Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata*

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Edited by Thomas Eisner, Cornell University, Ithaca, NY, and approved February 5, 2008 (received for review December 12, 2007)

Phytohormones mediate the perception of insect-specific signals and the elicitation of defenses during insect attack. Large-scale changes in a plant's transcriptome ensue, but how these changes are regulated remains unknown. Silencing of RNA-directed RNA polymerase 1 (*RdR1*) makes *Nicotiana attenuata* highly susceptible to insect herbivores, suggesting that defense elicitation is under the direct control of small-RNAs (smRNAs). Using 454-sequencing, we characterized *N. attenuata*'s smRNA transcriptome before and after insect-specific elicitation in wild-type (WT) and *RdR1*-silenced (irRdR1) plants. We predicted the targets of *N. attenuata* smRNAs in the genes related to phytohormone signaling (jasmonic acid, JA-Ile, and ethylene) known to mediate resistance responses, and we measured the elicited dynamics of phytohormone biosynthetic transcripts and phytohormone levels in time-course experiments with field- and glasshouse-grown plants. *RdR1* silencing severely altered the induced transcript accumulation of 8 of the 10 genes, reduced JA, and enhanced ethylene levels after elicitation. Adding JA completely restored the insect resistance of irRdR1 plants. irRdR1 plants had photosynthetic rates, growth, and reproductive output indistinguishable from that of WT plants, suggesting unaltered primary metabolism. We conclude that the susceptibility of irRdR1 plants to herbivores is due to altered phytohormone signaling and that smRNAs play a central role in coordinating the large-scale transcriptional changes that occur after herbivore attack. Given the diversity of smRNAs that are elicited after insect attack and the recent demonstration of the ability of ingested smRNAs to silence transcript accumulation in lepidopteran larvae midguts, the smRNA responses of plants may also function as direct defenses.

454-sequencing | herbivore resistance | JA signaling | phytohormone regulation

When herbivores attack plants, large-scale metabolic changes occur, which can be mimicked by applying herbivore-specific elicitors to mechanically produced wounds (1, 2). These rearrangements are preceded by a large-scale transcriptional response, both in angiosperms and gymnosperms (3, 4). How these large-scale transcriptional responses, which presumably change the metabolism and defense status, are so rapidly activated in both attacked and unattacked systemic tissues remains largely unknown. However, it is clear that phytohormones, especially those involved in oxylipin and ethylene signaling, mediate plant defense responses to herbivore attack: mutants defective in oxylipin and ethylene biosynthesis are impaired in many herbivore-elicited transcriptional responses (5, 6). Conversely, the speed and the magnitude of the transcriptional responses, some of which can precede changes in phytohormone levels, suggest that other regulatory mechanisms are involved.

RNA silencing is emerging as a fundamental regulatory process (7); small-RNAs (smRNAs) regulate processes as diverse as plant resistance to viruses, and development and differentiation. All RNA-silencing pathways require the genesis of 18- to 26-nt smRNAs from the cleavage of double-stranded RNA (dsRNA) (8). These smRNAs can be classified as micro-RNAs (miRNAs) and small-interfering RNAs (siRNAs). RNA-directed RNA polymerases [*RdRs* (9)] are critical for generating dsRNAs; these are cleaved to produce siRNAs that can be transmitted throughout the plant to mediate systemic signaling (10). Additionally, because *RdR*s and miRNAs may coordinate their actions, the miRNAs may set the phase for the *RdR*-dependent generation of siRNAs (11).

Three functionally distinct *RdR*s have been reported: *RdR1* and *RdR6* appear to function specifically in viral defense and posttranscriptional gene silencing (12, 13), and *RdR2* is involved in paramutation (14). The transitivity of the RNA signal depends on *RdR*s (15); during virus infection, *RdR6* is required for the cell to perceive the silencing signal but not to produce or transport it (16).

We have recently shown that silencing *Nicotiana attenuata* endogenous *RdR1* made plants susceptible to herbivores in both the glasshouse and the plant's native environment (17). These observations suggest that smRNAs, especially the siRNAs generated in an *RdR1*-dependent manner, are involved in regulating plant defense responses; these responses are, in turn, regulated mainly by phytohormones. Phytohormone signaling is activated in *N. attenuata* after herbivore attack: attack by *Manduca sexta* larvae dramatically amplifies the wound-induced jasmonate (JA) burst that is elicited by herbivore-specific signals from the larvae's oral secretions (OS). Applying *M. sexta* OS to standardized puncture wounds (OS elicitation) mimics all of the herbivore-specific responses measured to date (18–20). This simplifies the analysis of the rapid dynamics in elicited responses, because the timing of herbivore feeding behavior is difficult to standardize. The central importance of JA-dependent defense responses for herbivore resistance has been clearly demonstrated by silencing the JA-signaling cascade (21–23).

M. sexta attack also triggers an ethylene burst (24), which is elicited by the same herbivore-specific signals in OS that elicit the JA burst. The ethylene burst negatively regulates the wound- and JA-dependent increase in nicotine production that allows plants to adjust their allocation of resources to nicotine-adapted insects (25); nicotine is an effective defense against herbivores in nature but is costly for plants to produce (17).

Using high-throughput 454-sequencing, we examine the OS-elicited changes in smRNA populations in both wild-type (WT) *N. attenuata* plants with intact *RdR1*-dependent siRNA biosynthesis

Author contributions: I.T.B. designed research; S.P.P., K.G., and I.T.B. performed research; K.G. contributed new reagents/analytic tools; S.P.P., P.S., and I.T.B. analyzed data; and S.P.P. and I.T.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EU475965–EU476005).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0711363105/DC1.

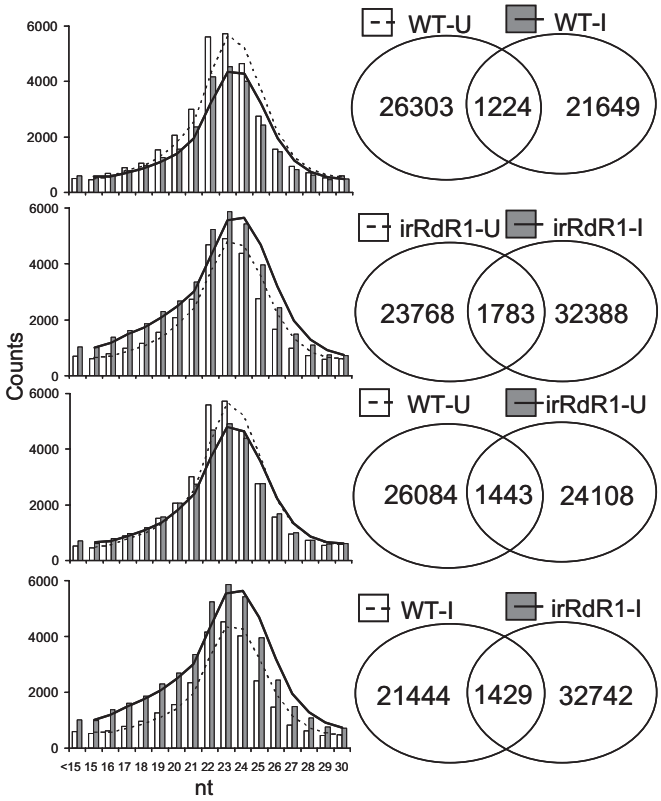

Fig. 1. Changes in the smRNA transcriptome in response to OS elicitation in WT and irRdR1 plants. 454-Sequencing of the smRNA component of the OS-elicited transcriptome reveals large-scale changes. (*Left*) Size distribution of smRNAs in WT (open bars) and irRdR1 (filled bars) plants. Twenty-two- to 25-nt smRNAs are the most abundant size class of the smRNA-transcriptome. Line graphs represent the moving averages for WT (broken lines) and irRdR1 (solid lines) plants. (*Right*) Venn diagrams depicting the overall lack of similarity between the smRNA populations in WT and irRdR1 plants. WT-U, irRdR1-U, WT-I, and irRdR1-I represent smRNA populations in uninduced and OS-elicited plants of WT and irRdR1 genotypes, respectively.

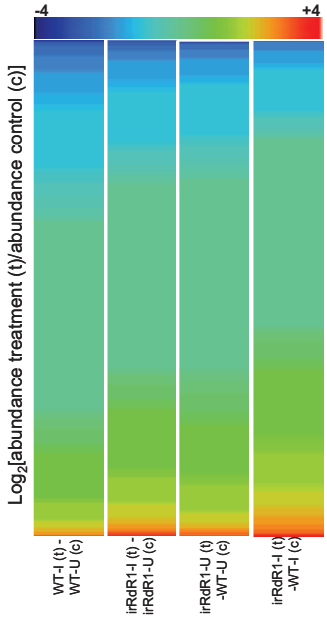

Fig. 2. Heat map of OS- and genotype-associated changes in abundance of commonly expressed smRNAs. Abundance threshold ratios were >1.5 or <0.67 and expressed on $\log_2$-transformed scale. An up/down regulation of 4 is a 16-fold change in comparison with control. More than 50% of the commonly expressed smRNAs were differentially regulated.

and plants in which *RdR1* has been silenced (irRdR1). We examine the role of *RdR1* silencing in phytohormone signaling and plant defense by comparing JA and ethylene signaling in irRdR1 and WT plants and examine the associations among the changes in smRNAs and the genes known to mediate the rapid elicitation of phytohormone signals during herbivore attack.

Results

***RdR1* Silencing Influences OS-Elicited Changes in smRNA Populations.** *M. sexta* attack and OS elicitation result in large-scale changes in the plant mRNA transcriptome (4). If these changes are regulated by smRNAs, herbivory must lead to similar changes in the smRNA transcriptome. Because 454-sequencing, in contrast to other methods such as MPSS (massive parallel signature sequencing), provides quantitative data about the number and length of the sequenced smRNAs (26, 27), we were able to evaluate how *N. attenuata*'s smRNA populations responded to OS elicitation in both WT plants and plants with *RdR1*-silenced (irRdR1) isogenic backgrounds [supporting information (SI) Fig. 7]. We generated 132,239 reliable sequences in the range of 15–30 nt from unelicited and OS-elicited WT and irRdR1 plants, of which 110,122 were unique sequences (SI Table 1). We annotated these sequences against the nonredundant nucleotide database of the National Center for Biotechnology Information (NR-DB) and the miRBase sequence database (SI Tables 2 and 3) (28). Large-scale changes associated with both OS elicitation and *RdR1* silencing were found (Fig. 1). Forty-three percent of the smRNAs in OS-elicited WT plants were not found in WT plants and only 2.4% were common to both smRNA transcriptomes (Fig. 1). Of these 1,224 common smRNAs, 380 were down-regulated and 264 were up-regulated in OS-elicited WT plants (Fig. 2 and SI Table 4). The appearance of so many new smRNAs is consistent with previous observations that *RdR1* transcripts increase dramatically in response to OS elicitation (17). Silencing of *RdR1* also had dramatic effects on the smRNA transcriptome. Compared with levels in untreated WT plants, levels of 21- to 24-nt smRNAs in untreated irRdR1 plants were reduced by 12%, but there was little overlap (1,443; 2.7%) between the two smRNA populations (Fig. 1). Of these shared sequences, 334 smRNAs were down-regulated and 415 smRNAs were up-regulated in irRdR1 plants (Fig. 2 and SI Table 5). Surprisingly, OS elicitation completely changed the smRNA profile of irRdR1 plants, resulting in an overall increase of 14.4%, and only 3.0% of the sequences were common to both control and OS-elicited plants (Fig. 1). Of the 1,783 commonly expressed smRNAs in irRdR1 plants, 554 were up-regulated and 442 were down-regulated (Fig. 2 and SI Table 6). Interestingly, only 1,429 sequences were common to both OS-elicited WT and irRdR1 plants; of these, 254 were down-regulated and 555 were up-regulated in OS-elicited irRdR1 plants (Fig. 2 and SI Table 7).

We annotated the *N. attenuata* smRNAs by BLASTing them (e = 0, mismatches = 0) against the NR-DB and found 34% of the sequences to have matches in the database; these matches were classified into seven categories of structural, regulatory, and coding RNAs (SI Table 2), suggesting that sequence conservation across different plant species at the (sm)RNA levels is probably not >40%. Next, we identified the conserved miRNAs by comparing the smRNAs from *N. attenuata* to all of the known miRNAs present in miRBase. A total of 41 miRNAs distributed in 17 families were identified (SI Table 3 and SI Fig. 8). Of these 41 miRNAs, 11 miRNAs were present in all four treatment groups and 9 miRNAs were differentially regulated between the OS-elicited WT and irRdR1 genotypes (Fig. 3). It was apparent that all of the members of a given family were regulated in the same direction upon

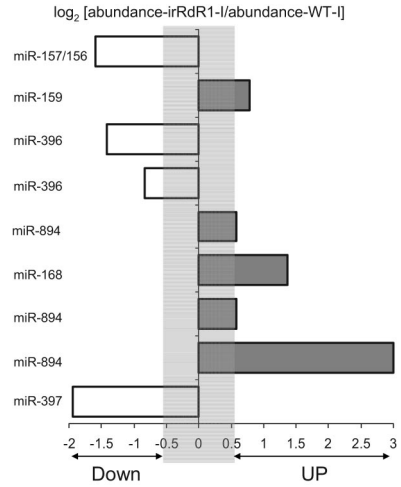

Fig. 3. Nine miRNAs were differentially regulated in response to OS elicitation and *RdR1* silencing. Two criteria were used to determine the differential regulation of miRNAs: (*i*) the miRNAs should be present in both treatment groups of OS-elicited WT (WT-I) and irRdR1 (irRdR1-I) genotypes and (*ii*) the log-transformed abundance ratio of irRdR1-I and WT-I should be less than −0.58 (down-regulated) or greater than 0.58 (up-regulated). All of the members of a given family were regulated in the same direction.

RdR1-silencing—e.g., all three members of miR-894 were up-regulated in irRdR1 plants, whereas both the members of miR-396 family were down-regulated (Fig. 3). In addition, we predicted smRNA targets in genes related to phytohormone signaling (analyzed below) because silencing of *RdR1* rendered plants susceptible to herbivore attack and intact phytohormone signaling is required for defense activation. A detailed hit-map of identified miRNAs and *N. attenuata*-specific smRNAs targeting these genes is presented in SI Tables 8 and 9, respectively.

Silencing of *RdR1* Influences OS-Elicited JA Signaling. Silencing of *RdR1* expression made *N. attenuata* plants highly susceptible to herbivore attack, in part because irRdR1 plants were unable to increase nicotine levels (17). Because nicotine induction requires intact JA signaling (18) and is negatively regulated by ethylene after OS elicitation and herbivore attack (25), we examined OS-elicited phytohormones in irRdR1 plants. Bioinformatic analysis (seed-pairing) revealed that many smRNAs had the potential to target genes related to phytohormone signaling (SI Tables 8 and 9). To determine whether phytohormone signaling is under smRNA control, we measured the OS-elicited changes in transcripts of 10 genes intimately involved in the OS-elicited changes in phytohormones (JA and ethylene) by quantitative real-time PCR (qPCR) in WT and irRdR1 plants.

The transcript levels of six of the eight genes related to JA biosynthesis or signaling were different in irRdR1 plants than in WT plants. We studied two members of the *N. attenuata* lipoxygenase gene family, *NaLOX2* and *NaLOX3*. *LOX2/3* are essential for the biogenesis of C-6 green leaf volatiles (GLVs) and JA, respectively, and are responsible for the regio- and stereospecific dioxygenation of linolenic acid, the first committed step in GLV and JA biogenesis. Unlike in WT plants, where a 3-fold increase in transcript accumulation was observed 45 min after OS elicitation, in irRdR1 plants, transcripts of the *NaLOX2* gene were suppressed (Fig. 4). We studied transcript accumulation of the *HPL* gene, essential for the biogenesis of GLVs. These can act as indirect defenses by attracting predators and feeding stimulants (22). No differences in the dynamics of *HPL* transcripts were observed (SI Fig. 9). That levels of *HPL* remain unchanged suggests that GLV production does as well, especially given that no differences in

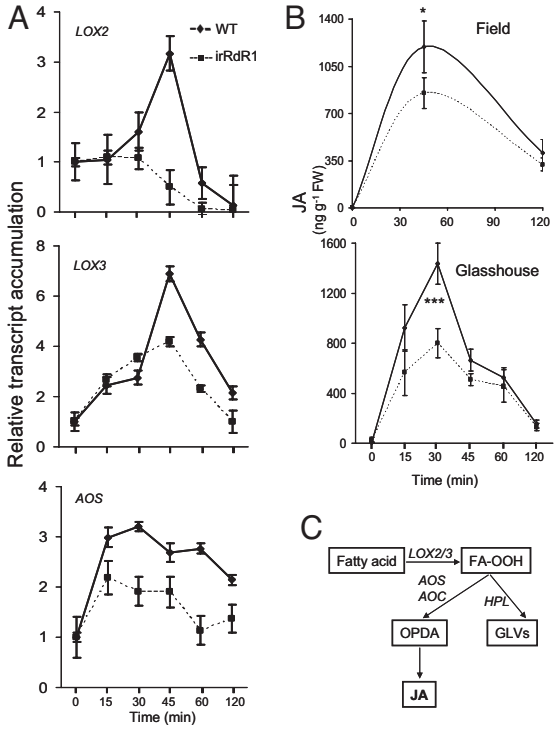

Fig. 4. Silencing of *RdR1* suppresses OS-elicited changes in JA biosynthesis. (*A*) Time course analysis of the dynamics of transcripts of genes required for JA biosynthesis in irRdR1 and WT plants using qPCR. At time 0, plants were OS-elicited by creating puncture wounds with a fabric pattern wheel and immediately treating the wounds with 20 μl of *M. sexta* OS. Transcript accumulation was normalized to the unregulated reference transcript (sulfite reductase, *ECI*), and OS-elicited transcript accumulations were calibrated to constitutive levels at the time of elicitation (0 min). (*B*) Silencing of *RdR1* diminishes the OS-elicited accumulation of JA in plants growing in native habitats in Utah and in the glasshouse. (*C*) A simplified scheme of JA biosynthesis. *, significantly different at $P < 0.05$. ***, repeated-measures ANOVA, significantly different at $P < 0.005$.

predation rates were observed between the WT and irRdR1 plants when they were studied in nature (17). On the other hand, 45 min after OS elicitation, *NaLOX3* levels were only 4 times their constitutive levels in irRdR1 plants, compared with 7 times those in WT plants (Fig. 4); in other words, the OS-elicited increase in *NaLOX3* transcript levels in irRdR1 plants was 57% that of WT plants. We studied the *AOS* gene, which is located downstream of *LOX* genes; this gene is essential for JA biosynthesis, forms an epoxide, and is involved in direct defense (22). Like those of *LOX* genes, the induced transcript levels of the *AOS* gene were also reduced by one-third in irRdR1 compared with WT plants (Fig. 4).

Reduced transcript accumulations of *LOX2/3* and *AOS* indicated reduced JA biosynthesis. We asked whether the differences in transcript levels translated into changes in elicited JA levels in WT and irRdR1 plants. In samples from field-grown plants, JA levels in irRdR1 plants were 28.8% lower than in WT plants at 45 min after OS elicitation (Fig. 4; paired t test, $n = 4$ pairs, $t = 3.53$, $P < 0.05$). We performed a detailed analysis over time of the OS-elicited dynamics in glasshouse-grown plants and found JA levels in irRdR1 plants to be 45% lower than in WT plants at 30 min after OS elicitation (Fig. 4; repeated-measures ANOVA, $F_{1,46} = 22.94$, $P < 0.005$).

Another oxylipin playing a role in defense signaling is JA-Ile (20). Maximum OS-induced JA-Ile/Leu levels are typically only 10–20% of the maximum induced JA levels (21). JA-Ile/Leu levels were unaltered in field-grown (SI Fig. 10; paired t test, $n = 4$ pairs, $t = 0.46$, $P > 0.05$) and glasshouse-grown plants after OS elicitation in

irRdR1 plants (SI Fig. 10; repeated-measures ANOVA, $F_{1,46} = 4.93$, $P > 0.05$). To understand how JA levels could be reduced without affecting JA-Ile levels, we measured transcripts (SI Fig. 10) of threonine deaminase (*TD*), which supplies Ile at the attack site required for JA-Ile biogenesis (20), and of two members of the *JAR* gene family, which adenylates JA so that Ile can be conjugated to JA to produce JA-Ile (20, 29). In WT plants, *TD* levels reached their maximum at 45–60 min after elicitation and were maintained 120 min later; but in irRdR1 plants, *TD* was rapidly elicited just 30 min after OS treatment and levels started to decline at 60 min after OS treatment (SI Fig. 10). This indicated that Ile was available from early on as a substrate for JA-Ile conjugation in irRdR1 plants. To make use of the early availability of Ile, *JAR* transcription would also be expected to be altered. So we measured the transcript accumulation of *JAR*s. In irRdR1 plants, levels of *JAR6* were elevated within 30 min of OS treatment (SI Fig. 10). This early elicitation of *TD* and *JAR6* (despite *JAR4* levels in WT plants being 3 times those in irRdR1 plants) correlated with similar levels of JA-Ile/Leu in WT and irRdR1 plants, even when JA levels in irRdR1 plants were reduced (Fig. 4; because only 10–20% of the total induced JA is conjugated to JA-Ile). Clearly, the two members of the *JAR* family in *N. attenuata* have redundant functions (29).

In addition, we studied the accumulation of *COI1* transcripts because this F-box protein plays a central role in herbivore resistance by mediating JA-Ile perception (30). When *COI1* is silenced in *N. attenuata*, as in irRdR1 plants, OS-elicited JA levels are reduced but JA-Ile/Leu levels were at WT levels (A. Paschold, G. Bonaventure, M. Kant, and I.T.B., unpublished data). This suggests that *COI1* may be down-regulated in irRdR1 plants. No differences were found, however, in levels of *NaCOI1* transcripts in WT and irRdR1 plants (SI Fig. 10), which suggests that if differences in *COI1* are responsible, the regulation occurs posttranslationally.

Silencing of *RdR1* Affects Ethylene Biosynthesis. In addition to JA, the other phytohormone known to modify the outcome of the wound response during herbivory is ethylene. JA and ethylene may act cooperatively or antagonistically; ethylene may modulate the sensitivity of the elicitation signal (which can be JA-dependent) for downstream defense responses. We therefore studied the elicitation kinetics of transcripts of an *ACS* gene (*ACS3a*) and of an *ACO* gene, both of which are known to be elicited by OS and to function in herbivore-induced ethylene biosynthesis (24). The transcript levels of the *ACS3a* gene in irRdR1 plants were lower than those in WT plants but attained their highest value within 15 min of OS induction compared with 45 min in WT plants (Fig. 5). Levels of *ACO3* were higher and more rapidly elicited in irRdR1 than in WT plants (Fig. 5). Silencing of *ACO3* expression in *N. attenuata* results in the strongest reductions in OS-elicited ethylene production (24), underscoring its importance in herbivory-elicited ethylene biosynthesis.

We measured ethylene levels at 300 min after OS elicitation and found them to be 25% higher in irRdR1 plants than in WT plants (Fig. 5; paired *t* test, $n = 5$ pairs, $t = 2.93$, $P < 0.05$). Increased ethylene production may be due to the different transcription levels of biosynthesis genes (Fig. 5) or to alterations in how irRdR1 plants perceive ethylene. We compared ethylene perception in irRdR1 and WT plants with the triple response assay and found no differences between the two genotypes (SI Fig. 11) in the length of their hypocotyls (ANOVA, $F_{1,76} = 0.949$, $P > 0.05$) or epicotyls (ANOVA, $F_{1,76} = 2.95$, $P > 0.05$). Because silencing of *ACO3* reduces ethylene production in *N. attenuata*, we propose that up-regulating this gene increases total elicited ethylene. Our results are consistent with this expectation: Increases in the speed and magnitude of OS-elicited *ACO3* transcript accumulation were correlated with a 25% increase in ethylene emissions (Fig. 5), indicating that ethylene production during herbivory is under *RdR1*/smRNA control.

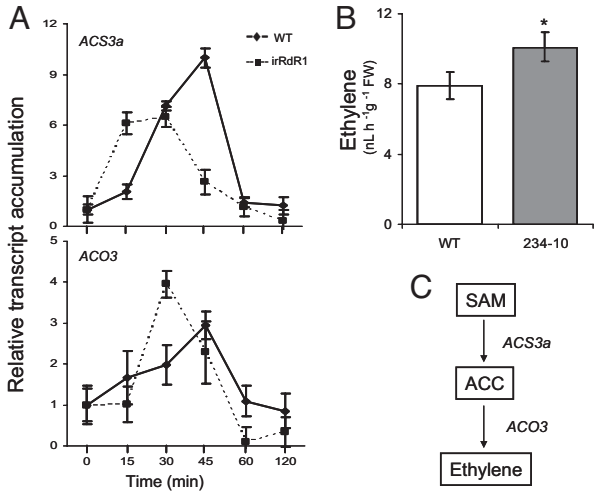

Fig. 5. Ethylene biosynthesis is also affected by *RdR1* silencing. (*A*) OS-elicitation dynamics of ethylene biosynthetic genes in irRdR1 and WT plants using qPCR. At time 0, plants were OS-elicited. Induced transcript accumulations were compared with constitutive levels at the time of elicitation (0 min). *ECI* was used as an endogenous reference. (*B*) When measured at 300 min after OS elicitation, ethylene levels were 25% higher in irRdR1 plants. (*C*) Simplified scheme of OS-elicited ethylene biosynthesis. *, significantly different at $P < 0.05$.

Silencing of *RdR1* Does Not Affect Photosynthesis, Growth, or *RCA* Transcript Accumulation. The effects of *RdR1* silencing on smRNA changes and JA signaling might simply be side effects of fundamental changes in plant growth or photosynthesis. To test this hypothesis, we measured (*i*) the photosynthetic rates of WT and irRdR1 plants over a range of internal CO_2 concentrations (C_i): no differences were found at any C_i or in the rates of carboxylation as measured by the A/C_i relationships (repeated-measures ANOVA, $F_{1,52} = 0.66$, $P > 0.05$); and (*ii*) parameters related to plant growth (SI Fig. 12): rosette diameter (repeated-measures ANOVA, $F_{1,48} = 2.71$, $P > 0.05$), petiole length (ANOVA, $F_{1,8} = 1.45$, $P > 0.05$), and stalk length (repeated-measures ANOVA, $F_{1,48} = 1.45$, $P > 0.05$), which confirmed previous results using field-grown plants (17). To determine whether more subtle changes had occurred in the abundance of key growth-related transcripts, we measured the kinetics of *RCA* (RuBPCase activase) transcripts after OS elicitation. *RCA* functions as an important regulator of photosynthesis by modulating the activity of RuBPCase. *RCA* in *N. attenuata* is down-regulated during herbivory and OS elicitation, and silencing of *RCA* results in reduced photosynthetic rates and decreased plant biomass (19). No difference in the elicitation kinetics of the *RCA* was observed between WT and irRdR1 plants (SI Fig. 13). This lack of difference suggests that the susceptibility of irRdR1 plants to herbivores was due not to altered metabolism but likely to altered phytohormone signaling.

Exogenous Addition of JA Restores Resistance in irRdR1 Plants. To determine whether the susceptibility of irRdR1 plants to insect herbivores was due to their attenuated JA levels, we performed complementation experiments (Fig. 6 and SI Fig. 14), in which JA was supplied to irRdR1 plants and neonate *M. sexta* larvae were allowed to feed for 11–12 days on JA-supplemented or water-supplemented (as a control) plants. *M. sexta* larvae grew faster on irRdR1 plants not supplemented with JA (Fig. 6; ANOVA, $F_{2,33} = 6.72$, $P < 0.005$) than on WT control or JA-supplemented irRdR1 plants; but *M. sexta* larvae allowed to feed on JA-supplemented irRdR1 plants performed similarly to those that fed on WT control plants (Fig. 6; Fisher's PLSD > 0.5). This suggests that irRdR1

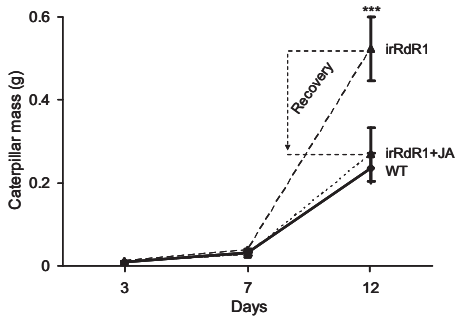

Fig. 6. The lack of resistance of irRdR1 plants to *M. sexta* larvae attack can be restored to WT levels by JA supplementation. Spraying of irRdR1 plants with 1 mM JA until runoff restores the resistance of *N. attenuata* plants as reflected in the mass gain of *M. sexta* larvae. ***, significantly different at $P < 0.005$.

plants are susceptible because of insufficient amounts of JA for normal defense activation.

Discussion

Here, we extend the molecular regulatory arena of plant direct defenses from the biosynthesis of phytohormones to the smRNA-mediated regulation of defense by demonstrating that phytohormone signaling is regulated by *RdR1*, a central component of the RNA-silencing pathway. RNA silencing is a part of the defense system against viral and transposon invaders. Here, we place RNA silencing at the center of plant defense against insect herbivores. We not only report the constitutive smRNA transcriptome of *N. attenuata* but also elucidate the dynamics of smRNA transcriptome when *RdR1* is silenced and when WT and irRdR1 plants are OS-elicited. The functional relevance of large-scale changes in smRNA-transcriptomes after different *RdR*s are silenced has not been determined until now; nor have biotic stresses (e.g., herbivory) been shown to elicit changes in the smRNA transcriptome. We verify the effects of the changes in the smRNA transcriptome by profiling transcript accumulations of genes in the signaling pathways that are central to herbivore resistance. Silencing of *NaRdR1* deregulated the transcriptional response of 8 of the 10 phytohormone-signaling-related genes studied here. This transcriptional response was associated with changes in the balance of OS-elicited phytohormones, which in turn likely contributed to the susceptibility of irRdR1 plants to herbivores. We were also able to exclude the possibility that the susceptibility of the irRdR1 plants is an indirect effect of changes in photosynthesis and growth.

JA and ethylene play central roles in the induced defenses that are elicited after *M. sexta* larvae attack. Attack from this herbivore elicits a rapid JA burst, which is associated with increases in the transcript levels of JA-biosynthesis genes (21, 22). In *N. attenuata*, this JA burst is required to elicit chemical defenses, of which nicotine is one of the most important (17). *M. sexta* attack also elicits an ethylene burst that attains maximum levels after the JA burst has waned and negatively regulates nicotine production (25), presumably to save resources, prevent autotoxicity, and prevent this nicotine-tolerant herbivore from sequestering nicotine for defense against its own natural enemies. Both field- and glasshouse-grown irRdR1 plants had significantly reduced JA levels after OS elicitation, and glasshouse-grown plants had enhanced ethylene emissions. As such, the OS-elicited responses found in WT plants are reversed in irRdR1 plants: the positive regulator (JA) is reduced and the negative regulator (ethylene) is increased.

In irRdR1 plants, the transcript levels of phytohormone-signaling related-genes were either reduced (*LOX2*, *LOX3*, *AOS*, *JAR4*, and *ACS3a*) or rapidly enhanced (*TD*, *JAR6*, and *ACO3*) to attain levels that were higher than in elicited WT plants. We propose that the smRNAs themselves or some repressor(s) (under smRNA control) prevents the genes from being expressed when the plants are not being attacked. The rapid elicitation of *RdR1* (17) could generate and amplify the smRNAs that degrade the repressor mRNA(s) and activate the phytohormone-signaling cascade. As in auxin signaling, where miRNAs regulate *TIR1* expression (31), the most promising "repressor" candidate is *COI1* or an unknown protein whose degradation is mediated by this F-box protein [e.g., JAZ (32)]. *COI1* plays a central role in JA signaling: By ubiquitin-mediated protein degradation, it regulates JA signaling (30). Interestingly, when the phenotypes of plants silenced for *COI1* expression (irCOI1) are compared with those of irRdR1 plants, several commonalities are apparent: irCOI1 plants are also susceptible to native herbivores (23), and compared with WT plants they had reduced levels of elicited JA but higher JA-Ile/Leu levels (A. Paschold, G. Bonaventure, M. Kant, and I.T.B., unpublished data). However, the expression kinetics of *COI1* in irRdR1 plants do not differ from those in WT plants. Therefore, we propose that whereas *COI1* is probably not the *RdR1*-dependent repressor, perhaps there is another *RdR1*-dependent repressor.

The *RdR1*-dependent generation of smRNAs may increase transcript accumulation, as was most recently shown in humans (33). Li *et al.* designed dsRNAs that target the promoter regions of the *p21* genes in humans. When these dsRNAs were transfected into human cell lines, instead of silencing genes, they caused prolonged and sequence-specific increases in the transcripts of the targeted genes. Because we use *RdR1*-silenced transgenic plants to study phytohormone signaling, parallels may exist between the studies. The down-regulation of the dsRNA-synthesizing gene (*RdR1*) results in the down-regulation of phytohormone-signaling-related genes. Genes that are activated early during elicitation may be under the direct control of *RdR1*-generated smRNAs. In addition, just as smRNAs have a stimulatory effect on bacteria (34), so might si/miRNAs. Similarities between transcription factors and miRNAs have recently been highlighted (35). The rapid elicitation of the *RdR1* gene by OS, the susceptibility of irRdR1 plants to herbivores (17), and the insufficient elicitation of genes related to phytohormone signaling along with the deregulation of phytohormone signaling (Figs. 2 and 3 and SI Fig. 10) all suggest the possibility that smRNAs both repress and activate gene expression.

The differential regulation of phytohormone-signaling genes in irRdR1 may be due to the surprising appearance of new smRNAs in elicited irRdR1 plants. These smRNAs, which were not present in the elicited WT plants, may also regulate transcriptional responses during herbivory by regulating gene transcription directly or by regulating transcription factors and/or repressors. A combination of the above-mentioned mechanisms is likely, because biological systems tend to be regulated at several levels. Currently, we lack sufficient knowledge of the transcription factors, repressors, and activators of the phytohormone-signaling network to fully interpret the role that smRNAs play in these changes. The complexity of the signaling network generates the expectation of large-scale changes in the smRNA transcriptome. We propose that during herbivore attack, the *RdR1*-mediated smRNA inductions take center stage in coordinating the changes.

Because the genome sequence for *N. attenuata* is lacking, much more work in the small-scale annotation of the sequenced smRNAs remains to be done; this study lays the foundation for this work. A similar large-scale analysis of herbivore-induced changes in smRNAs has not to our knowledge been carried out in any other plant. Profiling of smRNAs has been limited to comparing constitutive states of different *RdR* and *DCL* mutants (26, 27). When the smRNA profiles of WT and *RdR2*-mutated *Arabidopsis* were compared (26), many new smRNAs were found, as were many up-regulated miRNAs. This suggests that silencing of *RdR*s triggers pathways that may generate new smRNAs. Only 41 sequences (of 110,122 unique sequences) could be annotated as miRNAs in *N. attenuata*. From this result, it is becoming apparent that miRNAs

are not as well conserved in plants as has been previously assumed and that smRNA profiles may be even more diverse.

Species-specific smRNAs may play central roles in plant adaptation and defense, not only in the plant but also perhaps in the organisms that attack plants. *N. attenuata* plants are under constant attack from many different guilds of herbivores and pathogens, and it has been recently shown that dsRNAs synthesized in the host plants may trigger RNAi in the midgut of insects attacking them (36), which can in turn reduce insect performance. *N. attenuata* may employ similar strategies; for example, *RdR1*-dependent smRNAs or their dsRNA precursors may help protect plants by targeting genes in the insect midgut because host-derived dsRNAs are capable of silencing targets in insects (36). Further research into these molecular mechanisms will help us appreciate the ecological sophistication that underlies the arms race in plant–herbivore interactions.

Methods

Plant Materials and Treatment. WT *N. attenuata* plants were from the 22nd inbred generation of seeds originally collected from a native population in southwestern Utah, United States. Plants silenced in *RdR1* expression (irRdR1) are described in ref. 17 and in *SI Materials and Methods*. Plants were elicited with *M. sexta* oral secretions (OS treatment) to activate herbivore-specific plant responses as described in ref. 18 and in *SI Materials and Methods*. For the JA complementation experiments, 1 mM aqueous solutions of JA (Sigma) were used as detailed in *SI Materials and Methods*.

Analysis of smRNA Transcriptome of *N. attenuata*. Leaves growing at node +2 were harvested from three biological replicates, each from untreated and OS-elicited (after 45 min) WT and irRdR1 plants, and equal amounts of samples were pooled. RNA species of <200 bp were enriched, smRNAs were isolated, and 454-sequencing was performed after normalization by Vertis Biotechnologie as explained in *SI Materials and Methods*. All analyses were done with custom-written Perl scripts. A summary of methods adopted for analyzing and annotating the 454-sequence data is presented in SI Fig. 7. To determine whether the smRNAs targeted phytohormone biosynthesis and signaling genes from *N. attenuata*, an analysis of seed pairing (37, 38) was conducted as described in detail in *SI Materials and Methods*.

Transcript Accumulation by Real-Time PCR (qPCR). Time course analysis of 11 genes related to phytohormone signaling was conducted as stated in *SI Materials and Methods*. Gene-specific primers and probes are listed in SI Table 10. To analyze the data, the $2^{-\Delta\Delta CT}$ method was used.

Phytohormone Analysis of Field- and Glasshouse-Grown Plants. To determine phytohormone levels of irRdR1 plants subjected to attack from native herbivores, we analyzed samples from the plant population described in ref. 17 and in *SI Materials and Methods*. A kinetic analysis was performed with glasshouse-grown plants to verify the differences observed in JA and JA-Ile/Leu levels between the genotypes in field-grown plants, as described in *SI Materials and Methods*. Ethylene emissions were quantified with a photoacoustic laser spectrometer (INVIVO) as described in ref. 24 and explained in *SI Materials and Methods*.

Photosynthetic Measurements. Net photosynthetic rates and intercellular CO_2 concentrations were measured as described by Giri *et al.* (19) and are explained in *SI Materials and Methods*.

Statistical Analysis. Data were suitably transformed wherever needed to meet the assumption of homoscedasticity. For determining fold change, a cutoff of 1.5-fold regulation was used as stated in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank E. Wheeler for editorial comments; Dr. A. Berg, E. Rothe, Dr. M. Schöttner, and Dr. C. C. von Dahl for their help with the phytohormone analysis; Dr. E. Gaquerel for help with the field work; P. Srivastava for help with 454-sequence analysis; and the Max Planck Society for funding.

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