Herbivory in the Previous Generation Primes Plants for Enhanced Insect Resistance[1][W][OA]

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Inducible defenses, which provide enhanced resistance after initial attack, are nearly universal in plants. This defense signaling cascade is mediated by the synthesis, movement, and perception of jasmonic acid and related plant metabolites. To characterize the long-term persistence of plant immunity, we challenged Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) with caterpillar herbivory, application of methyl jasmonate, or mechanical damage during vegetative growth and assessed plant resistance in subsequent generations. Here, we show that induced resistance was associated with transgenerational priming of jasmonic acid-dependent defense responses in both species, caused caterpillars to grow up to 50% smaller than on control plants, and persisted for two generations in Arabidopsis. Arabidopsis mutants that are deficient in jasmonate perception (*coronatine insensitive1*) or in the biogenesis of small interfering RNA (*dicer-like2 dicer-like3 dicer-like4* and *nuclear RNA polymerase d2a nuclear RNA polymerase d2b*) do not exhibit inherited resistance. The observation of inherited resistance in both the Brassicaceae and Solanaceae suggests that this trait may be more widely distributed in plants. Epigenetic resistance to herbivory thus represents a phenotypically plastic mechanism for enhanced defense across generations.

To offset their sessile life, plants have evolved diverse strategies to survive and adapt to a broad range of biotic and abiotic stresses, including insect herbivory (Howe and Jander, 2008). Resistance to herbivory is mediated by preexisting physical and chemical barriers, rapidly induced defense mechanisms (Karban and Baldwin, 1997), and priming for stronger responses to subsequent attack (van Hulten et al., 2006). Given the adaptive value of such responses, there has been tremendous interest in unraveling the mechanisms of induced defenses. The hormonal signaling cascade triggering the production of antiherbi-vore defenses is largely mediated by the synthesis, movement, and perception of jasmonic acid (JA) and related metabolites (Chini et al., 2007; Thines et al., 2007; Howe and Jander, 2008). Interconversion of JA with the volatile methyl jasmonate (MeJA) may allow more rapid propagation of the defense signal to other plant parts. JA-Ile conjugates are the active ligands for CORONATINE INSENSITIVE1 (COI1), the F-box component of an E3-ubiquitin ligase complex, SCF^{COI1} (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). Upon binding of JA-Ile, the SCF^{COI1} protein complex targets JA ZIM domain (JAZ) proteins for degradation by the 26S proteasome. Removal of JAZ proteins, which act as repressors of JA-responsive transcription factors (e.g. MYC2), allows the transcription of these target genes. This induced gene expression leads to elevated resistance, systemic signal transmission, and defense priming (Howe and Jander, 2008).

Evidence of inherited responses to herbivory comes from research with wild radish (*Raphanus raphanistrum*), where insect-damaged plants produced more resistant seedlings than undamaged plants (Agrawal et al., 1999), and the demonstration of increased trichome production in yellow monkeyflower (*Mimulus guttatus*) that had been fed upon in the previous generation (Holeski, 2007). Transgenerational effects in plant responses to other environmental stresses have been described in several recent publications. Treatment of Arabidopsis (*Arabidopsis thaliana*) with UV light or bacterial flagellin caused an increased recombination frequency that persisted in subsequent

generations (Molinier et al., 2006). Infection with *Tobacco mosaic virus* increased tobacco (*Nicotiana tabacum*) recombination frequency and provided increased resistance for at least two subsequent generations (Kathiria et al., 2010). In the case of abiotic stress, exposure to elevated temperature led to increased heat tolerance for at least three subsequent generations in Arabidopsis (Whittle et al., 2009), and progeny of salt-stressed plants exhibited higher salt tolerance in the next generation (Boyko et al., 2006).

Epigenetic processes, which include inherited DNA methylation and histone modifications (Molinier et al., 2006; Chinnusamy and Zhu, 2009), are a likely mechanism for retaining stress memory in subsequent generations. Small interfering RNAs (siRNAs), approximately 24-nucleotide RNAs (Hamilton and Baulcombe, 1999) that are processed from double-stranded RNA by dicer-like RNase III enzymes (Lee et al., 2004), can lead to transcriptional regulation or, in some cases, mRNA decay (Matzke et al., 2001). Since siRNAs are able to move between cells and through the plant vasculature (Chitwood and Timmermans, 2010), they represent a possible mechanism for the inheritance of acquired resistance traits.

The mechanisms of transgenerational resistance to insect herbivory remain uninvestigated. In the few published examples of this phenomenon (Agrawal et al., 1999), it was not determined whether the progeny generations are constitutively more herbivore resistant or whether they are primed to respond more vigorously if there is a subsequent attack. It is also not known whether the inheritance of acquired herbivore resistance is a maternal effect transmitted through the seed, for instance through the storage of jasmonates or defensive secondary metabolites, or a longer term effect that is inherited in an epigenetic manner. To address these questions, we designed a series of experiments to investigate the role of jasmonates and siRNA in transgenerational induced resistance to herbivory in two well-studied model species, Arabidopsis and tomato (*Solanum lycopersicum*).

RESULTS

Specificity of Transgenerational Increased Resistance

Tomato and Arabidopsis plants were challenged with caterpillar feeding, MeJA exposure, or mechanical damage. Next, progeny from control and elicited plants were grown in a common environment to identify inherited increases in herbivore resistance. All three treatments decreased the growth of *Helicoverpa zea* (corn earworm) on progeny of treated tomato plants by about 40% relative to controls (Fig. 1A; $F_{3,116} = 11.269$, $P < 0.0001$). Preliminary experiments also showed that the small amount of ethanol used to dissolve MeJA for elicitation experiments has no effect on subsequent herbivory. In the case of Arabidopsis, prior-generation feeding by *Pieris rapae* (white cabbage
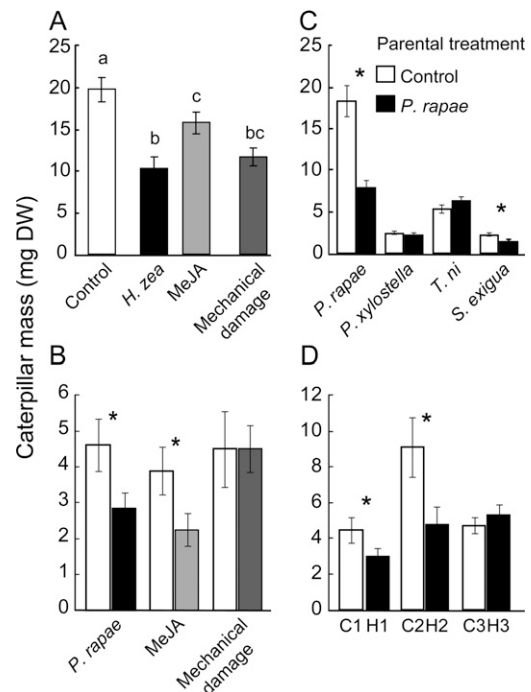

Figure 1. Transgenerational resistance in tomato and Arabidopsis. A, *H. zea* growth on tomato originating from parents that were either left undamaged (control) or subjected to caterpillar feeding, MeJA treatment, or mechanical damage. B, *P. rapae* caterpillar growth on first-generation Arabidopsis progeny originating from parents that were either left undamaged (white bars) or subjected to *P. rapae* feeding, MeJA elicitation, or mechanical damage (black or gray bars). C, Effect of parental generation *P. rapae* caterpillar feeding on *P. rapae*, *P. xylostella*, *S. exigua*, and *T. ni* caterpillar growth on progeny plants. D, *P. rapae* caterpillar growth on three generations of Arabidopsis after *P. rapae* feeding (black bars) or undamaged controls (white bars). C1, C2, and C3 = generations after control treatments; H1, H2, and H3 = generations after *P. rapae* herbivory. Error bars represent SE. Asterisks and different letters above bars represent posthoc Student's *t* tests comparing means between parental treatments ($P < 0.05$). DW, Dry weight.

butterfly) reduced caterpillar weight gain by 40% (Fig. 1B; $F_{1,24} = 26.848$, $P < 0.0001$), MeJA treatment had a similar 27% effect (Fig. 1B; $F_{1,18} = 4.108$, $P = 0.058$), but mechanical damage alone did not affect caterpillar growth (Fig. 1B; $F_{1,27} < 0.0001$, $P = 0.995$). The specificity of transgenerational resistance in Arabidopsis was demonstrated by exposing progeny from a new parental population to *P. rapae* and three additional lepidopteran herbivores, the crucifer-specialist diamondback moth (*Plutella xylostella*) and two generalists, the cabbage looper (*Trichoplusia ni*) and the beet armyworm (*Spodoptera exigua*). In addition to *P. rapae*, only *S. exigua* showed reduced performance on plants that were exposed to *P. rapae* herbivory in the previous generation (Fig. 1C; $F_{1,16} = 7.517$, $P = 0.015$), likely due to the fact that there is species-specific variation in lepidopteran sensitivity to Arabidopsis defenses (Müller et al., 2010). Both *P. rapae* and *S. exigua* also grew less well on plants whose parents were fed upon by *P. xylostella* compared with control plants whose

parents were left undamaged (Fig. 2). This indicates that the type of parental damage does not affect inherited herbivore resistance, which is consistent with observations of similar Arabidopsis transcriptional responses to different lepidopteran herbivores (Bidart-Bouzat and Kliebenstein, 2008).

Persistence of Transgenerational Resistance over Time

Further research to investigate the mechanisms of transgenerational insect resistance was focused primarily on the more genetically tractable Arabidopsis system. To measure the stability of the transgenerational resistance signal, we planted additional C1 (control) and H1 (herbivory in prior generation) seeds without further exposure to herbivores in the second generation. C2 and H2 seeds were harvested, and the procedure was repeated to obtain C3 and H3 seeds (third generation after control and herbivory treatments). Transgenerational resistance to *P. rapae* persisted in the H2 generation (Fig. 1D; $F_{1,33} = 4.634$, $P = 0.039$) but not in the H3 generation (Fig. 1D; $F_{1,36} = 0.032$, $P = 0.858$).

Seed size and provisioning of resources could affect the robustness of plants in the H1 progeny generation (Agrawal et al., 1999). Similar to what has been observed previously in using this plant-herbivore system (van Loon et al., 2003), 3 d of *P. rapae* feeding reduced Arabidopsis seed set by 50% (Fig. 3A; $F_{1,18} = 49.123$, $P < 0.0001$). However, the mass of individual seeds (Fig. 3B; $F_{1,19} = 1.944$, $P = 0.180$) and plant size in the progeny generation (Supplemental Fig. S1) were not altered, suggesting that there are no differences in seed provisioning. Reduced seed set could result from the smaller plant size (loss of leaf area due to herbivory) or could reflect a reallocation of resources from seed production
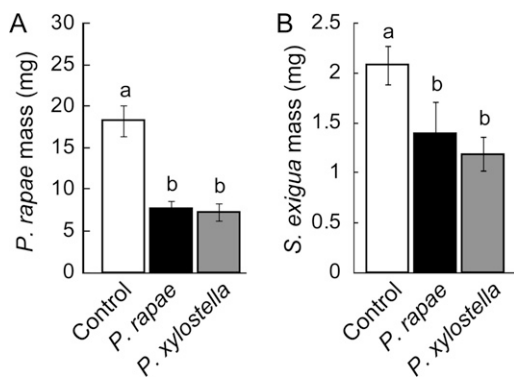

Figure 2. Specificity of resistance in H1 Arabidopsis plants. Shown are average dry mass ± SE of *P. rapae* (A) and *S. exigua* (B) caterpillars on Arabidopsis progeny plants with parents that were undamaged (white bars), *P. rapae* damaged for 3 d (black bars), or *P. xylostella* damaged for 3 d (gray bars). Both *P. rapae* and *P. xylostella* feeding increased resistance in the next generation (for *P. rapae*, $F_{2,37} = 20.949$, $P < 0.0001$; for *S. exigua*, $F_{2,22} = 4.039$, $P = 0.032$). Letters represent posthoc Student's *t* tests comparing means between parental treatments ($P < 0.05$).
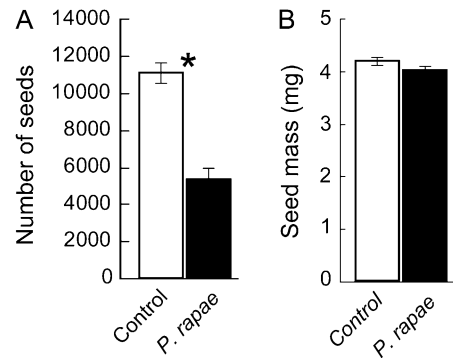

Figure 3. Effects of *P. rapae* feeding on Arabidopsis seed numbers (A) and seed mass (B). Shown are averages ± SE. The asterisk indicates a difference between parental treatments ($P < 0.05$, Student's *t* test).

to plant defense (Mauricio, 1998). Since Arabidopsis dies shortly after seed set, it is unlikely that plants are "saving" resources for later growth and reproduction, as might be expected from a perennial plant.

Mechanisms of Transgenerational Resistance

We determined whether parental generation herbivory inherently alters known defense traits in the seeds or the progeny generation. Seed content of the plant hormones JA, salicylic acid (SA), abscisic acid, and indole-3-acetic acid was not significantly affected by caterpillar feeding (Fig. 4), showing that H1 plants are not primed for insect resistance through the storage of these defense signaling molecules in the seeds. Glucosinolates, a crucifer-specific class of defensive secondary metabolites, also did not differ between seeds of control and *P. rapae*-treated plants (Fig. 5A). Among leaf glucosinolates in undamaged H1 plants, only 1-methoxyindol-3-ylmethylglucosinolate (1MI3M) was increased relative to C1 plants without *P. rapae* caterpillar feeding in the parental generation (Fig. 5B). Furthermore, the overall phenotype of the progeny plants was similar, irrespective of the parental treatment (Supplemental Fig. S1), suggesting that plant size is not growth limiting for the caterpillars. Rosette leaf trichome density, which is associated with insect resistance (Levin, 1973; Mauricio and Rausher, 1997), was not significantly altered by prior-generation herbivory (Supplemental Fig. S2).

JA Perception Is Required for Transgenerational Resistance

Since JA signaling plays a central role in the induction of plant defense responses (Howe and Jander, 2008), additional experiments were designed to determine whether inherited caterpillar resistance depends on the perception of JA via COI1 in the parental and/or progeny generation (Fig. 6A). Arabidopsis *coi1-1* mutants have normal growth patterns but are unable to perceive JA-Ile conjugates and therefore fail to initiate defense-related gene expression changes in
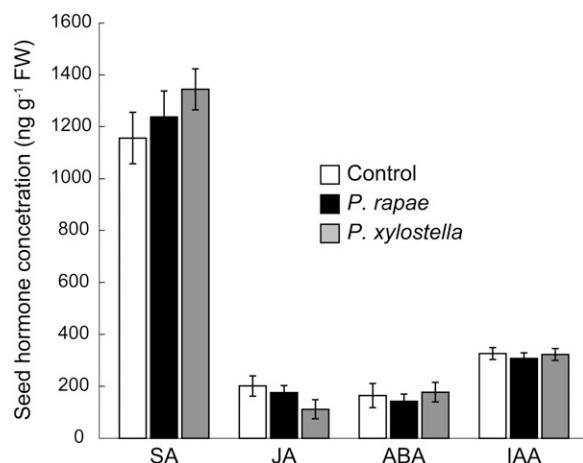
Figure 4. Concentrations of phytohormones in Arabidopsis seeds collected from plants with and without herbivory. Shown are averages ± SE of SA, JA, abscisic acid (ABA), and indoleacetic acid (IAA) in seeds of undamaged Col-0 Arabidopsis plants (Control) or of plants that were damaged during 3 d with one caterpillar per plant of the specialist lepidopteran herbivores *P. rapae* or *P. xylostella*. No differences were found across treatments (ANOVAs for SA, $F_{2,25} = 1.066$, $P = 0.359$; for JA, $F_{2,25} = 1.703$, $P = 0.313$; for ABA, $F_{2,25} = 0.333$, $P = 0.666$; for IAA, $F_{2,25} = 272$, $P = 0.544$). FW, Fresh weight.

response to herbivory (Chini et al., 2007; Thines et al., 2007; Howe and Jander, 2008). Consistent with this known defense signaling function of COI1, homozygous *coi1-1* mutant parent plants, which were induced by *P. rapae* feeding and pollinated with wild-type pollen, did not show increased resistance in the H1 generation (Fig. 6B; test for treatment effect; $F_{1,53} = 0.023$, $P = 0.880$). Functionally wild-type heterozygous *COI1/coi1-1* plants were also subjected to *P. rapae* herbivory. Segregating H1 progeny from *P. rapae*-induced parents were more resistant to herbivory, irrespective of their genotype (Fig. 6C; for treatment effect, $F_{1,37} = 13.143$, $P = 0.001$; for progeny genotype effect, $F_{1,37} = 0.656$, $P = 0.423$; for the interaction between treatment and progeny genotypes, $F_{1,37} = 0.025$, $P = 0.875$). Therefore, in contrast to the parental generation (Fig. 6B), perception of JA-Ile by COI1 in the progeny generation (Fig. 6C) is not required for inherited *P. rapae* resistance.

Consistent with the observed increase in 1MI3M (Fig. 5), which is typically induced by insect feeding and jasmonates (Agerbirk et al., 2009), *P. rapae* feeding in the parental generation caused JA levels to be 2-fold higher in H1 plants compared with C1 plants in the absence of caterpillar damage (dashed lines in Fig. 6D, and parental treatment effect below). Additionally, after 72 h of feeding by neonate caterpillars, JA levels were significantly elevated in H1 plants, which had been fed upon in the previous generation, relative to C1 controls (Fig. 6D; for parent treatments, $F_{1,58} = 5.706$, $P = 0.020$; for induction, $F_{1,58} = 46.117$ $P < 0.001$; for time, $F_{3,58} = 4.880$, $P = 0.004$), suggesting priming for a more robust defense response. On the other hand, SA, a phytohormone that is primarily associated with

pathogen defense, did not exhibit parental treatment effects. In fact, *P. rapae* herbivory reduced SA levels by 15% over a period of 96 h (Fig. 6D). Consistent with the elevated JA levels, the expression of *LIPOXYGENASE2* (*LOX2*), a well-studied JA-responsive gene in Arabidopsis (Howe and Jander, 2008), was increased in plants subjected to caterpillar feeding in the prior generation (Fig. 6E; for *LOX2* parental treatment effect, $F_{1,18} = 3.135$, $P = 0.094$; for induction effect, $F_{1,18} = 5.389$, $P = 0.032$; for interaction, $F_{1,18} = 4.078$, $P = 0.058$). *ALLENE OXIDE SYNTHASE* (*AOS*), a JA biosynthesis
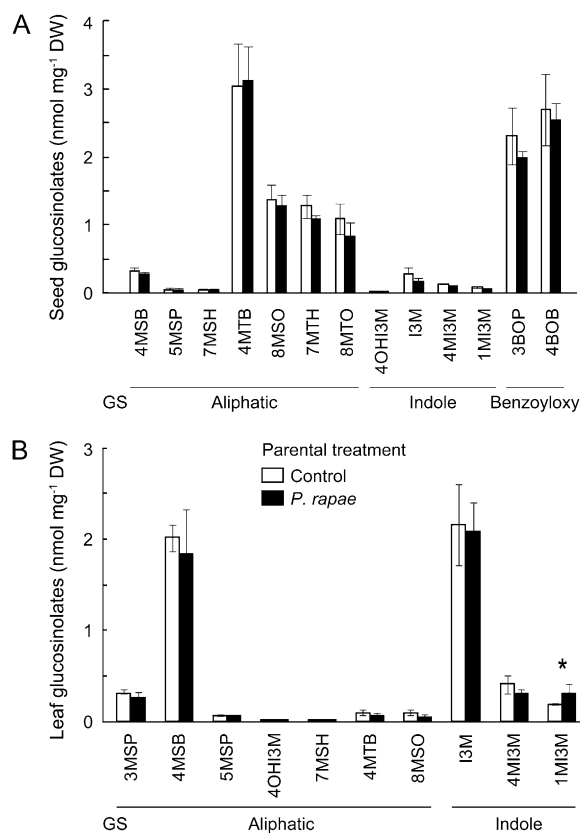

Figure 5. Glucosinolate content of control and *P. rapae*-damaged Arabidopsis plants. A, Averages ± SE abundance of seed aliphatic, indole, and benzoyloxy glucosinolates (GS). Seed originated from either control plants (white bars) or mature plants that were induced by 3 d of *P. rapae* feeding (black bars). B, Averages ± SE abundance of indole and aliphatic glucosinolates in leaves from Arabidopsis plants with parents that were either left undamaged (C1; white bars) or fed upon by *P. rapae* caterpillars for 3 d (H1; black bars). Glucosinolates were measured on damaged leaves from *P. rapae* treatments and on equivalent leaf amounts in the control treatments. Plants originated from the seed batches that were used for glucosinolate assays. Wilcoxon test for 1MI3M, $\chi^2 = 3.857$, degrees of freedom = 1, $P = 0.049$. *$P < 0.05$. Glucosinolate side chain abbreviations not defined in the text are as follows: 3MSP, 3-methylsulfinylpropyl; 4MSB, 4-methylsulfinylbutyl; 5MSP, 5-methylsulfinylpentyl; 4OHI3M, 4-hydroxyindol-3-ylmethyl; 7MSH, 7-methylsulfinylheptyl; 4MTB, 4-methylthiobutyl; 8MSO, 8-methylsulfinyloctyl; I3M, indol-3-ylmethyl; 7MTH, 7-methylthioheptyl; 8MTO, 8-methylthiooctyl; 3BOP, 3-benzoyloxypropyl; 4BOB, 4-benzoyloxybutyl. DW, Dry weight.
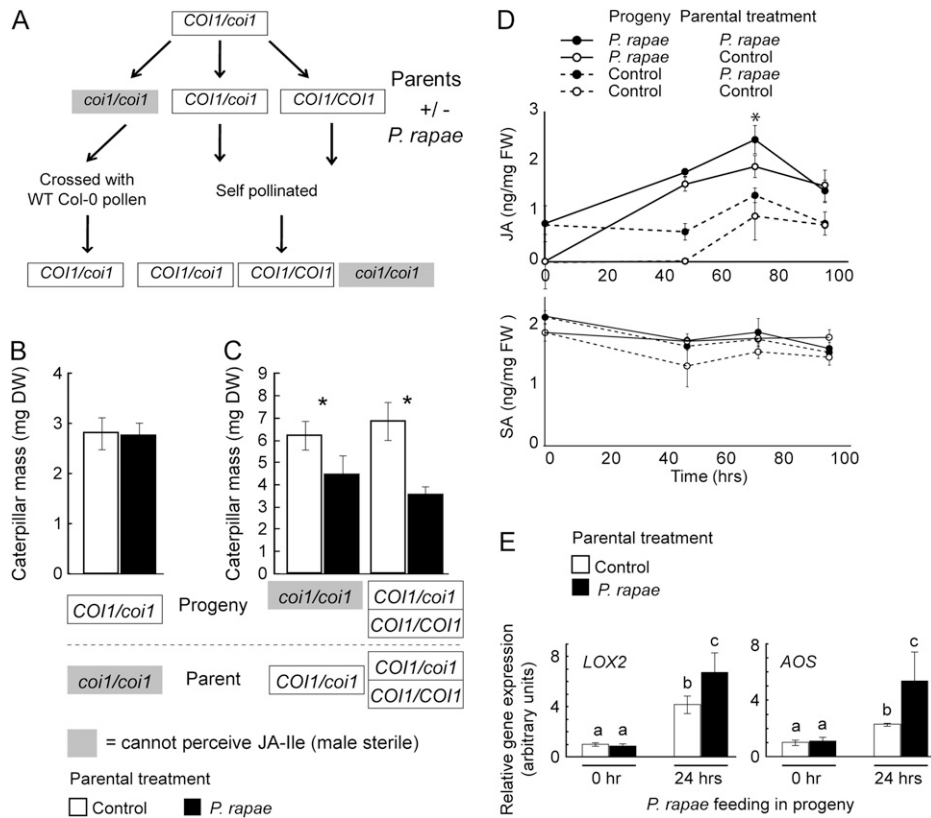
Figure 6. The role of jasmonate signaling and gene expression changes due to herbivory in the parental generation. A, Schematic of crosses used to determine when JA-Ile detection by COI1 is required. Progeny from heterozygous *COI1/coi1-1* plants were raised with and without *P. rapae* herbivory. Homozygous *coi1-1/coi1-1* plants were pollinated with wild-type (WT) Col-0. Heterozygous and wild-type plants were allowed to self-pollinate. C1 and H1 progeny were used for *P. rapae* growth experiments. B, *P. rapae* caterpillar growth on plants with jasmonate-insensitive *coi1-1/coi1-1* parents. C, *P. rapae* caterpillar growth on jasmonate-insensitive *coi1-1/coi1-1* C1 (control) and H1 (*P. rapae* on parents) progeny of functionally wild-type Arabidopsis (*COI1/coi1-1* or *COI1/COI1*). D, Accumulation of JA (top) and SA (bottom) in leaves of Arabidopsis with and without *P. rapae* feeding in the parental and progeny generations. E, Zero- and 24-h induction of *LOX2* (left) and *AOS* (right) gene expression in Arabidopsis with *P. rapae*-damaged (H1; black bars) or undamaged (C1; white bars) parents. Gene expression is in arbitrary units, with control plant expression set to 1. Error bars represent se. Asterisks and letters above bars represent differences between bars ($P < 0.05$, *t* test). DW, Dry weight; FW, fresh weight.

gene (Howe and Jander, 2008), was also more highly induced by *P. rapae* if the previous generation had been subjected to herbivory (Fig. 6E; for *AOS* parental treatment effect, $F_{1,20} = 4.349$, $P = 0.050$; for induction effect, $F_{1,20} = 31.242$, $P < 0.0001$; for interaction, $F_{1,20} = 1.402$, $P = 0.250$). Similar priming of defense gene expression was observed with the JA-regulated tomato *PROTEASE INHIBITOR2* (*PIN2*) gene (Miersch and Wasternack, 2000), which was induced to a higher level by wounding if the previous generation had been exposed to MeJA (Supplemental Fig. S3).

Small RNAs Are Required for Transgenerational Resistance

The elevated caterpillar resistance (Fig. 1) and JA-mediated defenses (Fig. 6, D and E) suggest defense priming via an inherited signal from the parental plant. To test the hypothesis that this effect requires a

siRNA signal, we conducted experiments with two Arabidopsis mutants that have a normal growth pattern but are deficient in small RNA biogenesis: a *nuclear RNA polymerase d2a nuclear RNA polymerase d2b* (*nrpd2a nrpd2b*) double mutant, which lacks nuclear RNA polymerases required for the synthesis of siRNAs (Pontes et al., 2006), and a *dicer-like2 dicer-like3 dicer-like4* (*dcl2 dcl3 dcl4*) triple mutant, which is defective in siRNA processing by dicer-like enzymes (Henderson et al., 2006). Whereas feeding in the previous generation reduced the growth of *P. rapae* on wild-type Arabidopsis, this was not the case on the *nrpd2a nrpd2b* or *dcl2 dcl3 dcl4* mutants (Fig. 7A; treatment effect for wild-type *P. rapae*-fed landrace Columbia-0 [Col-0], $F_{1,30} = 10.198$, $P = 0.003$; for *nrpd2a nrpd2b*, $F_{1,36} = 1.405$, $P = 0.244$; for *dcl2 dcl3 dcl4*, $F_{1,26} = 0.363$, $P = 0.552$). Similarly, application of MeJA to the parent plants was ineffective at inducing transgenerational resistance in the mutants but not on wild-type

plants (Fig. 7B; treatment effect for Col-0, $F_{1,35} = 13.034$, $P = 0.001$; for *nrpd2a nrpd2b*, $F_{1,30} = 0.001$, $P = 0.971$; for *dcl2 dcl3 dcl4*, $F_{1,31} = 0.009$, $P = 0.307$).

Both the *nrpd2a nrpd2b* and *dcl2 dcl3 dcl4* mutants have reduced amounts of some glucosinolates relative to wild-type Col-0, with or without *P. rapae* feeding (Fig. 8; for total glucosinolate genotype effect, $F_{2,300} = 8.772$, $P = 0.0002$; treatment effect, $F_{1,300} = 2.330$, $P = 0.128$; genotype-treatment interaction, $F_{2,300} = 1.034$, $P = 0.357$). In particular, 4-methoxyindol-3-ylmethyl-glucosinolate (4MI3M), which has been associated with pathogen defense responses (Bednarek et al., 2009; Clay et al., 2009), is constitutively less abundant in the mutants. Caterpillar feeding induces the production of 4MI3M and 1MI3M in wild-type plants, but there was no significant increase in the mutant lines. Reduced caterpillar size on the *nrpd2a nrpd2b* and *dcl2 dcl3 dcl4* mutants (Fig. 7) is correlated with a lower abundance of glucosinolates, which are *P. rapae* feeding stimulants (Barth and Jander, 2006; Müller et al., 2010).

DISCUSSION

Our results demonstrate that both Arabidopsis and tomato plants that were subjected to herbivory are more resistant to subsequent attack in the next gener-
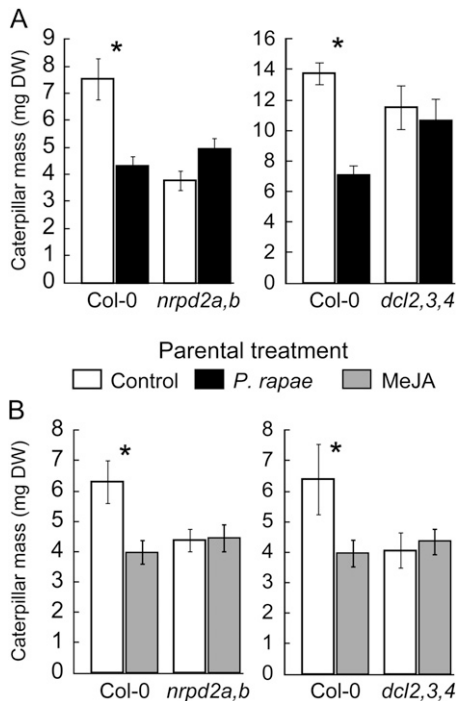
Figure 7. Transgenerational resistance to insect herbivores is absent in small RNA-deficient mutants. Shown are average mass ± SE of *P. rapae* caterpillars on wild-type Arabidopsis compared with *nrpd2a nrpd2b* and *dcl2 dcl3 dcl4* mutants with parent plants that were damaged by *P. rapae* (black bars; A), sprayed with MeJA (gray bars; B), or left as controls (white bars). Asterisks indicate differences between parental treatments ($P < 0.05$, *t* test). DW, Dry weight.
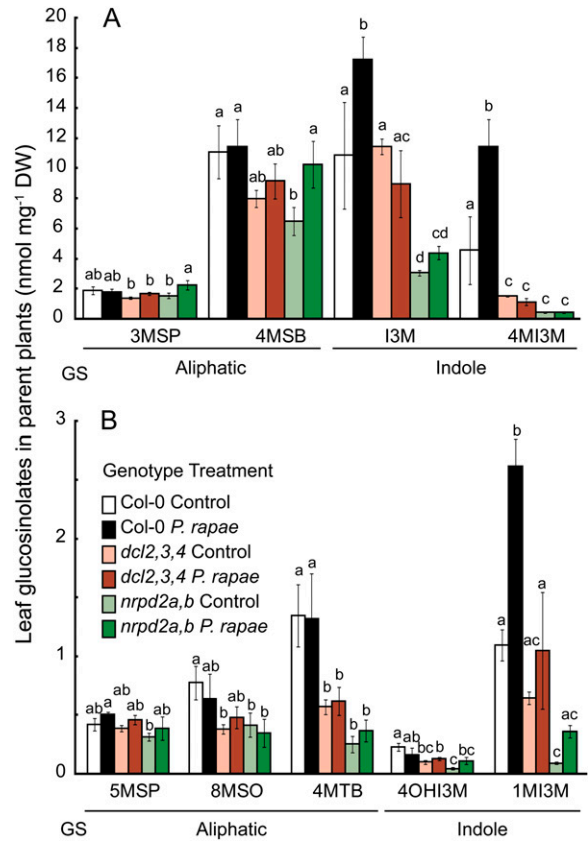
Figure 8. Constitutive and induced levels of indole and aliphatic glucosinolates (GS) in leaves of wild-type Col-0 Arabidopsis and RNA polymerase-deficient (*nrpd2a nrpd2b*) and dicer-like (*dcl2 dcl3 dcl4*) mutants. Shown are averages ± SE of more abundant glucosinolates (A) and less abundant glucosinolates (B). Glucosinolates were measured in equivalent amounts of damaged (*P. rapae*) and undamaged (Control) leaves. Different letters above bars indicate significant differences ($P < 0.05$, *t* test) for each glucosinolate type. Glucosinolate side chain abbreviations are as in Figure 5. DW, Dry weight.

ation. In the case of Arabidopsis, this transgenerational resistance against chewing herbivores includes the priming of JA-related defense responses and requires siRNA biogenesis.

JA-Mediated Increased Transgenerational Resistance

Numerous studies show that jasmonates, SA, and ethylene have important functions in orchestrating plant responses to tissue damage or wounding (Howe, 2004; De Vos et al., 2005). Previous defense induction can cause plants to be primed for a more robust or rapid defense response upon subsequent attack (van Hulten et al., 2006), and treatment of seeds with JA primes plants for enhanced herbivore resistance weeks later (Worrall et al., 2011). Here, we have taken these observations a step further to show that MeJA induction in the previous generation can prime progeny plants for enhanced resistance. Additionally, using *coi1* mutant plants, we demonstrate that the perception

of JA is required in mother plants for increased resistance in the next generation. Further work using JA signaling-deficient mutants (e.g. *jai1*) will be needed to determine whether tomato also requires JA perception for increased transgenerational resistance. Unlike in the case of Arabidopsis, wounding alone primed tomato for increased caterpillar resistance in H1 plants. This could reflect inherent differences between Arabidopsis and tomato, or it could be the result of different experimental methods and perhaps insufficient mechanical wounding of the Arabidopsis plants. For instance, only frequent and controlled repetitive wounding triggered herbivore-like defense elicitation in lima bean (*Phaseolus lunatus*; Mithöfer et al., 2005).

Accumulating evidence demonstrates cross talk between different phytohormone signaling pathways. For example, JA-mediated defense signaling is strongly activated upon chewing herbivore attack, whereas SA and ethylene are more specific to piercing-sucking herbivores or pathogen attack (De Vos et al., 2005). Consistent with these prior reports, we observed priming for faster and higher JA induction in the H1 generation, but we did not observe increased SA accumulation in response to *P. rapae* attack on H1 or C1 plants. These results are consistent with a specific activation of JA signaling during chewing herbivore attack as well as with possible cross talk with the SA signaling pathway (De Vos et al., 2005).

siRNA and Transgenerational Resistance

siRNA is phloem mobile (Chitwood and Timmermans, 2010) and could provide a signal that is passed from vegetative tissue to developing seeds in response to biotic or abiotic stress. In the developing seeds and/or progeny plants, siRNA could alter gene expression through targeted mRNA degradation, regulation of translation, or DNA methylation. The inheritance of resistance over two generations (Fig. 1D) suggests that DNA methylation, which is impacted by PolIV- and DCL2-dependent siRNA production and can be inherited through meiosis, is a possible mechanism for transgenerational inheritance. Increased resistance over two generations also indicates that the signal is likely to be propagated in the embryo rather than in maternal tissue, which makes up the Arabidopsis seed coat.

Two other articles in this issue show that similar transgenerational priming of defense signaling pathways is involved in plant responses to pathogen infection. Infection with avirulent *Pseudomonas syringae* and treatment with β-aminobutyric acid primed Arabidopsis for the induction of SA-mediated defense responses in the subsequent generation (Slaughter et al., 2012). In another study, the descendants of primed plants showed a faster and stronger SA-mediated defense response relative to controls (Luna et al., 2012). Comparable effects in a *drm1 drm2 cmt3* DNA methylation mutant suggest that hypomethylation is required to prime progeny plants for these SA-mediated defense responses.

Future Prospects

Like all forms of phenotypic plasticity, transgenerational resistance to herbivory will only be effective if the response in one generation protects seedlings in the next generation (Karban et al., 1999). A plant that employs transgenerational resistance but whose progeny are not subject to attack may suffer energetic and ecological costs without benefits (Agrawal, 2001). Many natural populations of Arabidopsis have more than one generation per growing season (Donohue, 2009; Bentsink et al., 2010), which would expose parents and progeny to similar herbivore pressures. Therefore, rapid-cycling Arabidopsis genotypes would benefit from priming of insect resistance in progeny of plants that were subjected to herbivory. A testable prediction is that defense priming should be reduced in response to longer seed dormancy, which would expose progeny plants to a likely different herbivore environment in subsequent years. Such effects have been seen in the release of gene silencing due to seed aging (Lang-Mladek et al., 2010).

Unlike most plants in natural ecosystems, the Arabidopsis and tomato lines used for these experiments had been growing in the complete absence of herbivores for several generations. Since inherited resistance in Arabidopsis attenuates over three generations (Fig. 1D), herbivore-free laboratory rearing may account for the consistent transgenerational effects that we have observed. However, even in a growth chamber setting, there are variables than cannot completely be controlled. Out of the nine independent positive control experiments testing first-generation progeny from *P. rapae*-treated and control plants, seven showed
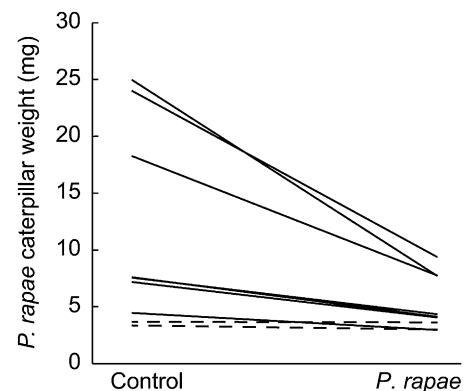

Figure 9. Independent replicates of Arabidopsis transgenerational resistance to *P. rapae* herbivory. Shown are average *P. rapae* caterpillar mass after 7 d of feeding on progeny from control and *P. rapae*-induced Arabidopsis plants from nine independent experiments. Parent lines were either left undamaged (Control) or were induced by a *P. rapae* caterpillar feeding. Out of nine experiments, which include those shown in Figures 1, 2, 6, and 7, seven showed increased transgenerational resistance (solid lines) and two showed no effect (dashed lines). Paired *t* test between treatments across the nine experiments, $t_9 = -2.806$, $P = 0.012$.

variable levels of increased resistance, two showed no significant differences, and none showed a negative effect (i.e. caterpillar growth was never reduced on C1 plants compared with H1 plants; Fig. 9). Similar to the protected existence of our laboratory-grown plants, some plants in agricultural settings may grow in the almost complete absence of herbivory for several generations, particularly with the heavy use of insecticides in seed production fields. Just as the application of jasmonates to seeds can promote long-term herbivore resistance (Worrall et al., 2011), defense elicitation prior to seed set could be used to prime progeny plants for increased herbivore and pathogen resistance, thereby providing a defensive benefit to tomato and other crops that are particularly vulnerable to herbivory at the seedling stage.

MATERIALS AND METHODS

Plants and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana*) landrace Col-0 was obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Seeds of *nrpd2a nrpd2b* (Henderson et al., 2006) and *dcl2 dcl3 dcl4* (Henderson et al., 2006) Arabidopsis mutants were obtained from Eric Richards (Boyce Thompson Institute), and *coi1-1* seeds (Xie et al., 1998) were obtained from Gregg Howe (Michigan State University). Plants were grown in Conviron growth chambers in 20- × 40-cm nursery flats using Cornell Mix (by weight, 56% peat moss, 35% vermiculite, 4% lime, 4% Osmocoat slow-release fertilizer [Scotts], and 1% Unimix [Peters]) at 23°C, and 60% relative humidity, with a light intensity of 180 μmol m^{-2} s^{-1} photosynthetic photon flux density and a 16-h/8-h light/dark photoperiod. Plants were grown for 3 weeks and were used in experiments before flowering.

Tomato (*Solanum lycopersicum*) cv Micro-Tom was used in all tomato experiments. Seeds were originally purchased from Tomato Growers Supply. Seedlings were grown in Metromix 400 potting mix (Griffin Greenhouse & Nursery Supplies) in a greenhouse at Pennsylvania State University. The greenhouse was maintained on a 16-h/8-h light/dark photoperiod. Six-week-old plants were used for insect feeding, mechanical damage, or MeJA treatment.

Insects and Rearing Conditions

A *Pieris rapae* colony was established with approximately 20 adult butterflies that were collected on the Cornell University campus in 2008. Caterpillars were raised on cabbage (*Brassica oleracea* var Wisconsin Golden Acre (Seedway) under the same conditions as those used for growing Arabidopsis. Adult butterflies were fed with a 20% Suc solution. *Trichoplusia ni*, *Plutella xylostella*, *Spodoptera exigua*, and *Helicoverpa zea* eggs were obtained from Benzon Research. For induction in the parental generation, individual caterpillars were allowed to feed for 3 d on each plant, and seeds were harvested 5 to 6 weeks after planting. We used about 25 parental plants for induction, and progeny were obtained from a random sampling of the mother plants. For bioassays in subsequent generations, insects were confined on the leaves of 3-week-old Arabidopsis with mesh-covered cups. A single neonate lepidopteran larva per plant was allowed to feed for 7 d before being collected and lyophilized for 1 d. Larval dry weight was determined using a precision balance.

For experiments with *H. zea*, neonates were fed a wheat germ- and casein-based artificial diet (Bio-Serv) for 1 d and then transferred to tomato leaves for 4 d (one larva per plant). Larval fresh weight was determined using a precision balance.

COI1 Experiments

Seeds from *COI1/coi1-1* Arabidopsis were planted and grown as described above to produce a segregating population of *COI1/coi1-1*, *COI1/COI1*, and *coi1-1/coi1-1* plants. Half of all plants were induced with *P. rapae* for 3 d as described above. Homozygous mutants (*coi1-1/coi1-1*), which are male sterile, were visually identified by the absence of seed pods after flowering. Homozygous *coi1-1/coi1-1* mutants were pollinated with wild-type Col-0 to obtain viable heterozygous seeds. Subsequently, one neonate *P. rapae* larva per plant was allowed to feed for 7 d on 3-week-old, next-generation (H1 and C1) plants, which either originated from functionally wild-type parents (heterozygous *COI1/coi1-1* or homozygous *COI1/COI1*) or from the cross between the jasmonate-insensitive mutants (*coi1-1/coi1-1*) and a wild-type Col-0 plant. The design of the *COI1* experiments is illustrated in Figure 6A.

Phytohormone Analysis

Arabidopsis Col-0 wild-type seeds were planted as described above, and after 3 weeks of growth, half of the plants were subjected to 3 d of *P. rapae* feeding. Plants were allowed to self-pollinate, and seeds were collected 5 to 6 weeks after initial planting. Harvested seeds were grown as described above and divided into four treatments (with and without *P. rapae* feeding in the parental and progeny generations). In the progeny generation, damage was imposed by placing one first instar *P. rapae* caterpillar on each plant and allowing it to feed continuously for 4 d. Full-grown, visibly damaged leaves were harvested after 0, 48, 72, and 96 h. Plants from which a leaf had been harvested were discarded and not used for further experiments. Harvested leaves were weighed and placed in tubes containing 0.9 g of FastPrep matrix (BIO 101) before being flash frozen in liquid nitrogen and stored at −80°C until further use. One milliliter of extraction buffer (2:1:0.005, isopropanol:water:HCl) was added to each sample. d_4-SA and d_5-JA (CDN Isotopes) were added as internal standards, and samples were homogenized in a FastPrep homogenizer (MP Biomedicals) at 6 m s^{-1} for 45 s. Samples were dissolved in 200 μL of methanol after extraction with dichloromethane and solvent evaporation, and 15 μL was analyzed using a triple-quadrupole liquid chromatography-tandem mass spectrometry system (Quantum Access; Thermo Scientific). Analytes were separated on a C18 reverse-phase HPLC column (3 μm, 150 × 2.00 mm; Gemini-NX; Phenomenex) using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 μL min^{-1}. The initial condition of 10% B was kept for 2 min and increased to 100% solvent B at 20 min. Phytohormones were analyzed by negative electrospray ionization (spray voltage, 3.5 kV; sheath gas, 15; auxiliary gas, 15; capillary temperature, 350°C), collision-induced dissociation (argon gas pressure, 1.3 mTorr; energy, 16 V), and selected reaction monitoring of compound-specific parent/product ion transitions (SA, 137→93; d_4-SA, 141→97; JA, 209→59; d_5-JA, 214→62).

Glucosinolate Assays

Arabidopsis leaves were collected, frozen in liquid nitrogen, and lyophilized. Extraction of plant tissue and preparation of desulfoglucosinolates were done as described previously (Barth and Jander, 2006; Kim et al., 2008). Desulfoglucosinolates were separated using a Waters 2695 HPLC device and detected using a Waters 2996 photodiode array detector. For HPLC separation, the mobile phases were water (A) and 90% acetonitrile (B), with a flow rate of 1 mL min^{-1} at 23°C. Column linear gradients for samples were as follows: 0 to 1 min, 98% A; 1 to 6 min, 94% A; 6 to 8 min, 92% A; 8 to 16 min, 77% A; 16 to 20 min, 60% A; 20 to 25 min, 0% A; 25 to 27 min, hold at 0% A; 27 to 28 min, 98% A; 28 to 37 min, 98% A.

Gene Expression Analysis

Total RNA was extracted from frozen tissue samples using the SV Total RNA Isolation system with on-column DNase treatment (Promega). RNA integrity was verified using a 1.2% formaldehyde agarose gel. Transcript abundance of *LOX2* (At3G45140), *AOS* (AT5G42650), and *PIN2* (K03271) was analyzed by quantitative real-time reverse transcription (qRT)-PCR. *Elongation Factor1-α* (AT5G60390), which was used as an internal standard for Arabidopsis experiments, was identified from publicly available microarray data as stably expressed after herbivory, and this stable expression was verified across samples using qRT-PCR. Ubiquitin (X58253) was used as an internal standard for tomato qRT-PCR. After RNA extraction and DNase treatment, 1 μg of total RNA was reverse transcribed with SMART Moloney murine leukemia virus reverse transcriptase (Clontech) using an oligo(dT)$_{12-18}$ primer. Gene-specific primers used for qRT-PCR were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) with the following criteria: melting temperature of 60°C; PCR amplicon lengths of 90 to 150 bp yielding

primer sequences with lengths of 18 to 24 nucleotides, with an optimum at 21 nucleotides; and GC contents of 40% to 60%. Primer sequences can be found in Supplemental Table S1. Reactions were carried out using 5 μL of the SYBR Green PCR master mix (Applied Biosystems), with 800 nM primer, in the 7900HT instrument (Applied Biosystems). The PCRs were initiated by incubation at 95°C for 10 min to activate the enzyme. Then, the following cycle was repeated 40 times: 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The cycle threshold values were quantified and analyzed according to the standard curve method.

Statistical Analysis

All larval growth, seed set, seed mass, glucosinolate, and trichome count data were analyzed on a parental treatment comparison (such as *P. rapae* induction, *P. xylostella* induction, MeJA induction, mechanical damage versus undamaged control plants) with one-way ANOVA. Caterpillar mass for the *coi1-1* experiment was analyzed as for the parental treatments if parents were homozygous recessive *coi1/coi1-1* but with two-way ANOVA for the heterozygous parent side of the experiment to test interaction between parental treatments and segregating genotypes in the progeny generation. Phytohormone induction data were analyzed with one-way ANOVA with induction (control plants versus *P. rapae*-fed plants), parental treatment (control versus *P. rapae* induction), and time as main factors. Gene expression data were analyzed with two-way ANOVA, with parental treatment and timing of induction as main effects. Because of the small sample size, leaf and seed glucosinolates of Col-0 plants were individually analyzed with nonparametric tests. Data were checked for normality and log transformed where needed. In addition, as needed, data for caterpillar growth were blocked for plant position or batch to control for position effect in growth chamber experiments. All analyses were performed with JMP 8 software (SAS Institute).

Supplemental Data

The following materials are available in the online version of this article

Supplemental Figure S1. Herbivory in the parental generation does not have a significant effect on the overall size and morphology of progeny plants.

Supplemental Figure S2. Trichome production on C1 and H1 Arabidopsis rosette leaves.

Supplemental Figure S3. Expression of *PIN2* in tomato with and without MeJA treatment in the previous generation.

Supplemental Table S1. Primers used for qRT-PCR in this study.

ACKNOWLEDGMENTS

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