RNA-Directed RNA Polymerase3 from *Nicotiana attenuata* Is Required for Competitive Growth in Natural Environments[1][W][OA]

Shree P. Pandey[2], Emmanuel Gaquerel, Klaus Gase, and Ian T. Baldwin*

Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Jena 07745, Germany

SDE1/SGS2/RdR6, a putative RNA-directed RNA polymerase, maintains plant defenses against viruses in Arabidopsis (*Arabidopsis thaliana*) and *Nicotiana benthamiana*, but its function has not been examined in natural habitats or with respect to other ecological stresses. We evaluated the organismic-level function of this gene (Na*RdR3*) in an ecological model species, *Nicotiana attenuata*, by transforming plants to stably silence *RdR3* (irRdR3). Minor morphological changes (elongated leaves and reduced leaf number) and increased susceptibility to tobamoviruses typical of *RdR6* silencing in other species were observed, but these changes did not alter the reproductive performance of singly grown plants (measured as seed and capsule production) or herbivore resistance in laboratory trials. 454-sequencing of irRdR3's small RNA (smRNA) transcriptome revealed that 21- and 24-nucleotide smRNAs were not affected, but the abundance of 22- to 23-nucleotide smRNAs was reduced. When planted in pairs with wild-type plants in *N. attenuata*'s natural habitat in the Great Basin Desert, irRdR3 plants produced shorter stalks with significantly reduced flower and capsule numbers, but did not influence the ability of plants to resist the native herbivore community, indicating that silencing *RdR3* reduced a plant's competitive ability. We tested this hypothesis in the glasshouse by planting irRdR3 and wild-type pairs in communal containers; again irRdR3 plants had severely reduced stalk elongation and reproductive measures. The reduced competitive ability of irRdR3 plants was associated with altered phytohormone homeostasis, especially as reflected in the distribution of auxin. We suggest that *RdR3* helps to regulate hormone balance when plants compete with conspecifics in natural environments.

According to the conventional wisdom about the encoding of genetic information, DNA is replicated from DNA and then transcribed to RNA to make proteins; these proteins ultimately regulate cellular processes. The pathway that governs how RNA is replicated from itself has gained importance outside this central scheme. Self-replication of RNA, which is important for RNA viruses, is mediated by RNA-dependent/directed RNA polymerases. Mechanistically similar enzymes are also present in plants that participate in RNA-mediated gene silencing. RNA silencing regulates processes affecting many layers of endogenous gene expression (Voinnet, 2002). All RNA-silencing pathways involve the cleavage of double-stranded RNA (dsRNA) into short, 21- to 26-nucleotide RNAs (Baulcombe, 2004). The dsRNA molecules are produced by the plant's *RNA-directed RNA polymerase* genes (*RdR*s; Wassenegger and Krczal, 2006). *RdR*s have also been reported in several other life forms such as nematodes (Sijen et al., 2001) and fungi (Cogoni and Macino, 1999; Makeyev and Bamford, 2002). In plants, the functions of the *RdR*s have remained elusive; until now they have been mainly associated with antiviral defenses.

Three functionally distinct *RdR*s have been identified from tomato (*Solanum lycopersicum*), tobacco (*Nicotiana* spp.), and Arabidopsis (*Arabidopsis thaliana*; Schiebel et al., 1998; Dalmay et al., 2000; Mourrain et al., 2000; Yang et al., 2004). *RdR1* has been implicated in defense against viruses and herbivores (Xie et al., 2001; Yu et al., 2003; Yang et al., 2004; Pandey and Baldwin, 2007), whereas *RdR2* has been associated with de novo methylation and paramutation (Xie et al., 2004; Alleman et al., 2006). The third *RdR* (referred to as *RdR3* in *Nicotiana attenuata*), the *RdR6* (or *SGS2/SDE1*), is essential for posttranscriptional gene silencing (PTGS) and antiviral defense (Dalmay et al., 2000; Mourrain et al., 2000).

In Arabidopsis (Mourrain et al., 2000) and in *Nicotiana benthamiana*, *RdR6* makes plants resistant to a broad spectrum of viruses, and its action is temperature dependent (Qu et al., 2005). The transitivity of the RNA-silencing signal depends on the activity of the *RdR*s (Himber et al., 2003). The activity of *RdR6* in transitive gene silencing in *N. benthamiana* can be primed or unprimed (Petersen and Albrechtsen, 2005). In *N. benthamiana* during virus resistance, the *RdR6* homolog is required for the cell to perceive the silencing signal but not to produce or transport it in a systemic manner (Schwach et al., 2005).

[1] This work was supported by the Max Planck Society.

[2] Present address: Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany.

* Corresponding author; e-mail baldwin@ice.mpg.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ian T. Baldwin (baldwin@ice.mpg.de).

[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.121319

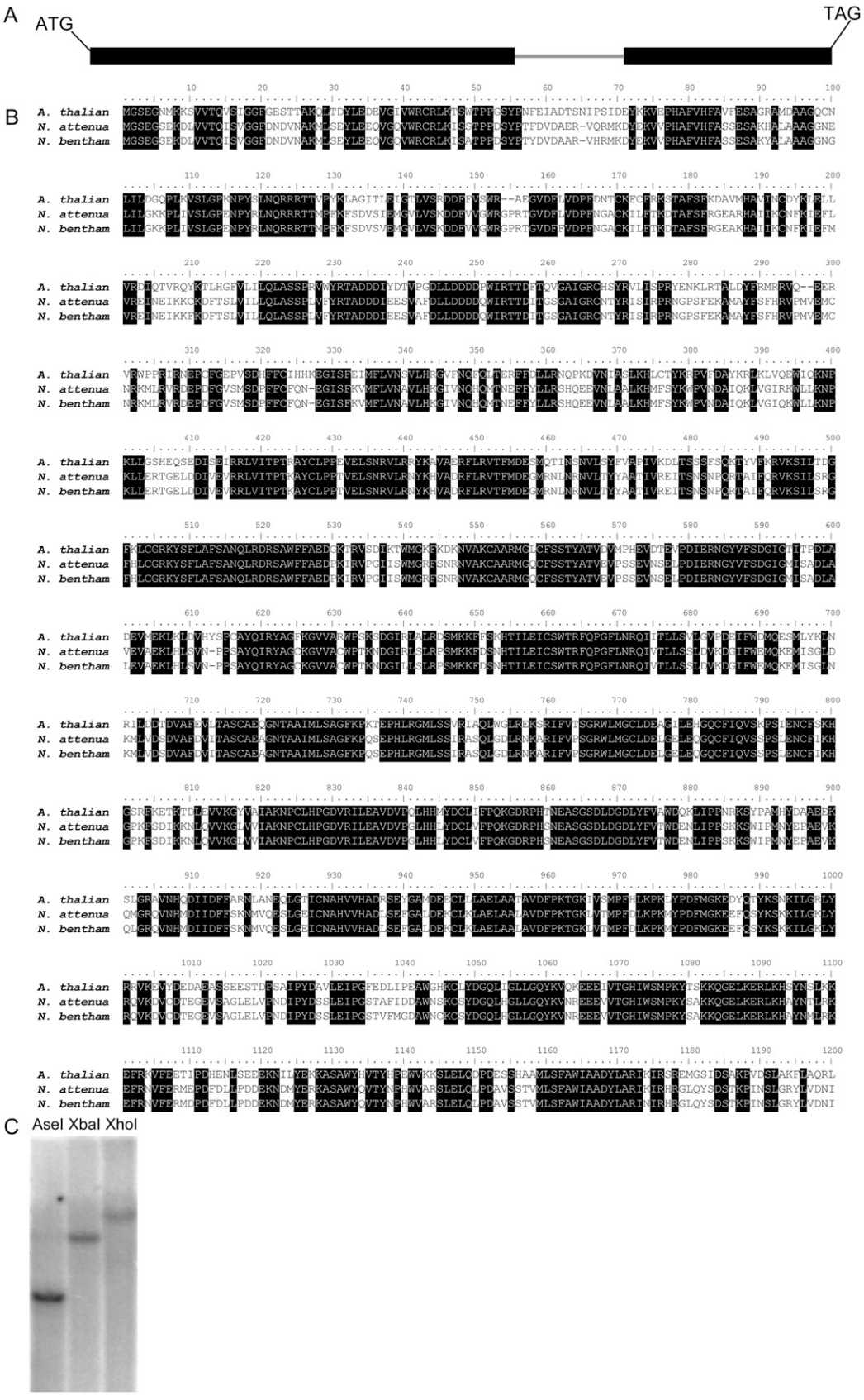

Figure 1. (*Legend appears on following page.*)

Although *RdR6*'s antiviral role under controlled laboratory conditions has been intensively studied, its association with defense against viruses in native habitats has not. Hardly anything is known about the ecological relevance of *RdR6* when plants respond to other challenges in their native environments. We address these questions in a well-studied ecological model system, *N. attenuata. N. attenuata* is a native of the southwestern United States and grows in the immediate postfire environment. Its peculiar germination behavior in the postfire environment, from long-lived seed banks to produce ephemeral populations in the first two growing seasons after fires (Preston and Baldwin, 1999), governs its association with other organisms. Burnt patches provide nutrient-rich environments, but soil resources may vanish rapidly due to rising temperatures and intra- and interspecific competition as the burned areas become colonized by other species. Therefore, plants are compelled to grow quickly and in close proximity to each other. Under such ephemeral postfire environments, the herbivore community, which represents an important biotic stress for *N. attenuata* in the Great Basin Desert of the United States (Baldwin, 2001), is constantly being reestablished. *N. attenuata* produces self-compatible flowers, which mature into seed capsules: The limiting factor for seed production is not the pollen load but the amount of resources that are available to sustain plant growth. Plants, which have to manage their resources, especially the rapidly depleting supply of nitrogen (N)—allocating them between plant growth and defense (Lynds and Baldwin, 1998)—thus display high phenotypic plasticity.

In this study, we explore the ecological role of the *RdR6* homolog in *N. attenuata* by cloning the gene from *N. attenuata* (Na*RdR3*) and characterizing the transformants silenced for this gene. The introduction of silenced plants into natural habitats is a valuable means of determining the whole-plant function of a gene. Traditionally, functions have been assigned based on elicitor studies or sequence similarity, or by silencing the gene and challenging mutants with defined lab-based stresses. Relying only on any one of these approaches may result in a misleading understanding of how a gene functions in *N. attenuata*, due to the particular ecological stresses this species encounters, namely, fast-depleting, limited available resources and herbivory. In this study, therefore, we generated inverted-repeat *RdR3*-silenced plants and introduced them into natural habitats in Utah, to study the role of Na*RdR3* gene in *N. attenuata*. Inferences drawn from the field work were examined further with experiments in the glasshouse.

RESULTS

Isolation and Characterization of *RdR3* from *N. attenuata*

The complete Na*RdR3* gene was isolated using a PCR-based approach. When sequences from amplified genomic DNA and cDNA were compared, and after Fourier transformation, the Na*RdR3* gene appeared to contain a single intron (Fig. 1). *RdR3* from *N. attenuata* had high levels of sequence similarity (Fig. 1) with its corresponding homologs from *N. benthamiana* and Arabidopsis. When the putative protein sequences were compared, NaRdR3 and NbRdR6 were 98% identical, whereas Arabidopsis RdR6 homolog was >65% identical to NaRdR3 or NbRdR6 protein sequences. DNA gel-blot analysis was performed to determine how many *RdR3* loci were present in the *N. attenuata* genome. Southern analysis (Fig. 1C) revealed that the endogenous *RdR3* occurs as a single-copy gene.

The patterns of transcript accumulation of genes after induction with stresses or elicitors that mimic them have been used as reliable means of inferring gene function. To determine the function of the *RdR3* gene in *N. attenuata*, we studied the elicitation dynamics of transcript accumulation after applying elicitors that mimic herbivory or pathogen attack. A slight increase (over 1.5-fold) in transcript levels was recorded (Supplemental Fig. S1) when the leaves were mechanically wounded and the puncture wounds immediately treated with *Manduca sexta* oral secretions (OS elicitation). OS elicitation has been shown to mimic all of the herbivore-specific phytohormone, transcriptome, proteome, metabolome, and resistance responses measured to date (McCloud and Baldwin, 1997; Halitschke et al., 2001; Roda et al., 2004; Giri et al., 2006; Kang et al., 2006). Similarly, applying salicylic acid (SA) had little effect on the accumulation of Na*RdR3* transcripts (Supplemental Fig. S1). SA accumulates after herbivore and pathogen attacks, and orchestrates induced plant defense responses (Rayapuram and Baldwin, 2007). Also, no significant diurnal patterns of *RdR3* transcript accumulation were detected (Supplemental Fig. S1).

RdR3 Silencing Reduces Leaf Number, But Not Reproductive Performance or Herbivore Resistance

Plants stably silenced for *RdR3* expression (irRdR3) were produced by transforming *N. attenuata* with an *RdR3*-specific gene fragment in an inverted-repeat orientation (Supplemental Fig. S2), using *Agrobacterium*-mediated transformation (Kruegel et al., 2002). After a

Figure 1. Characterization of *RdR3* from *N. attenuata*. A, *RdR3* gene structure showing a single intron. B, Putative Na*RdR3* (EU327187) protein is highly similar (>90% identity) to *RdR6* homologs from *N. benthamiana* (AY722008). Protein identity of Arabidopsis *RdR6* homolog (AF239718) to Na*RdR3* or Nb*RdR6* is >65%. Regions marked in black are identical in all the three sequences. C, DNA gel blot reveals that the *RdR3* gene occurs as a single copy in the *N. attenuata* genome.

high-throughput phenotypic screening (Kruegel et al., 2002) and verification by Southern analysis, two independently transformed lines harboring single inserts (244-4 and 282-3; Supplemental Fig. S2) were analyzed for relative transcript accumulation of the *RdR3* gene 1 h after OS elicitation: Neither had accumulated more than one-fifth of the transcript levels found in wild-type plants (Fig. 2A).

Single mutants of *RdR6* in Arabidopsis display minor developmental phenotypes, which typically include elongated leaves and reduced leaf numbers that have little effect on plant fitness (Peragine et al., 2004; Li et al., 2005). We observed similar phenotypes in *RdR3*-silenced *N. attenuata* plants: elongated leaves due to increased petiole lengths, as well as reductions in the production of one to two leaf nodes (Fig. 2, B and C). These alterations in plant morphology did not affect stalk elongation of irRdR3 plants, which was indistinguishable from that of wild-type plants when plants were grown singly in pots in the glasshouse (repeated-measures ANOVA, $F_{2,51} = 0.5$, $P > 0.05$; Fig. 2D). More importantly, differences in rosette morphology did not affect the reproductive output of irRdR3 plants when they were grown without competitors in the glasshouse: irRdR3 plants produced similar numbers of seed capsules and the average mass of the seed capsules could not be distinguished from that of wild-type plants (ANOVA, $P > 0.05$; Fig. 2E). This indicated that the reductions in rosette growth did not translate into fitness differences when plants were grown in single pots in the glasshouse.

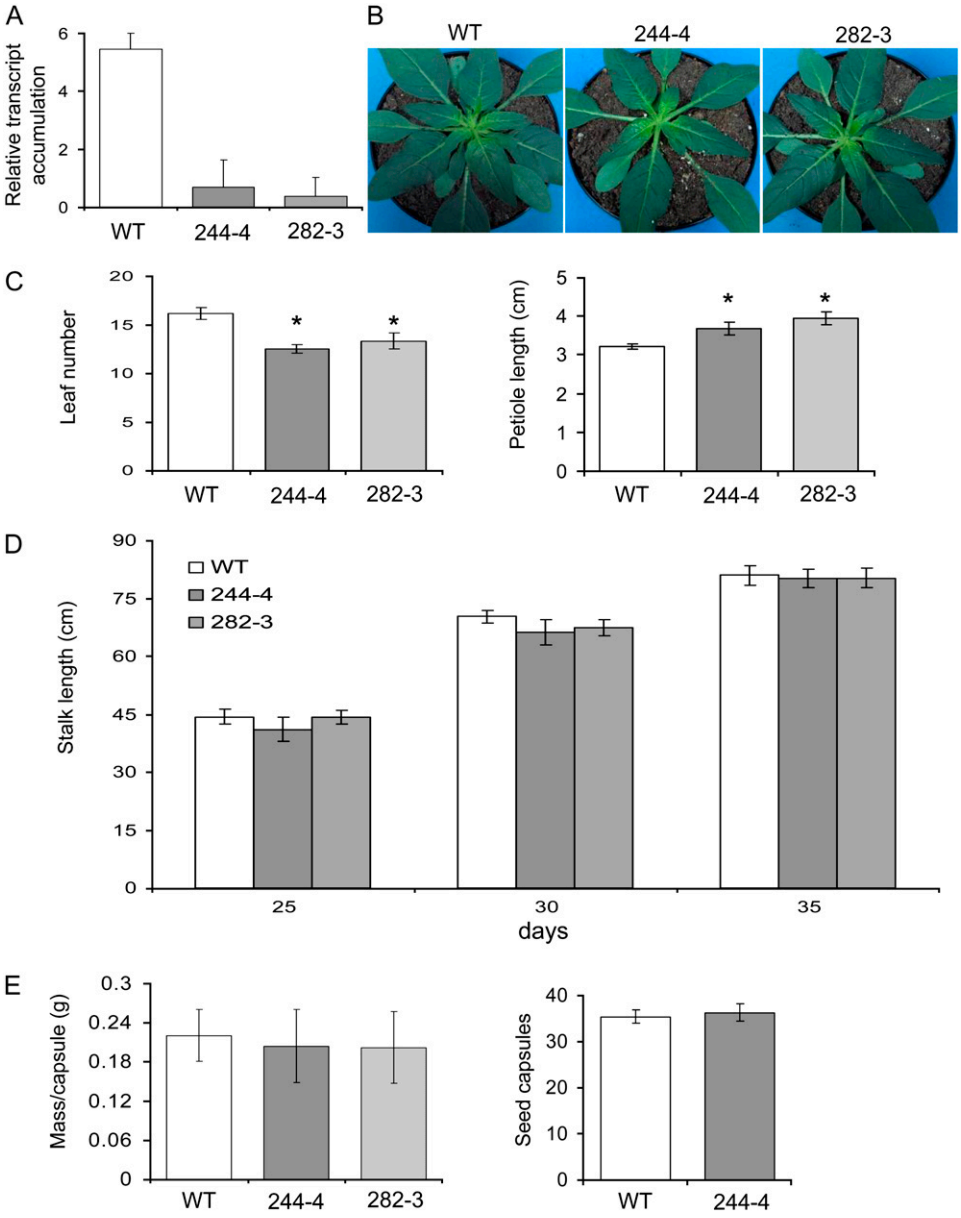

Figure 2. Silencing *RdR3* does not affect plant fitness when plants are grown in individual pots in the glasshouse. A, *RdR3* transcript accumulation in two independently transformed homozygous lines harboring a single copy of a fragment of Na*RdR3* in an inverted-repeat orientation. Wild type and the two irRdR3 lines were OS elicited and *RdR3* transcript accumulation was analyzed 1 h after OS elicitation. Both lines (244-4 and 282-3) accumulated more than 5 times fewer transcripts than did the wild-type plants. B, Minor phenotypic changes in the rosette morphology after stably silencing *RdR3*. C, *RdR3*-silenced plants display growth phenotypes that have been reported in Arabidopsis plants silenced in SGS2/SDE1/RDR6 expression: elongated leaves and reduced leaf number (Peragine et al., 2004). Asterisk (*) indicates significantly different at $P < 0.05$. D, Silencing *RdR3* transcript accumulation did not affect stalk elongation of virus-free plants. The stalks (cm) of irRdR3 (gray bars) and wild-type (white bars) plants grown in single pots were measured 25, 30, and 35 d after transplanting ($P > 0.05$). E, Silencing *RdR3* did not affect plant fitness, measured in terms of capsule mass or number of seed capsules produced, when plants were grown in single pots ($P > 0.05$).

Because *RdR6* homologs in *N. benthamiana* and Arabidopsis have been previously associated with virus resistance (Mourrain et al., 2000; Qu et al., 2005; Schwach et al., 2005), we performed virus-susceptibility screens as phenotypic tests to determine if the antiviral function of *RdR3* had been silenced. Compared to inoculated wild-type plants, both lines were severely affected by tobamoviruses, resulting in highly impaired growth, rapid senescence, and death of *RdR3*-silenced plants (Fig. 3A).

Because the accumulation of *RdR3* transcripts increases after OS elicitation and herbivores are an important biotic stress in *N. attenuata*'s natural habitats, we investigated the possible role of *RdR3* in resistance to insect herbivores. We challenged wild-type and irRdR3 plants with *M. sexta* larvae and compared insect performance: No differences were found (ANOVA, $P > 0.05$; Fig. 3B), demonstrating that silencing *RdR3* had no effect on resistance to insect herbivores.

Changes in smRNA Transcriptome after *RdR3* Silencing

We next investigated how the small RNA (smRNA) profiles change after silencing *RdR3* in *N. attenuata*. Arabidopsis *RdR6* homolog is associated with trans-acting small interfering RNA (siRNA) production (Peragine et al., 2004). We evaluated the changes in smRNA profiles after *RdR3* silencing and compared them with wild-type profiles using 454-sequencing. 454-sequencing can provide quantitative information about the sequenced smRNA as well as its length (Lu et al., 2006; Kasschau et al., 2007; Molnar et al., 2007). Although we were unable to perform an in-depth analysis similar to that reported for Arabidopsis *RdR6* mutants (Kasschau et al., 2007) due to the unavailability of a sequenced *N. attenuata* genome, we compared the smRNA transcriptomes of wild-type and irRdR3 plants. smRNAs in the range of 22 to 23 nucleotides were specifically reduced after *RdR3* silencing in *N. attenuata* (Supplemental Fig. S3). Of 31,256 unique smRNAs from irRdR3 plants, only 5% overlapped with the wild-type smRNA sequences (Supplemental Fig. S4A). This may be due to the increased abundance of smRNAs in size classes <21 nucleotides and >24 nucleotides (Supplemental Fig. S3). Analysis of the commonly regulated 1,529 smRNAs between wild-type and irRdR3 plants revealed that 315 smRNAs were down-regulated, whereas 580 smRNAs were enriched in irRdR3 plants (Supplemental Fig. S4B). We annotated these sequences against the nonredundant nucleotide database (NR-DB) of the National Center for Biotechnology Information (NCBI) and the miRBase Sequence Database (Supplemental Tables S1 and S2). Of 31,256 unique smRNAs from irRdR3 plants, 12,792 sequences matched different categories of RNA sequences (Supplemental Fig. S4C), including 21 microRNAs. Some differences between the patterns of changes of smRNAs when *RdR6* is mutated in Arabidopsis (Kasschau et al., 2007) and when *RdR3* is silenced in *N. attenuata* may result from differences in the depth of the analyses. Moreover, the generation of new smRNAs in response to *RdR3* silencing resulting from the activation of (other) smRNA pathways, not requiring *RdR*s and *DCL*s, cannot be ruled out.

Silencing *RdR3* Reduces Reproductive Performance in Native Habitats without Affecting Herbivore Resistance

Silencing *RdR3* had no effect on reproductive output and resistance when plants are grown without competitors in the glasshouse. To determine the ecological relevance of the *RdR3* gene in *N. attenuata* and to understand how changes in the smRNA transcriptome after *RdR3* silencing affect the ecophysiology of plants, we assessed the performance of irRdR3 lines in their native habitat. In nature, *N. attenuata* plants face two major stresses: an unpredictable herbivore population and high intraspecific competition due to their synchronized germination from seed banks after fires. To simulate the intraspecific competition, we planted wild-type and irRdR3 plants in pairs in close proximity (approximately 20 cm apart; Supplemental Fig. S5). In natural habitats, irRdR3 plants were able to defend themselves as successfully as the wild-type plants did against the community of herbivores attacking plants at the time (Fig. 4B). Because the plants were highly susceptible to tobamoviruses when tested in the glasshouse (Fig. 3), we expected the irRdR3 plants to act as viral biosensors, to show viral infection symptoms even if only the slightest inoculum was present. Yet, no viral infections were observed on wild-type or irRdR3 plants.

However, the irRdR3 plants did not perform as well as their wild-type counterparts (Fig. 4A). Total plant canopy area damaged and plant heights were recorded 10 d after wild-type and irRdR3 plants were released in the field. No differences were observed in herbivory (wild type-244-4 pairs; $n = 10$ pairs; paired t test, $P > 0.05$) or plant performance (wild type-244-4 pairs; $n = 10$ pairs; paired t test, $P > 0.05$) between the wild-type and irRdR3 plants. After 5 d, herbivory rates remained the same (Fig. 4B; $n = 10$ pairs; paired t test, $P > 0.05$), but irRdR3 plants elongated more slowly than did the wild-type plants (Fig. 4B; $n = 10$ pairs; paired t test, $t = 6.16$, $P < 0.005$). A third and final observation, recorded 5 d after the second, revealed that irRdR3 plants continued to lag behind wild-type plants in their stalk elongation ($n = 10$ pairs; paired t test, $t = 2.51$, $P < 0.05$) but suffered similar amounts of herbivore damage (Fig. 4B; $n = 10$ pairs; paired t test, $P > 0.05$). Similarly, decreased performance (Fig. 4B; paired t test, $t = 2.69$, $P < 0.05$) and unaltered herbivore resistance (Fig. 4B; paired t test, $P > 0.05$) were also observed in a second line of transformed irRdR3 plants (282-3; Fig. 4B).

Although we were not able to measure lifetime fitness parameters completely, silencing irRdR3 clearly had deleterious effects on reproductive output. The total numbers of flowers produced during the study

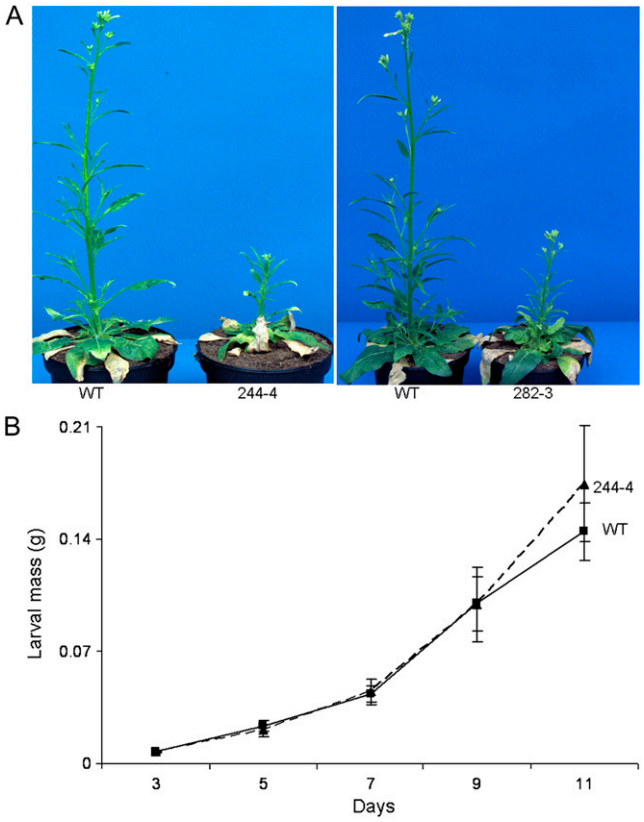

Figure 3. Silencing *RdR3* reduces resistance to viruses but not to an insect herbivore. A, Susceptibility of irRdR3 lines to viruses. When rosette leaves were inoculated with the tobamovirus, growth of the two irRdR3 lines was severely impaired and plants of both lines rapidly senesced and died. B, Performance of *M. sexta* larvae feeding on *N. attenuata* wild-type and irRdR3 plants. Neonates were placed on the first fully expanded source leaves and their mass (g) was measured after 3, 5, 7, 9, and 11 d. No differences were observed ($P > 0.05$), indicating that irRdR3 plants had intact direct defenses.

were significantly fewer in line 244-4 (Fig. 4A; paired *t* test, $t = 4.45$, $P < 0.005$) as well as in line 282-3 (Fig. 4A; paired *t* test, $t = 2.55$, $P < 0.05$) compared to their wild-type counterparts. These results suggested that irRdR3-silenced plants were impaired in their ability to compete with their wild-type neighbors.

Silencing *RdR3* Reduces Reproductive Performance When Plants Are Grown with Wild-Type Competitors in the Glasshouse

To verify the reduced plant performance observed in nature, we grew plants in the glasshouse: (1) without resource competition (in single pots), and (2) with resource competition (plants were grown in close proximity, approximately 7–10 cm apart in 2-L pots). For the competition experiments, the irRdR3 and wild-type plants were combined as follows: wild type-wild type, 244-4-244-4, wild type-244-4, 282-3-282-3, and wild type-282-3. No differences in stalk elongation or reproductive output were recorded between the wild-type and irRdR3 plants when they were grown singly in pots (Fig. 2, D and E). In contrast, wild-type plants outcompeted their irRdR3 neighbors when grown in communal 2-L pots (Fig. 5; repeated-measures ANOVA; wild type-244-4 combination, $F_{1,118} = 256.1$, $P < 0.0001$; wild type-282-3 combination, $F_{1,138} = 89.0$, $P < 0.0001$). No differences in plant performance were observed when plants from the same genotype competed with each other (Supplemental Fig. S6; repeated-measures ANOVA; $P > 0.05$). Lifetime seed capsule numbers (Fig. 5; paired *t* test; for wild type-244-4, $t = 6.42$, $P < 0.0001$; for wild type-282-3, $t = 10.70$, $P < 0.0001$) of irRdR3 plants were strongly reduced as was plant dry mass (Fig. 5; paired *t* test; for wild type-244-4, $t = 8.01$, $P < 0.0001$; for wild type-282-3, $t = 15.48$, $P < 0.0001$). Seed capsule numbers (Supplemental Fig. S6; paired *t* tests, $P > 0.05$) and dry mass (Supplemental Fig. S6; paired *t* tests, $P > 0.05$) were the same when plants from the same genotype competed with each other.

Transcriptional Responses of *RdR3*-Silenced Plants When These Compete with Wild-Type Plants

To understand how transcriptional responses were altered in *RdR3*-silenced plants and to predict the potential targets of smRNAs, we performed microarray analysis with an unbiased potato (*Solanum tuberosum*) 10 K cDNA microarray (The Institute for Genomic Research), previously proven suitable for *N. attenuata* (Schmidt et al., 2005). Using RNA extracted from irRdR3 plants, we hybridized arrays against the corresponding wild-type plants; both genotypes had been growing in competition prior to leaf tissue harvest for RNA extraction. Three replicate chips were hybridized with biologically replicated RNA samples. Only 97 genes were differentially regulated (Supplemental Table S3) at a cutoff of 1.5-fold or greater, and the false discovery rate = 5%; 90 genes were down-regulated and seven were up-regulated. Two of the important down-regulated genes included a cytochrome P450-Q9LUC9 and leafy-cotyledon-1. Some members of primary metabolism were also down-regulated, as were some transport proteins. Many of the regulated genes were of unknown function. In Arabidopsis, when the transcript profiles of wild-type and *ZIP*-, *SGS3*-, and *RdR6*-mutated plants were compared with Affymetrix ATH1 arrays with 22,800 genes, at a cutoff as low as 1.3-fold, only 17 genes were found to be differentially regulated, most of which were false positives (Peragine et al., 2004). This suggested that the *RdR6*-dependent siRNAs may not act on the transcripts but may affect the end product, possibly regulating protein biosynthesis.

Phytohormone Analysis

To understand the mechanistic basis of the reduced competitive ability of the *RdR3*-silenced plants, we analyzed phytohormones from field and glasshouse studies. From the field-grown samples, which were

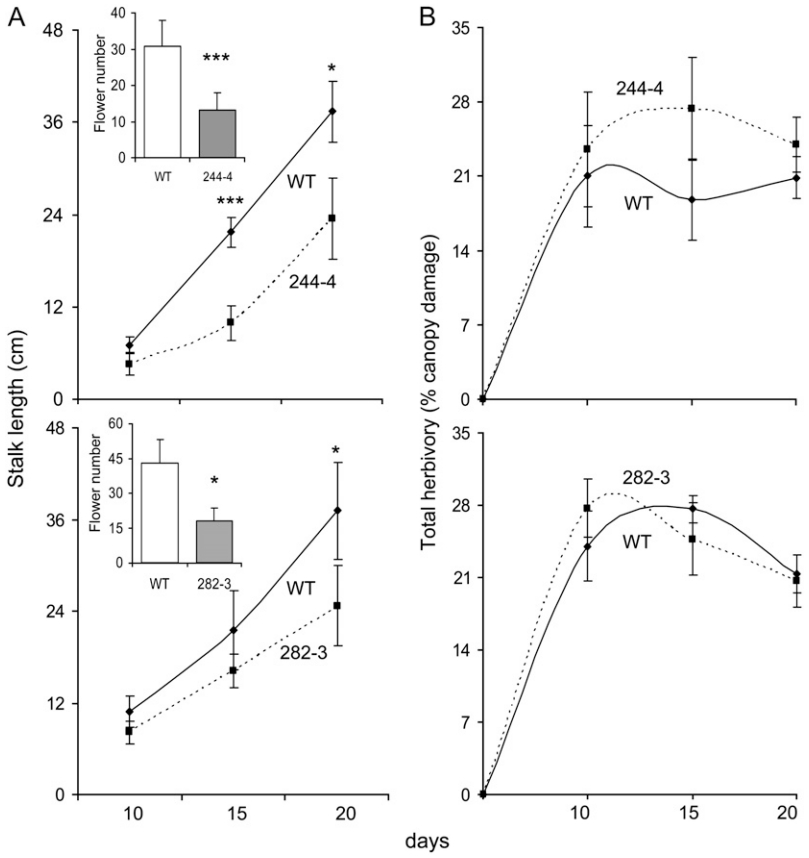

Figure 4. Silencing *RdR3* reduces growth and reproductive performance, but not herbivore resistance in the plant's native habitat. irRdR3 plants from each line (244-4 and 282-3) were individually paired with a wild-type plant and planted in close proximity (approximately 20 cm) to each other in a native population in the Great Basin Desert of Utah. A, Size-matched plants were transplanted to the field at day 0 and stalk lengths (cm) of irRdR3 (dotted lines) and wild-type (solid lines) plants were measured 10, 15, and 20 d later. Total numbers of flowers produced during 21 d of growth in the field are presented as insets. B, The cumulative herbivore damage to irRdR3 plants (dotted lines) and wild-type plants (solid lines) was measured and no significant differences in herbivory were observed between irRdR3 and wild-type plants. Asterisks indicate: ***, significantly different at $P < 0.005$; *, significantly different at $P < 0.05$.

elicited by natural herbivores and the application of OS, we tested the accumulation of four important phytohormone-signaling compounds known to be involved in plant growth, adaptation, and defense: abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and its Ile/Leu conjugate (JA-Ile/Leu). Whereas ABA may be regarded as a general abiotic stress marker, JA, JA-Ile/Leu, and SA are involved in defense against herbivores in *N. attenuata* (Halitschke and Baldwin, 2003; Kang et al., 2006; Paschold et al., 2007; Rayapuram and Baldwin, 2007). JA and JA-Ile/Leu levels in irRdR3 were similar to those in wild-type plants (Supplemental Fig. S7; paired *t* test, $P > 0.05$), indicating that irRdR3 plants had intact defense signaling. Compared to wild-type plants, irRdR3 plants had enhanced SA levels 120 min after OS elicitation (Supplemental Fig. S7; ANOVA, $F_{1,6} = 5.68$, $P < 0.05$). When we compared the constitutive ABA levels in the samples not elicited with OS (time 0 min), ABA levels were slightly higher in irRdR3 plants than in their wild-type counterparts (Supplemental Fig. S8). High SA and ABA levels may contribute to reducing their growth when plants are grown in competing, stressful conditions of their natural environment.

To reexamine this hypothesis, we measured the profiles of phytohormones under controlled glasshouse conditions, when irRdR3 and wild-type plants were grown in competition with each other. In line with the observations from field samples, similar JA (paired *t* test, $P > 0.05$) and JA-Ile/Leu levels (paired *t* test, $P > 0.05$) were observed in wild-type and irRdR3 plants (Supplemental Fig. S9). As in the field, levels of SA (paired *t* test, $P < 0.05$) and ABA (paired *t* test, $P < 0.05$) in the irRdR3 plants were higher than those in wild-type plants.

Auxin is the phytohormone most associated with the modulation of plant growth (Teale et al., 2006) and most responsive to competition-related stresses (Morelli and Ruberti, 2000; Hoecker et al., 2004). We measured auxin levels in the first stem leaves of competing irRdR3 and wild-type plants at different stages of growth, starting just after stems began to elongate, until the plants entered the reproductive phase and started to produce seed capsules. Overall, irRdR3 plants had reduced auxin levels (Supplemental Fig. S10A), with maximum differences occurring during the early vegetative phase, 18 d after transplanting (repeated-measures ANOVA, $F_{1,22} = 13.62$, $P < 0.01$). We performed auxin-complementation assays using lanolin paste as previously described by Reinhardt et al. (2000); in these, auxin was exogenously supplied to the apical meristem and plant performance was measured. Such complementation assays are commonly used for restoration studies. Exogenous supply of auxin (1 mM) could not restore the growth of irRdR3 plants to wild-type levels (Supplemental Fig. S10B;

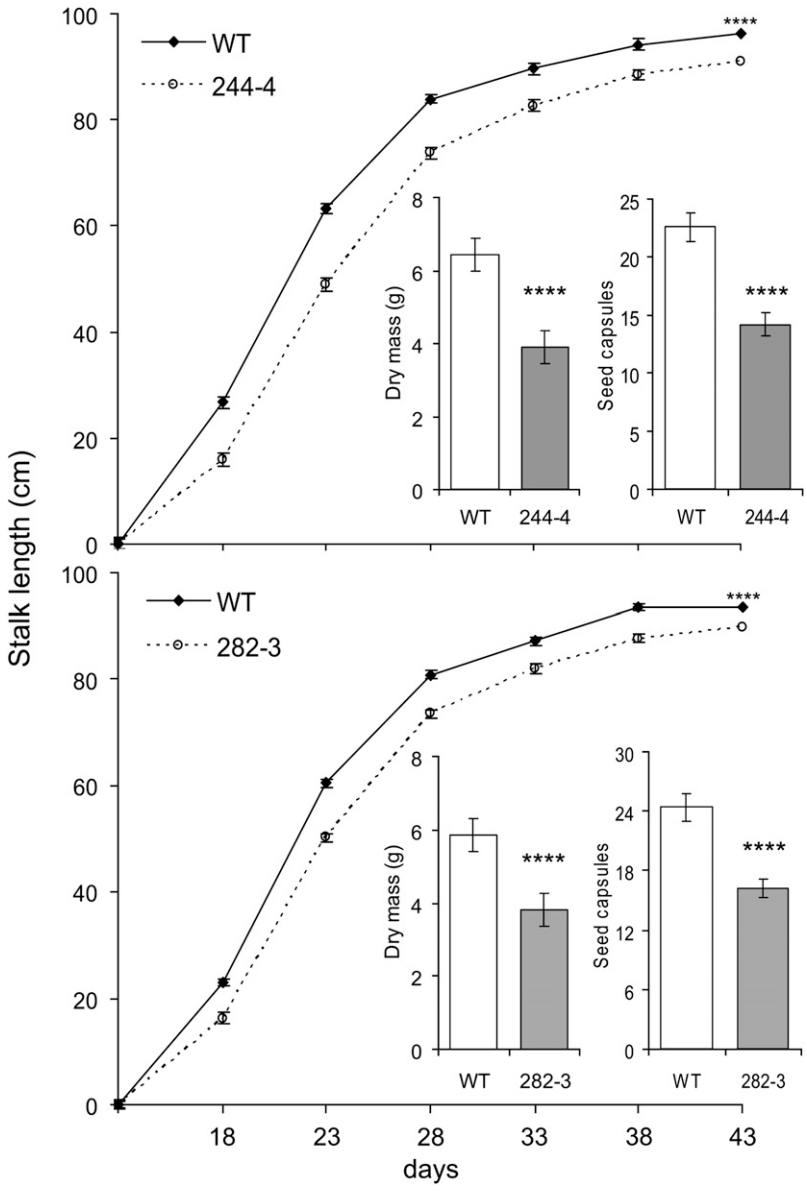

Figure 5. Silencing *RdR3* reduces the plant's ability to compete with wild-type plants. Size-matched irRdR3-wild type plant pairs were grown in close proximity (7–10 cm) in 2-L pots. Stalk lengths (cm) of competing irRdR3 (dotted lines) and wild-type (solid lines) plants were measured 18, 23, 28, 33, 38, and 43 d after planting. Plant dry mass (g) and (lifetime) seed capsule numbers produced during the duration of the study are presented as insets. Asterisks (****) indicate significantly different at $P < 0.0005$.

repeated-measures ANOVA, $F_{1,34} = 41.64$, $P < 0.0001$); rather, the additional auxin slowed the elongation of competing irRdR3 plants, suggesting that distribution or regulation of its homeostasis, rather than biosynthesis, may have been altered in irRdR3 plants during competitive growth. To test if the biosynthesis of auxin was altered in irRdR3 plants, we measured auxin levels in the apical meristem of competing irRdR3 and wild-type plants: no differences were found (Supplemental Fig. S10C; paired *t* test, $P > 0.05$).

DISCUSSION

In this study, we examined the real-world relevance of phenotypes associated with the silencing of the *RdR6* homolog in *N. attenuata* (Na*RdR3*). *RdR3*-silenced *N. attenuata* plants displayed phenotypes similar to the previously characterized phenotypes of Arabidopsis *RdR6* mutants, which include elongated and reduced numbers of leaves and susceptibility to virus attack. We show that Na*RdR3* is essential for the optimization of reproductive output when plants grow with competitors, as they frequently do in their natural environments. *N. attenuata*, one of the first species to become established in postfire environments, germinates from a long-lived seed bank (Preston and Baldwin, 1999). The ability of a genotype to represent itself in the seed bank depends on its ability to grow rapidly and reproduce in environments with initially unlimited resources that are rapidly depleted as burns are revegetated. The plants allocate their resources, especially their rapidly depleting N supply, between plant growth and defense (Lynds and Baldwin, 1998).

Initial differences in vegetative growth strongly influence reproductive performance and fitness (number of seed capsules), and thus their representation in seed banks. In this study, we show that the *RdR3* gene mediates part of phenotypic plasticity in *N. attenuata* and that the impaired competitive ability is associated with altered phytohormone concentrations.

*RdR*s form an important component of the RNA-silencing/PTGS machinery by synthesizing dsRNAs; these are progenitors of siRNAs. Six *RdR*s are predicted in Arabidopsis, but only three have been functionally described (Wassenegger and Krczal, 2006). *RdR*-dependent RNA-silencing pathways seem to be functionally specialized in *N. attenuata*: In previous work, we demonstrated that *RdR1* mediates resistance to insect herbivores without influencing growth or competitive ability (Pandey and Baldwin, 2007; Pandey et al., 2008), while *RdR2* mediates resistance to UV stress without affecting herbivore resistance (Pandey and Baldwin, 2008). Here we show that *RdR3* mediates competitive ability without influencing herbivore resistance. These different functions were discovered by introducing the independently silenced lines into native habitats and the results highlight the value of field releases in allowing for the rapid identification of phenotypes. During a field release, plants are exposed to a gauntlet of abiotic and biotic stresses, and a researcher intimately familiar with the plant's natural history is able to rapidly identify the stress responsible for decreases in fitness and performance. A particular stress can be manipulated in subsequent experiments in the field or laboratory to test the inferences gleaned from the field experiments.

To understand the mechanistic basis underlying the reduced competitive ability of the irRdR3 plants, we adopted a two-tiered strategy. First, we performed an unbiased comparative analysis of the transcriptomes of irRdR3 and wild-type plants grown in competition. This analysis was not very helpful for obtaining mechanistic inferences. Similar observations were made in Arabidopsis, where microarray studies with a much larger array (of 22,800 clones) and low cutoffs of 1.3-fold showed negligible changes in transcript levels (just 17, most of which were false positives) when *ZIP*-, *SGS3*-, and *RdR6*-mutated plants were compared (Peragine et al., 2004). It may be that *RdR3*-dependent siRNAs regulate the protein levels of their targets, rather than the transcript accumulation.

Second, we profiled a set of phytohormones known to relay signaling networks that fine-tune plant growth and chemical adaptive changes. The levels of JA and its amino acid conjugate, JA-Ile, the two well-described regulators of most of *N. attenuata*'s responses to herbivory (Halitschke and Baldwin, 2003; Kang et al., 2006), were unchanged in irRdR3 plants, which is consistent with the data excluding a role for *RdR3* in antiherbivore defense. On the other hand, under competing conditions, mature leaves of irRdR3 plants had higher SA and lower indole-3-acetic acid (IAA) concentrations than wild type did. In contrast to the well-documented role of IAA in shade avoidance (Morelli and Ruberti, 2000; Hoecker et al., 2004), the influence of SA during light competition is not well understood (Genoud et al., 2002). It is noteworthy that plants accumulating SA frequently display morphological phenotypes that are reminiscent of IAA-deficient or IAA-insensitive mutants, such as reduced apical dominance and stunted growth (Bowling et al., 1997; Clarke et al., 1998; Li et al., 2001). Recently published data have associated this phenotype with the repression by SA of the *TIR1* IAA-receptor gene (Wang et al., 2007). IAA, which is synthesized in young leaves of the shoot system and transported downward to the root tip through the vasculature, regulates many different aspects of plant development. Our results suggest that altering IAA's distribution or homeostasis rather than its biosynthesis might affect the decreased vegetative fitness of irRdR3 plants. Forming and maintaining IAA gradients in planta is known to require a specific polar auxin-transport system. Carrier proteins involved in such a process have been recently identified in Arabidopsis (Galweiler et al., 1998; Chen et al., 1999); however, how they influence developmental plasticity in the real world is still unknown. Glasshouse-based studies of the shade-avoidance phenomenon have shown that the spatial reconfiguration of IAA gradients, more than of its production, plays an essential coordinating function (Morelli and Ruberti, 2000). Treatment with *N*-1-naphthylphthalamic acid, an IAA transport inhibitor, significantly reduces hypocotyl elongation of wild-type seedlings in response to far-red-rich light (Steindler et al., 1999).

In conclusion, we have identified some of *RdR3*'s roles in plant adaptation to intraspecific competition. Silencing *RdR3* in *N. attenuata* changed the homeostasis of SA and IAA, which could be correlated with the reduced competitive ability of the *RdR3*-silenced plants in nature and glasshouse. At the same time, this study opens doors for investigating SA-IAA cross talk as well as how IAA signaling is regulated during competition.

MATERIALS AND METHODS

Plant and Insect Material

Wild-type *Nicotiana attenuata* plants were from the 17th or 22nd inbred generation of seeds originally collected from a native population in Utah. All plants were grown under conditions described earlier (Kruegel et al., 2002; Halitschke and Baldwin, 2003). Eggs of *Manduca sexta* (Lepidoptera) were acquired from North Carolina State University (Raleigh, NC).

Isolating *N. attenuata*'s *RdR3* Gene

A PCR-based strategy was used to clone *RdR3* from *N. attenuata*. To isolate *RdR3*, identical or complementary PCR primers were designed from the homologous sequences (*RdR6*) from close relatives of *N. attenuata*, *Nicotiana benthamiana*, and *Nicotiana tabacum*. PCR was conducted with genomic DNA (extracted with procedures described earlier; Bubner et al., 2004) and cDNA (described below). Single bands were gel purified with GFX PCR DNA and gel band purification kit (Amersham Biosciences) according to the manufacturer's instructions, and sequenced directly (if amplified from genomic DNA)

or cloned in pJET vectors and sequenced. Sequences were aligned with the corresponding cDNA sequence from *N. benthamiana* and *N. tabacum*. Fragments giving a positive alignment were considered to be exons. Sequences that did not match the corresponding homologs were tentative introns and subjected to Fourier analysis to determine if they were noncoding (Tiwari et al., 1997). Gene sequences from different *RdR*s (accession nos. given below) were aligned with MegAlign (DNASTAR).

Transcript Analysis by Quantitative Real-Time PCR

RdR3 homologs (*RdR6*) in Arabidopsis (*Arabidopsis thaliana*) and *N. benthamiana* are elicited by SA treatments and virus attack, and their role in defense against viruses has been demonstrated (Yu et al., 2003; Yang et al., 2004). To determine if herbivore attack changes transcript accumulation of the *RdR3* gene, the second fully expanded (+2 van; Van Dam et al., 2001) leaves of three to four rosette-stage *N. attenuata* plants were wounded with a fabric pattern wheel and puncture wounds were immediately treated with 20 μL (diluted 1:1 with distilled water) *Manduca sexta* OS, which are known to activate the herbivore-specific responses in *N. attenuata* (Halitschke et al., 2001). This procedure is hereafter referred to as OS elicitation. We also determined the effect of SA on *RdR3* transcript levels by spraying plants with a 2 mM SA solution until runoff (Yang et al., 2004; Pandey and Baldwin, 2007). OS- or SA-treated +2 leaves were harvested from three to four replicate plants at 0, 1, 4, 12, and 48 h after each treatment. To determine diurnal changes in *RdR3* transcript accumulations, we harvested leaves from untreated +2 nodes of four replicate plants at 4:00 h, 8:00 h, 12:00 h, 16:00 h, 20:00 h, and 24:00 h.

Total RNA was extracted following the TRIZOL method and reverse transcribed to prepare first-strand cDNA with the SuperScript first-strand synthesis system for reverse transcription-PCR, with oligo(dT) as primers (Invitrogen), following the manufacturer's protocol. SYBR Green assays were developed (qPCR core kit for SYBR Green I, Eurogentec; following the manufacturer's protocol); all the qPCR assays were performed with cDNA corresponding to 100 ng RNA before transcription and gene-specific primers and probes. Each biological replicate was used twice on the qPCR plate. The $2^{-\Delta\Delta CT}$ method was used for data analysis (Bubner et al., 2004). To simplify data interpretation, expression levels in control plants (time point of 0 h treatment) were fixed to 1. *N. attenuata* sulfite reductase (*ECI*), a house-keeping gene involved in sulfur metabolism whose transcript abundance is unchanged from constitutive levels in response to OS elicitation, was used as an endogenous reference. To determine the *RdR3* levels in the silenced lines, gene-specific primers were designed outside the region used in the silencing constructs. All the gene-specific primers were designed with Primer Express software (http://www.appliedbiosystems.com).

Silencing *RdR3* of *N. attenuata*

An *RdR3* gene-specific fragment (353 bp; DQ988992) was cloned in an inverted-repeat orientation in a pRESC5 transformation vector as described earlier (Steppuhn et al., 2004; Bubner et al., 2006). *Agrobacterium tumefaciens*-mediated transformation was done as described in Kruegel et al. (2002). T_1 plants were screened for hygromycin resistance and homozygosity was determined by segregation analysis of T_2 plants. qPCR was used to quantify transcript accumulations, as described above, and Southern analysis was used to determine transgene copy number. Two independently transformed homozygous lines (244-4 and 282-3), each containing a single insertion of the transgene, were further characterized in the T_2 generation. Since *RdR3* is required for resistance to viruses, a virus susceptibility screen assay was used as an additional positive control for *RdR3* silencing (Pandey and Baldwin, 2007). Two tobamoviruses, *Tomato mosaic virus* and *Bell pepper mosaic virus*, were inoculated into +1, 2, and 3 leaves of the wild-type and transgenic plants. Leaves of three replicate plants of wild type and both transgenic lines in the rosette stage (28 d after germination) were rubbed with corborundum powder, and 50 μL of viral material suspended in phosphate buffer was applied to the abraded leaves. As mock controls, equal numbers of plants from each line were rubbed with corborundum powder and treated with 50 μL of phosphate buffer without any virus. Plants were monitored for the development of symptoms for 12 to 14 d.

Insect Performance and Competition Assays

Insect performance assays were conducted by challenging irRdR3 and wild-type plants with *M. sexta* larvae. A freshly hatched larva was placed on the +2 leaf of each of the genotypes. Eleven to 15 replicate plants were used for each genotype. Starting from the third day, caterpillar mass was measured every 2 d for 11 d.

To compare the competitive ability of irRdR3 and wild-type plants, we grew initially size-matched seedlings in 2-L pots as described earlier (Zavala et al., 2004). The seeds were germinated as previously described (Kruegel et al., 2002), and 20 d after germination, size-matched seedling pairs were transplanted, approximately 7 cm apart in 2-L pots in the following combinations: wild type-wild type, 244-4-244-4, 282-3-282-3, wild type-244-4, and wild type-282-3. Stalk length was recorded 18 d after transplanting, when all the plants had started to elongate. Six subsequent observations were made at 7-d intervals.

Performance under Native Conditions

The planting of *RdR3*-silenced plants (irRdR3) into the natural habitats of *N. attenuata* in the Great Basin Desert in southwestern Utah under APHIS notification number 06–003–08 was as described by Pandey and Baldwin (2007). Ten days after germination, seedlings were transferred to borax-soaked Jiffy 703 pots (AlwaysGrows) and 3 to 4 weeks later they were transferred to field plots. Ten irRdR3-wild type pairs of same-size adapted seedlings from both transgenic lines were transplanted. Seedlings were watered every second day for 2 weeks until the roots had established themselves. The plants were colonized by native herbivores for 3 weeks and the study was terminated after 28 d. All the capsules were removed and destroyed along with all the plants in and around the plantation to comply with 7CFR 340.4. In three consecutive observations at 5-d intervals, stalk length was recorded and the leaves of irRdR3-wild type pairs were scrutinized for characteristic damage caused by attack from natural herbivores, which included mirids, grasshoppers, and beetles; total herbivory was estimated as a percentage of total canopy area (Pandey and Baldwin, 2007).

Microarray Analysis

Microarray analysis for samples derived from the above-described competition study was performed with unbiased potato (*Solanum tuberosum*) 10 K-cDNA microarray chips (The Institute for Genomic Research; Schmidt et al., 2005). First stem leaves were harvested from three biological replicates of wild type-irRdR3 pairs, grown in competition for 18 d. Total RNA was extracted following the TRIZOL method from three biological replicates and an equal amount of RNA from each replicate was used for one chip. RNA from the irRdR3 plants was labeled with Cy_3; RNA from the wild-type counterparts was labeled with Cy_5. Around 400 μg total RNA was used in each labeling reaction. Three such chips were made from independent biologically replicated samples. Microarray data were lowess-normalized with R statistical package, differential regulation was determined at a cutoff of 1.5-fold, and false discovery rates were set at 5% (SAM statistics for identification of significantly regulated genes).

Phytohormone Analysis

JA, JA-Ile/Leu, SA, and ABA were extracted with ethyl acetate following the phytohormone procedure described previously (Wu et al., 2007). Briefly, approximately 150 mg of leaf tissue was homogenized and extracted in FastPrep tubes containing 0.9 g of FastPrep Matrix (BIO 101) and 1 mL ethyl acetate spiked with 200 ng JA-$^{13}C_2$, SA-D_4, and ABA-D_6 as internal standards (IS). Samples were centrifuged at 13,000 rpm for 15 min at 4°C, and supernatants were collected. Each pellet was reextracted with 0.5 mL of ethyl acetate and centrifuged; supernatants were combined and then evaporated to dryness on a vacuum concentrator (Eppendorf). The residue was resuspended in 0.5 mL of 70% methanol (v/v).

Endogenous IAA was extracted according to Edlund et al. (1995). Between 75 to 100 mg of tissue per sample was incubated with 1 mL of extraction buffer (50 mM Na_2HPO_4, pH 7, and 0.02% diethyldithiocarbamic acid) spiked with 50 ng IAA-D_5 for 2 h at 4°C in the dark. Samples were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were acidified with 1 M HCl to pH 2.7, and 60 mg of Amberlite XAD-7 was added. After 1 h of incubation, supernatants were removed, XAD-7 resin was washed with 1% acetic acid, and absorbed compounds were eluted twice with 1 mL of dichloromethane. Eluants were combined and evaporated to dryness on a vacuum concentrator (Eppendorf). The residue was resuspended in 50 μL of 70% methanol (v/v).

Hormone extracts (10-μL aliquot) were analyzed by reverse-phase HPLC coupled to tandem mass spectrometry (MS) as described earlier (Wu et al., 2007). Multiple reaction monitoring (MRM) was conducted with a 1,200 L MS/MS/MS system (Varian), after negative ionization, with parent-ion/daughter-ion selections: 209/59 (JA), 211/61 (JA-$^{13}C_2$), 322/130 (JA-ILE/LEU), 137/93 (SA), 141/97 (SA-D_4), 263/153 (ABA), 269/159 (ABA-D6), 174/130 (IAA), and 179/135 (IAA-D_5). The area beneath the MRM product ion peak was determined for each analyte and IS. The quantity of the analyte was calculated according to the formula: analyte product ion peak area $\times$ (IS concentration/IS product ion peak area). For JA-Ile/Leu, JA-$^{13}C_2$ was used as IS: Calibration curves were created by plotting the known concentration of synthetic JA-Ile in dilution series against the quantity calculated using the above formula.

Analyzing the smRNA Portion of the Transcriptome

The smRNA portion of the transcriptome of the irRdR3 was sequenced by 454-sequencing (Lu et al., 2006; Kasschau et al., 2007; Molnar et al., 2007) along with the other genotypes described elsewhere (Pandey et al., 2008). The 454-sequencing was performed by Vertis Biotechnologie AG. Briefly, leaf material was ground under liquid nitrogen; RNA species <200 bp were enriched (mirVana microRNA isolation kit, Ambion); smRNAs were separated on a denaturing 12.5% polyacrylamide gel and stained with SYBR green II; and size fractions of 15 to 30 nucleotides were isolated, precipitated with ethanol, and dissolved in water. RNA was first poly(A)-tailed with poly(A) polymerase; RNA adapters were ligated to 5′-P; and first-strand cDNA synthesis was performed using oligo(dT) primer linker and PCR amplified to about 30 ng/μL with 22 cycles using high-fidelity Taq DNA polymerase. The bar codes, which were attached to a 5′ flanking sequence for the two genotypes, were ACTA (wild type) and CAGC (irRdR3). 5′ and 3′ flanking sequences were: 5′-GCCTCCCTCGCGCCATCAGCTNNNNGACCTTGGCTGTCACTCA-3′ and 5′-GCCTTGCCAGCCCGCTCAGACGAGACATCGCCCCGC($T)_{25}$-3′. cDNAs were pooled in equal amounts, gel fractionated, eluted, and purified. The gel-purified cDNA pool was submitted to 454-sequencing at Vertis Biotechnologie AG.

After initial cleaning steps, the data were parsed into two groups according to the bar codes. Sequences were rejected for further analysis if they lacked bar codes or adequate 5′ and 3′ flanking sequences. Adapter sequences, 5′, and 3′ flanking sequences were identified and removed from each bin, and sequences shorter than 15 nucleotides were discarded. According to manufacturer's instructions, the technology is not able to distinguish more than eight identical nucleotides in a stretch; therefore all the A's from the 3′ end (or any continuous run of single nucleotide at the 5′ end) were removed. This may cause sequences to be underrepresented by one nucleotide at the 5′ or 3′ end. Total abundance and number of distinct sequences were determined in each case. All the analyses were performed with custom-written programs in Perl.

Statistical Analysis

Data (suitably transformed, wherever they did not meet assumptions of normality) were analyzed with StatView (Abacus Concepts, Inc.). Assays in the glasshouse were analyzed for the ANOVA or repeated-measures ANOVA. All the field data or data derived from samples from field and from competition experiment were tested with paired *t* tests because all the field and competition experiments were conducted in pairs. A transgenic line and wild-type control plant were planted as a single pair.

The accession number of the *N. attenuata RdR3* submitted to NCBI is EU327187. The accession numbers for the other *RdR*s used in this study are: AY722008 NbSDE1 and AF239718 AtRdR6/SGS2.

Supplemental Data

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

Supplemental Figure S1. Characterization of *RdR3* transcription from *N. attenuata*.

Supplemental Figure S2. Southern analysis (A) of the two independently transformed irRdR3 lines; B shows the map of the irRdR3 transformation vector.

Supplemental Figure S3. Qualitative and quantitative smRNA profiles obtained using 454-sequencing.

Supplemental Figure S4. Changes in the smRNA transcriptome after *RdR3* silencing.

Supplemental Figure S5. Morphology of irRdR3 lines and wild-type plants competing in their natural habitat.

Supplemental Figure S6. No differences in growth and fitness were observed when plants from the same genotypes competed with each other.

Supplemental Figure S7. Phytohormone analysis in the field.

Supplemental Figure S8. Analysis of ABA levels in the field-grown wild-type and irRdR3 plants.

Supplemental Figure S9. Phytohormone analysis of plants grown in the glasshouse with competitors.

Supplemental Figure S10. The distribution rather than the biosynthesis of IAA is altered in irRdR3 plants competing with wild-type plants.

Supplemental Table S1. Annotations of smRNAs after BLASTing them against NR-DB at NCBI.

Supplemental Table S2. smRNAs identified as microRNAs (after matching them against Sanger DB).

Supplemental Table S3. Genes differentially regulated during competition, as revealed by microarray analysis.

ACKNOWLEDGMENTS

We thank E. Wheeler for editorial comments, P. Shahi and P. Srivastava for help with 454-sequence analysis and assistance in microarray analysis, W. Kroeber, S. Allmann, and S. Kutschbach for invaluable assistance with the microarray hybridization and scanning and plant transformation, Brigham Young University for use of their awesome field station, the Lytle Ranch Preserve, and APHIS for constructive regulatory oversight.

Received April 16, 2008; accepted May 5, 2008; published May 14, 2008.

LITERATURE CITED

Alleman M, Sidorenko L, McGinnis K, Seshadri V, Dorweiler JE, White J, Sikkink K, Chandler VL (2006) An RNA-dependent RNA polymerase is required for paramutation in maize. Nature **442:** 295–298

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiol **127:** 1449–1458

Baulcombe D (2004) RNA silencing in plants. Nature **431:** 356–363

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell **9:** 1573–1584

Bubner B, Gase K, Baldwin IT (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. BMC Biotechnol **4:** 14

Bubner B, Gase K, Berger B, Link D, Baldwin IT (2006) Occurrence of tetraploidy in *Nicotiana attenuata* plants after *Agrobacterium*-mediated transformation is genotype specific but independent of polysomaty of explant tissue. Plant Cell Rep **25:** 668–675

Chen R, Rosen E, Masson PH (1999) Gravitropism in higher plants. Plant Physiol **120:** 343–350

Clarke JD, Liu Y, Klessig DF, Dong X (1998) Uncoupling PR gene expression from NPR1 and bacterial resistance: characterization of the dominant *Arabidopsis* cpr6-1 mutant. Plant Cell **10:** 557–569

Cogoni C, Macino G (1999) Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature **399:** 166–169

Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for post-transcriptional gene silencing mediated by a transgene but not by a virus. Cell **101:** 543–553

Edlund A, Eklof S, Sundberg B, Moritz T, Sandberg G (1995) A microscale technique for gas-chromatography mass-spectrometry measurements of picogram amounts of indole-3-acetic-acid in plant-tissues. Plant Physiol **108:** 1043–1047

Galweiler L, Guan C, Muller A, Wisman E, Mendgen K, Yephremov A, Palme K (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science **282:** 2226–2230

Genoud T, Buchala AJ, Chua NH, Metraux JP (2002) Phytochrome signalling modulates the SA-perceptive pathway in *Arabidopsis.* Plant J **31:** 87–95

Giri AP, Wunsche H, Mitra S, Zavala JA, Muck A, Svatos A, Baldwin IT (2006) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata.* VII. Changes in the plant's proteomes. Plant Physiol **142:** 1621–1641

Halitschke R, Baldwin IT (2003) Antisense LOX expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata.* Plant J **36:** 794–807

Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata.* III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. Plant Physiol **125:** 711–717

Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J **22:** 4523–4533

Hoecker U, Toledo-Ortiz G, Bender J, Quail PH (2004) The photomorphogenesis-related mutant red1 is defective in CYP83B1, a red light-induced gene encoding a cytochrome P450 required for normal auxin homeostasis. Planta **219:** 195–200

Kang JH, Wang L, Giri A, Baldwin IT (2006) Silencing threonine deaminase and JAR4 in *Nicotiana attenuata* impairs jasmonic acid-Isoleucine-mediated defenses against *Manduca sexta.* Plant Cell **18:** 3303–3320

Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Carrington JC (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. PLoS Biol **5:** e57

Kruegel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. Chemoecology **12:** 177–183

Li H, Xu L, Wang H, Yuan Z, Cao X, Yang Z, Zhang D, Xu Y, Huang H (2005) The putative RNA-dependent RNA polymerase RDR6 acts synergistically with ASYMMETRIC LEAVES1 and 2 to repress BREVIPEDICELLUS and MicroRNA165/166 in *Arabidopsis* leaf development. Plant Cell **17:** 2157–2171

Li X, Clarke JD, Zhang Y, Dong X (2001) Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. Mol Plant Microbe Interact **14:** 1131–1139

Lu C, Kulkarni K, Souret FF, MuthuValliappan R, Tej SS, Poethig RS, Henderson IR, Jacobsen SE, Wang W, Green PJ, et al (2006) MicroRNAs and other small RNAs enriched in the *Arabidopsis* RNA-dependent RNA polymerase-2 mutant. Genome Res **16:** 1276–1288

Lynds GY, Baldwin IT (1998) Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata.* Oecologia **115:** 531–540

Makeyev EV, Bamford DH (2002) Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Mol Cell **10:** 1417–1427

McCloud ES, Baldwin IT (1997) Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris.* Planta **203:** 430–435

Molnar A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC (2007) miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii.* Nature **447:** 1126–1129

Morelli G, Ruberti I (2000) Shade avoidance responses: driving auxin along lateral routes. Plant Physiol **122:** 621–626

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, et al (2000) *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101:** 533–542

Pandey SP, Baldwin IT (2007) RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. Plant J **50:** 40–53

Pandey SP, Baldwin IT (2008) Silencing RNA-directed RNA polymerase 2 (RdR2) increases *Nicotiana attenuata*'s susceptibility to UV in the field and in the glasshouse. Plant J **54:** 845–862

Pandey SP, Shahi P, Gase K, Baldwin IT (2008) Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata.* Proc Natl Acad Sci USA **105:** 4559–4564

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore-induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. Plant J **51:** 579–591

Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis.* Genes Dev **18:** 2368–2379

Petersen BO, Albrechtsen M (2005) Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. Plant Mol Biol **58:** 575–583

Preston CA, Baldwin IT (1999) Positive and negative signals regulate germination in the post-fire annual, *Nicotiana attenuata.* Ecology **80:** 481–494

Qu F, Ye X, Hou G, Sato S, Clemente TE, Morris TJ (2005) RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana.* J Virol **79:** 15209–15217

Rayapuram C, Baldwin IT (2007) Increased SA in NPR1-silenced plants antagonizes JA and JA-dependent direct and indirect defenses in herbivore-attacked *Nicotiana attenuata* in nature. Plant J **52:** 700–715

Reinhardt D, Mandel T, Kuhlemeier C (2000) Auxin regulates the initiation and radial position of plant lateral organs. Plant Cell **12:** 507–518

Roda A, Halitschke R, Steppuhn A, Baldwin IT (2004) Individual variability in herbivore-specific elicitors from the plant's perspective. Mol Ecol **13:** 2421–2433

Schiebel W, Pelissier T, Riedel L, Thalmeir S, Schiebel R, Kempe D, Lottspeich F, Sanger HL, Wassenegger M (1998) Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell **10:** 2087–2101

Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT (2005) Specificity in ecological interactions: attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. Plant Physiol **138:** 1763–1773

Schwach F, Vaistij FE, Jones L, Baulcombe DC (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. Plant Physiol **138:** 1842–1852

Sijen T, Fleenor J, Simmer F, Thijssen KL, Parrish S, Timmons L, Plasterk RHA, Fire A (2001) On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107:** 465–476

Steindler C, Matteucci A, Sessa G, Weimar T, Ohgishi M, Aoyama T, Morelli G, Ruberti I (1999) Shade avoidance responses are mediated by the ATHB-2 HD-zip protein, a negative regulator of gene expression. Development **126:** 4235–4245

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PLoS Biol **2:** 1074–1080

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nat Rev Mol Cell Biol **7:** 847–859

Tiwari S, Ramachandran S, Bhattacharya A, Bhattacharya S, Ramaswamy R (1997) Prediction of probable genes by Fourier analysis of genomic sequences. Comput Appl Biosci **13:** 263–270

Van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in Nicotiana attenuata. J Chem Ecol **27:** 547–568

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. Curr Opin Plant Biol **5:** 444–451

Wang D, Pajerowska-Mukhtar K, Culler AH, Dong X (2007) Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr Biol **17:** 1784–1790

Wassenegger M, Krczal G (2006) Nomenclature and functions of RNA-directed RNA polymerases. Trends Plant Sci **11:** 142–151

Wu J, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly

activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata.* Plant Cell **19:** 1096–1122

Xie ZX, Fan BF, Chen CH, Chen ZX (2001) An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. Proc Natl Acad Sci USA **98:** 6516–6521

Xie ZX, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol **2:** 642–652

Yang SJ, Carter SA, Cole AB, Cheng NH, Nelson RS (2004) A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana.* Proc Natl Acad Sci USA **101:** 6297–6302

Yu DQ, Fan BF, MacFarlane SA, Chen ZX (2003) Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense. Mol Plant Microbe Interact **16:** 206–216

Zavala JA, Patankar AG, Gase K, Baldwin IT (2004) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata.* Proc Natl Acad Sci USA **101:** 1607–1612