hybrids were created by pollinating *Nmi* stigmas with *Na* pollen. The hybrid seedlings were induced to a tetraploid state with colchicine treatment, and the resulting unreduced gametes were inbred to create the F_2 polyploids. Natural *Nicotiana* polyploids are most likely formed by fusing two spontaneously occurring unreduced gametes from one individual. They therefore differ from our synthetic system in that natural polyploids presumably arise from a meiotic chromosome non-reduction as opposed to a somatic chromosome doubling (Ramsey and Schemske, 1998). However, the somatic origins of polyploid individuals cannot be entirely discounted in *Nicotiana*, due to the high frequency of spontaneous autopolyploids found after cell culture in particular *Na* genotypes (Bubner *et al.*, 2006).

Genotype analysis of *Nma* confirmed that it is an allotetraploid and does not exhibit a high rate of non-Mendelian genomic changes (discussed in Comai *et al.*, 2000; Liu and Wendel, 2002). Chromosome counts from individuals from the F_2 of line 1 demonstrated that the allopolyploids were indeed euploid ($2n = 48$) with a total genome size consistent with polyploidy (Figure S1). *Nma* polyploids displayed entirely predictable AFLP fragment patterns that were additive of the parental lines, confirming the hybrid nature of *Nma* and indicating that genetic rearrangements are infrequent in *Nma.* In this respect, *Nma* approximates tetraploid cotton, where no genetic rearrangements were found (Liu *et al.*, 2001); however, further studies that assay a larger number of AFLP loci may uncover rearrangements in *Nma*. Likewise, AFLP screening of multiple lines of *Nma* may uncover rearrangements unique to individual crosses. Whereas non-Mendelian structural genetic differences may be rare in *Nma* neopolyploids, DNA methylation or other forms of epigenetic silencing, which have been shown to be prevalent after polyploid formation (reviewed in Osborn *et al.*, 2003), may also be responsible for phenotypic differences among individuals, although they were not assayed in this study.

Consistent aspects of *Nma* morphology (Figure 2) appear either similar to one parent, intermediate between the two parents, or different from either parent, as many descriptions of polyploids have shown (Levin, 1983). Although polyploids appeared physiognomically consistent, a higher degree of variation was found in *Nma* than in either parent species when individual traits were quantified (Figure 3).

Novel quantitative trait variation and phenotypic instability have been observed in multiple synthetic polyploids (Comai *et al.*, 2000; Schranz and Osborn, 2000), and correlate with structural and epigenetic genome changes (Madlung *et al.*, 2005; Pires *et al.*, 2004). A similar spectrum of phenotypic variance was observed in the expression of the polygenetic anti-herbivore defense complex.

The ability of *Nmi, Na* and *Nma* lines to recognize attack from *M. sexta* larvae was determined by comparing responses elicited from wounds that were treated with water to those that were treated with *M. sexta* OS. These comparisons identified responses that are probably regulated by endogenous JA signaling, which, in turn, are activated by elicitors in OS (i.e. fatty acid-amino acid conjugates (FACs); Halitschke *et al.*, 2001). Treatments with MeJA identified traits that are jasmonate-inducible but independent of the plant's ability to elicit endogenous JA increases in response to OS elicitation. Such traits allowed us to identify responses that had become uncoupled from endogenous JA signaling networks during polyploidization. Constitutive metabolite levels were measured in plants induced with only lanolin (LC) or left untreated (Con).

Many *Nicotiana* species respond to attack with a JA burst in which JA concentrations increase to as much as 20 times their constitutive levels in the hours following attack (Halitschke and Baldwin, 2005). The JA burst, which is part of a signaling cascade, elicits direct and indirect defenses, but the quantitative relationships between the magnitude of the burst and the elicited responses are complex. When *Na* wounds are treated with *M. sexta* OS, their JA levels transiently increase to twice those of plants that have only been wounded (Figure 4; Lou and Baldwin, 2003). When *Na* is transformed to silence the expression of the lypoxygenase gene (NaLOX3) that supplies lipid hydroperoxides for JA biosynthesis, both the W- and OS-elicited JA bursts are reduced by approximately 50%. Such a dramatic reduction not only completely abolishes the OS-elicited VOC release, it also reduces TPI and nicotine elicitation significantly (Halitschke and Baldwin, 2003). These experiments demonstrate that JA signaling is essential for the elicited defense responses, but extrapolating the quantitative relationships between the OS-elicited JA dynamics and the OS-elicited defense responses among different species is at best a tenuous proposition: hormonal signaling involves a complex interplay of dynamics among and sensitivity to the hormones.

In contrast to the response in *Na*, OS treatment of *Nmi* did not elicit a higher JA maximum, although it did prolong the JA burst so that JA levels at 60 min were significantly elevated above those found in W-treated plants. The maximum level of JA in *Nmi* was only 500 ng/g FW, less than half the concentration of JA in the non-OS-treated *Na* plant, suggesting that *Nmi* does not 'recognize' the elicitors in *M. sexta* OS. This result is consistent with *Nmi*'s lack of OS-elicited TPI activity (Figure 5), caffeoylputrescine concentrations (Figure 7) or VOC release (Figure 9), as these are all elicited by MeJA treatment. Hence, in *Nmi*, the direct (TPI) and indirect (VOC release) defenses that help *Na* resist *M. sexta* attack are not elicited by OS because the elicitors in OS do not amplify the wound-induced JA burst. The fact that MeJA elicitation in *Nmi* did not significantly reduce *M. sexta* larvae performance in a laboratory trial (Figure 10) suggests that the MeJA-elicited TPI response does not provide *Nmi* with the same resistance to this adapted herbivore that it does *Na* (Zavala *et al.*, 2004a). Whether the MeJA-elicited increases in *trans*-caryophyllene emissions (Figure 9) function as an indirect defense remains unknown. Extensive field work in *Nmi*'s native habitats, as performed for OS-elicited *cis*-α-bergomotene emissions in *Na* (Kessler and Baldwin, 2001), will be required before this can determined. However, OS treatment is not completely without effect in *Nmi*. The OS-elicited prolongation of the wound-induced JA burst (Figure 4) is associated with a decrease in chlorogenic acid, which is also decreased by MeJA treatment (Figure 7). Collectively, these results suggest that OS treatment of wounds does not directly elicit JA signaling in *Nmi*, but may influence another signaling pathway, perhaps ethylene, which may prolong the JA burst and downregulate the accumulation of some phenolic secondary metabolites.

In both *Nmi* and *Nma* (line 1), although OS treatment of wounds did not amplify the wound-induced JA burst, they did prolong the waning of the wound-induced response (Figure 4). Otherwise, the JA burst observed in the allopolyploid *Nma* was intermediate in magnitude between its two parent species. Interestingly, the natural allopolyploids *N. quadrivalvis* and *N. clevelandii*, compared with their parent species (Lou and Baldwin, 2003), also have prolonged JA bursts. As in *Nmi* plants, the absence of an OS-elicited amplification of the JA burst suggested a lack of OS recognition, which was confirmed by the lack of OS-elicited TPI activity (Figure 5), VOC releases (Figure 9), DTG accumulations (Figure 8) and caffeoylputrescine increases (Figure 7), all of which are elicited by MeJA treatment. However, in contrast to the results from *Nmi*, MeJA elicitation significantly reduced *M. sexta* performance in *Nma* (Figure 10). This MeJA-elicited increase in resistance is probably attributable to the large increases in TPIs and DTGs, increases that were comparable to those observed in *Na*. TPIs are well-established defenses (Zavala *et al.*, 2004b). DTGs, on the other hand, although insufficiently studied in terms of structure and physiological function, are thought to decrease the growth rates of tobacco budworm (Snook *et al.*, 1997) on cultivated tobacco (*N. tabacum*). The levels of DTGs across species correspond to differences in *M. sexta* larvae growth rate in the naturally occurring *Nicotiana* polyploids, *N. quadrivalvis* and *N. clevelandii* (Lou and Baldwin, 2003). In the latter study, the growth rate of larvae that fed on the polyploid species, *N. clevelandii*, which

lacked DTGs, was higher than that of the other two species. In summary, the synthetic allopolyploid lineages appear to have retained enough aspects of *Na*'s JA signaling to recognize OS and increase the production of direct defenses to impair the growth of *Manduca* larvae. However, the different lines of *Nma* revealed substantial variation in the elicited regulation of TPIs, nicotine, phenolics and VOCs that they inherited from their phylogenetically similar parents *Nmi* and *Na* (Wu *et al*., 2006). The differences among the *Nma* lines provided insights into patterns observed in the natural allopolyploids.

The induced levels of TPIs in *Nma* lines did not exceed those of either parents, as was observed in the natural *Nicotiana* polyploids *N. quadrivalvis* and *N. clevelandii* (Lou and Baldwin, 2003). This suggests that the higher level of TPIs in these species is not a novelty of polyploidy potentially arising from gene dosage, but a result of the subsequent evolution of TPI expression in either the polyploids or the parents. OS treatment elicited a higher concentration of TPIs than did water treatment in *Na* and *Nma* lines 2 and 3, but not in *Nmi* or *Nma* line 1. The lack of response in *Nma* line 1 is unlikely to be due to the uncoupling of JA signaling from TPI elicitation, as is probably the case in *Nmi*; most likely, wounding alone triggers an OS-elicited response, suggesting cross-talk between W- and OS-mediated signaling. No changes in nicotine concentrations were observed in *Na* with OS, although previous studies have indicated the downregulation of nicotine upon OS treatment due to the suppression of putrescine *N*-methyl transferase by OS-induced ethylene (Kahl *et al*., 2000; Winz and Baldwin, 2001). Such downregulation of nicotine was observed only in *Nma* line 2, and probably reflects an amplification of this system in this allopolyploid line.

Phenolic compounds with a caffeic acid moiety (chlorogenic acid and caffeoylputrescine) were regulated fundamentally differently in the parental species *Na* and *Nmi* and among the different allopolyploid lines (Figure 7). Concentrations of chlorogenic acid (multiple isomers) increased with MeJA treatment in *Na*, decreased in *Nmi*, and decreased even more in the *Nma* lines. Wounding decreased chlorogenic acid concentrations in all lines. OS further decreased chlorogenic acid concentrations in *Nma* line 3. Caffeoylputrescine was upregulated by MeJA treatment in *Nmi, Na* and *Nma* lines 1 and 2. That levels of caffeoylputrescine were lower than in either parent suggests that the combined differences in metabolic flux and regulation between parent species create a seemingly unique polyploid phenotype. When elicited by MeJA, *Nmi* either decreases the flux of caffeic acid or mobilizes the caffeic acid moiety for purposes other than chlorogenic acid biosynthesis, while *Na* increases flux moderately towards chlorogenic acid and highly towards caffeoylputrescine. A more detailed analysis of key regulatory enzymes in phenolic biosynthesis, such as phenylalanine ammonium lyase or hydroxycinnamoyl transferase, which catalyzes the last and controlling step in chlorogenic acid biosynthesis (Niggeweg *et al*., 2004), may clarify how parental differences in metabolic fluxes are inherited in the allopolyploids.

VOCs are often elicited after herbivore attack and can attract predators of the attacking herbivores, as has been described for *Na* (Kessler and Baldwin, 2001). As such, the plume of specific VOCs released by a plant may be tailored to the specific mutualistic relationships it has evolved with predators and parasitoids, but may also be co-opted as a feeding attractant (Pichersky and Gershenzon, 2002). Such ploys and counter-ploys have probably contributed to the great diversity and specificity of VOCs in nature. Induced levels of *cis*-α-bergamotene have been shown in native North American populations of *Na* to attract the generalist predator *Geocoris pallens*, which in turn increases predation on *M. sexta* larvae and eggs (Kessler and Baldwin, 2001). *Nmi* and *Na* emit unique patterns of inducible VOCs. In *Nmi*, caryophyllene and a small amount of *cis*-α-bergamotene are the major sesquiterpenes emitted after MeJA elicitation, whereas *Na* releases a higher concentration of *cis*-α-bergamotene and germacrene A (Figure 9). *Nmi* also emits linalool and another unidentified monoterpene after MeJA elicitation; *Na* lacks these compounds. Linalool, however, has been found in *Na* genotypes other than those used in the current study (Halitschke *et al*., 2000). *Nma* inherited all compounds from *Nmi*, except germacrene A, which was found only in *Na*. Generally, most compounds found in one or both parents were also present in the polyploid, but, compared with the parents, the polyploids generally produced lower amounts and these were constitutively released. This suggests a constitutive regulation of metabolite flux toward total volatile terpene synthesis, which decreases the levels of specific compounds in the polyploids due to the higher diversity of compounds produced by these plants. This higher structural diversity in elicited volatiles may allow the polyploids to exploit the insect mutualists of both parental species, resulting in novel ecological interactions.

In summary, our comparisons of JA signaling and JA- and OS-elicited chemical defenses among the diploid parents and different lines of the synthetic allopolyploid demonstrate that physiological systems that may have no immediate benefit to the initial generations of polyploid individuals are functionally retained in most polyploid offspring. All polyploid individuals assayed retained components of the JA burst as well as the same major downstream metabolite groups as their parental species. Inheritance of individual compounds from parental species to polyploidy offspring, however, was complex, and in some cases may be explained by differences in metabolite flux. This study also demonstrates that these systems are flexible, resulting in the rapid divergence of individuals over few generations. Line 1 of *Nma*, for example, did not produce TPIs after OS elicitation, whereas lines 2 and 3 did. Allopolyploids

appear able to integrate two differentiated physiological systems into a single unique and plastic system.

These physiological alterations support a model of allopolyploid speciation in which the initial neoallopolyploids function ecologically as generalists, but due to increased plasticity are able to exploit different niches than those used by the more locally adapted parents (Levin, 1983; Levin, 2003; Otto and Whitton, 2000). Such a model for allopolyploid speciation is supported by the observation that polyploids tend to inhabit different – although not necessarily intermediate – niches compared with parent species (Levin, 2002). The synthetic allopolyploid *Nma* can be used to falsify functional predictions of this model by examining attack rates and fitness parameters of *Nma* compared with parents in their native habitats.

Experimental procedures

Plant material

Plant growth. Plants were germinated and grown as described previously (Krügel *et al.*, 2002). Briefly, seeds of all species were sterilized, induced to germinate by a 1 h treatment with 0.1 M GA3, and germinated on sterile agar in a Percival growth chamber (Perry, IA, USA) with 26°C/16 h 100% light and 24°C/8 h dark. *N. attenuata* (*Na*) seeds received additional 'smoke' germination cues (see Krügel *et al.*, 2002), but this was not required to synchronize germination of *N. miersii* (*Nmi*) or the allopolyploid *N.* × *mierata* (*Nma*). After 10 days of growth, seedlings were transferred to soil-based growth medium in Teku pots (Waalwijk, The Netherlands), and after an additional 10 days, transplanted to soil in 1 l pots and grown in a glasshouse at 26–28°C under 16 h supplemental light from Philips Sun-T Agro 400 Na lights (Eindhoven, The Netherlands). Plants in the rosette stage of growth were used for all experiments, except for morphological characterizations and flow cytometry, which required a variety of life stages: seed, seedling, bolting and flowering.

Polyploid formation, confirmation and breeding. *Nicotiana attenuata Na* Torrey (Syn: *N. torreyana* Nelson & J. F. Macbr) seeds were collected from a native Utah population (Baldwin *et al.*, 1994) and subsequently inbred for 16 generations. *Nicotiana miersii* J. Rémy seeds were obtained from the Oxford Tobacco Research Station, North Carolina (GenBank accession for NTS sequence: AJ8499824), having originated from a collection by Goodspeed (1954), and further inbred for two generations. Homozygosity of both parent species was confirmed by AFLP fingerprinting.

A single flower of *Nmi* was emasculated and fertilized with pollen from an excised dehisced stamen from *Na*. The resulting hybrid seeds (approximately 60) were germinated, and seedlings were treated while in the cotyledon stage with 0.3% colchicine for 24 h to induce polyploidization. Other hybrid seeds grown to adult plants without colchicine treatment resulted in entirely sterile primary hybrids. Seeds from the colchicine-treated plants were collected and assigned to individual capsules to avoid sampling differences from the potentially chimeric F_1 polyploids. In subsequent generations, however, seeds were pooled from multiple capsules from individual plants. Three lines from the 60 allopolyploid (hereafter *Nicotiana* × *mierata* Krügel or *Nma*) lines were selected and further bred (Figure 1a) for use in all experiments. *C* values for individuals from these lines confirmed the plants' polyploidy (Table 1). Selected individuals from the F_3 generation from line 1 were AFLP-genotyped to examine the additivity of the two parental genomes. The association of certain morphological traits, i.e. trichome morphology, seed mass, and flower and leaf shape for all plants with a confirmed allopolyploid state, allowed for high-throughput screening of *Nma* plants for potential ploidy shifts or potentially aneuploid individuals. No morphologically aberrant individuals were used in this study.

Morphology. Photographs were taken with a Canon D30 digital camera, and seed micrographs were taken using SPOT software (Visitron System, Puchheim, Germany) on a dissecting microscope (Axioscope, Zeiss, Jena, Germany). Stalk height at first flowering and seed mass (from samples of 10 seeds weighed to the nearest 10 μg) were measured on approximately 300 *Nma* individuals (line 1, F_3) and approximately 30 *Nmi* and *Na* individuals.

Genome analysis

Flow cytometry. The genome size (*C* value) of individuals from parent species and allopolyploids was determined on a Partec CCA-II flow cytometer (Partec, Münster, Germany) as described by Bubner *et al.* (2006). Fresh tissue from either the apical part of a bract on an 8-week-old plant or from a cotyledon was finely chopped, and the released nuclei were stained using the Partec UV-precise P chromatin stain kit. The absorption of extracted nuclei was monitored (at 435 nm) after flow cytometric separation for a chromatin/nucleus measurement. Comparison with an internal standard of known genome size (*Hordeum vulgaris* 'Sultan': 5.56 pg DNA/nucleus) provided an estimate of the total genome size of individuals (in pg).

AFLP genotyping. Leaf material (approximately 2 g) was collected from rosette-stage leaves of *Nmi, Na* and *Nma* (line 1, F_3 from two separate F_2 parents) individuals (four individuals per species plus four *Nma* individuals from a separate F_2 parent). DNA was extracted using the CTAB method. DNA quality and quantity were determined by ethidium bromide staining after electrophoresis. Genomic DNA (0.5 μg) was prepared for AFLP fragment analysis according to the Applied Biosystems Plant Mapping kit protocol (Applied Biosystems/Perkin Elmer, Foster City, CA, USA), using the selective nucleotide primer pairs MSEI-CTT JOE-EcoR1-AGG, MSEI-CAA FAM-EcoR1-ACT, and MSEI-CAG NED-EcoR1-AGC. Briefly, DNA was digested with MSEI and *Eco*R1 and simultaneously ligated to ABI MSEI- and *Eco*R1-specific adaptor pairs. Incremental PCR was run with pre-selective primers complementary to those adaptors. Samples were diluted and a subsequent round of PCR was run with primers differing only by the three selective nucleotides. Each *Eco*R1-specific primer was labeled with a different fluorescent dye (JOE, FAM or NED). These PCR products were run on an Applied Biosystems 310 Genetic Analyzer set to the Genescan STR POP4 F module, and compared with ROX-500 internal size standards to determine absolute fragment length. Peaks were identified using GeneScan software if above 50 intensity units. Duplicate samples were taken from four arbitrary *Nma* individuals to ensure consistency of banding patterns. Three combined samples of *Nmi* and *Na* DNA were analyzed to control for PCR differences between samples from plants with different genome sizes. Bands were evaluated for consistency across replicated samples and scored manually.

Plant elicitation. The sink-source leaf from rosette-stage plants was wounded on both sides of the central vein with a fabric pattern

wheel. Either 20 μl of water (W) or 1:1 water: oral secretions (OS) collected from fourth- or fifth-instar *Manduca sexta* larvae were immediately added to the puncture wounds produced by the pattern wheel. For MeJA treatments, 20 μl of lanolin paste (LC) with or without 150 μg MeJA were added to non-wounded leaves. Untreated control plants (Con) were included in every experiment.

Detection of elicited defense responses

JA kinetics. Plants at uniform stages of growth from each species were randomly assigned to OS or water treatments, and leaves were collected 0, 0.5, 1.0, 1.5, 3.0 and 8.0 h after elicitation (five plants/treatment/harvest time). JA was extracted for analysis by LC-MS as described by Wu *et al.* (2006) for plants from line 1 of *Nma*. The JA responses of *Na* have been characterized in numerous other studies (e.g. Halitschke and Baldwin, 2003; Lou and Baldwin, 2003), and, to ensure comparability with previous research, several *Na* samples were analyzed in parallel with the *Nmi* and *Nma* samples; all fell within the range of values reported by Lou and Baldwin (2003).

Secondary metabolites. Plants of each species or lineage were randomly assigned to one of the five treatments (nine or ten plants/treatment). Leaf material (approximately 150 mg) was collected 36 h after elicitation and extracted for the analysis of alkaloids, caffeoylputrescine, chlorogenic acid isomers and diterpene glycosides (DTGs) as described previously (Keinänen *et al.*, 2001).

Volatile organic compounds. Plants of each species or lineage were randomly assigned to each of the five treatments (four or five plants/treatment). At 24 h after elicitation, headspace volatiles were trapped from plants in 50 l glass chambers for 8 h starting at 10:30 a.m. in an open-flow trapping design as described by Halitschke *et al.* (2000), and analyzed by GC-MS. All samples were compared with control traps in chambers lacking plants and corrected for background contamination levels. Caryophyllene was measured in *Nmi*, although not described by Halitschke *et al.* (2000), and identified from an 88% match in a NIST database search and by comparing retention times with an authentic standard. VOCs from treatment–control pairs (MeJA and LC; OS and W) were collected simultaneously to ensure comparability of values. Differences between non-paired treatments may be due to slight variations in air flow rates and a 1-day difference in plant age. Chromatograms of *Na* samples from Lou and Baldwin (2003) were quantified for all *Na* data. Two *Na* plants from each treatment were included in each experiment to ensure direct comparability between this study and that of Lou and Baldwin (2003).

Trypsin protease inhibitors. Plants of each species or lineage were randomly assigned to one of the five treatments (six or seven plants/treatment). Leaf material (approximately 150 mg) was collected 36 h after induction and prepared for TPI quantification normalized to total protein amount and expressed as nmol TPI/mg protein as described by van Dam *et al.* (2001). The minimum detection limit of this technique was determined to be approximately 0.2 nmol TPI. In order to have statistically analyzable treatment pairs in which one value was below the detection limit, values that fell below the detection limit were set at 0.2 nmol TPI and normalized to the sample's total protein measurement.

M. sexta *performance.* On day 1, plants from each species were randomly assigned to MeJA or LC treatments (20 plants/treatment). Both the sink = source transition leaf and the leaf at the next oldest position were elicited to ensure a high level of systemic defense induction. Freshly hatched *M. sexta* larvae (eggs from North Carolina State University Insectary, Raleigh, NC, USA) were placed on the leaf, one position below the youngest induced leaf on day 0. Larvae were allowed to feed freely on the plant and were weighed on days 4, 6, 8 and 10. From *Nma*, only plants from line 1 were used in order to allow for sufficient treatment replication.

Statistical analysis

Treatment–control pairs were analyzed with unpaired *t*-tests. In order to compare induced metabolite levels to constitutive levels for samples that were below the detection limit for a technique, the detection limit was used in the analysis. Seed mass and height means were compared using a Games–Howell ANOVA *post hoc* test, while the variances were compared with an *F*-test corrected by hand using the Bonferroni correction. All analyses were conducted in Statview (SAS Institute, Cary, NC, USA).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Mean parent values, chromosome squashes, and *N.* × *mierata* botanical description.

This material is available as part of the online article from http://www.blackwell-synergy.com

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