The role of small-RNAs in regulating stress-induced responses in *Nicotiana attenuata*

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1. Introduction

Plants, which are mostly autotrophic, appear to be sessile. However, within the plants are numerous pathways running to give them a stabilized appearance in nature. These pathways allow the plants to develop, grow, reproduce, disperse, adapt and evolve. On the other hand, cast-iron presence in the space puts plants under tremendous pressure from the environment. Environmental heterogeneity is the most important selective force of nature. Biotic and abiotic agents of environment constantly test plants: abiotic agents include limited water and soil resources, salinity, cold, high temperatures, wind, mechanical damage, etc.; biotic stresses include invading pathogens (viruses, bacteria, fungi), nematodes, herbivores (e.g. insects, mammals), etc. Such abiotic and biotic stresses require the plants to make maxium use of the available resources, and to design strategies for tolerance, defense and escape. Whereas pathogens colonize and spread across plant parts, damage by herbivores removes appreciable plant area and biomass. To check the loss of photosynthetic area, plants need to evolve defense strategies; to compensate for the loss of photosynthetic area, they need regeneration strategies.

In order to respond and adapt to their dynamic environments, plants derive “cues” (or detectable information) from their abiotic and biotic surrounding. In abiotic environments, these cues include changes in ionic strength, disruption of membranes, etc. (Bray, 1997; Braam, 2005) , whereas in biotic environments, cues include chemical compounds, wounding patterns, and volatile organic compounds emitted by neighboring plants, etc., (Alborn et al., 1997; Schmelz et al., 2006; Gershenzon, 2007). In their natural habitats (and also elsewhere), plants are constantly exposed to such cues. This exposure presents plants with challenges: which changes in their environment should they respond to? More important, how should they decide if a change is “a stress”?

1.1 Stress and Stress responses

Stress and Stress responses: conquering to adapt and adapting to conquer

Biological stress has been defined as any change in environment that might reduce or adversely change a plant’s growth or development; biological strain refers to reduced or changed function (Levitt, 1972, 1980). In their natural habitats, plants respond to a plethora of

stresses that impose high fitness costs. These responses involve the perception, processing, and integration of external information into the cellular and physiological machinery. These responses may be (i) a strategy to defend and/or to adapt to the changes in environment or (ii) an outcome of an adaptation strategy evolved over time due to the presence of a persistent stress across generations in the eco-physiological niche of the organism. As an outcome of first the responses are more induced, and the second, more of constitutive nature.

Phytohormones are important compounds having multiple functions: they act as signals, integrate different cellular processes, and sometimes regulate the biosynthesis of other phytohormones. These chemical compounds amalgamate the externally perceived information into regulatory processes such as stimulated or suppressed growth, apoptosis, immune responses, metabolism, and reproduction. For a chemical compound to be a phytohormone signal, it should (1) be synthesized or secreted, (2) be transported and perceived at the receiver location, (3) activate an enzymatic reaction or process, and (4) be metabolized or excluded from the site of action, so as to terminate the response at the end. Phytohormones traditionally described as growth regulators are abscisic acid, auxins, cytokinins, ethylene, and gibberellins. Phytohormones associated with plants' defenses responses, especially to herbivores and pathogens, are jasmonic acid and salicylic acid. Figuring out how these phytohormones act in regulatory circuits and, further, how plant phenotypes evolve, are challenges plant researchers face.

1.2 Phenotypic plasticity

Phenotypic plasticity as the driving force in circumventing stress

The ability of an individual to alter its physiology, morphology, and/or behavior in response to a change in environment is called phenotypic plasticity. Phenotypic plasticity can be defined simply as environmentally dependent phenotypic expression. Plants have the capacity to respond differently to different environments (Figure 1), in a manner appropriate to their specific needs, matching the functional specificity to environment response (adaptive plasticity), which separates these responses from the inevitable effects of responses to limited resources and other suboptimal environments (Sultan, 2000 and 2003).

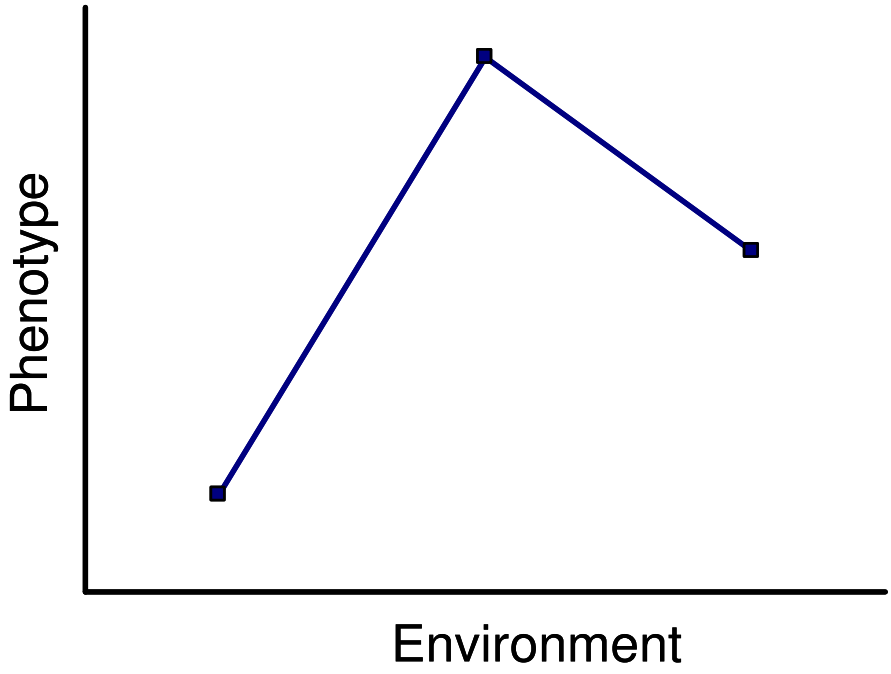

Figure 1. Variation of expression of a genotype as a function of organism's (external and internal) environment. Plants' genetic makeups allow them to respond to different environments differently to match their specific functional needs.

Adaptive plasticity allows a genotype to grow successfully in different environments, to play a major role in ecological distribution, and to maintain the evolutionary variability. Adaptive plasticity forms an important aspect of phenotypic variability, contributing to the performance of organisms in different environments. A genotype can change its chemistry, physiology, development, morphology, and even behavior in response to environmental cues to adapt and thus evolve. Therefore, it may be concluded that phenotypic plasticity helps organisms maximize their fitness in variable environments.

As phenotypic plasticity involves variation in expression of traits as a function of differences in environments (Figure 1), the traits in question should be separated from the plasticity of the trait itself. A genetic basis exists for plasticity of particular traits, as does the genetic basis for the traits themselves. In other words, different genes control different processes so that the trait and its plasticity may evolve independently. *Nicotiana attenuata*, a native of southwestern United States, provides an ideal model system for studying phenotypic plasticity, its genetic basis, and the genetic basis of regulation of the traits providing such plasticity.

1.3 *Nicotiana attenuata*

Nicotiana attenuata: phenotypic plasticity allows plants to adapt to highly variable and stressful environments

Nicotiana attenuata is a diploid, largely selfing tobacco species native to southwestern United States. *N. attenuata*'s eco-physiological strategies, which are highly plastic in nature, have probably facilitated its adaptation to natural habitats. *N. attenuata* grows at altitudes of 200-3000m in ambient UV-B environments. The habitat selection of *N. attenuata* is largely determined by its peculiar germination behavior. Seeds from a long-lived seed bank germinate in post-fire environments (Figure 2) by responding to germination stimulants in wood-smoke (Baldwin et al., 1994).

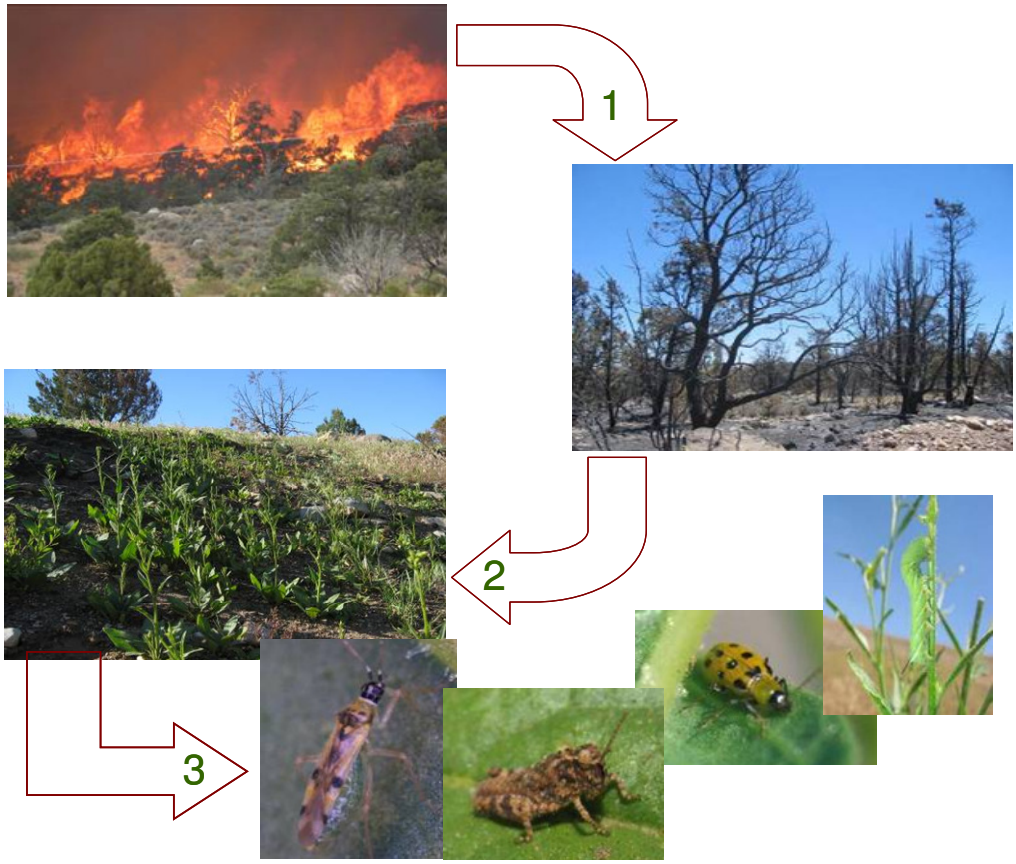

Figure 2. *N. attenuata* acts as pioneering species in natural habitats in post-fire environments. (1) In post-fire environments, *N. attenuata* seeds germinate in patches with high resources, especially nitrogen (N). This may lead to development of monocultures (2). This is followed by establishment of an unpredictable herbivore community (3), which has to re-colonize itself with every new population of *N. attenuata* after fires.

In post-fire burns with high nitrogen (N) content in the soil, these plants act as pioneering species and often result in monocultures. With highly synchronized germination behavior and living in a habitat with fast rising temperatures and resources being lastly depleted, *N. attenuata* plants have been selected for rapid growth. An unpredictable herbivore community follows the germination and growth of *N. attenuata* in post-fire environments. Herbivores from more than 20 different texa attack these plants, and specific herbivore populations vary from year to year because they also recolonize the habitat after fire. The representation of a genotype of *N. attenauta* in the seed banks is determined by how successfully a particular genotype alters its phenotype to respond to these highly variable biotic selection regimes, translating vegetative growth into reproductive output, i.e. seed (Baldwin, 2001). Therefore, *N. attenuata* caters to the needs of an ideal model system for understanding the genetic basis of regulation of phenotypic plasticity. Such an understanding must underlie any understanding of the ecological sophistications of plant adaptation, defense, and evolution.

1.4 The world of small-RNAs

The world of small-RNAs: exploring possibilities of their involvement in regulating traits of phenotypic plasticity in N. attenuata

When plants are stressed, they rearrange their metabolome to produce defense responses. Depending upon the nature of the stress, these may be constitutive or inducible, direct or indirect, for escape or tolerance, or (in most cases) a combination. At the same time, many responses are tailored to co-opt different stresses. Such metabolomic rearrangements are probably preceded by large-scale transcriptional rearrangements. How these responses are regulated remains poorly understood. Chemicals that have appeared on the forefront of regulatory molecules are phytohormones (described above). Looking at the magnitude of defense responses (e.g. during herbivory, the transcripts of hundreds to thousand genes are rearranged, including those of phytohormone signaling), it does not seem likely that responses are regulated only by phytohormones. Moreover, how the transcription of phytohormone biosynthesis and signaling is regulated remains poorly known.

Molecular regulators should fulfill some basic properties: (a) they should be essential and, in the case of inducible, direct and indirect defense responses, rapidly elicited; (b) they

should be amplifiable and also capable of systemic transport, an ability which is essential for executing induced defense responses, as well as tolerance and escape responses; (c) their action should not be masked by the environment; and (d) most importantly, they should be able to co-ordinate different pathways, because in nature, plants encounter many stresses that act in parallel. Plants need to respond with a defense which is both effective as well as less costly. Small, regulatory RNAs seem to fulfill these requirements.

Small-RNAs have appeared as important players on regulatory scene. These 18-24 nucleotide (nt) long regulatory molecules regulate gene expression post-transcriptionally (Voinnet, 2002) in a process often called RNA interference (RNAi) or post-transcriptional gene-silencing (PTGS). Small-RNAs target transcripts by pairing their complementary sequence to the transcripts. Depending on how they are generated, they can be broadly classified as micro-(mi) RNAs or small-interfering-(si) RNAs. The siRNAs may be classified into primary siRNA, secondary siRNAs, trans-acting-siRNAs and natural antisense derived siRNAs [Table 1; (Chapman and Carrington, 2006)]. In animals another type of smRNAs, piRNAs are also found.

Table 1. Origin and functions of different classes of eukaryotic smRNAs

smRNA class	Origin and function
microRNA (miRNA)	Processed from fold-back miRNA-gene transcripts, and regulates transcript accumulation post-transcriptionally
Primary small-interfering (si) RNAs	Processed from dsRNAs, binds complementary target RNAs, guide for RdR-dependent generation of secondary siRNAs
Secondary siRNAs	After processing of RdR-derived dsRNAs, post-ranscriptional regulation of transcripts, heterochromatin formation
Trans-acting siRNAs (tasiRNA)	Conversion of TAS gene transcripts into dsRNAs in miRNA- and RdR-dependent manner, post-transcriptional regulation of transcripts
Natural antisense derived siRNAs (natsiRNAs)	Arising from sense-antisense transcript pairs, involved in pathogen defense and stress responses
Piwi-interacting RNA (piRNA)	Transposon and reroelement suppression in animals

In general, miRNAs are transcribed in the nucleus as primary transcripts in a RNA-polemerase II dependent manner; they are then transported into the cytoplasm as folded, stem-loop structures, from which mature miRNAs are generated (He and Hannon, 2004). The siRNAs are synthesized in the cytoplasm (He and Hannon, 2004). siRNAs are also generated from double-stranded (ds) RNAs, dsRNAs are generated by a special class of RNA-polymerases, RNA-directed (or dependent)-RNA polymerases (RdRs or RdRps). Mechanistically, both mi- and si- RNAs act similarly: the preferred mode of action for siRNAs is transcriptional cleavage, for miRNAs, translational inhabitation. In plants, miRNAs often act as siRNAs, performing transcriptional cleavage (Tang et al., 2003).

miRNAs have established themselves as a new layer of regulators, especially in development, where large-scale transcriptional changes have to be timed to match different developmental events. Similarly siRNAs have been established as main defenses against viruses. In the current investigation, we adopt a holistic approach to the study of these regulatory small-RNAs in different ecological processes requiring substantial phenotypic, metabolomic, and transcriptional plasticity. Here, the role of small-RNAs (mainly the siRNAs) is explored, with respect to the traits that provide phenotypic plasticity for three main stresses *N. attenuata* encounters in its natural habitat: (a) herbivores; (b) ambient UV-B levels; and (c) high intra-species competition. In the first two manuscripts, the role of small-RNAs in plant defense against herbivores is described, and in the third and fourth manuscripts, the role of small-RNAs in plant adaptation to ambient UV-B levels and high intra-species competition, respectively, is described.

Manuscript Overview

Manuscript I

RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature

Shree P. Pandey, Ian T. Baldwin

The Plant Journal (2007) 50 (1), 40–53

In this manuscript we explore the possibility if the small-RNAs may mediate plant defense responses after herbivore attack. We silenced the expression of three RNA-directed RNA polymerases (RdRs) by virus-induced gene silencing in *Nicotiana attenuata* and determined the RdR (RdR1) which is responsible for herbivore resistance in plants. Stably transformed plants, silenced for RdR1 expression (irRdR1) showed high susceptibility to herbivores in glasshouse as well as nature. irRdR1 plants were reduced in elicited nicotine levels, due to reduced transcription of nicotine biosynthesis genes.

Ian T. Baldwin had the idea; I designed all the experiments, conducted them, and analyzed the data. Ian T. Baldwin generated resources for field release and was also involved in field experiments. I and Ian T. Baldwin wrote the manuscript.

Manuscript II

Herbivory-induced changes in the Small-RNA Transcriptome and Phytohormone Signaling in *Nicotiana attenuata*

Shree P. Pandey, Priyanka Shahi, Klaus Gase and Ian T. Baldwin

Proceeding of National Academy of Sciences (2008) In Press

This manuscript describes how small-RNA populations change after herbivory in WT and *RdR1*-silenced plants. We elucidate the smRNA transcriptome of *N. attenuata* with 454-sequencing and smRNAs are annotated with respect to available information in the public non-redundant nucleotide and miRNA databases. Conserved miRNAs are identified, and targets of these smRNAs in phytohormone signaling related genes are predicted. Herbivory induced transcript analyses, using quantitative real-time PCR in time course manner, are made and phytohormone levels are measured in WT and irRdR1 plants. Finally we show that the *RdR1*-silenced plants had WT levels of plant growth, but were susceptible to herbivores partly due to reduced jasmonic acid.

Under the supervision of Ian T. Baldwin, I designed all the experiments, I and Klaus Gase were involved in 454-sequencing, and I and Priyanka Shahi were involved in analyzing the 454-sequence data. I carried out transcript and phytohormone measurements and bioassays and analyzed the data. I and Ian T. Baldwin wrote the manuscripts. All the authors read the manuscript and provided their suggestions.

Manuscript III

Silencing RNA-directed RNA polymerase 2 (RdR2) increases *Nicotiana attenuata*'s susceptibility to UV in the field and in the glasshouse

Shree P. Pandey, Ian T. Baldwin

The Plant Journal (2008) In Press

In this manuscript we identified a previously unknown function of RNA-directed RNA polymerase 2 (RdR2). Transcripts of RdR2 accumulate fast after herbivore attack, but silencing its expression does not affect herbivore performance. When RdR2-silenced plants (irRdR2) were released in nature, their growth was significantly reduced when compared to wild type plants. irRdR2 plants had reduced accumulation of plant phenolics, which act as sunscreens in ambient UV-B environments. This indicated that RdR2 may be involved in protection from UV-B, which was confirmed in glasshouse experiments. Silencing RdR2 reduces the small-RNA populations, as well as generated new ones. RdR2-dependint smRNAs may be involved in all the three processes of UV-B protection and repair, as was evident in our analysis of transcripts, proteins and metabolites.

Ian T. Baldwin suggested conducting loss of function analysis of RdRs in nature; I designed all the experiments, conducted them, and analyzed the data. Ian T. Baldwin generated resources for field release and was also involved in field experiments. I and Ian T. Baldwin wrote the manuscript.

Manuscript IV

Functional characterization of RNA-directed RNA polymerase (RdR) 3 from *Nicotiana attenuata*

Shree P. Pandey, Emmanuel Gaquerel, Klaus Gase and Ian T. Baldwin

Plant Physiology (2008) In Review

In this manuscript we identified a previously unknown function of RNA-directed RNA polymerase 3 (RdR3) in *Nicotiana attenuata*. The homolog of this gene (RdR6) in other species is involved in virus resistance and post-transcriptional gene silencing. Viruses are not a natural threat in *N. attenuata*. To determine the eco-physiological role of this gene, we introduced the plants silenced in RdR3 expression (irRdR3) in the natural habitats in south-western United States. No viral symptoms were recorded on irRdR3 plants or wild type (WT) plants. Also, the irRdR3 plants and WT levels of plant defense against insect herbivores, but were severely reduced in their growth. Glasshouse studies showed that irRdR3 plants were severely reduced in their ability to compete with WT neighbors, therefore were severely reduced in growth as well as reproductive output when competed with WT plants which could be attributed to altered auxin transport.

Ian T. Baldwin had the idea of field experiments with RdR3-silenced plants, I designed all the experiments, I and Klaus Gase isolated the gene, I and Emmanuel Gaquerel conducted field experiments, Emmanuel Gaquerel developed method for auxin analysis, I screened the transgenic lines, characterized them, conduced all the competition experiments and other assays, Klaus Gase provided the silencing construct and Ian T. Baldwin generated resources for field release. I and Ian T. Baldwin wrote the manuscript, all authors gave read the manuscript and gave their comments.

2. Manuscripts

2.1 Manuscript I

The Plant Journal (2007) 50 (1), 40–53

RNA-directed RNA Polymerase 1 (RdR1) Mediates *Nicotiana attenuata's* Resistance to Herbivore Attack in Nature

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Running title: RdR1's involvement in defense against insects

Keywords: herbivore resistance, *N. attenuata,* RNA-directed RNA polymerase, transcriptional regulation, RdR1, nicotine biosynthesis

Summary

Small-RNAs are important regulators of plant development and resistance against viruses. To determine if small-RNAs mediate defense responses to herbivore attack, we silenced the expression of three RNA-directed RNA-polymerases (*RdRs*) in the native tobacco *Nicotiana attenuata* by virus-induced gene silencing. Larvae of the leaf-chewing solanaceous specialist *Manduca sexta* grew faster on the *RdR1*-silenced plants than on empty vector (EV) controls; silencing *RdR3* and *2* had little to no effect on larval performance. *NaRdR1* transcripts were strongly elicited when puncture wounds were treated with *M. sexta* oral secretions (OS) to simulate herbivore attack, and with SA and JA, phytohormones elicited by herbivore attack. Stably silencing *RdR1* by transforming *N. attenuata* with an inverted-repeat *RdR1* construct produced plants (irRdR1) that grew normally but were highly susceptible to both *M. sexta* larvae and the cell-content feeder *Tupiocoris notatus.* When irRdR1 lines were planted into *N. attenuata's* native habitat in the Great Basin Desert, they were highly susceptible to herbivore attack, due to deficiencies in direct rather than indirect defenses. Microarray analysis revealed the down-regulation of *ADC* and *ODC* genes, which supply substrates for synthesizing the chemical defense, nicotine, which irRdR1 lines failed to accumulate after attack. We conclude that *RdR1* mediates herbivore resistance and infer that the small-RNAs produced by RdR1 are likely involved in orchestrating some of the rapid metabolic adjustments required for plants to survive herbivore attack in their natural habitats. The experiment highlights the value of carrying out real-world tests of gene function early in the discovery process.

Introduction

Attack from herbivorous insects elicits a large-scale reconfiguration of plant metabolism (Walling, 2000; Kessler and Baldwin, 2004). Because insects are highly mobile, the responses spread rapidly throughout a plant and are frequently elicited by herbivore-specific elicitors introduced into wounds during feeding (Korth, 2003; Voelckel and Baldwin, 2004b). The responses include the production and activation of direct defenses, such as toxins, digestibility reducers, and anti-feedants that directly protect plants (Duffey and Stout, 1996), as well as indirect defenses that recruit natural enemies from the plant's surroundings to attack feeding herbivores (Turlings and Tumlinson, 1992; De Moraes *et al.*, 1998; Kessler and Baldwin, 2001). A large-scale, transcriptional response, which precedes and presumably

mediates many of the induced defense responses, is also elicited by herbivore attack. For instance, in *Populus*, 1,728 genes are differentially regulated after attack from forest tent caterpillars, *Malacosoma disstria* (Ralph *et al.*, 2006). On the other hand, in *Arabidopsis*, only 114 genes are differentially regulated after attack from *Pieris rapae* larvae (Reymond *et al.*, 2004). Although this transcriptional response did not differ substantially among plants attacked by lepidopteran larvae that are known to be *Brassica* specialists and generalists, the transcriptional responses to herbivore attack in the *Solanaceous* taxa is known to be highly herbivore-specific (Voelckel and Baldwin, 2004b; 2004a; Voelckel *et al.*, 2004) and species-specific (Qu *et al.*, 2004; Schmidt *et al.*, 2005). The transcriptional responses, such as the defense responses, are known to spread from the attack site throughout the plant rapidly and systemically following vascular connections between attacked and unattacked plant parts (van Dam *et al.*, 2001; Schittko and Baldwin, 2003). How these large and rapidly dispersed transcriptional responses to herbivore attack are coordinated remains unknown, except that phytohormone signaling, in particular oxylipin signaling, is involved (Howe *et al.*, 1996; Ryan and Pearce, 2003; Halitschke *et al.*, 2004; Reymond *et al.*, 2004). Both the speed and extent of the responses suggest that transcriptional regulation by the movement of proteins or phytohormones may not be the only mechanism.

RNA silencing is emerging as a fundamental regulatory process affecting many layers of endogenous gene expression (Voinnet, 2002); non-coding small-RNAs appear to be important regulators of gene expression in both plants and animals (Bartel, 2004; Mallory and Vaucheret, 2004). RNA silencing has been shown to be essential for plant development and differentiation processes, such as embryonic and vegetative organ formation, leaf morphogenesis, and flower development (Llave *et al.*, 2002; Emery *et al.*, 2003; Palatnik *et al.*, 2003; Chen, 2004; Mallory *et al.*, 2004). Small-RNAs are now regarded as key elements that, depending on their source, on the RNA, and on the nature of the interaction with the target nucleic acid, trigger chromosomal modifications (Mette *et al.*, 2000; Aufsatz *et al.*, 2002; Martienssen, 2003), post-transcriptional gene silencing (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Yu and Kumar, 2003), and a translational blockade. Of the small-RNAs, a large class of 18-26-nucleotide-long RNAs (small-interfering (si)RNAs and micro (mi)RNAs) is involved in defense against viruses; in post-transcriptional gene silencing; and in regulating developmental genes through mRNA degradation or translational repression (Pickford andCogoni, 2003; Bartel, 2004). The role of endogenous RNA interference in the mediation of

responses to herbivore attack remains unexplored. Moreover, none of the small-RNA mediated phenotypes have been examined in organisms living in real-world settings. Given that components of the endogenous RNAi system are sensitive to abiotic stresses (Borsani *et al.*, 2005), it's not clear how consistently the responses will be expressed in organisms growing in complex environments.

All of the RNA-silencing pathways involve cleaving double-stranded (ds) RNA into short 21-26-nucleotide RNAs (Baulcombe, 2004). The dsRNA molecules are produced by the RNA-directed RNA polymerases (RdRps) (Pickford and Cogoni, 2003); or RdRs according to the new nomenclature (Wassenegger and Krczal, 2006) in *C. elegans* (Sijen *et al.*, 2001), fungi (Cogoni and Macino, 1999), and plants (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000). So far these RdRs have not been reported in humans and higher animals, suggesting that they mediate mechanisms important for genetic regulation. Since plant miRNAs are similar to siRNAs, miRNAs may be serving as primers that allow RdRs to generate dsRNA (Tang *et al.*, 2003); but see (Petersen and Albrechtsen, 2005). Alternatively, miRNAs (along with RdRs) may be involved in additional processes (Allen *et al.*, 2005): miRNAs may mediate the formation of pre-siRNAs; using these pre-siRNAs, RdR may then form dsRNAs; and after degradation, the remaining strand of dsRNA, siRNA, regulates the mRNA targets (Allen *et al.*, 2005).

Three functionally distinct *RdRs* have been reported in *Arabidopsis,* tomato, and *Nicotiana* (Schiebel *et al.*, 1998; Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Yang *et al.*, 2004). In separate but overlapping processes (Pickford and Cogoni, 2003), *RdR1* and *6* (the *SDE1*/*SGS2* and their natural variants) are thought to be involved in virus resistance and post-transcriptional gene-silencing mechanisms; however, the role of the third RdR (*RdR2*) is not understood (reviewed in (Wassenegger and Krczal, 2006). The transitivity of the RNA-silencing signal depends on the activity of the RdRs (Himber *et al.*, 2003). The *RdR6* homolog is required for the cell to perceive the silencing signal but not to produce or transport it (Schwach *et al.*, 2005). *RdR1* and *6* are elicited by salicylic acid (SA) treatment in tobacco and *Arabidopsis*, but these responses appear to be confined specifically to viral defense (Yu *et al.*, 2003; Yang *et al.*, 2004). There is clearly much more to be learned about the role that RdRs play in RNA silencing, and silencing the expression of these key enzymes in order to examine the phenotypes of RdR-silenced plants represents a valuable means of uncovering the function of RdRs.

Here we explore the role of RdRs in mediating defense responses to herbivore attack in a model ecological expression system whose defense responses are well characterized. *Nicotiana attenuata* is a native of the southwestern United States and grows in the immediate post-fire environment by timing seed germination from a long-lived seed bank by responding to pyrolysis products found in wood smoke (Preston and Baldwin, 1999). Because the plant "chases" the ephemeral post-fire environment in time, the herbivore community is forced to re-establish itself with each new plant population (Baldwin, 2001). The resulting unpredictability of the composition of herbivore communities has likely selected for the plant's vast array of inducible defenses. These allow the plant to shape its resistance traits according to the herbivore community that attacks it at a given location (Kessler and Baldwin, 2004; Voelckel and Baldwin, 2004a). How the plant tailors its defense responses is best understood in the case of attack from the larvae of the specialist lepidopteran herbivore *Manduca sexta,* the species that regularly accounts for the majority of the leaf area lost to herbivores in native populations (Kessler and Baldwin, 2002).

Attack from this larvae results in the differential regulation of about 500 *N. attenuata* genes, which can be crudely classified into functional categories such as photosynthesis, electron transport, primary metabolism, signaling, cytoskeleton, secondary metabolism, DNA-binding proteins, stress responsive factors, etc. (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003; Heidel and Baldwin, 2004; Schmidt *et al.*, 2005). The large-scale, rapid, herbivore-specific transcriptional responses can be elicited by applying *M. sexta* oral secretions (OS) to puncture wounds (Halitschke *et al.*, 2001; Roda *et al.*, 2004). Eight fatty acid-amino acid conjugates present in *M. sexta* OS are necessary and sufficient to elicit the response (Halitschke *et al.*, 2001; Roda *et al.*, 2004), which in turn requires jasmonate (JA) signaling. The importance of JA signaling is apparent from the highly attenuated response in plants transformed to silence the specific lypoxygenase (NaLox3) that supplies hydroperoxide substrates for JA biosynthesis (Halitschke and Baldwin, 2003). JA signaling transcriptionally elicits a number of potent direct defenses including the neurotoxin nicotine (Winz and Baldwin, 2001). Once its genes for nicotine biosynthesis have been silenced, *N. attenuata* is highly vulnerable to herbivores in nature (Steppuhn *et al.*, 2004). Yet how these rapidly activated responses are regulated at molecular levels remains unknown.

Here we explore the involvement of RNA-interference in regulating herbivore-induced plant-defense responses by independently silencing the expression of the three RdRs present in

the *N. attenuata* genome. Our study highlights the value of testing the functional significance of a gene under real-world circumstances at an early stage in the discovery process. In the study of traits mediating pathogen resistance, tests of the real-world significance are not usually conducted until most of the mechanistic details mediating the response are understood. Using an herbivore susceptibility screen with virus-induced gene silenced (VIGS) plants in glasshouse experiments, we identified an *RdR* (*RdR1*) which, when silenced, increased the susceptibility of plants to *M. sexta* attack. Plants were produced that were stably silenced in *RdR1* expression by *Agrobacterium*-mediated transformation and susceptible to *M. sexta* attack as well as to attack from a cell-content feeding herbivore, *Tupiochoris notatus*. The stably silenced plants were planted into *N. attenuata*'s native habitat and further characterized.

Results

***Isolation of* RdR1 *from* N. attenuata**

We used a PCR-based approach with *N. attenuata* DNA and isolated the complete coding region of *RdR1*. NaRdR1 has high sequence similarity (>90%) with its corresponding homologue from *N. tabacum* and *N. benthamiana* but no similarity with the other RdRs from closely related species. Screening a cDNA library prepared from *N. attenuata* leaves after 24h of continuous *M. sexta* attack did not yield any positive clones, indicating *RdR1* is expressed at very low levels (Yang *et al.*, 2004). The probes used in the screening were obtained by PCR amplification of 434 bp fragments of RdR1 from *N. attenuata* genomic DNA. Similarly, partial *NaRdR2* and *3* sequences were obtained by PCR amplification of 350, and 353 bp fragments from genomic DNA of *N. attenuata*. The primer sequences are given in the Methods section. The *RdR1* and partial *RdR2* and *3* sequences from *N. attenuata* were aligned with the other characterized *RdRs* from closely related solanaceous species, namely, *N. benthamiana*, *N. tabacum*, and *Lycopersicon esculentum*, and the six putative RdRs from *Arabidopsis,* and a phylogenetic tree was prepared (Figure 1).

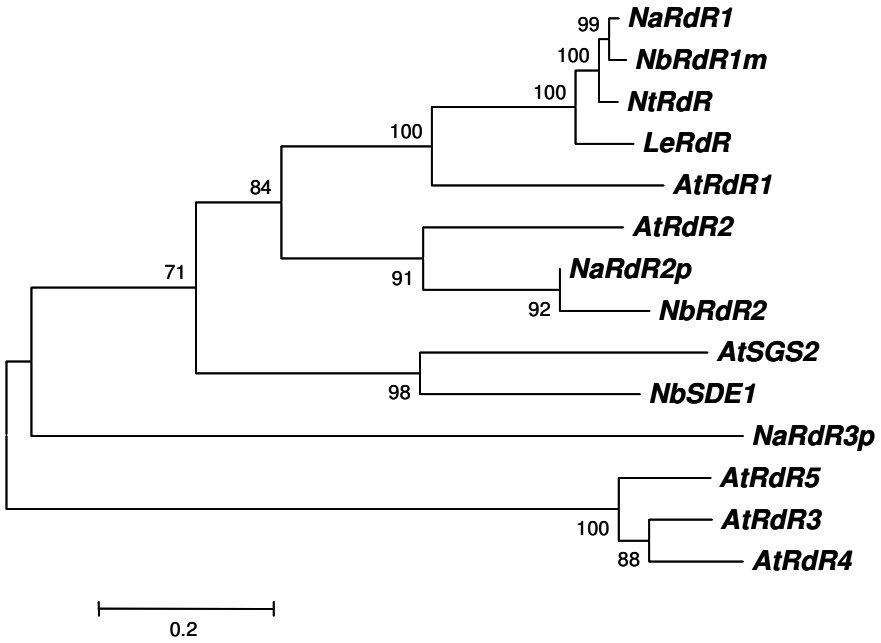


Figure 1. Phylogenetic analysis of *N. attenuata* RdR1. The full-length NaRdR1 and partial NaRdR2 and 3 sequences were aligned with sequences of 6 RdRs from *Arabidopsis* (AtRdR1, 2, 3, 4, 5 and AtSGS2), three RdRs from *N. benthamiana, N. tabacum* and *L. esculentum* RdRs. Calculated distance values are according to Neighbor Joining method with 1,000 bootstrap replicates.

Virus-Induced Gene Silencing (VIGS) of RdRs *and* M. sexta *performance*

In order to study the role of the three *RdRs* in defense against herbivores, we silenced the expression the three *RdRs* independently with a VIGS system optimized for *N. attenuata* (Saedler and Baldwin, 2004), in which unique fragments of the three *RdRs* (Figure S1) were expressed in a TRV vector and *Agro*-inoculated into the plants (Ratcliff *et al.*, 2001). We monitored the progress of silencing by observing the bleaching in plants inoculated with pTVPDS constructs containing a 206 bp fragment of the *N. benthamiana* phytoene desaturase gene (Figure S2). The first bleaching symptoms appeared 9d after inoculation, and all five replicate plants showed bleaching symptoms at 11d. A qRT-PCR analysis revealed that all three *RdRs* were silenced (Figure 3).

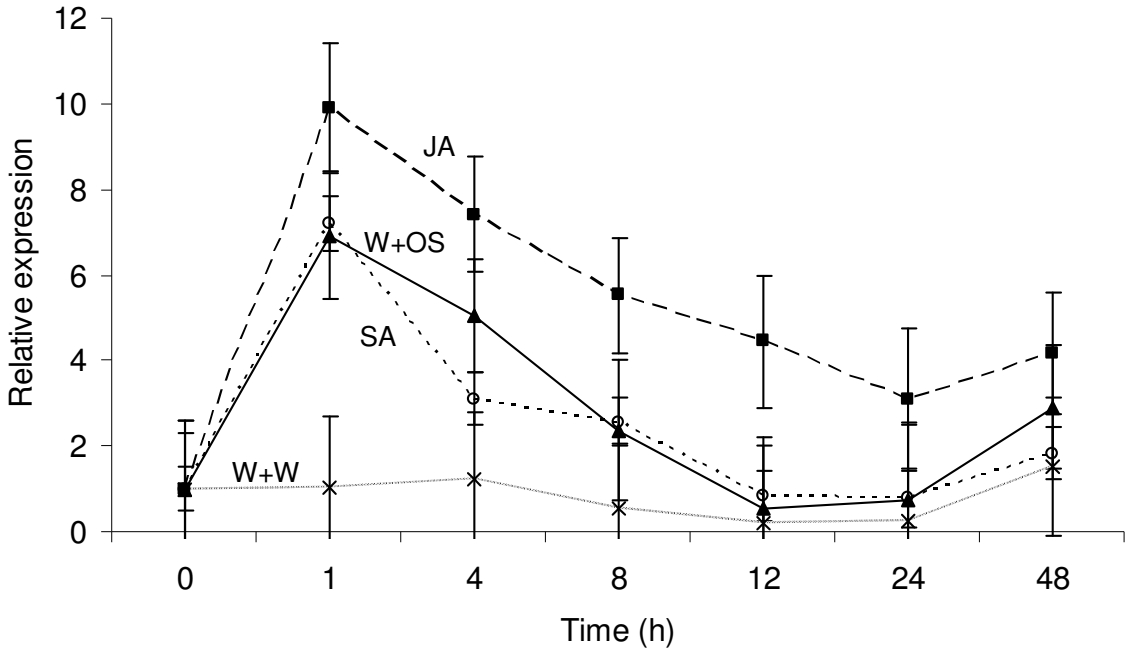


Figure 2. Characterization of RdR1 from *N. attenuata*. Time course analysis of RdR1 transcript induction with different elicitors. High accumulation of induced NaRdR1 was recorded within 1-4h of elicitation with 1mM JA (broken line, solid squares), 2mM SA (broken lines, open circles), and simulated herbivory (wounding with fabric pattern wheel and immediately treated with 20µl *M. sexta* OS; solid lines, solid triangles), but not after only mechanical wounding (broken line with cross). At time 0, plants were induced with different elicitors and induced NaRdR1 levels were compared to constitutive levels at the time of elicitation.

In RdR1-silenced plants, *RdR1* transcript levels were reduced by more than 50%, while levels of *RdR2* and *3* were unaffected. In RdR2-silenced plants, *RdR2* transcript levels were reduced by 81%, while RdR1 transcript levels were unchanged and *RdR3* transcripts increased by 58%. When transcripts of *RdR3* were silenced, levels were reduced by 64%, while *RdR2* transcript levels were unaltered and *RdR1* levels were reduced by 28%. Thus, quantitatively, the RdR1-silencing was completely specific, effecting no change in non-target *RdRs* and largely specific for *RdR2* and *3*, with only minor changes in the non-target *RdRs*. Just as there were no apparent abnormalities in plant morphology, there were no any significant variations in plant height compared to empty vector (EV) control plants even 30 days after inoculation with the silencing constructs (Figure S2; ANOVA, $F_{3,93} = 2.628$; P= 0.0549).

To examine the roles of *RdRs* in mediating direct defenses against *M. sexta* attack, we measured larval growth on the three RdR-silenced lines as well as on the EV control plants in an experiment which included 20 replicate plants per line (Figure 3). After 12 days of continuous feeding, the larvae growing on the RdR1-silenced plants gained more than twice as

much as those growing on EV control plants (ANOVA, $F_{1,38}$ = 17.28; P=0.0002), and those growing on RdR3-silenced lines gained 1.5 times as much as those growing on EV control plants (ANOVA, $F_{1,38}$=10.461; P=0.0025). Larval performance on the RdR2-silenced plants compared to EV controls did not differ significantly (ANOVA, $F_{1,38}$=3.685; P=0.0625). Since the TRV-RdR3 construct also attenuated the accumulation of RdR1 transcripts (by 28%), the significant increase in larval performance could be due to the silencing of *RdR1* rather than *RdR3*.

RdR1-expression patterns

Because *M. sexta* larvae performed best on RdR1-silenced plants, we characterized *RdR1* transcript accumulation in WT plants by qRT-PCR (Figure 2, Figure S3). Enhanced *RdR1* levels were found when the WT plants were subjected to various elicitors. Elicitation with jasmonic acid (JA), salicylic acid (SA), and OS increased *RdR1* transcript accumulation up to ten-fold within 1h. But no change in transcript levels was recorded when plants were only wounded (Figure 2). This suggests that the *RdR1* is involved in direct plant defense against herbivores.

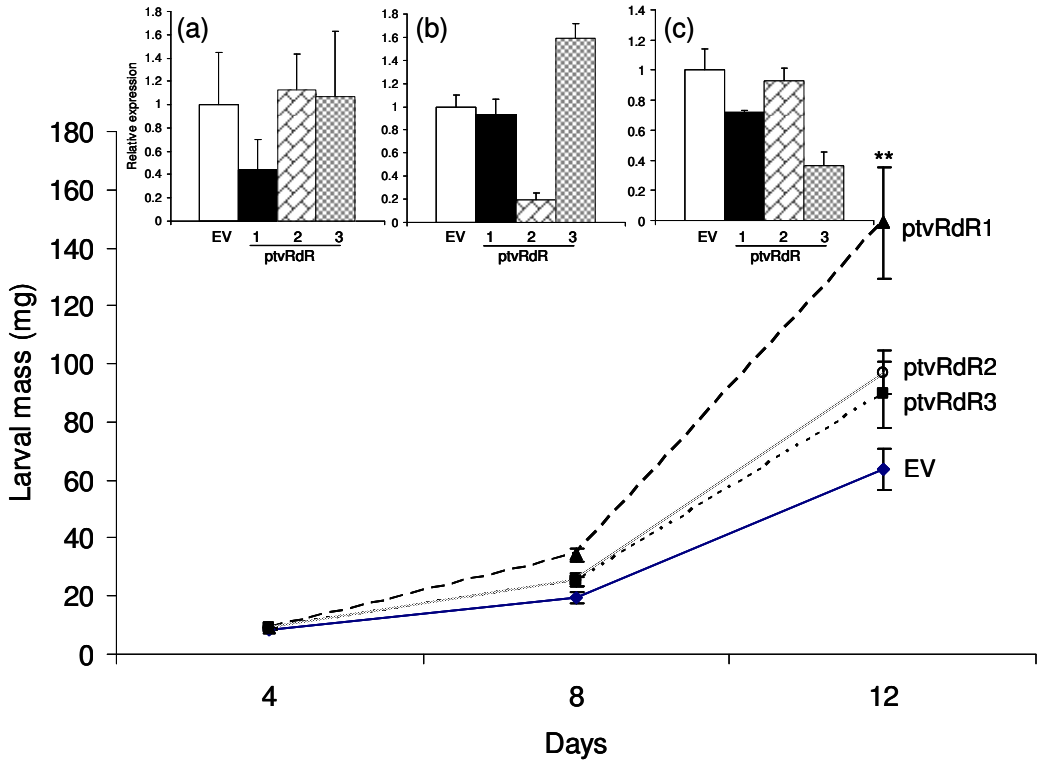


Figure 3. *M. sexta* performance after virus-induced gene silencing (VIGS) of the three *N. attenuata* RdRs. Bar graphs in the upper panel represent the relative transcript levels of (a) RdR1, (b) RdR2, and (c) RdR3 in empty

vector (EV) and the three ptvRdR lines. All comparisons were made relative to transcript levels in EV (set to 1). The line graph represents the performance of *M. sexta* larvae on EV and different RdR lines. Larval mass was measured at 4-day intervals. ** correspond to significant differences from EV at $p \leq 0.001$.

Stably silencing* RdR1 *increases performance of herbivores from two feeding guilds

Plants stably silenced in *RdR1* expression were produced by transforming *N. attenuata* with the same *RdR1* sequence used in the TRV-RdR1 construct in an inverted repeat orientation by *Agrobacterium*-mediated transformation (Kruegel *et al.*, 2002). Transformed plants were subjected to high-throughput phenotype screening (Kruegel *et al.*, 2002), and transgene incorporation in a single copy was verified by Southern analysis (Figure S4). Two independently transformed lines harboring a single insert (234-10 and 265-7; Figure S5) were analyzed for *RdR1* transcript levels after OS elicitation: neither had accumulated any elicited transcripts (Figure 4). Since *RdR1* has been associated with resistance to plant viruses in *N. tabacum* (Xie *et al.*, 2001), we used a virus susceptibility screen as a phenotypic test of *RdR1* silencing at a functional level. Both the lines were highly susceptible to tobamo-viruses, whose consequences include highly impaired growth, and rapid senescence and death (Figure S6).

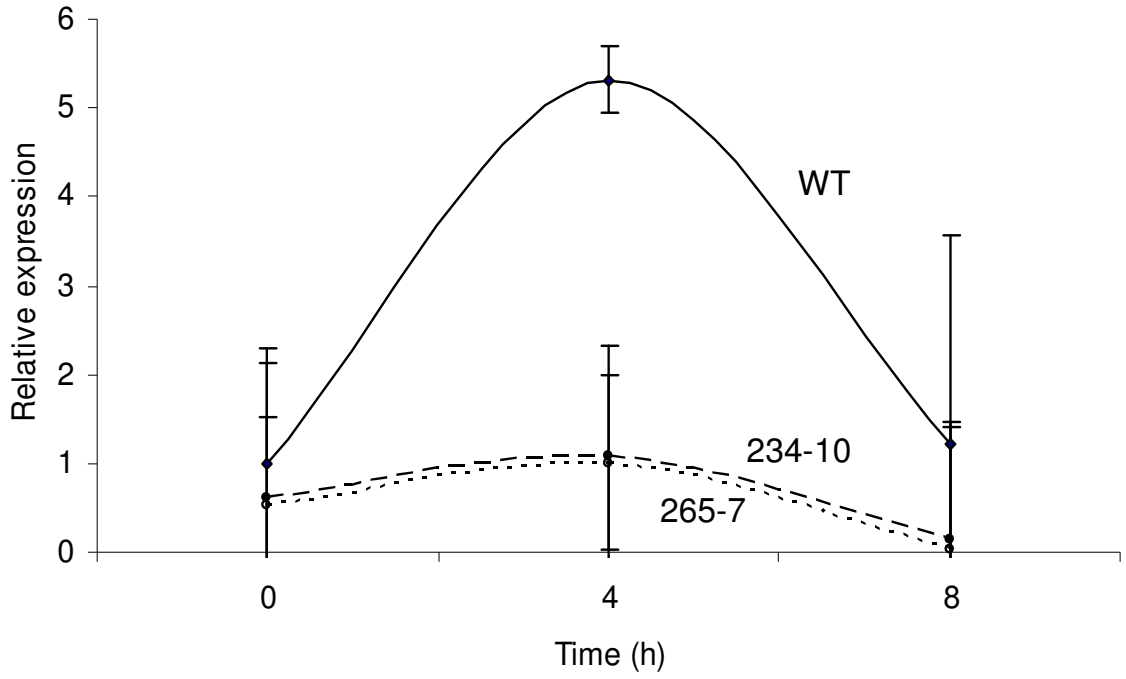
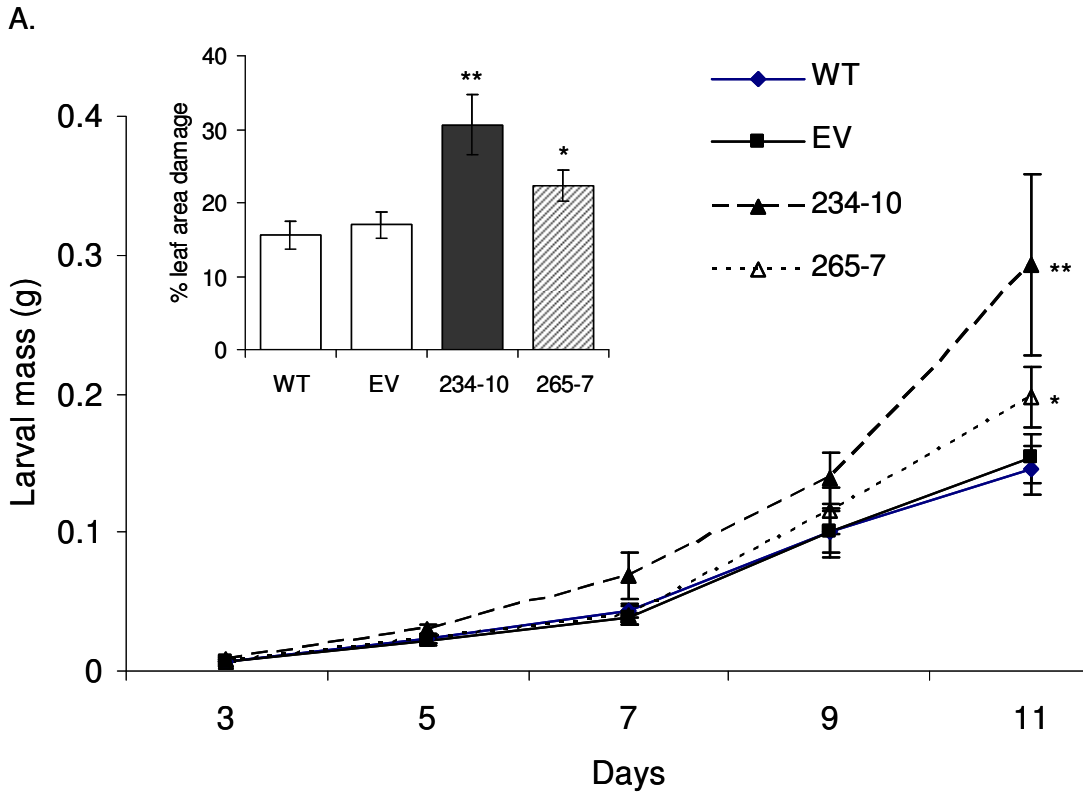


Figure 4. NaRdR1 expression in two independently transformed homozygous lines harboring a single copy of a fragment of NaRdR1in an inverted-repeat construct. Both lines (234-10, broken lines with solid circle, and 265-7, broken lines with open circles) failed to accumulate NaRdR1 transcripts after OS elicitation.

To investigate the role of *RdR1* in plant defense against insect herbivores, we challenged the irRdR1 plants with two different herbivores that regularly attack *N. attenuata* in its native habitat, *M. sexta* and *T. notatus,* and compared their performance to that on WT or stably transformed empty vector (EV) plants. No difference was recorded in the mass of *M. sexta* larvae growing on WT or EV plants (Figure 5a; ANOVA, $F_{1,30}$ = 0.117; P=0.735; furthermore, a comparison of WT and EV plants is presented in Table S1: no differences were observed). *M. sexta* larvae grew faster on 234-10 plants (Figure 5a; ANOVA, $F_{2,38}$ = 4.32; P=0.02) than on WT (Fisher's PLSD = 0.0068) or EV (Fisher's PLSD = 0.0256) control plants. Similarly, *M. sexta* larvae performed better on 265-7 (ANOVA, $F_{2,44}$ = 4.487; P=0.016) than on WT (Fisher's PLSD = 0.0064) or EV (Fisher's PLSD = 0.033). Also, both irRdR1 lines were more severely damaged than were WT or EV plants (Figure 5a; ANOVA, $F_{3,50}$ = 6.9; P=0.0006). When all three genotypes were challenged with *T. notatus*, both irRdR1 lines suffered significantly more damage than did the controls (Figure 5b; ANOVA, $F_{3,50}$ = 3.846; P=0.0149). The damage to WT did not differ from the damage to EV plants (Fisher's PLSD=0.7882).



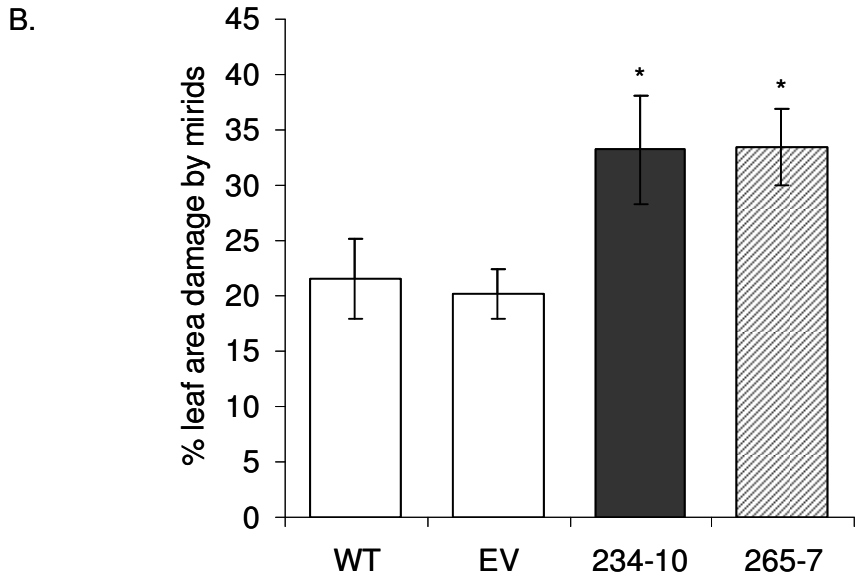


Figure 5. irRdR1 lines were highly susceptible to herbivores from two different feeding guilds. (A) The performance of *M. sexta* larvae on WT (-♦-), EV (-■-) and two irRdR1 lines (-▲-, 234-10; --Δ--, 265-7) was recorded during 11 days of continuous feeding. Larval mass was recorded at an interval of two days. The bar graph in the inset represents the total amount of damage (% of leaf area) caused at the end of 11 days. (B) The performance of *T. notatus* on different lines in terms of % damage after 6 days of insect attack. ** and * correspond to significant differences from WT and EV plants at $p \leq 0.01$ and 0.05, respectively.

Silencing RdR1 *increases* N. attenuata's *vulnerability to herbivores in their native habitats due to deficiencies in direct rather than indirect defenses*

In order to determine the ecological relevance of *RdR1* silencing, we examined the performance of both transformed lines in the plant's native habitat in the Great Basin Desert. irRdR1 plants did not defend themselves well against the native herbivore community. Mirids, beetles, and grasshoppers were the main herbivores attacking the plants at their release sites, with mirids causing the most damage. Total canopy area damaged was recorded 10 days after their release in the field. Because comparisons in the laboratory showed that the EV behaved like WT, and no differences between the WT and EV were observed (showed above and in Table S1), we used WT plants as controls in the field experiments. Plants from line 234-10 suffered damage to 15% more leaf area than did WT plants (Figure 6; n=10 pairs, paired t-test, t=3.672; P=0.0051). In a subsequent reading after 5 days, herbivory started to decline, but irRdR1 plants still suffered more damage than did WT plants (n=10 pairs, paired t-test, t=2.435, P=0.0377). A third and final reading was recorded 5 days after the second reading. The decline in herbivory continued, with consistently more damage to irRdR1 plants than to

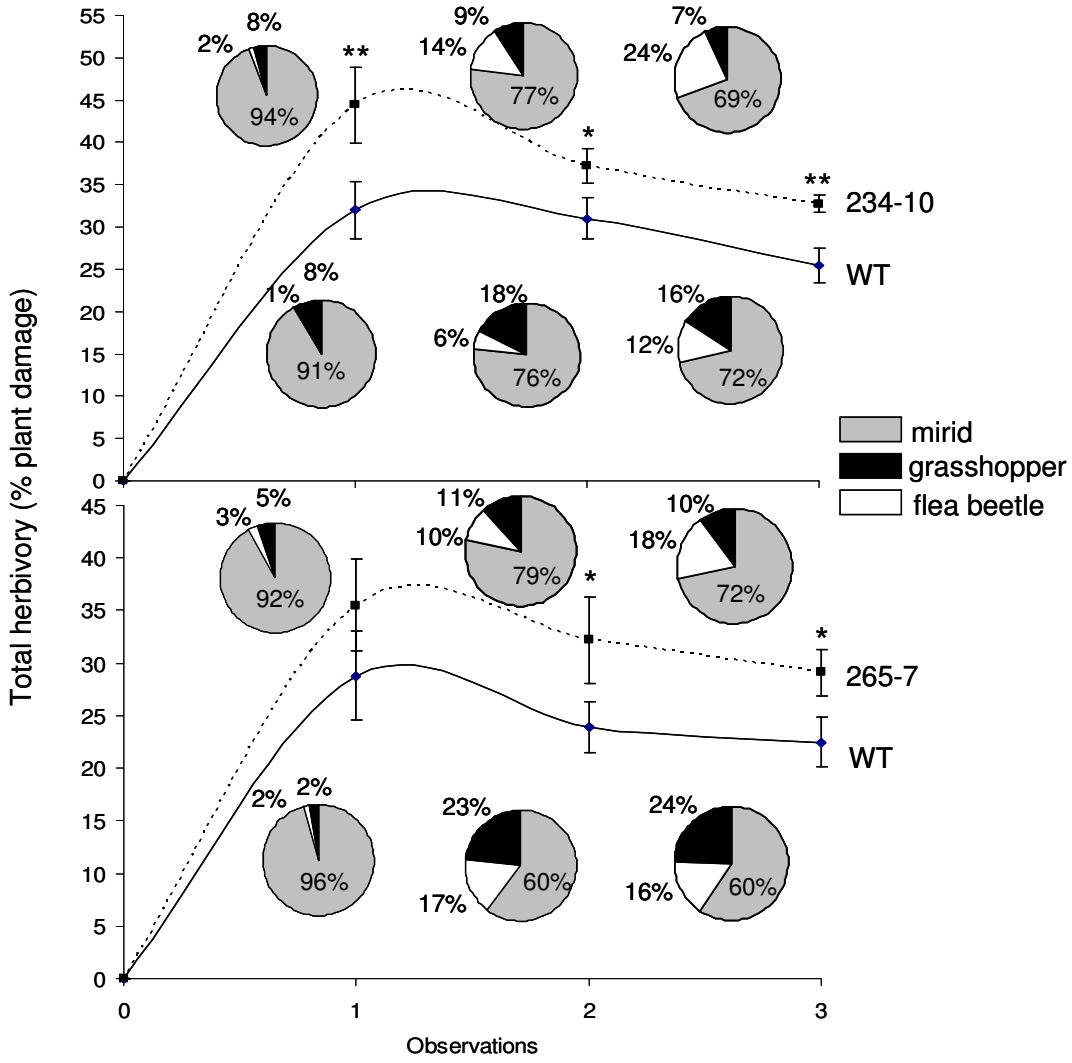


Figure 6. Performance of irRdR1 lines in natural habitats. Every irRdR1 plant from each line (234-10 and 265-7) was individually paired with a WT plant. Plants were transplanted to the field at day 0 and were undamaged. Cumulative damage on irRdR1 plants (broken) and WT plants (solid) was monitored three times, 10, 15, and 20 days after transplanting to the field. The individual pie charts represent the contribution of particular herbivores to the total herbivory (measured in terms of total plant area damaged). Pie charts in the upper panel of the line graph are for irRdR1 lines and in the lower panel for WT. ** and * correspond to significant differences between irRdR1 lines and WT plants at $p \leq 0.01$ and 0.05, respectively.

WT controls (Figure 6; n=10 pairs, paired t-test, t=3.744; P=0.0046). Similar patterns were seen for the plants of line 265-7 in comparison to WT controls (Figure 6). The contribution of different herbivorous taxa to the total canopy damage at a given time is presented in pie diagrams in Figure 6. There were no apparent abnormalities in plant morphology, or significant differences in plant height between irRdR1 and WT plants even in the field (for

234-10, n=10 pairs, paired-t test, t=0.0556; P=0.592; for 265-7, n=9 pairs, paired t-test, t=1.604; P=0.1474).

Because *Geocoris pallens* predators have been shown to be attracted to herbivore-attacked plants by the release of volatile organic compounds (VOCs) into the atmosphere, making VOCs an effective indirect defense in nature (Kessler and Baldwin, 2001), we performed a *Manduca*-egg predation assay with the field-grown lines. As with *M. sexta* caterpillar attack, when *N. attenuata* leaves are elicited with OS, they release VOCs that attract *G. pallens* from the surroundings; these insects then predate the affixed eggs. Predation on *M. sexta* eggs was <0.05% (both on WT and 234-10) before OS elicitation just 48 h after the eggs were affixed. Since OS elicitation is known to attract *G. pallens* predators, we elicited plants by mechanically wounding the first stem leaf and immediately applying OS. A 12-16% increase in predation was observed after 48h of elicitation; but there were no differences between WT and 234-10 (paired t test, n= 10 pairs, t = 0.56, P= 0.591). After 72h of elicitation the predation rates increased to 42-44%; still no differences in *M. sexta* egg predation between WT and 234-10 were observed (paired t test, n= 10 pairs, t = 0.19, P= 0.853).

Transcriptional responses of RdR1-silenced plants

In order to understand how the herbivore-induced transcriptional responses are altered in RdR1-silenced plants, we performed microarray analysis with a custom microarray which was developed to characterize the *M. sexta*-induced responses in *N. attenuata* (Halitschke and Baldwin, 2003; Voelckel and Baldwin, 2004b; 2004a). We hybridized arrays using RNA extracted from irRdR1 and WT plants grown in their natural habitat in Utah, and elicited the plants by treating puncture wounds with *M. sexta* OS 2h prior to tissue harvest. The microarray analysis was replicated with independent biological samples. For each of the two microarrays, RNA was extracted from three replicate WT and 234-10 plants. For 45 genes the OS-induced transcripts levels were significantly altered in irRdR1 plants compared to WT plants (Figure S7).

NaLOX3 is the specific lipoxygenase in *N. attenuata* required for JA signaling, which in turn mediates many of *N. attenuata*'s induced defense responses. Because silencing the *NaLOX3* gene also decreases the resistance of *N. attenuata* to *M. sexta* attack (Halitschke and Baldwin, 2003), as was observed for RdR1-silenced plants, we compared the transcriptional

responses of irRdR1 to OS elicitation with those of as-lox3 plants (Halitschke and Baldwin, 2003). There was very little overlap in the transcriptional responses of the two genotypes. Photosynthetic genes were the most commonly down-regulated genes. *Sn-1* and dioxygenase-like proteins (stress-responsive genes) were up-regulated in both irRdR1 and as-lox3 lines. On the other hand, as-lox3 responses substantially differed from irRdR1 responses. The signature JA-responsive genes such as *HPL* (hydroperoxide lyase, responsible for production of volatiles), *TD* (threonine deaminase), and *NaPI* (proteinase inhibitor) were down-regulated in as-lox3 but not in irRdR1. This suggested that the susceptibility of irRdR1 plants was likely not due to impaired JA signaling.

The microarray analysis revealed that the cell wall extensin precursor and other stress-responsive genes (phosphatase 2C, superoxide dismutase, and pathogenesis-related proteins) commonly up-regulated by OS elicitation in WT plants were down-regulated in RdR1-silenced plants and may account for the greater susceptibility of these plants to herbivore attack. Aldolase like protein and threonine synthase (primary metabolism) as well as gibberellin-2-oxidase, GAI-like protein (hormone metabolism) and polyphenol oxidase, *S. nigrum* prosystemin (secondary metabolism and signaling) were up-regulated. Serine protease inhibitors (*Pin2*) along with the subtilisin-like proteinase were also up-regulated. How these relate to the herbivore phenotype is not known.

The key biosynthetic genes for putrescine, *arginine decarboxylase* (*ADC*) and *ornithine decarboxylase* (*ODC*) were strongly down-regulated in irRdR1 compared to WT plants, as were *nitrate reductase* and *inorganic phosphatase*. Since these genes supply the nicotine biosynthetic pathway, we hypothesized that OS-elicited nicotine production was impaired by RdR1 silencing.

RdR1-silencing reduces elicited nicotine production

Nicotine is one of the most important induced direct defense compounds in *N. attenuata* (Steppuhn *et al.*, 2004). Nicotine was measured in non-wounded and OS-elicited WT and irRdR1 plants. Compared to WT plants, nicotine levels in irRdR1 plants were reduced by 51% and 35% in lines 234-10 and 265-7, respectively, 72h after elicitation—the time of maximum wound-induced nicotine accumulation (Figure 7a; ANOVA, $F_{5,24}$=4.485, P=0.005, Fisher's PLSD < 0.05). The induced nicotine levels in WT were more than twice those of

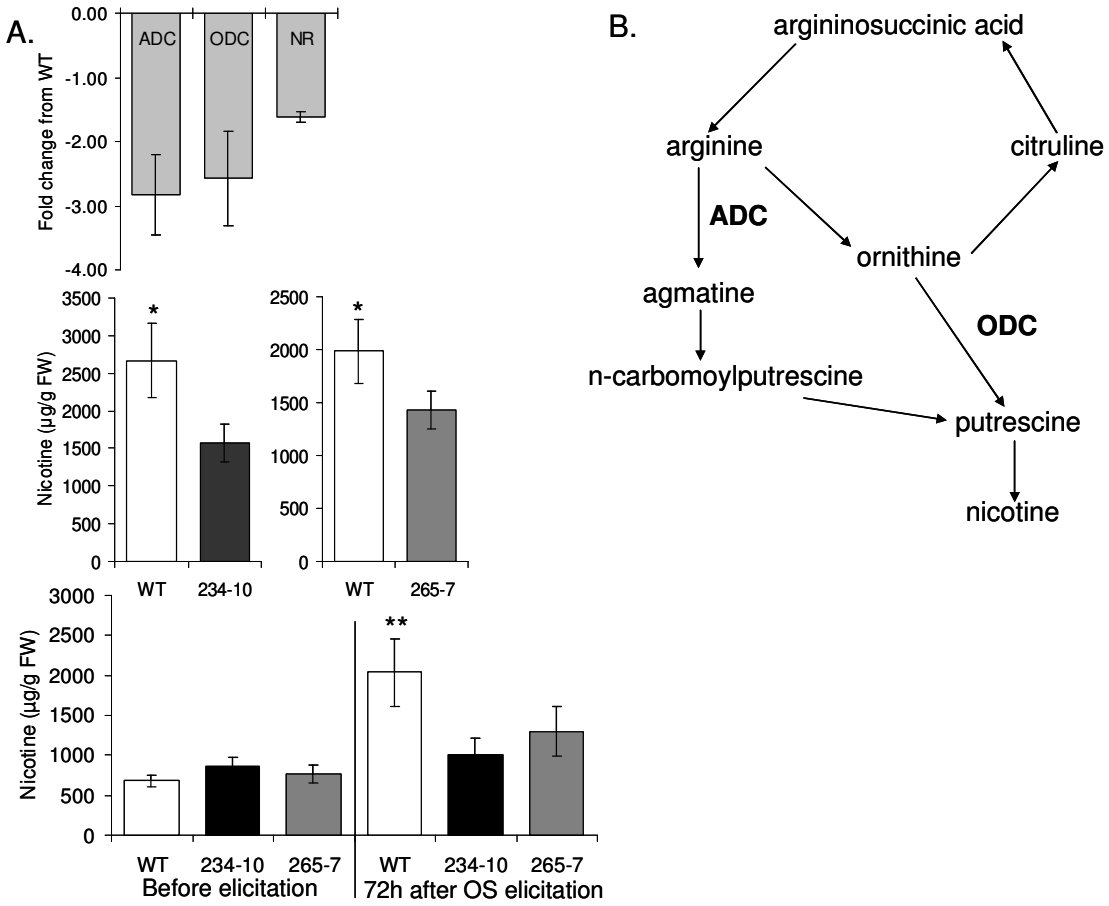


Figure 7. Silencing RdR1 inhibits elicited nicotine production. (A) Bar graphs in upper panel represent the fold down-regulation of putrescine biosynthesis enzymes, ODC, and ADC, along with nitrate reductase (NR). Levels of nicotine from field- (middle panel) and glasshouse-grown (lower panel) WT and irRdR1 lines. (B) A simplified scheme for nicotine production. ** and * show significant differences between irRdR1 lines and WT at $p \leq 0.01$ and 0.05, respectively.

uninduced WT plants (Fisher's PLSD ≤ 0.0005); whereas the elicited nicotine levels in irRdR1 lines did not differ significantly from those in untreated irRdR1 lines or untreated WT plants (Fisher's PLSD>0.05). Similarly, under field conditions, the elicited nicotine levels in line 234-10 were only 41% of WT plants (paired t test, n= 5 pairs, t = 3.02, P= 0.039) and in line 265-7 only 28% of WT plants (paired t test, n= 5 pairs, t = 3.518, P= 0.024).

Discussion

Here we expand the functional arena of plant *RNA-directed RNA polymerase 1* (*RdR1*) from post-transcriptional gene silencing and virus resistance (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Xie *et al.*, 2001; Yu *et al.*, 2003; Yang *et al.*, 2004) to the regulation of traits

mediating the direct defenses of plants against herbivores; *N. attenuata* plants whose *RdR1* gene was silenced with VIGS or by stable transformation were highly susceptible to insect herbivores from different feeding guilds in the glasshouse as well as in their native habitats. The demonstration of "real-world" relevance of the *RdR1* phenotype is significant from both mechanistic and pedagogical perspectives.

RdRs form an important component of the small-RNA-regulatory machinery in siRNA biogenesis (Xie *et al.*, 2004). RdRs' role in plant defense against viruses and PTGS has been studied with mutant screens and challenges with known viruses in the laboratory (Dalmay *et al.*, 2000; Vaistij *et al.*, 2002; Schwach *et al.*, 2005); but to our knowledge none of the studies has evaluated plants whose *RdRs* or any other component of the small-RNA-regulatory system have been silenced in their natural habitats. *RdRs* act in the biogenesis and transport of siRNAs, which may function epigenetically. In maize, *RdR2* has recently been shown to be essential for paramutation, a process by which epigenetic information is transferred to the next generation (Alleman *et al.*, 2006). Although *RdR1* has not been strictly associated with paramutations, the function of siRNA in cells may be regarded as an epigenetic process. Epigenetic traits are highly affected by the environment; accordingly, plants silenced for some components of small-RNA production or activity may behave differently in natural environments than under controlled conditions. This difference is why it is important to determine whether small-RNA-driven phenotypes can be reproducibly identified in organisms growing in complex environments.

A subtext to this study is that it highlights the value of carrying out "real-world" tests of gene function early in the discovery process. Such "real world" tests typically are conducted after most of the mechanistic details of a problem have been resolved. Postponing proof-of-function studies can lead to some interesting situations. For example, the pathogen resistance protein, PR1, which has been used for more than 3 decades of research in plant-pathogen interactions as a "reliable marker for pathogen resistance," has never been shown to be necessary or sufficient for resistance to any pathogen in a "real world" setting (Niderman *et al.*, 1995). Postponing real world functional tests is an artifact of the educational chasm that several decades ago split the biology departments of most universities into cell and molecular and organismic sub-divisions. As was eloquently illustrated in a recent proteomic analysis of *Pseudomonas fluorescens* (Knight *et al.*, 2006), organismic-level functional understanding is

essential for understanding gene function and an intensive period of cross-chasm training will thus be required for the next generation of biologists.

While this work establishes the real-world significance of *RdR1* function, much additional work is needed to understand the mechanisms by which *RdR1* mediates herbivore resistance. Herbivore-specific elicitors in OS, or the endogenous signals they elicit, rapidly increase the accumulation of *RdR1* transcripts. Microarray analysis revealed very little overlap between the OS-elicited transcriptional signatures in NaLOX3- and RdR1-silenced plants, and *N. attenuata*'s indirect defenses, which also require intact jasmonate signaling (Halitschke and Baldwin, 2003), are unaffected in RdR1-silenced plants. These results suggest that RdR1 mediates processes that are either jasmonate independent or down-stream of jasmonate signaling. In the microarray analysis, alkaloid biosynthesis genes were found to be down-regulated, indicating that the nicotine biosynthetic pathway was affected. Experiments with nicotine-silenced *N. attenuata* plants grown in natural habitats have demonstrated the importance of this direct defense (Steppuhn *et al.*, 2004). Induced nicotine production may be affected in two ways: either the regulators of ADC and ODC may be direct targets, or the siRNAs regulate the influx of nitrogen in the metabolism of defense-related compounds. As evidence, in addition to ADC and ODC, nitrate reductase (NR) was also found to be down-regulated. The molecular basis of induced alkaloid biogenesis is not well understood, because little is known about OS-inducible transcription factors and repressors. We propose that herbivory elicits *RdR1* activity, which then amplifies siRNA genesis. These siRNAs target constitutive repressors of alkaloid biosynthesis, which induce nicotine production. But when *RdR1* is silenced, these repressors are not degraded effectively; therefore irRdR1 plants cannot produce sufficient nicotine and are, in turn, susceptible to herbivores.

Experimental procedures

Plant and insect material

Wild type *N. attenuata* plants were from the 17th or 22nd generation inbred line of seeds originally collected from a native population in Utah. All the plants were grown under conditions described in (Kruegel *et al.*, 2002) and (Halitschke andBaldwin, 2003). Twenty-eight- to thirty-two-day-old plants in the rosette stage were used for all experiments. Eggs of *Manduca sexta L.* (Lepidoptera) were from North Carolina State University (Raleigh, NC, USA) and *Tupiocoris notatus* (Hemiptera: Miridae) were collected from the field station in

Utah in the southwestern United States and maintained at the Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena, Germany.

***Isolating* N. attenuata RdRs**

A PCR-based strategy was used to clone the *RdRs* from *N. attenuata*. Genomic DNA was extracted with procedures described in (Bubner *et al.*, 2004). For isolating *RdR1*, identical or complementary PCR primers were designed from the corresponding *RdR1* sequences from relatives of *N. attenuata*, *N. benthamiana,* and *N. tabaccum*. Single bands were gel-purified with GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Buckinghamshire, England) according to the manufacturer's instructions and sequenced. Sequences were aligned with the corresponding cDNA sequence. Fragments giving a positive alignment were considered to be exons. Sequences that did not match the corresponding homologues were tentative introns and subjected to Fourier analysis to determine if they were non-coding (Tiwari *et al.*, 1997). The following primer pairs were designed for the PCR amplification of gene fragments of RdR2 and RdR3 from *N. attenuata*: RDR3-32 (5'-GCGGCGGTCGACGTGCTGCAAGGATGGGTCAG-3') and RDR4-34 (5'-GCGGCGGGATCCCTTGGTAATATTAAGCATCCTG-3') for *RdR2*; RDR31-32 (5'-GCGGCGGTCGACTGAACCGGCAAATAGTAACC-3') and RDR32-31 (5'-GCGGCGGGATCCAAGCTCACCTAATTCATCC-3') for *RdR3* (*Sal*I and *Bam*HI sites underlined). Gene sequences from different *RdRs* were aligned with MegAlign (DNASTAR, Madison, WI, USA). Using MEGA 3 (Molecular Evolutionary Genetic Analysis; (Kumar *et al.*, 2004)) a neighbor-joining tree was built. Support for the groups was evaluated with 1,000 bootstrap replicates (Wu *et al.*, 2006).

Expression analysis by quantitative real time PCR (qRT-PCR)

RdR1 and *RdR6* in *Arabidopsis* and *N. benthamiana* are known to be elicited by SA treatments and virus attack, and their role in viral defense has been demonstrated (Yu *et al.*, 2003; Yang *et al.*, 2004). To determine if herbivore attack elicits RdR1 transcriptional changes, we wounded plants and immediately treated the puncture wounds with *M. sexta* OS, which are known to activate the herbivore-specific responses in *N. attenuata* (Halitschke *et al.*, 2001). The second fully expanded (+2, (van Dam *et al.*, 2001)) leaves of three to four rosette-stage plants were wounded by rolling a fabric pattern wheel down the leaf lamina and

immediately treating the resulting puncture wounds with 20µl OS (diluted 1:1 with distilled water) as described in (Halitschke *et al.*, 2001) or with water for each time point (described below). Because JA treatment also elicits herbivore-specific responses, we measured *RdR1* transcript pools in WT plants sprayed until runoff with 1mM JA. We also determined the effect of SA on *RdR1* levels by spraying plants with a 2mM SA solution until runoff (Yang *et al.*, 2004). To determine the kinetics of *RdR1* transcript accumulation, treated +2 leaves were harvested from 3-4 replicate plants at 0, 1, 4, 8, 12, 24, and 48 h after each treatment. To determine if *RdR1* transcripts accumulated diurnally, we harvested leaves from untreated +2 nodes of four replicate plants at 4:00, 8:00, 12:00, 16:00, 20:00, and 24:00.

Total RNA was extracted following the TRIZOL method. Total RNA was reverse-transcribed to prepare first-strand cDNA with the "SuperScript first-strand synthesis system for RT-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) to test the efficiency of the amplicon. All the qRT-PCR assays were performed with cDNA corresponding to 100ng RNA before transcription as a template, and amplified using qPCR^{TM} core reagent kit (Eurigentec, Seraing, Belgium) 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 0h treatment) were fixed to 1 and relative expression levels were calculated with respect to this reference value. To determine the *RdR* levels in the silenced lines, gene-specific primers were designed outside the region used in the silencing constructs. All the gene-specific primers and probes were designed with "Primer Express" software.

Silencing the RdRs *of* N. attenuata

The "virus-induced gene-silencing" (VIGS) system based on the tobacco rattle virus (TRV) was used to independently silence the three RdRs in *N. attenuata* (Ratcliff *et al.*, 2001; Saedler and Baldwin, 2004). PCR fragments of 434, 350, and 353 bp for *RdR1*, *RdR2*, and *RdR3*, respectively (Figure S2), obtained using the primer pair combination of RDR1-31 (5'-GCGGCGGTCGACTATGATCCAGTGAGGTGGC-3') and RDR2-31 (5'-GCGGCGGGATCCATCCACACTGAATTATCCC-3') for *RdR1* (*Sal*I and *Bam*HI sites underlined); RDR3-32 and RDR4-34 for RdR2; RDR31-32 and RDR32-31 for RdR3 (primer

sequences mentioned above) were digested with *Sal*I and *Bam*HI (site also in the amplified *RdR1* sequence) and cloned in plasmid pTV00 (Ratcliff *et al.*, 2001) cut with the same enzymes. The inserts of the resulting TRV-based vectors pTVRDR1, pTVRDR2 and pTVRDR31 (all 5.9 kb) for *RdR1*, *RdR2,* and *RdR3*, respectively, were sequenced. Empty vector (EV; pTV00) constructs served as controls for this experiment. Inoculating plants with a TRV vector containing a 206bp fragment of phytoene desaturase (PDS) gene from *N. benthaminana* (pTVPDS), which bleaches plants as the *PDS* gene is silenced, allowed us to monitor the progress of the VIGS (Figure S2). Forty-five replicate plants per construct were inoculated with each of the three RdRs and an EV control construct; and five replicate plants were inoculated with PDS constructs. Twenty-five to twenty-eight-day-old *N. attenuata* WT plants, in the rosette stage, were used to inoculate the VIGS constructs. Growth conditions at the start of the experiment were 20 ^{0}C, 65% relative humidity, and no light for two days, after which light levels were returned to normal (400-1000 µmol $m^{-2}s^{-1}$, 16/8h light/dark).

In order to produce plants stably silenced in RdR1 expression, a RdR1 gene fragment was cloned in an inverted repeat orientation in a pRESC5 transformation vector as described in (Steppuhn *et al.*, 2004) and (Bubner *et al.*, 2006). Transformation using *Agrobacterium tumefaciens* was done as described in (Kruegel *et al.*, 2002). T_1 plants were screened for hygromycin resistance. Homozygosity was determined by segregation analysis of T_2 plants. qRT-PCR was used to quantify transcript accumulation, as described above, and Southern analysis was used for copy number determination of the transgene. Two independently transformed homozygous lines (234-10 and 265-7), each containing a single insertion of the transgene, were further characterized in the T_2 generation. Since *RdR1* is thought to be required for resistance to viruses, we used a virus assay as an additional positive control for RdR1-silencing. +1, 2, and 3 leaves of the WT and transgenic plants were inoculated with tomato mosaic virus. Leaves of 3 replicate plants of each line in the rosette-stage of growth (28 days after germination) were rubbed with corborundem powder and 50µl of viral material suspended in phosphate buffer was applied to the abraded leaves. Equal numbers of plants from each line were rubbed with corborundem powder and treated with 50µl of phosphate buffer without any virus as mock control. Plants were monitored for symptom development for 12-14 days.

Insect performance assays

The performance of *M. sexta* larvae was evaluated for the VIGS-silenced plants and the stably transformed lines in separate experiments. A freshly enclosed larva was placed on the +2 leaf of each replicate plant, 14 to 15 days after inoculation with the four pTRV constructs (three *RdR* constructs and the one empty vector control) under the temperature and light conditions required for VIGS described above. The bleaching symptoms appeared in all five replicate plants 11 days after inoculation with pTVPDS. Larval mass was recorded every fourth day for 12 days and 20 replicate plants per construct were used. Performance assays were also done for each transgenic line (234-10, 265-7, EV) and for WT plants in a separate experiment with 10-16 replicate plants per line, grown under normal glasshouse conditions. In this experiment, larvae were allowed to feed for 11 days and data were recorded every 2 days for 11 days starting from day 3. The percentage of total damage on each transgenic line and on WT plants was estimated at the end of the assay.

T. notatus performance assays were conducted on stably transformed lines. 12-14 replicate plants from four genotypes (234-10, 265-7, EV, and WT), all in the early elongation stage of growth, 35 days after germination, were enclosed in a completely randomized manner in a mesh tent. Mirids are highly mobile and readily move among the plants in a tent. Approximately 250 adult mirids were released into the tents and allowed to feed on the plants. After 6 days, the percentage of leaf area damaged was measured.

Performance under field conditions

WT and transgenic irRdR lines were planted into natural habitats of *N. attenuata* in the southwestern United States. Plants were placed in a watered field plot at the Lytle Preserve research station (Santa Clara, UT, USA) in a paired design. Seeds of WT and irRdR1 plants were germinated on the agar plates. The plates were then kept at 25°C/16h (200 µm/s/m^2 light) and 20°C/8h dark. After 10 days, seedlings were transferred to jiffy 703 pots (1 ¾ inch x 1 ¾ inch, AlwaysGrows, Newark, OH, USA) which had been soaked in borax solution (0.4mg/ 45mL water). The seedlings were fertilized with iron solution (stock solution: 2.78 g $FeSO_4.7H_2O$ and 3.93g Titriplex in 1L H_2O, diluted 100 fold for fertilization) after 7 days. After 3-4 weeks, plants were transferred to the field plot. They were allowed to gradually adapt to the environmental conditions of the Great Basin desert (high sun exposure and low relative humidity) over 2 weeks in a mesh tent. 10-12 irRdR1-WT pairs of adapted seedlings

of the same size were transplanted to a nearby, watered field plot. Seedlings were watered every other day for 2 weeks until roots were established in the native soil. Releases of the transformed plants were conducted under APHIS notification numbers 06-003-08n. The plants were colonized by the natural herbivore community for three weeks and the study was terminated after 28 days. All the capsules were plucked off and destroyed and all the plants in and around the plantation plot were removed and destroyed to comply with 7CFR 340.4, the legal statute which governs the release of transgenic organisms. The leaves were scrutinized at intervals of five days for the characteristic damage of the various herbivores that commonly attack *N. attenuata* in Utah (Supporting Method, Figure 1), such as mirids, grasshoppers, beetles, etc., and total herbivory was estimated as a percentage of the total canopy area.

M. sexta eggs are laid singly as well as in clusters of four to seven on the underside of the leaf surface. In order to determine if *N. attenuata*'s indirect defense, specifically its ability to attract predators with OS-elicited VOCs, was altered in irRdR1 plants, we conducted a predation assay (Kessler and Baldwin, 2001): five *M. sexta* eggs were glued using natural glue (known to have no effects on VOC production) on the second stem leaf of ten replicate WT and irRdR1 plant pairs which had not been previously attacked by *M. sexta*. Predation rates were measured twice at an interval of 24h after the *M. sexta* eggs were attached. Because OS elicitation mimics the release of VOCs that normally occurs after larval attack and the VOCs attract *Geocoris pallens* predators which preys on *M. sexta* eggs and larvae, the first stem leaves were elicited with *M. sexta* OS and the number of eggs predated 24, 48, and 72h after elicitation was determined.

Microarray analysis

Microarray analysis for samples derived from the above-described field study was performed with microarrays enriched with *M. sexta*-responsive *N. attenuata* genes which had been previously used to characterize the insect-induced responses in *N. attenuata* (Voelckel and Baldwin, 2004b; 2004a) in accordance with the MIAMI guidelines. A similar hybridization strategy was adapted as described in (Halitschke and Baldwin, 2003), in which samples from NaLOX3-silenced plants (as-lox3) were Cy3-labeled and hybridized against WT Cy5-labeled samples. In the field, for each chip**,** the second fully expanded (+2) leaves of three plants of the 234-10 line and the WT control, respectively, were elicited with *M. sexta* OS as described above. The treated leaves were harvested after 2h. Total RNA was extracted from

three biological replicate plants and an equal amount of RNA from each replicate was used for each chip. RNA from the treated 234-10 plants was labeled with Cy3; RNA from the treated WT control was labeled with Cy5. Approximately 400 µg total RNA was used in each labeling reaction. The whole procedure was replicated and two arrays were hybridized. Microarray data were lowess-normalized with the MIDAS package (Microarray data analysis system, Institute for Genome Research, Washington, D.C., USA). The quadruplicate spots for each gene were analyzed for significant differences using a t-test at confidence level (α) 0.05; and a threshold of a 1.5-fold change in expression ratio was used. A gene was regarded as differentially regulated if it met both the criteria in both microarrays. In some genes where the values were present only for one channel, the data were evaluated for differences from signal to noise ratio, and if the intensity was more than 2.5 times the signal to noise ratio, the gene was regarded as differentially regulated.

Analysis of secondary metabolites

Secondary metabolites were analyzed using HPLC as described in (Steppuhn *et al.*, 2004). Briefly, leaf samples from the field (zero or first stem leaves) as well as glasshouse-grown plants (second fully expanded leaves) from 5-6 replicate plants for each line and WT were isolated 72h after OS elicitation. Samples (~100mg) were extracted with 2:3 methanol:0.5% acetic acid (v/v) and injected into HPLC. A standard curve was made using a dilution series of nicotine and nicotine levels were quantified.

Statistical analysis

Data (arcsine transformed, wherever they did not meet assumptions of normality) were analyzed with StatView (Abacus Concepts, Inc., Berkeley, CA, USA). Insect assays in the glasshouse were analyzed with an Analysis of Variance (ANOVA). All the field data or data derived from samples from field were tested with a paired t-test because in all the field experiments, a transgenic line and WT control plant were planted as a single pair.

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Supplementary Material

The following supplementary material is available for this article:

Supporting Method Figure 1. Characteristic damage symptoms of different herbivore species that attack *N. attenuata* plants in Utah (pictures by D. Kessler).

Figure S1. Partial sequences of the three *N. attenuata* RdRs used to make silencing constructs

Figure S2. No morphological abnormalities were seen after silencing the three RdRs with VIGS during (A) rosette- (B) flowering-stage growth. Bleaching symptoms resulting from silencing phytoene desaturase (PDS) show the spread of the VIGS throughout the plant.

Figure S3. Relative expression of RdR1 in WT *N. attenuata.* (A) RdR1 expression was detected in all plant parts; (B) The constitutive levels did not exhibit a diurnal pattern of accumulation.

Figure S4. Southern analysis of the two independently transformed irRdR1 lines showing single insertion. Genomic DNA (10 μg) from individual plants was digested with ECoR1 and blotted onto a nylon membrane. The blot was hybridized with a PCR fragment of the *hygromycin phosphotransferase II* gene, specific for the selective marker on the T-DNA.

Figure S5. Growth phenotypes of the two independently transformed lines. No differences in rosette size (A; ANOVA, $F_{3,16} = 1.396$, P=0.2803) and stalk length (B and C; ANOVA, $F_{3,20} = 0.268$, P=0.847) were observed.

Figure S6. irRdR1 silenced lines are highly susceptible to viruses. When rosette leaves were inoculated with tomato-mosaic virus, growth of the irRdR1 lines was severely impaired and plants of both lines rapidly senesced and died.

Figure S7. Microarray analysis of significantly (from two replicate arrays hybridized with cDNA from 3 pooled plants of each line) differentially OS-regulated genes in irRdR1 compared to WT plants. +/- from zero represent up- or down-regulation.

Table S1. Analysis of Variance (ANOVA) for growth phenotypes of WT and EV – transformed plants used in this study.

References

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

Allen, E., Xie, Z.X., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, **121**, 207-221.

Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, A.J.M. and Matzke, M. (2002) RNA-directed DNA methylation in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 16499-16506.

Baldwin, I.T. (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. *Plant Physiology*, **127**, 1449-1458.

Bartel, D.P. (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-297.

Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356-363.

Borsani, O., Zhu, J.H., Verslues, P.E., Sunkar, R. and Zhu, J.K. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell*, **123**, 1279-1291.

Bubner, B., Gase, K. and Baldwin, I.T. (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *Bmc Biotechnology*, **4**.

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

Chen, X.M. (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science*, **303**, 2022-2025.

Cogoni, C. and 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. and Baulcombe, D.C. (2000) An RNA-Dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell*, **101**, 543-553.

De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T. and Tumlinson, J.H. (1998) Herbivore-infested plants selectively attract parasitoids. *Nature*, **393**, 570-573.

Duffey, S.S. and Stout, M.J. (1996) Antinutritive and toxic components of plant defense against insects. *Archives of Insect Biochemistry and Physiology*, **32**, 3-37.

Emery, J.F., Floyd, S.K., Alvarez, J., Eshed, Y., Hawker, N.P., Izhaki, A., Baum, S.F. and Bowman, J.L. (2003) Radial patterning of Arabidopsis shoots by class IIIHD-ZIP and KANADI genes. *Current Biology*, **13**, 1768-1774.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W. and Baldwin, I.T. (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 Physiology*, **125**, 711-717.

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

Halitschke, R., Ziegler, J., Keinanen, M. and Baldwin, I.T. (2004) Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in Nicotiana attenuata. *Plant Journal*, **40**, 35-46.

Heidel, A.J. and Baldwin, I.T. (2004) Microarray analysis of salicylic acid- and jasmonic acid-signalling in responses of Nicotiana attenuata to attack by insects from multiple feeding guilds. *Plant Cell and Environment*, **27**, 1362-1373.

Hermsmeier, D., Schittko, U. and Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiology*, **125**, 683-700.

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

Howe, G.A., Lightner, J., Browse, J. and Ryan, C.A. (1996) An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell*, **8**, 2067-2077.

Hui, D.Q., Iqbal, J., Lehmann, K., Gase, K., Saluz, H.P. and Baldwin, I.T. (2003) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata: V. Microarray analysis and further characterization of large-scale changes in herbivore-induced mRNAs. *Plant Physiology*, **131**, 1877-1893.

Kessler, A. and Baldwin, I.T. (2001) Defensive function of herbivore-induced plant volatile emissions in nature. *Science*, **291**, 2141-2144.

Kessler, A. and Baldwin, I.T. (2002) Manduca quinquemaculata's optimization of intra-plant oviposition to predation, food quality, and thermal constraint's. *Ecology*, **83**, 2346-2354.

Kessler, A. and Baldwin, I.T. (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco Nicotiana attenuata. *Plant Journal*, **38**, 639-649.

Knight, C.G., Zitzmann, N., Prabhakar, S., Antrobus, R., Dwek, R., Hebestreit, H. and Rainey, P.B. (2006) Unraveling adaptive evolution: how a single point mutation affects the protein coregulation network. *Nat Genet*, **38**, 1015-1022.

Korth, K.L. (2003) Profiling the response of plants to herbivorous insects. *Genome Biology*, **4**, 221.

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

Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150-163.

Llave, C., Xie, Z.X., Kasschau, K.D. and Carrington, J.C. (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. *Science*, **297**, 2053-2056.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G.L., Zamore, P.D., Barton, M.K. and Bartel, D.P. (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5 ' region. *Embo Journal*, **23**, 3356-3364.

Mallory, A.C. and Vaucheret, H. (2004) MicroRNAs: something important between the genes. *Current Opinion in Plant Biology*, **7**, 120-125.

Martienssen, R.A. (2003) Maintenance of heterochromatin by RNA interference of tandem repeats. *Nature Genetics*, **35**, 213-214.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A. and Matzke, A.J.M. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *Embo Journal*, **19**, 5194-5201.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T.A. and Vaucheret, H. (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101**, 533-542.

Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B. and Mosinger, E. (1995) Pathogenesis-Related Pr-1 Proteins Are Antifungal - Isolation and Characterization of 3 14-Kilodalton Proteins of Tomato and of a Basic Pr-1 of Tobacco with Inhibitory Activity against Phytophthora-Infestans. *Plant Physiology*, **108**, 17-27.

Palatnik, J.F., Allen, E., Wu, X.L., Schommer, C., Schwab, R., Carrington, J.C. and Weigel, D. (2003) Control of leaf morphogenesis by microRNAs. *Nature*, **425**, 257-263.

Petersen, B.O. and Albrechtsen, M. (2005) Evidence implying only unprimed RdRP activity during transitive gene silencing in plants. *Plant Molecular Biology*, **58**, 575-583.

Pickford, A.S. and Cogoni, C. (2003) RNA-mediated gene silencing. *Cellular and Molecular Life Sciences*, **60**, 871-882.

Preston, C.A. and Baldwin, I.T. (1999) Positive and negative signals regulate germination in the post-fire annual, Nicotiana attenuata. *Ecology*, **80**, 481-494.

Qu, N., Schittko, U. and Baldwin, I.T. (2004) Consistency of Nicotiana attenuata's herbivore- and jasmonate-induced transcriptional responses in the allotetraploid species Nicotiana quadrivalvis and Nicotiana clevelandii. *Plant Physiology*, **135**, 539-548.

Ralph, S., Oddy, C., Cooper, D., Yueh, H., Jancsik, S., Kolosova, N., Philippe, R.N., Aeschliman, D., White, R., Huber, D., Ritland, C.E., Benoit, F., Rigby, T., Nantel, A., Butterfield, Y.S.N., Kirkpatrick, R., Chun, E., Liu, J., Palmquist, D., Wynhoven, B., Stott, J., Yang, G., Barber, S., Holt, R.A., Siddiqui, A., Jones, S.J.M., Marra, M.A., Ellis, B.E., Douglas, C.J., Ritland, K. and Bohlmann, J. (2006) Genomics of hybrid poplar (Populus trichocarpa x deltoides) interacting with forest tent caterpillars (Malacosoma disstria): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. *Molecular Ecology*, **15**, 1275-1297.

Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant Journal*, **25**, 237-245.

Reymond, P., Bodenhausen, N., Van Poecke, R.M.P., Krishnamurthy, V., Dicke, M. and Farmer, E.E. (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell*, **16**, 3132-3147.

Roda, A., Halitschke, R., Steppuhn, A. and Baldwin, I.T. (2004) Individual variability in herbivore-specific elicitors from the plant's perspective. *Molecular Ecology*, **13**, 2421-2433.

Ryan, C.A. and Pearce, G. (2003) Systemins: A functionally defined family of peptide signal that regulate defensive genes in Solanaceae species. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 14577-14580.

Saedler, R. and Baldwin, I.T. (2004) Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in Nicotiana attenuata. *Journal of Experimental Botany*, **55**, 151-157.

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

Schittko, U. and Baldwin, I.T. (2003) Constraints to herbivore-induced systemic responses: Bidirectional signaling along orthostichies in Nicotiana attenuata. *Journal of Chemical Ecology*, **29**, 763-770.

Schmidt, D.D., Voelckel, C., Hartl, M., Schmidt, S. and Baldwin, I.T. (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. *Plant Physiology*, **138**, 1763-1773.

Schwach, F., Vaistij, F.E., Jones, L. and Baulcombe, D.C. (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 Physiology*, **138**, 1842-1852.

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

Steppuhn, A., Gase, K., Krock, B., Halitschke, R. and Baldwin, I.T. (2004) Nicotine's defensive function in nature. *PLoS Biology*, **2**, 1074-1080.

Tang, G.L., Reinhart, B.J., Bartel, D.P. and Zamore, P.D. (2003) A biochemical framework for RNA silencing in plants. *Genes & Development*, **17**, 49-63.

Tiwari, S., Ramachandran, S., Bhattacharya, A., Bhattacharya, S. and Ramaswamy, R. (1997) Prediction of probable genes by Fourier analysis of genomic sequences. *Computer Applications in the Biosciences*, **13**, 263-270.

Turlings, T.C.J. and Tumlinson, J.H. (1992) Systemic Release of Chemical Signals by Herbivore-Injured Corn. *Proceedings of the National Academy of Sciences of the United States of America*, **89**, 8399-8402.

Vaistij, F.E., Jones, L. and Baulcombe, D.C. (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell*, **14**, 857-867.

Van Dam, N.M., Horn, M., Mares, M. and Baldwin, I.T. (2001) Ontogeny constrains systemic protease inhibitor response in Nicotiana attenuata. *Journal of Chemical Ecology*, **27**, 547-568.

Voelckel, C. and Baldwin, I.T. (2004a) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. *Plant Journal*, **38**, 650-663.

Voelckel, C. and Baldwin, I.T. (2004b) Generalist and specialist lepidopteran larvae elicit different transcriptional responses in Nicotiana attenuata, which correlate with larval FAC profiles. *Ecology Letters*, **7**, 770-775.

Voelckel, C., Weisser, W.W. and Baldwin, I.T. (2004) An analysis of plant-aphid interactions by different microarray hybridization strategies. *Molecular Ecology*, **13**, 3187-3195.

Voinnet, O. (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. *Current Opinion in Plant Biology*, **5**, 444-451.

Walling, L.L. (2000) The myriad plant responses to herbivores. *Journal of Plant Growth Regulation*, **19**, 195-216.

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

Winz, R.A. and Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. IV. Insect-induced ethylene reduces jasmonate-induced nicotine accumulation by regulating putrescine N-methyltransferase transcripts. *Plant Physiology*, **125**, 2189-2202.

Wu, J., Hettenhausen, C. and Baldwin, I.T. (2006) Evolution of proteinase inhibitor defenses in North American allopolyploid species of Nicotiana. *Planta*, **224**, 750-760.

Xie, Z.X., Fan, B.F., Chen, C.H. and Chen, Z.X. (2001) An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 6516-6521.

Xie, Z.X., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E. and Carrington, J.C. (2004) Genetic and functional diversification of small RNA pathways in plants. *Plos Biology*, **2**, 642-652.

Yang, S.J., Carter, S.A., Cole, A.B., Cheng, N.H. and Nelson, R.S. (2004) A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by Nicotiana benthamiana. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 6297-6302.

Yu, D.Q., Fan, B.F., MacFarlane, S.A. and Chen, Z.X. (2003) Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. *Molecular Plant-Microbe Interactions*, **16**, 206-216.

Yu, H. and Kumar, P.P. (2003) Post-transcriptional gene silencing in plants by RNA. *Plant Cell Reports*, **22**, 167-174.

Accession numbers

The NCBI accession numbers of the gene sequences of the *NaRdRs* are DQ988990, DQ988991, DQ988992 for *RdR1* and partial *RdR2* and *3* sequences respectively. The accession numbers for the other RdRs used in this study are: Y10403 LeRdR; AJ011576 NtRdR, AY574374 NbRdR1m; AY722009 NbRdR2; AY722008 NbSDE1; At1g14790 AtRdR1; At4g11130 AtRdR2; AF239718 AtRdR6/SGS2; At2g19910 AtRdR3; At2g19920 AtRdR4; At2g19930 AtRdR5.

2.2 Manuscript II

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Herbivory-induced changes in the Small-RNA Transcriptome and Phytohormone Signaling in *Nicotiana attenuata*

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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 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 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 *RdR1*-silenced plants. *RdR1*-silenced plants had photosynthetic rates, growth, and reproductive output indistinguishable from that of WT plants, suggesting unaltered primary metabolism in *RdR1*-silenced plants. We conclude that the susceptibility of *RdR1*-silenced 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.

Key words: herbivore resistance, JA, *Nicotiana attenuata*, phytohormone regulation, *RdR1*, small-RNA, transcriptional regulation, 454-sequencing

Introduction

When herbivores attack plants, large-scale metabolic changes occur, which can be mimicked by applying herbivore-specific elicitors to mechanically produced wounds (1-3). These rearrangements are preceded by a large-scale transcriptional response, apparently not only in angiosperms but also in gymnosperms, which regulate from hundreds to thousands of genes during insect attack (4). *Populus* recruits >1000 genes while defending itself against attack from forest tent caterpillars (5), and using a small phytohormone-focused microarray, *Arabidopsis* plants were found to regulate 114 genes when *Pieris rapae* larvae attacked (6). When the native tobacco *Nicotiana attenuata* is attacked by the larvae of its specialist herbivore, *Manduca sexta* larvae, >500 genes are differentially regulated (7). Furthermore, the transcriptional responses to herbivore attack are highly herbivore- and species-specific (7-9). How these large-scale transcriptional responses, which presumably result in changes in metabolism and defense status, are so rapidly activated in both attacked and unattacked systemic tissues remains largely unknown; however, it is clear that phytohormones play a central role in the signal transduction cascade (10-14).

Phytohormones, especially those involved in oxylipin and ethylene signaling, are known to mediate plant defense responses to herbivore attack, and mutants defective in oxylipin and ethylene biosynthesis are impaired in many herbivore-elicited transcriptional responses (10-14). However, 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 (15); and small-RNAs (smRNAs) have been shown to regulate processes as diverse as plant resistance to viruses, and development and differentiation (16, 17). All RNA-silencing pathways require the genesis of 18- to 26-nucleotide smRNAs from the cleavage of double-stranded (ds) RNA (18). These smRNAs can be classified as micro-(mi) and small-interfering (si) RNAs. RNA-directed RNA polymerases [referred to as *RdRps* (19) or *RdRs* (20)] are critical for generating dsRNAs; these are cleaved to produce siRNAs that can be transmitted throughout the plant to mediate systemic signaling (21). Additionally, as *RdR*s and miRNAs may coordinate their actions, the miRNAs may set the phase for the *RdR*-dependent generation of siRNAs (22).

Three functionally distinct *RdRs* have been reported from *Arabidopsis,* tomato, and *Nicotiana* (23-26): *RdR1* and *6* are elicited in tobacco and *Arabidopsis* by treatment with

salicylic acid and appear to function specifically in viral defense and post-transcriptional gene silencing (19, 26, 27); *RdR2* is involved in transcriptional gene silencing, paramutation, and heterochromatin formation (20, 28). The transitivity of the RNA signal depends on *RdR*s (29); during virus infection, *RdR6* is required for the cell to perceive the silencing signal but not to produce or transport it (30).

We have recently shown that *N. attenuata*'s *RdR1* is strongly elicited by herbivore-specific signals which are introduced into plants during feeding; moreover, silencing the endogenous *NaRdR1* gene by transforming plants with RNAi constructs containing a Na*RdR1* fragment in an inverted-repeat orientation (irRdR1) made *N. attenuata* susceptible to herbivores in both the glasshouse and the plant's native environment (31). 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 known to be activated in *N. attenuata* after herbivore attack: attack by *M. sexta* larvae dramatically amplifies the wound-induced jasmonate (JA) burst which is elicited by herbivore-specific signals from the larvae's oral secretions (OS). Applying *M. sexta* OS to standardized puncture wounds mimics all of the herbivore-specific phytohormone, transcriptome, proteome, metabolome, and resistance responses measured to date (32-36). This process, referred to as OS elicitation, simplifies the analysis of the rapid dynamics in elicited responses under both field and laboratory conditions, as 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 (37-39). When plants silenced in either JA biosynthesis (39) or perception (40) are planted into the plant's natural habitat in the Great Basin Desert, the plants were found to be not only vulnerable to adapted herbivores but also attractive to new ones (39).

M. sexta attack also triggers an ethylene burst (41), 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 (42, 43); nicotine is an effective defense against herbivores in nature (44) but is costly for plants to produce (45).

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 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 populations 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 (7). If these systemic 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 (46-48), we were able to evaluate how *N. attenuata*'s smRNA-populations responded to OS elicitation in both WT plants and plants with *RdR1*-silenced isogenic backgrounds (Supporting (S) Fig. 8). We generated 132,239 reliable sequences in the range of 15-30 nucleotides (nt) from unelicited and OS-elicited WT and *RdR1*-silenced (irRdR1) plants, of which 110,122 were unique sequences (Table S1). We annotated these sequences against the non-redundant nucleotide database (NR-DB) of NCBI and the miRBase Sequence Database (Tables S2 and S3) (49). Large-scale changes associated with both OS elicitation and *RdR1* silencing were found (Fig. 1).

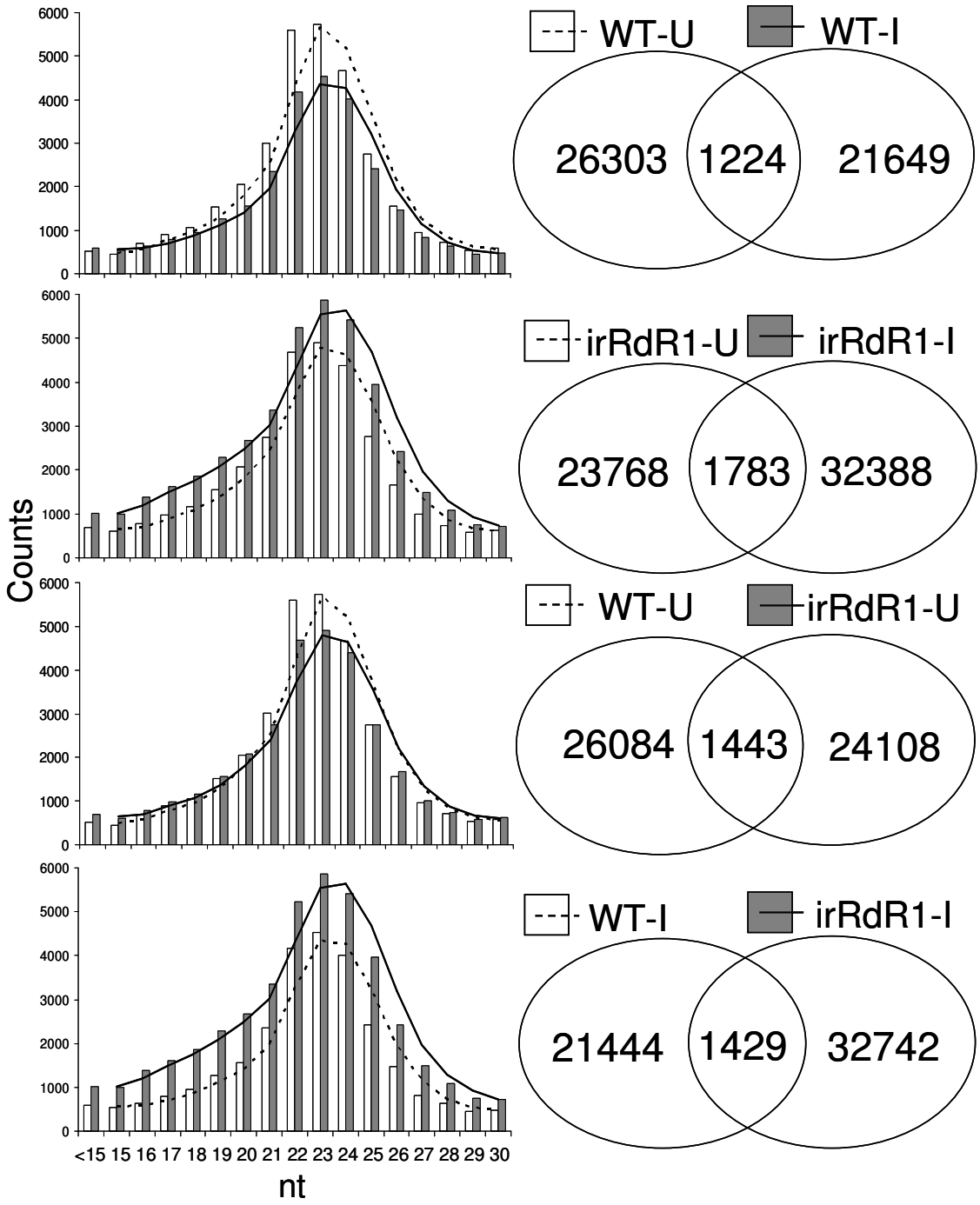

Fig. 1. Changes in the small-RNA transcriptome in response to OS elicitation in WT and *RdR1*-silenced *Nicotiana attenuata* plants. 454-sequencing of the smRNA component of the OS-elicited transcriptome reveal large-scale changes. Left panel depicts the size distribution of smRNAs in WT (open bars) and irRdR1 (solid bars) plants. 22-25 nt smRNAs are the most abundant size class of the smRNA-transcriptome. Line graph represents the moving average for WT (broken lines) and irRdR1 (solid lines) plants. Right panel: Venn diagrams depict 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.

Forty-three percent of the 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, Table S4).

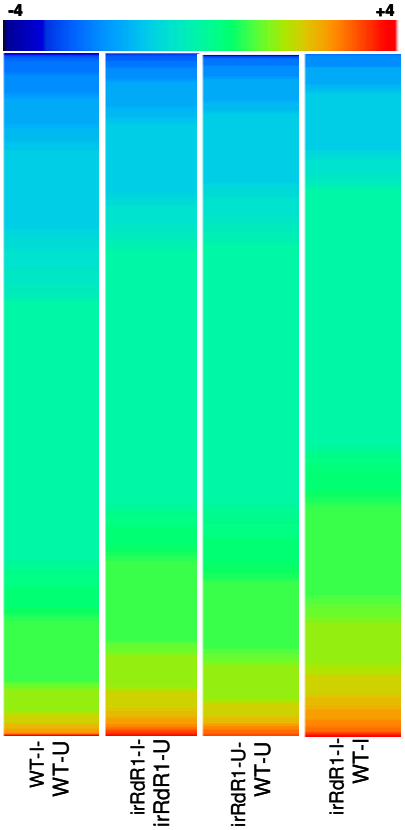

Fig. 2. Heat map of OS- and genotype-associated changes in abundance of commonly expressed smRNAs. Abundance threshold ratios were >1.5 or <0.67 and expressed on log_2-transformed scale. More than 50% of the commonly expressed smRNAs were differentially regulated.

The appearance of so many new smRNAs is consistent with previous observations that *RdR1* transcripts increase dramatically in response to OS elicitation (31). Silencing *RdR1* also had dramatic effects on the smRNA transcriptome. Compared to levels in untreated WT plants, levels of 21-24 nt smRNAs in untreated irRdR1 plants were reduced by 12%; but there was little overlap (1443; 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, Table S5). 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 1783 commonly expressed smRNAs in RdR1-silenced plants, 554 were up-regulated and 442 were down-regulated (Fig. 2, Table S6). Interestingly, only 1429 sequences were common to both OS-

elicited WT and irRdR1 plants; of these, 254 were down-regulated and 555 up-regulated in OS-elicited irRdR1 plants (Fig. 2, Table S7).

We annotated the *N. attenuata* smRNAs by BLASTing them against the NR-DB at NCBI and found 34% of the sequences to have matches in the database; these matches were classified into 7 categories of structural, regulatory and coding RNAs (Table S2), suggesting that sequence conservation across different plant species at the (sm)RNA levels is probably not more than 40%. Next, we identified the conserved miRNAs by comparing the smRNAs from *N. attenuata* to all the known miRNAs present in the miRBase Sequence Database at the Sanger Institute. A total of 41 miRNAs distributed in 17 families were identified (Table S3, S Fig. 9). Of these 41 miRNAs, 11 miRNAs were present in all 4 treatment groups, and 9 miRNAs were differentially regulated between the OS-elicited- WT and irRdR1 genotypes (Fig. 3).

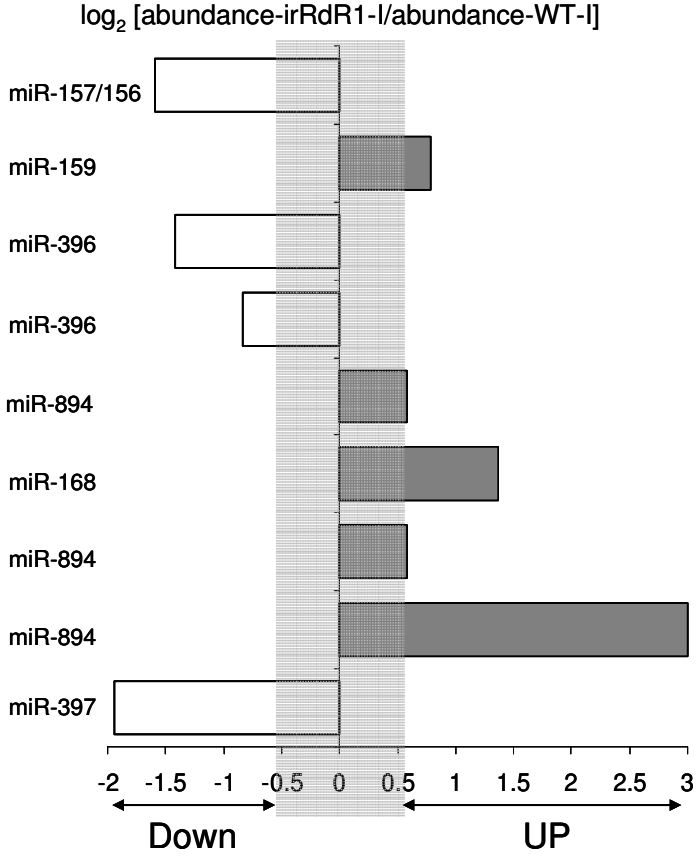

Fig. 3. Nine miRNAs were differentially regulated in response to OS elicitation and *RdR1* silencing. Two criteria were used to determine the differential regulation of miRNAs: (1) the miRNAs should be present in both treatment groups of OS-elicited WT (WT-I) and irRdR1 (irRdR1-I) genotypes, (2) the log-transformed abundance ratio of irRdR1-I and WT-I should be <- 0.58 (down-regulated) or > 0.58 (up-regulated). All the members of a given family were regulated in the same direction.

It was apparent that all the members of a given family were regulated in the same direction upon *RdR1*-silencing, e.g. all 3 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 the smRNA targets in genes would be related to phytohormone signaling (analyzed below), because silencing *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 Tables S8 and S9, respectively.

Silencing *RdR1* influences OS-elicited JA signaling

Silencing *RdR1* expression made *N. attenuata* plants highly susceptible to herbivore attack, in part because irRdR1 plants were unable to increase nicotine levels (31). Because nicotine induction requires intact JA signaling (37) and is negatively regulated by ethylene after OS elicitation and herbivore attack (42, 43), we examined OS-elicited phytohormones in *RdR1*-silenced plants. Our bioinformatic analysis revealed that many smRNAs had the potential to target genes related to phytohormone signaling (Tables S8, 9). To determine if 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 6 of the 8 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 stereo-specific 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, which is essential for the biogenesis of GLVs. These can act as indirect defenses by attracting predators and feeding stimulants (38, 50). No differences in the dynamics of *HPL* transcripts were observed (S Fig. 10). That levels of HPL remain unchanged suggests that GLV production does as well, especially given that no differences in predation rates were observed between the WT and irRdR1 plants when they were studied in nature (31). On the other hand, 45 min after OS elicitation *NaLOX3* levels were only 4 times their constitutive levels in irRdR1 plants, compared to 8 times those in WT plants (Fig. 4); in other words, the OS-elicited increase in *NaLOX3* transcript levels in irRdR1 plants was only half 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 involved in direct defense (38). Like those of *LOX* genes, the induced transcript levels of the *AOS* gene were also reduced by one-third in irRdR1 compared to WT plants (Fig. 4).

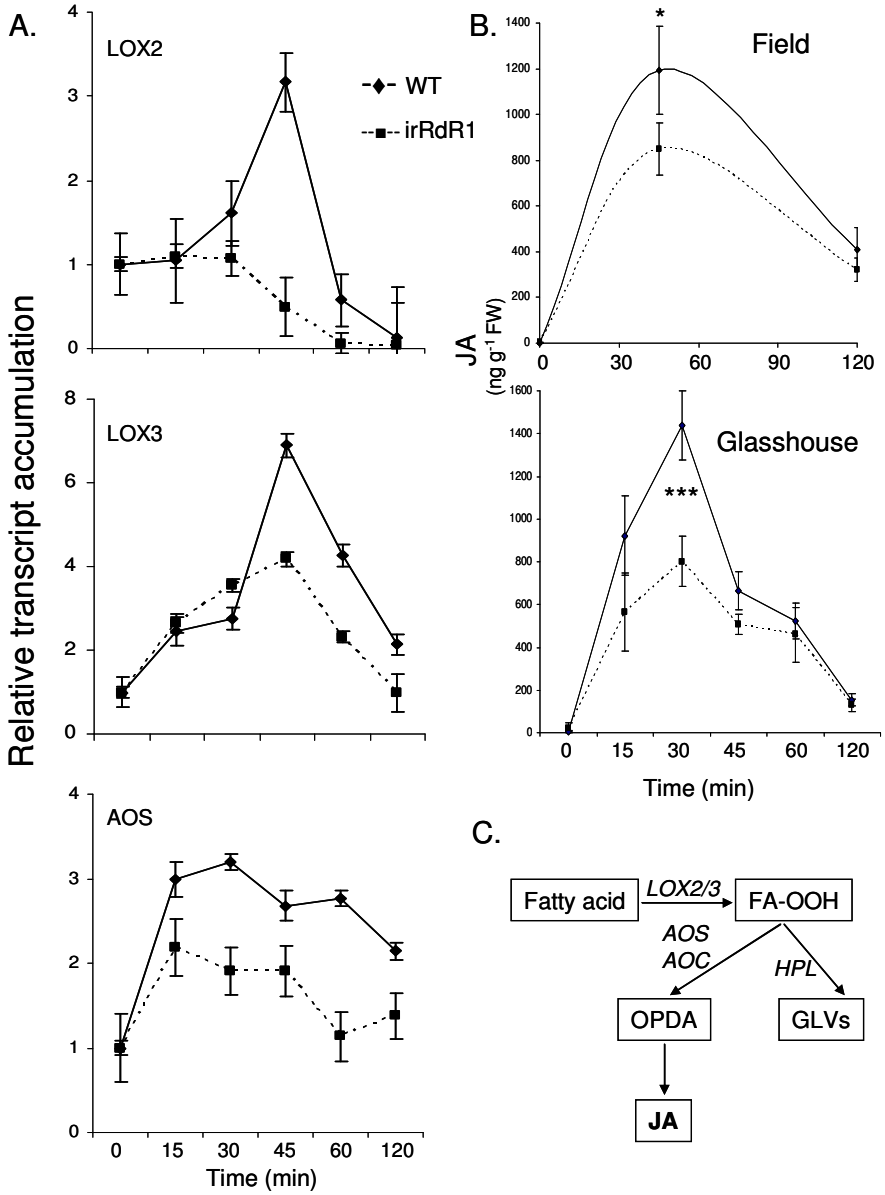

Fig. 4. Silencing *RdR1* suppresses OS-elicited changes in jasmonic acid (JA) biosynthesis. (**A**) Time course analysis of the dynamics of transcripts of genes required for JA biosynthesis in irRdR1 and WT plants using q-PCR. At time 0, plants were OS-elicited by creating puncture wounds with a fabric pattern wheel and immediately treating the wounds with 20 µL *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 *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.

Reduced transcript accumulations of *LOX*2/3 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, 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 30 min after OS elicitation (Fig. 4; repeated measures ANOVA, $F_{1,46}$=22.94, P<0.005).

Another oxylipin known to play a role in defense signaling is JA-Ile (36). The maximum OS-induced JA-Ile/Leu levels are typically only 10-20% of the maximum induced JA levels (36). JA-Ile/Leu levels were unaltered in field-grown (S Fig. 11; paired *t*-test; n=4 pairs; t=0.46; P>0.05) as well as in glasshouse-grown plants after OS elicitation in irRdR1 plants (S Fig. 11; 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 (S Fig. 11) of threonine deaminase (*TD*), which supplies the Ile at the attack site required for JA-Ile biogenesis (36), and of two members of the *JAR* gene family, which adenylates JA so that Ile can be conjugated to JA to produce JA-Ile (36, 51). In WT plants, *TD* levels reached their maximum 45-60 min after elicitation and were maintained 120 min later. High levels of *TD* expression from 6 to 30h after OS elicitation were observed in an earlier study of WT *N. attenuata* plants (35). But in *RdR1*-silenced plants, *TD* was rapidly elicited just 30 min after OS treatment, and levels started to decline 60 min after OS treatment (S Fig. 11). This indicated Ile was available from early on to serve as a substrate for JA-Ile conjugation in irRdR1 plants. In order 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 (S Fig. 11). This early elicitation of *TD* and *JAR6* (despite *JAR4* levels in WT plants being three 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; as 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 (51).

In addition, we studied the accumulation of *CO11* transcripts, since this F-box protein plays a central role in herbivore resistance by mediating JA-Ile perception (52). 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 (53). 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 (S Fig. 11), which suggests that if differences in *COI1* are responsible, the regulation occurs post-translationally.

Silencing *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; and 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 ethylene biosynthesis genes in *RdR1*-silenced plants. We studied an *ACS* gene (*ACS3a*) and an *ACO* gene, both of which are known to be elicited by OS and to function in herbivore-induced ethylene biosynthesis (41). The transcript levels of the *ACS3a* gene in irRdR1-silenced plants were lower than those in WT plants but attained their highest value within 15 min of OS induction compared to 45 min in WT plants (Fig. 5). Levels of the *ACO3* were higher and more rapidly elicited in *RdR1*-silenced than in WT plants (Fig. 5). Silencing *ACO3* expression in *N. attenuata* results in the strongest reductions in OS-elicited ethylene production (41), underscoring its importance in herbivory-elicited ethylene biosynthesis.

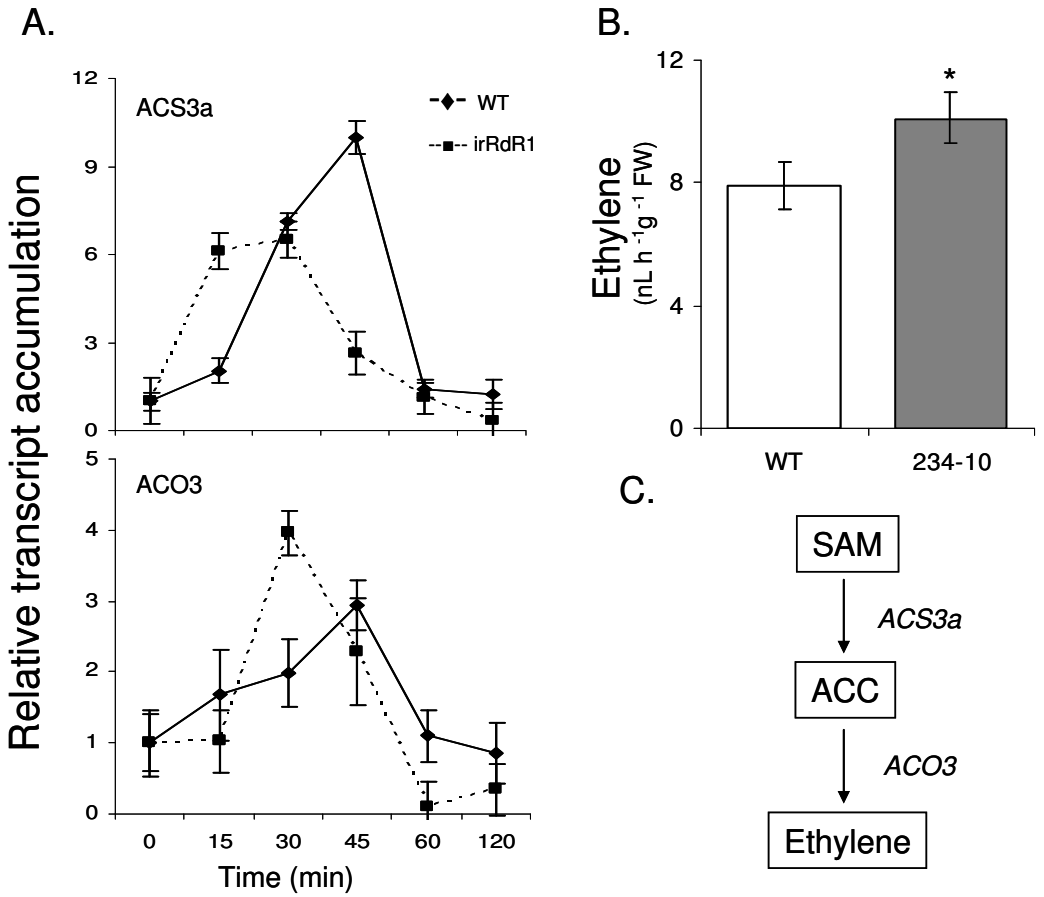

Fig. 5. Ethylene biosynthesis is also affected by *RdR1* silencing. (**A**) OS elicitation dynamics of ethylene biosynthetic genes in irRdR1 and WT plants using q-PCR. At time 0, plants were OS-elicited. Induced transcript accumulations were compared to constitutive levels at the time of elicitation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

We measured ethylene levels 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 (S Fig. 12) 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). Since silencing *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.

Silencing *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 both (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: rosette diameter (S Fig. 13; repeated measures ANOVA; $F_{1,48}= 2.71$; $P>0.05$), petiole length (S Fig. 13; ANOVA; $F_{1,8}= 1.45$; $P>0.05$), and stalk length (S Fig. 13; repeated measures ANOVA; $F_{1,48}= 1.45$; $P>0.05$), which confirmed previous results using field-grown plants (31). To determine if 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 *RCA* results in reduced photosynthetic rates and decreased plant biomass (35). No difference in the elicitation kinetics of the *RCA* was observed between WT and irRdR1 plants (S Fig. 14). 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 if the susceptibility of irRdR1 plants to insect herbivores was due to their attenuated JA levels, we performed complementation experiments (Fig. 6, S Fig. 15), in which JA was supplied to irRdR1 plants and neonate *M. sexta* larvae were allowed to feed for 11-12 days on JA- or water- (as a control) supplemented 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 plants are susceptible due to insufficient amounts of JA for normal defense activation.

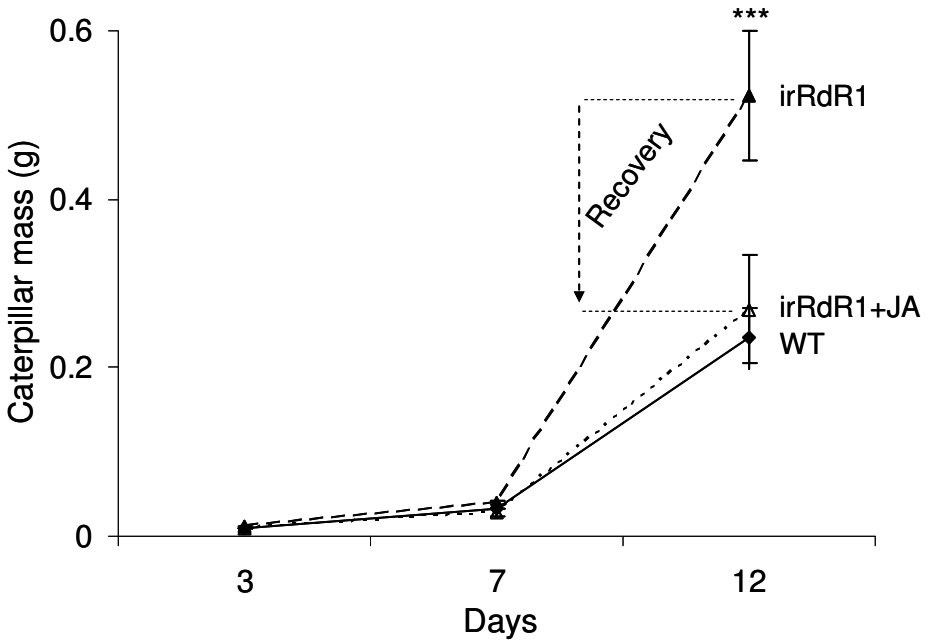

Fig. 6. The lack of resistance of irRdR1 plants to *M. sexta* larvae attack can be restored to WT levels by JA supplementation. Spraying irRdR1 plants with 1mM JA until runoff restores the resistance of *N. attenuata* plants as reflected in the mass gain of *M. sexta* larvae. ***Significantly different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

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 defense system against viral and transposon invaders (48). 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*, an ecological model plant (54), but also elucidate the dynamics of smRNA transcriptome when *RdR1* is silenced and when WT and *RdR1*-silenced 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 *NaRdR1* de-regulated 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 *RdR1*-silenced plants is an indirect effect of changes in photosynthesis and growth.

JA and ethylene play a central role in the induced defenses that are elicited after *M. sexta* larvae attack (Fig. 7A).

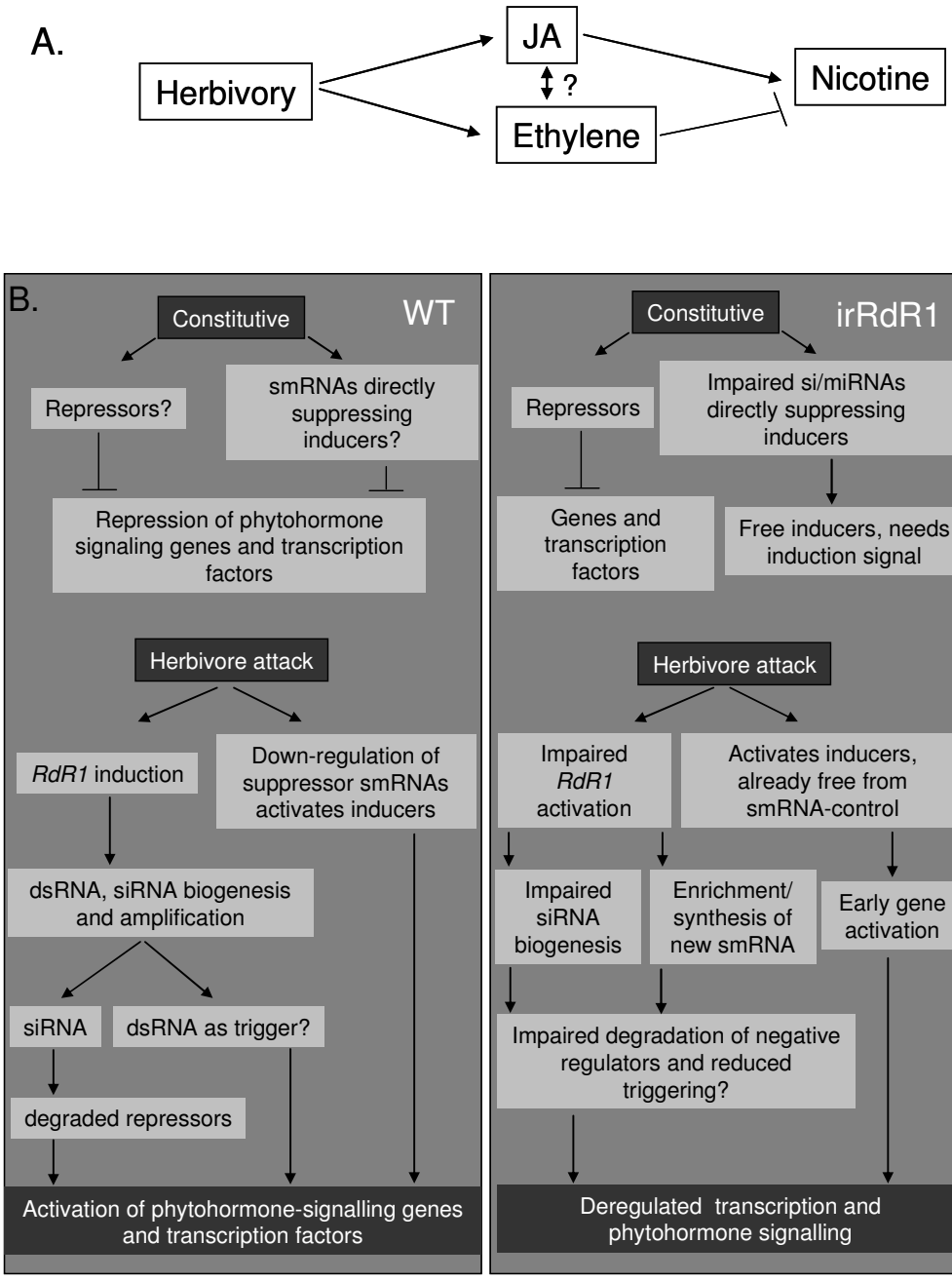

Fig. 7. A model of phytohormone and smRNAs function in plant defense responses against herbivores. (**A**) Interplay of JA and ethylene fine-tunes nicotine responses after herbivory. (**B**) Schematic representation of a model for *RdR1*-mediated phytohormone signaling after herbivore attack. Left panel applies to WT plants with intact small RNA machinery. siRNAs could increase transcript accumulation of genes after herbivore attack in two ways: either they degrade the negative regulators, or they directly increase transcript accumulation. Right

panel applies to *RdR1*-silenced plants that generate insufficient smRNAs as well as several new smRNAs that are enriched in an *RdR1*-independent manner. This could result in insufficient degradation of the repressors or improper gene activation.

Attack from this herbivore elicits a rapid JA burst, which is associated with increases in the transcript levels of JA-biosynthesis genes (37, 38). In *N. attenuata*, this JA burst is required to elicit chemical defenses, of which nicotine is one of the most important (45). *M. sexta* attack also elicits an ethylene burst which attains maximum levels after the JA burst has waned and negatively regulates nicotine production (43), presumably to save resources, prevent auto-toxicity, and prevent this nicotine-tolerant herbivore from sequestering nicotine for defense against its own natural enemies (45). 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 (Fig. 7A).

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) prevent the genes from being expressed when the plants are not being attacked (Fig. 7B). The rapid elicitation of *RdR1* (31) could generate and amplify the smRNAs that degrade the repressor mRNA(s) and activate the phytohormone-signaling cascade. The most promising “repressor” candidate is *COI1* or an unknown protein whose degradation is mediated by this F-box protein. *COI1* plays a central role in JA signaling: by ubiquitin-mediated protein degradation, it regulates JA signaling (52, 55). 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 (40), and compared to WT plants they had reduced levels of elicited JA but higher JA-Ile/Leu levels (53). 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 has been found to increase transcript accumulation, most recently in humans by Li *et al.* (56), who 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 (57), so might si/miRNAs. Similarities between transcription factors and miRNAs have recently been highlighted (58). The rapid elicitation of the *RdR1* gene by OS, the susceptibility of irRdR1 plants to herbivores (31), and the insufficient elicitation of genes related to phytohormone signaling along with the de-regulation of phytohormone signaling (Figs. 2, 3 and S4) 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, as 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, and present a simplified model for how smRNAs could mediate these changes (Fig. 7B).

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 smRNAs has been limited to comparing constitutive states of different *RdR* and *DCL* mutants (46, 47). When the smRNA profiles of WT and *RdR2*-mutated *Arabidopsis* were compared (47), many new smRNAs were found as were many up-regulated miRNAs. This suggests that silencing

*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 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 (59), which can in turn reduce insect performance. *N. attenauta* may employ similar strategies, for example, *RdR1*-dependent smRNAs or their dsRNA precursors may help protect plants by targeting genes in the insect midgut, as host-derived dsRNAs are capable of silencing targets in insects (59). Further research into these molecular mechanisms will help us appreciate the ecological sophistication that underlies the arms race in plant-herbivore interactions.

Methods

Plant and insect materials and treatments, qPCR analysis, phytohormone analysis, photosynthetic measurements, caterpillar assays and smRNA sequencing with “454” are as described in Supporting methods.

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References

1. Walling, L. L. (2000) *Journal of Plant Growth Regulation* **19,** 195-216.
2. Korth, K. L. (2003) *Genome Biology* **4,** 221.
3. Voelckel, C. & Baldwin, I. T. (2004) *Ecology Letters* **7,** 770-775.
4. Ralph, S. G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J. A., Nelson, C. C., Butterfield, Y. S. N., Kirkpatrick, R., Liu, J., Jones, S. J. M., Marra, M. A., Douglas, C. J., Ritland, K. & Bohlmann, J. (2006) *Plant Cell and Environment* **29,** 1545-1570.
5. Ralph, S., Oddy, C., Cooper, D., Yueh, H., Jancsik, S., Kolosova, N., Philippe, R. N., Aeschliman, D., White, R., Huber, D., Ritland, C. E., Benoit, F., Rigby, T., Nantel, A., Butterfield, Y. S. N., Kirkpatrick, R., Chun, E., Liu, J., Palmquist, D., Wynhoven, B., Stott, J., Yang, G., Barber, S., Holt, R. A., Siddiqui, A., Jones, S. J. M., Marra, M. A., Ellis, B. E., Douglas, C. J., Ritland, K. & Bohlmann, J. (2006) *Molecular Ecology* **15,** 1275-1297.
6. Reymond, P., Bodenhausen, N., Van Poecke, R. M. P., Krishnamurthy, V., Dicke, M. & Farmer, E. E. (2004) *Plant Cell* **16,** 3132-3147.
7. Schmidt, D. D., Voelckel, C., Hartl, M., Schmidt, S. & Baldwin, I. T. (2005) *Plant Physiol* **138,** 1763-1773.
8. Qu, N., Schittko, U. & Baldwin, I. T. (2004) *Plant Physiology* **135,** 539-548.
9. Voelckel, C. & Baldwin, I. T. (2004) *Plant J* **38,** 650-663.
10. Howe, G. A., Lightner, J., Browse, J. & Ryan, C. A. (1996) *Plant Cell* **8,** 2067-2077.
11. Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J. & Solano, R. (2003) *Plant Cell* **15,** 165-178.
12. Ryan, C. A. & Pearce, G. (2003) *Proceedings of the National Academy of Sciences of the United States of America* **100,** 14577-14580.
13. Halitschke, R. & Baldwin, I. T. (2004) *Journal of Plant Growth Regulation* **23,** 238-245.
14. von Dahl, C. C. & Baldwin, I. T. (2007) *Journal of Plant Growth Regulation* **(In Press)**.
15. Voinnet, O. (2002) *Current Opinion in Plant Biology* **5,** 444-451.
16. Bartel, D. P. (2004) *Cell* **116,** 281-297.
17. Mallory, A. C. & Vaucheret, H. (2004) *Current Opinion in Plant Biology* **7,** 120-125.
18. Baulcombe, D. (2004) *Nature* **431,** 356-363.
19. Pickford, A. S. & Cogoni, C. (2003) *Cellular and Molecular Life Sciences* **60,** 871-882.
20. Wassenegger, M. & Krczal, G. (2006) *Trends in Plant Science* **11,** 142-151.
21. Yoo, B. C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y. M., Lough, T. J. & Lucas, W. J. (2004) *Plant Cell* **16,** 1979-2000.
22. Allen, E., Xie, Z. X., Gustafson, A. M. & Carrington, J. C. (2005) *Cell* **121,** 207-221.
23. Schiebel, W., Pelissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H. L. & Wassenegger, M. (1998) *Plant Cell* **10,** 2087-2101.
24. Dalmay, T., Hamilton, A., Rudd, S., Angell, S. & Baulcombe, D. C. (2000) *Cell* **101,** 543-553.
25. Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J. B., Jouette, D., Lacombe, A. M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T. A. & Vaucheret, H. (2000) *Cell* **101,** 533-542.

26. Yang, S. J., Carter, S. A., Cole, A. B., Cheng, N. H. & Nelson, R. S. (2004) *Proceedings of the National Academy of Sciences of the United States of America* **101,** 6297-6302.
27. Yu, D. Q., Fan, B. F., MacFarlane, S. A. & Chen, Z. X. (2003) *Molecular Plant-Microbe Interactions* **16,** 206-216.
28. Alleman, M., Sidorenko, L., McGinnis, K., Seshadri, V., Dorweiler, J. E., White, J., Sikkink, K. & Chandler, V. L. (2006) *Nature* **442,** 295-298.
29. Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C. & Voinnet, O. (2003) *Embo Journal* **22,** 4523-4533.
30. Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. C. (2005) *Plant Physiol* **138,** 1842-1852.
31. Pandey, S. P. & Baldwin, I. T. (2007) *Plant J* **50,** 40-53.
32. McCloud, E. S. & Baldwin, I. T. (1997) *Planta* **203,** 430-435.
33. Halitschke, R., Schittko, U., Pohnert, G., Boland, W. & Baldwin, I. T. (2001) *Plant Physiol* **125,** 711-717.
34. Roda, A., Halitschke, R., Steppuhn, A. & Baldwin, I. T. (2004) *Molecular Ecology* **13,** 2421-2433.
35. Giri, A. P., Wunsche, H., Mitra, S., Zavala, J. A., Muck, A., Svatos, A. & Baldwin, I. T. (2006) *Plant Physiol* **142,** 1621-1641.
36. Kang, J. H., Wang, L., Giri, A. & Baldwin, I. T. (2006) *Plant Cell* **18,** 3303-3320.
37. Halitschke, R. & Baldwin, I. T. (2003) *Plant J* **36,** 794-807.
38. Halitschke, R., Ziegler, J., Keinanen, M. & Baldwin, I. T. (2004) *Plant J* **40,** 35-46.
39. Kessler, A., Halitschke, R. & Baldwin, I. T. (2004) *Science* **305,** 665-668.
40. Paschold, A., Halitschke R & Baldwin IT (2007) *Plant J* **51,** 579-91.
41. von Dahl, C. C., Winz, R., Halitschke, R., Kühnemann, F., Gase, K. & Baldwin, I. T. (2007) *Plant J* **51,** 293-307.
42. Winz, R. A. & Baldwin, I. T. (2001) *Plant Physiology* **125,** 2189-2202.
43. Kahl, J., Siemens, D. H., Aerts, R. J., Gabler, R., Kuhnemann, F., Preston, C. A. & Baldwin, I. T. (2000) *Planta* **210,** 336-342.
44. Steppuhn, A., Gase, K., Krock, B., Halitschke, R. & Baldwin, I. T. (2004) *PLoS Biol* **2,** 1074-1080.
45. Steppuhn, A. & Baldwin, I. (2007) *In A Schaller, eds, Induced Plant Resistance to Herbivory, Springer Wien, NewYork* ***In Press***.
46. Kasschau, K. D., Fahlgren, N., Chapman, E. J., Sullivan, C. M., Cumbie, J. S., Givan, S. A. & Carrington, J. C. (2007) *PLoS Biol* **5,** e57.
47. Lu, C., Kulkarni, K., Souret, F. F., MuthuValliappan, R., Tej, S. S., Poethig, R. S., Henderson, I. R., Jacobsen, S. E., Wang, W., Green, P. J. & Meyers, B. C. (2006) *Genome Res* **16,** 1276-88.
48. Molnar, A., Schwach, F., Studholme, D. J., Thuenemann, E. C. & Baulcombe, D. C. (2007) *Nature* **447,** 1126-1129.
49. Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A. & Enright, A. J. (2006) *Nucleic Acids Res* **34,** D140-4.
50. Kessler, A. & Baldwin, I. T. (2001) *Science* **291,** 2141-2144.
51. Wang, L., Halitschke, R., Kang, J. H., Berg, A., Harnisch, F. & Baldwin, I. T. (2007) *Planta***,** PMID: 17273867.
52. Li, L., Zhao, Y. F., McCaig, B. C., Wingerd, B. A., Wang, J. H., Whalon, M. E., Pichersky, E. & Howe, G. A. (2004) *Plant Cell* **16,** 126-143.
53. Paschold, A., Bonaventure, G. & T, B. I. (2007) *Planta* **In Review**.

54. Baldwin, I. T. (2001) *Plant Physiol* **127,** 1449-1458.
55. Devoto, A., Nieto-Rostro, M., Xie, D. X., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M. & Turner, J. G. (2002) *Plant J* **32,** 457-466.
56. Li, L. C., Okino, S. T., Zhao, H., Pookot, D., Place, R. F., Urakami, S., Enokida, H. & Dahiya, R. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103,** 17337-17342.
57. Gottesman, S. (2002) *Genes & Development* **16,** 2829-2842.
58. Hobert, O. (2004) *Trends in Biochemical Sciences* **29,** 462-468.
59. Mao, Y. B., Cai, W. J., Wang, J. W., Hong, G. J., Tao, X. Y., Wang, L. J., Huang, Y. P. & Chen, X. Y. (2007) *Nat Biotechnol* **25,** 1307-1313.

2.3 Manuscript III

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Silencing RNA-directed RNA polymerase 2 (RdR2) increases *Nicotiana attenuata*'s susceptibility to UV in the field and in the glasshouse

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Summary

RNA-directed-RNA-polymerases (RdRs) are essential in siRNA biogenesis and appear to be functionally specialized. We examined the consequences of silencing *RdR2* in *Nicotiana attenuata* with a field release and transcriptional, 2-D proteomic and metabolite analyses. Na*RdR2*-silenced plants (irRdR2) had large reductions (46% of WT) in 22-24 nt small-RNAs and smaller reductions (35, 23, and 26% of WT) in the 19-21, 25-27, and 28-30 nt smRNAs, respectively. When planted into their native habitats in the Great Basin Desert, irRdR2 plants had impaired growth and reproductive output, which were associated with reduced levels of leaf phenolics (rutin and 4'chlorogenic acid) and *MYB* and *PAL* transcripts, but were unaffected in their herbivore resistance. These phenotypes were confirmed in glasshouse experiments but only when irRdR2 plants were grown with UV-B radiation. irRdR2 plants had WT levels of elicited phytohormones and resistance to *Manduca sexta* attack but when exposed to UV-B, had reduced growth, fitness, levels of *MYB* and *PAL* transcripts, and phenolics. Proteins related to protection against oxidative and physiological stresses, chromatin remodeling, and transcription were also down-regulated. Silencing the *MYB* gene by VIGS in WT plants reduced levels of *PAL* transcripts and phenolics, as it did in UV-exposed irRdR2 plants. Bioinformatic analysis revealed that genes involved in phenylpropanoid biosynthesis contained a large number of smRNA binding motives, suggesting that these genes are targets of smRNAs. We conclude that while Na*RdR2* transcripts are up-regulated in response to both UV-B and herbivore elicitation, the responses they regulate have been tailored to provide protection from UV-B radiation.

Introduction

Plants adapt to their habitats by dealing efficiently and flexibly with the plethora of stresses that they face. These stresses, which include biotic (microbes and herbivores) and abiotic (such as drought, salinity, and light) factors, require phenotypic and genotypic plasticity because the different stresses demand different solutions to optimize plant fitness. The energy plants require for all of their activities is derived from captured solar energy. The solar energy that plants use can contain substantial amounts of ultraviolet-B (UV-B: 280-320 nm). Levels of UV-B, which are rising due to continued depletion of the ozone layer, have been linked to increases in skin cancer (de Gruijl and Van der Leun, 1994) and decreases in crop yield (Searles *et al.*, 2001). The amount of UV-B radiation received on earth varies with

time and location, and is in direct proportion to elevation as a result of less air mass and greater atmospheric transparency at higher elevations (Madronich *et al.,* 1995). Therefore plants inhabiting higher altitudes require robust mechanisms to prevent UV-B damage, especially to their genomes. UV is a mutagenic agent that cross-links DNA, and plants mutated for specific DNA repair pathways are killed by even low UV-B doses (Landry *et al.,* 1997).

Exposure to high levels of UV-B radiation has profound effects on a plant's metabolism and physiology. For instance, *Picea asperata* seedlings exposed to high UV-B levels had inhibited growth, photosynthetic rates, stomatal conductance, transpiration rates, and accumulated fewer photosynthetic pigments (Yao and Liu, 2007). These effects on metabolism may be regulated at the transcriptional level. In UV-irradiated parsley cells, transcripts encoding enzymes for primary metabolism and flavonoid secondary product formation are selectively co-induced (Logemann *et al.,* 2000). When *Nicotiana longiflora* plants grown in the field under ambient UV-B were assayed with a microarray enriched in defense-related genes, 20% of the genes were differentially regulated (Izaguirre *et al.,* 2003). Maize plants exposed to elevated UV-B conditions differentially regulated 347 genes, with the greatest response observed in exposed tissues (Casati *et al.,* 2004). Several studies have revealed that exposure to UV-B results in large-scale transcriptional responses (in the irradiated tissues which are transmitted to shielded tissues), and metabolic changes as well. How these transcriptional responses are mediated molecularly remains largely unknown.

In maize, several genes implicated in chromatin remodeling are differentially expressed during UV-B exposure (Casati *et al.,* 2006). After these genes are silenced, the plants became hypersensitive to UV-B (Casati *et al.,* 2006). In response to UV-B exposure, the cell-cycle responses in fission yeast are highly delayed and develop a post-replication DNA damage checkpoint (Callegari and Kelly, 2006). Such results point to the existence of regulators that coordinate responses at several levels: the induced biosynthesis of "sunscreens," rapid photosynthetic remodeling, and the recruitment of genomic "guards" that maintain genomic integrity and remodel chromatin. A particular chromatin organization is necessary for appropriate gene regulation to occur. An RNA-directed RNA polymerase (*RdRP*; Pickford and Cojini, 2003) has been shown to be essential for transposon silencing and paramutations in maize (Alleman *et al.,* 2006, Woodhouse *et al.,* 2006), and in

heterochromatin formation in yeast (Sugiyama *et al.,* 2005). Taken together, these results suggest that *RdRps* may be involved in UV-B protection.

*RdRP*s or *RdR*s (Wassanager and Krezel, 2006) are essential components of the RNA-interference machinery. They have been reported in diverse organisms such as *Caenorhabditis elegans* (Sigen *et al.,* 2001), fungi (Cogoni and Macino, 1999), and plants (Dalmay *et al.,* 2000, Mourrain *et al.,* 2000) where they produce double-stranded RNA (dsRNA) essential for the biogenesis of small interfering RNAs (siRNAs). Based on sequence analysis, six *RdR*s are thought to exist in *Arabidopsis* (Wassanager and Krezel, 2006), but so far only three functionally distinct *RdR*s have been found in *Arabidopsis*, tomato (*Solanum lycopersicum*), and *Nicotiana* species (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000; Schiebel *et al.,* 1998; Yang *et al.,* 2004; Pandey and Baldwin, 2007). Although *RdR1* and *6* and their natural variants are known to play important roles in plants' defense against viruses and in post-transcriptional gene silencing mechanisms, the less-studied third *RdR* (*RdR2*) is involved in paramutation and transposon silencing (Chen *et al.,* 2004; Sugiyama *et al.,* 2005; Alleman *et al.,* 2006; Woodhouse *et al.,* 2006). In tobacco and *Arabidopsis*, *RdR1* is elicited by salicylic acid (SA) treatment and its role appears confined to viral defense (Yang *et al.,* 2004; Yu *et al.,* 2003). In native tobacco, *Nicotiana attenuata*, *RdR1* is elicited by herbivore-specific elicitors as well as by jasmonic acid (JA) and SA treatments, and mediates induced defense responses to insect attack (Pandey and Baldwin, 2007). *RdR6* is involved in post-transcriptional gene silencing and virus resistance pathways (Dalmay *et al.,* 2000; Mourrain *et al.,* 2000), and is required for cells to perceive the silencing signal but not to produce or transport it (Schwach *et al.,* 2005). In contrast to the relative wealth of information about *RdR1* and *6*, less is known about the molecular and ecological functions of the *RdR2*.

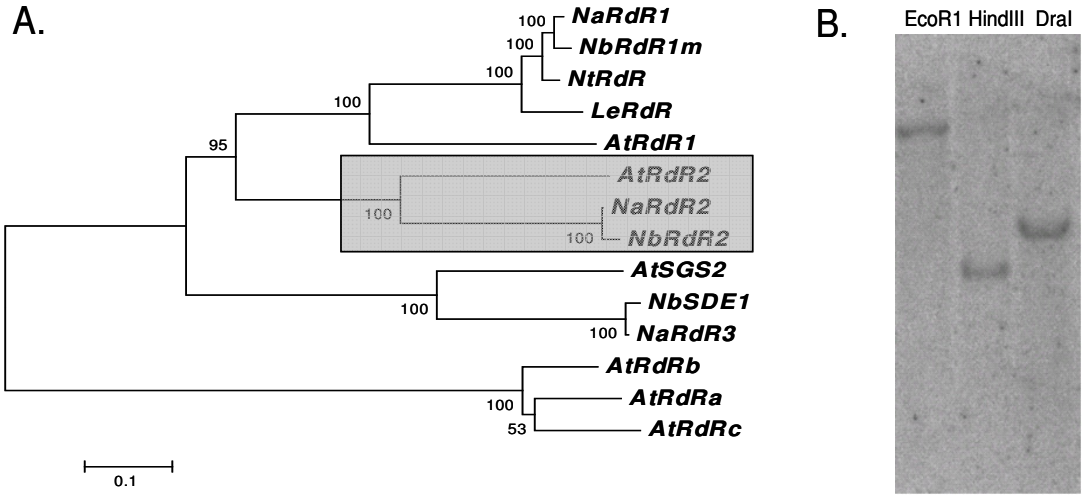
Here we explore the function of *RdR2* in an ecological model system, *N. attenuata,* an annual native of the southwestern United States which grows in post-fire environments by timing germination from its long-lived seed banks with fire-related germination cues (Preston and Baldwin, 1999). The plant can be found growing over a range of elevations from 200-3000m and is thus exposed to a range of UV-B fluences as well as unpredictable herbivore and pathogen populations and intra-species competition. To cope with these stresses, the plants have evolved a remarkable suite of plastic responses, some of which are commonly elicited (Izaguirre *et al.,* 2003 and 2007). Here we characterize the whole-plant function of the *RdR2* gene in *N. attenuata* by silencing its expression and comparing the performance of silenced

and WT plants when they are planted into the plants' native environment in the Great Basin Desert. When planted into their native habitats, irRdR2 plants exhibited a growth phenotype which was not observed when plants were grown in the glasshouse. Additional experiments identified UV-B as the environmental factor responsible for the growth phenotype and defects in the production of phenolics, which likely function as UV-B sunscreens, as the explanation for the reduced growth. The study provides an additional example of the value of a bottom-up approach in elucidating the function of *RdR*s.

Results

Isolation and characterization of RdR2 *from* N. attenuata

To isolate the complete coding sequence of the *N. attenuata RdR2* gene, we used a previously described PCR-based approach (Pandey and Baldwin, 2007). The Na*RdR2* gene has a high sequence similarity (>90% at nucleotide level) to its corresponding homologue in *N. tabacum* and *N. benthamiana* but little to no similarity to the other *RdRs* from closely related species. A phylogenetic tree was constructed after multiple-alignment of all the 6 *RdRs* of Arabidopsis, and other known *RdRs* of tobacco and tomato (Figure 1A). No positive clones were obtained after screening the cDNA library prepared from *N. attenuata* leaves following 24h of continuous *Manduca sexta* attack, indicating low levels of expression of *RdR2* (Yang *et al.,* 2004).



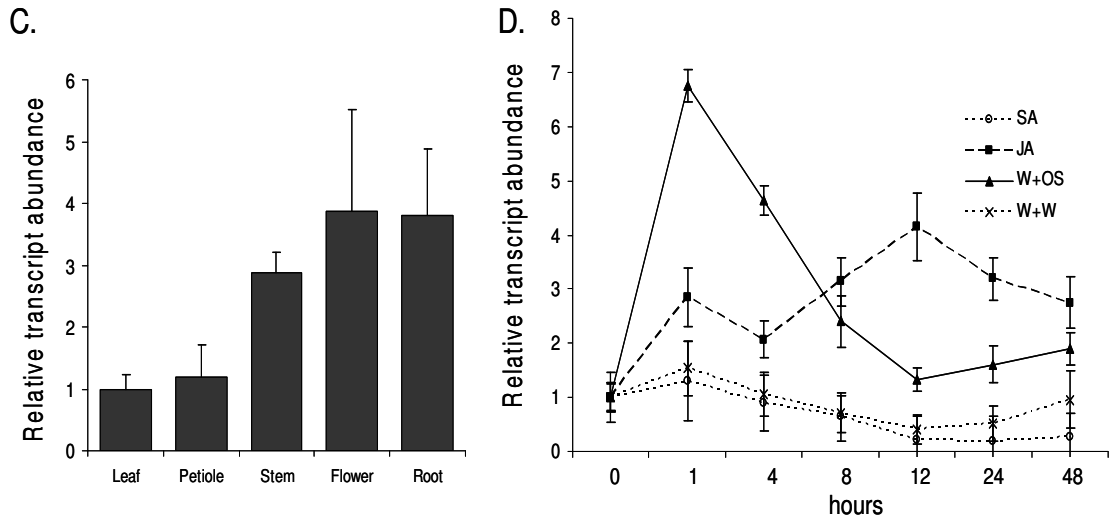


Figure 1. Characterization of *RdR2* from *N. attenuata*
(A) Phylogenetic analysis of Na*RdR2* shows its close relation to *RdR2*-orthologs from other species than to the other *RdR* members. Distance values are calculated according to neighbor-joining method with 1000 bootstraps. (**B**) DNA gel blot analysis reveals that endogenous *RdR2* gene is present in a single copy in *N. attenuata*. Relative levels of Na*RdR2* by qPCR analysis (**C**) in different tissues; and (**D**) in a time course experiment with different elicitors show that high levels of Na*RdR2* transcripts accumulate 1-4h after wounding and *Manduca sexta* oral secretion (OS) elicitation (straight lines and solid triangles; Repeated Measures ANOVA, $F_{1,52}$=12.81, P<0.01) and intermediate levels accumulate after 1mM JA is applied (broken lines, solid squares); only mechanical wounding (broken lines and crosses) or SA application (broken lines and open circles) has no effects on transcript accumulation. Induced transcript levels were compared to constitutive levels at the time of elicitation. The *ECI* gene which is not regulated under these conditions was used for internal reference in all the qPCR assays.

We performed the DNA gel blot analysis to determine the copy number of Na*RdR2* in the *N. attenuata* genome. Southern blot analysis showed that the Na*RdR2* gene is present as a single copy (Figure 1B); its transcripts were detected in all plant parts (Figure 1C) as shown by quantitative real-time PCR (qPCR) analysis.

To gain insight into the ecological processes in which Na*RdR2* may be involved, we characterized its expression in a time course experiment using qPCR and different elicitors in wild-type (WT) plants (Figure 1D). Elicitation with mechanical wounding followed by the immediate application of *M. sexta* oral secretions (OS) increased *RdR2* transcript accumulation by more than six-fold within 1h. Applying JA also triggered an increase in transcript accumulation, but transcript levels remained unchanged when plants were only wounded or treated with SA (Figure 1D). This suggests that *RdR2* may be involved in direct plant defense against herbivores or in processes that "share" the signals of herbivore defense such as UV-B exposure (Izaguirre *et al.,* 2003).

***Stably silencing* RdR2 *reduces small-RNA populations in* N. attenuata**

N. attenuata was transformed with a 350 bp Na*RdR2*-specific fragment in an inverted-repeat orientation by *Agrobacterium*-mediated transformation (Kruegel *et al.,* 2002) to produce plants stably silenced in their *RdR2* expression (irRdR2). Transformed plants were subjected to high-throughput phenotype screening (Kruegel *et al.,* 2002) and transgene incorporation in a single copy was verified by Southern blot analysis. Two independently transformed lines harboring a single insert (271-7 and 303-1; Figure S1) were analyzed for *RdR2* transcript levels after OS elicitation: neither accumulated any (Figure 2). *RdR2*-silenced plants were phenotypically similar to WT or empty vector (EV) plants (Figure S2) and no differences in their growth were observed when plants were grown in the glasshouse (Repeated Measures ANOVA; $F_{3,96}$=1.12, P>0.05).

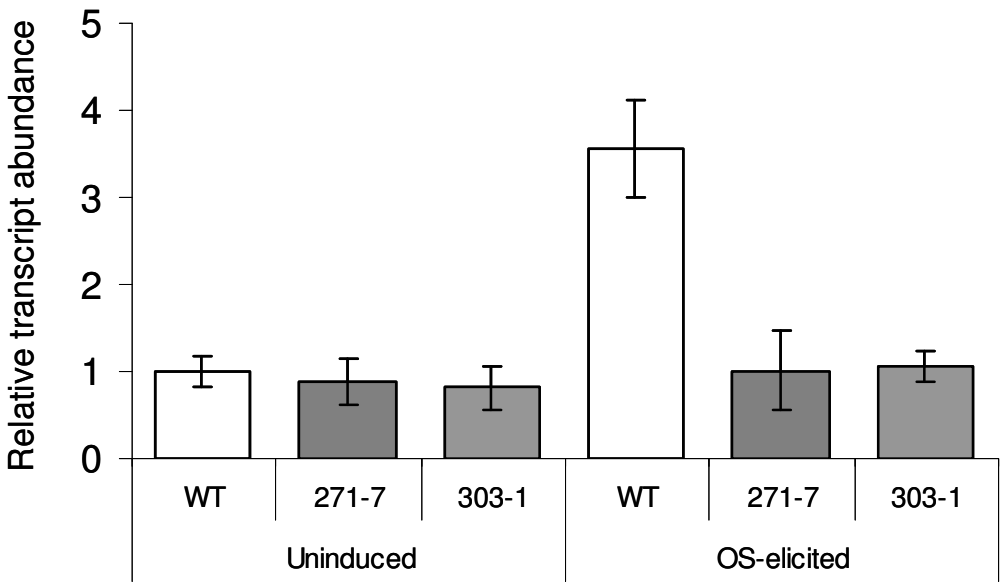
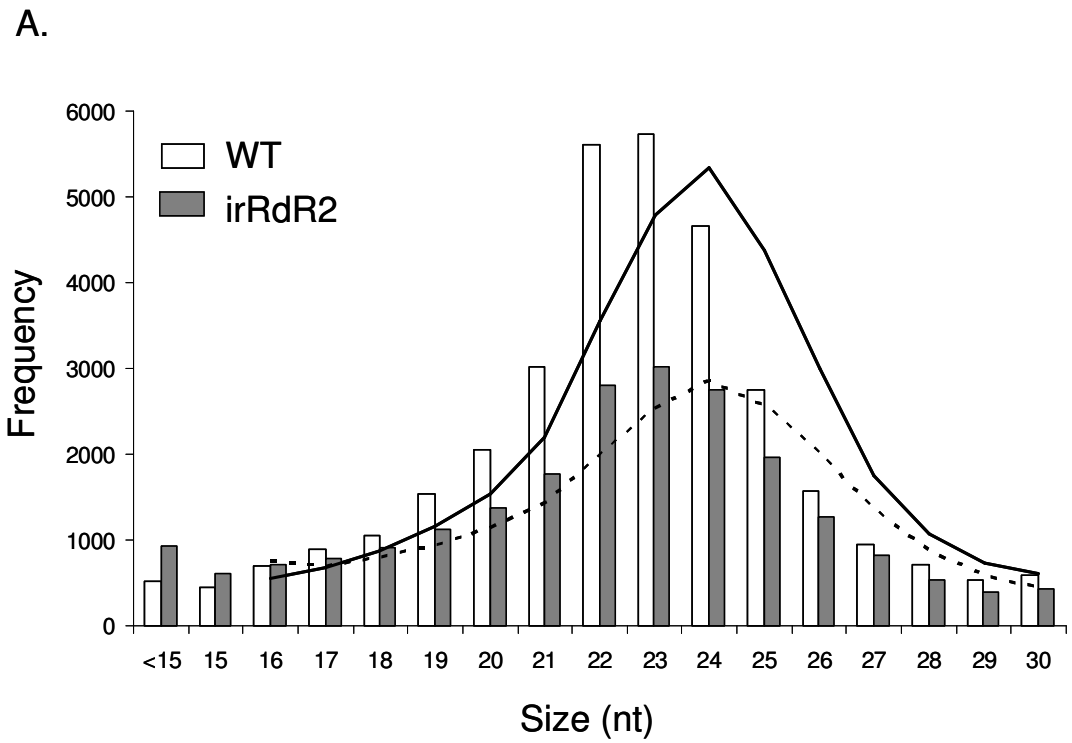


Figure 2. Na*RdR2* transcript accumulation in two independently transformed homozygous lines harboring a single copy of a fragment of Na*RdR2* in an inverted-repeat construct. Both the lines (271-7 and 303-1) failed to accumulate Na*RdR2* transcripts 1h after OS elicitation.

We examined the small-RNA (smRNA) components of the *N. attenuata* transcriptome to determine if their populations changed after *RdR2* silencing. 454-sequence analyses of the smRNA species (Kasschau *et al.,* 2007; Molnar *et al.,* 2007) in the range of 15-30 nucleotides (nt) from the WT and irRdR2 lines showed that silencing *RdR2* reduced the smRNA component of the transcriptome (Figure 3A). We generated 54,053 sequences of smRNAs in

the size range of 15-30 nt from the two genotypes, WT (32,769 sequences) and irRdR2 (21,284 sequences), for which both barcodes and 5' and 3' adapter sequences were identified and removed. Of these, 45,350 (27,527 for WT and 17,823 for irRdR2) were distinct sequences (where distinct refers to number of different sequences found within a set; Lu *et al.,* 2006). Unlike the results obtained from *Arabidopsis*, a 35% decrease in 19-21 nt smRNAs were observed in irRdR2 plants compared to WT *N. attenuata*. A 46% reduction was observed in 22-24 nt smRNAs, followed by 23 and 26% reductions in 25-27 and 28-30 nt smRNAs, respectively, in irRdR2 plants, compared to WT. Only 1,238 smRNAs were found in both genotypes (Figure 3B), indicating that silencing *RdR2* may have resulted in the accumulation of new smRNAs, as was also seen in *Arabidopsis* (Lu *et al.,* 2006). The largest reductions (46%) in the smRNA species were found in the 22-24 nt size class, which is consistent with work in *Arabidopsis*, in which 24 nt siRNAs are generated largely in an *RdR2*-dependent manner (Lu *et al.,* 2006; Kasschau *et al.,* 2007).



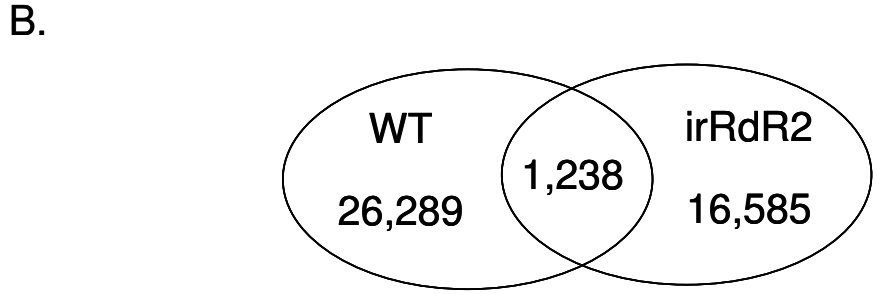


Figure 3. Small-RNAs in the transcriptome of WT and irRdR2 *N. attenuata* plants.
(**A**) Size distribution of the smRNAs in WT (open bars) and irRdR2 (solid bars) shows that the 22-24 nt smRNAs form the major component of the smRNA-transcriptome, which is strongly down-regulated in *RdR2* silenced plants. Other fractions such as 19-21 nt, 25-27 nt, and 28-30 nt were also down-regulated. Line graph represents the moving average for WT (solid lines) and irRdR2 (broken lines). (**B**) Venn diagram reveals that smRNA populations in the WT and irRdR2 plants have little similarity: of 44,112 distinct sequences, only 1,238 were found in both smRNA transcriptomes.

Loss-of-function analysis in native habitats

In order to determine the ecological relevance of *RdR2*, we examined the performance of both transformed lines in the plant's native habitat in the Great Basin Desert of southwestern Utah, USA, where an unpredictable native herbivore community, intra-species competition, and a high PAR and UV-B environment are among the main stresses the plants encounter. Total canopy area damaged by herbivores was recorded 10 days after plants were released in the field. 271-7 plants defended themselves against the native herbivore community as well as the WT plants did (Figure 4; n=10, paired t-test, t=0.61, P>0.05). Because comparisons in the laboratory had shown that WT plants behaved like the empty-vector transformants (EV) and no differences between the two were observed, we used WT plants as controls in the field experiments (Pandey and Baldwin, 2007). After 5 days, the growth of 271-7 plants was slower than that of the WT plants (n=9, paired t-test, t=2.91, P<0.05), but there were no differences in total damage between the two genotypes (n=9, paired t-test, t=1.26, P>0.05). After five additional days of growth, 271-7 plants continued to lag behind the WT controls (Figure 4; n=9, paired t-test, t=6.53, P < 0.0005), but no differences in amounts of damage from herbivores were observed (Figure 4; n=9, paired t-test, t=0.07, P>0.05). Total flower number was also reduced in 271-7 plants compared to WT plants (Figure 4; n=9, paired t-test, t=4.15, P<0.01). Similar patterns of reduced growth and reduced flower number, and comparable amounts of herbivory were seen for the plants of line 303-1 in comparison to their WT pairs (Figure 4). Additionally, we did not observe any

differences in bacterial or fungal diseases, or nematode infestations between irRdR2 or WT plants.

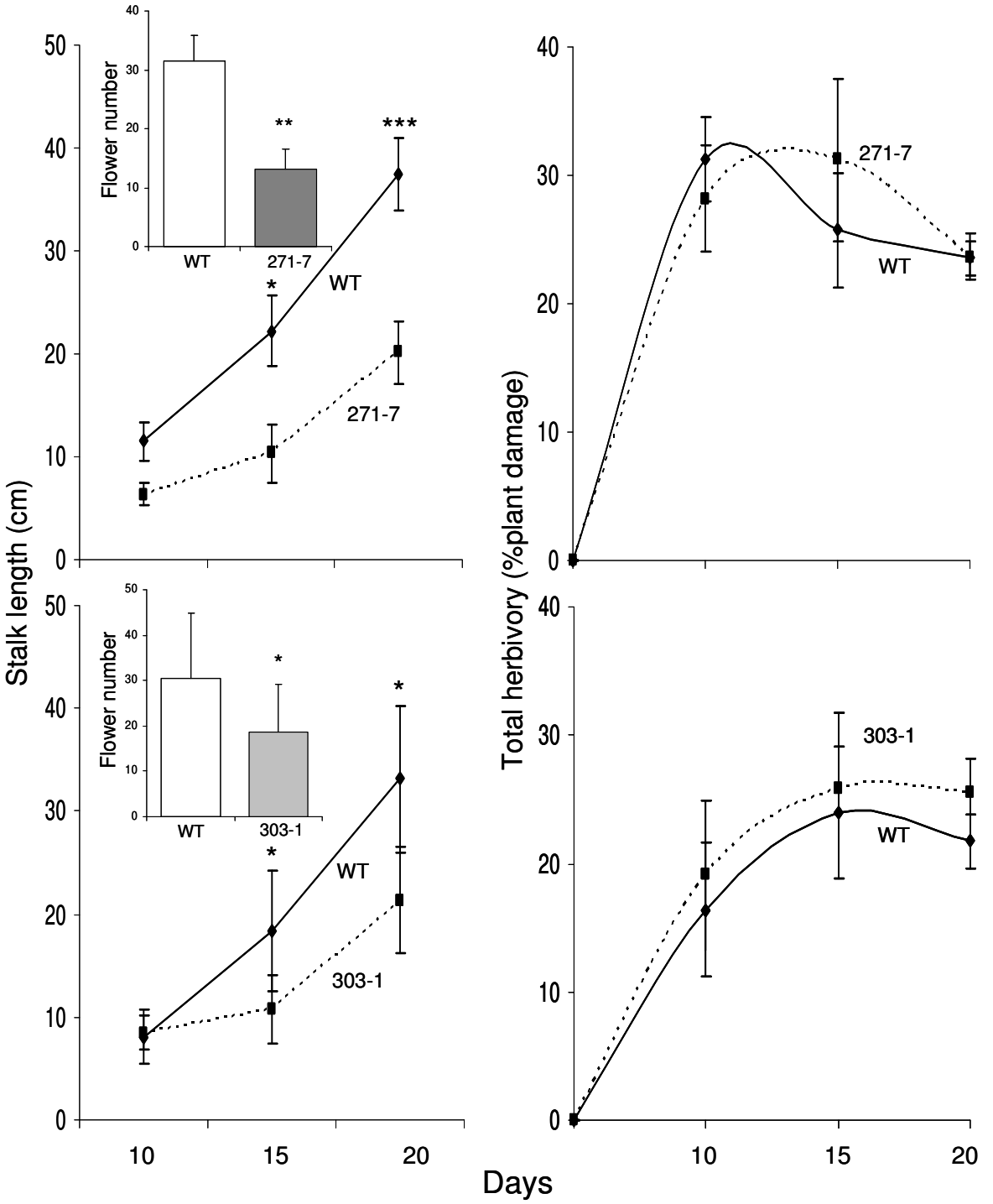


Figure 4. Performance of *RdR2*-silenced plants in native habitats.
All the irRdR2 plants (dotted lines) were individually paired with WT plants (solid lines). Undamaged plants were transplanted into the field at day 0. Cumulative damage (right panel) and plant growth (stalk length; left panel) were monitored three times, 10, 15, and 20 days after transplanting. irRdR2 plants grew slowly and had reduced flowers (inserts in the left panel), but defended themselves as well as the WT plants did against the

native community of herbivores attacking plants in these populations. Upper (271-7-WT) and the lower (303-1-WT) panel represent plant performance in two independently transformed line-WT pairs. * Significantly different at P<0.05; ** significantly different at P<0.01; *** significantly different at P<0.005.

In order to understand the reason for the reduced performance of irRdR2 plants in their natural habitat, we analyzed phytohormone profiles; 45 min after OS elicitation, no differences in JA, SA, or ABA levels were observed between WT and irRdR2 plants (Figure S3; paired t-test; n=5 pairs, t's=1.87, 0.24 and 0.28, respectively; P's>0.05). In contrast, two of the most abundant phenolics in *N. attenuata* leaves were strongly down-regulated: (1) rutin levels in 271-7 plants were >25% lower than those of WT (Figure 5; paired t-test, n=5 pairs, t=7.23, P<0.005). Similarly, a >22% reduction over WT was seen in 303-1 plants (Figure 5; paired t-test, n=4 pairs, t=10.72, P<0.005). (2) In 303-1 plants, 4'chlorogenic acid was 29% lower than in WT plants (Figure 5; paired t-test, n=4 pairs, t=3.47, P<0.05); a 17% reduction was observed in 271-7 plants (Figure 5; paired t-test, n=5 pairs, t=3.17, P<0.05).

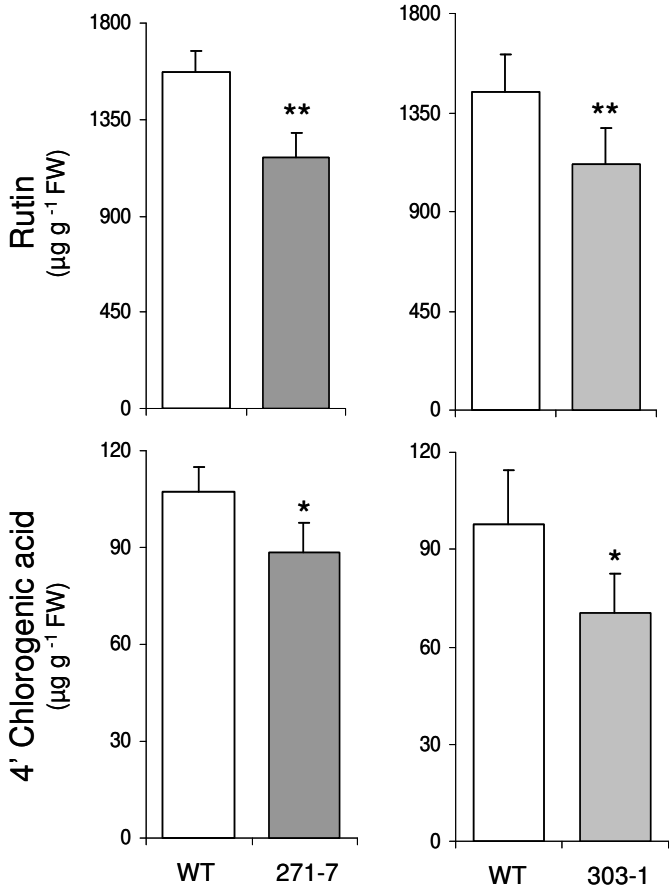


Figure 5. Phenolic compounds in field-grown irRdR2 and WT plants.
When irRdR2 plants were grown in their native habitat, they accumulated lower concentrations of rutin (upper panel) and 4'chlorogenic acid (lower panel). Left and right panels show data for WT-271-7 and WT-303-1 pairs, respectively. ** significantly different at P<0.01; * significantly different at P<0.05.

In the glasshouse, irRdR2 plants are more susceptible to enhanced UV-B but have the competitive abilities and herbivore resistance of WT plants

The reduced growth of irRdR2 plants in their native habitat suggested two possible explanations given that their resistance to herbivores and pathogens was not discernibly different from WT plants: silencing *RdR2* expression either made plants more susceptible to UV-B or inhibited their ability to compete with neighbors. In order to test the latter hypothesis, we planted 10 size-matched pairs of irRdR2 and WT plants into 2L pots and measured their growth at 5-day intervals. No differences in stalk lengths were measured (Figure S4; 271-7-WT, Repeated Measures ANOVA, $F_{1,58}$=2.63, P>0.05). In order to test the former hypothesis, we exposed irRdR2 and WT plants to enhanced UV-B radiation and compared their growth with that of plants of the same genotypes that had not been exposed to UV-B. When exposed to enhanced UV-B, irRdR2 plants grew less than WT plants (Figure 6A; Repeated Measures ANOVA; $F_{2,270}$=10.97, P<0.0005) and had lower above-ground dry mass (Figure 6A; ANOVA; $F_{2,36}$=10.38, P<0.0005) than their WT counterparts. The strongest effects of *RdR2*-silencing in supplemental UV environments were seen in the fitness estimates: irRdR2 plants produced fewer seed capsules (Figure 6A; ANOVA; $F_{2,36}$=12.56, P<0.0001) compared to WT plants. After being exposed to UV, irRdR2 plants also showed a rapid decrease in their chlorophyll contents compared to WT plants (Figure 6A; Repeated Measures ANOVA; $F_{2,270}$=60.41, P<0.0001). On the other hand, all plants grown without enhanced UV grew similarly (Figure 6B; Repeated Measures ANOVA; $F_{2,312}$=0.17, P>0.05). No differences in dry mass (Figure 6B; ANOVA; $F_{2,40}$=1.64, P>0.05), number of seed capsules (Figure 6B; ANOVA; $F_{2,40}$=0.06, P>0.05), or chlorophyll contents (Figure 6B; Repeated Measures ANOVA; $F_{2,312}$=2.56, P>0.05) were observed between the two genotypes grown without supplemental UV-B.

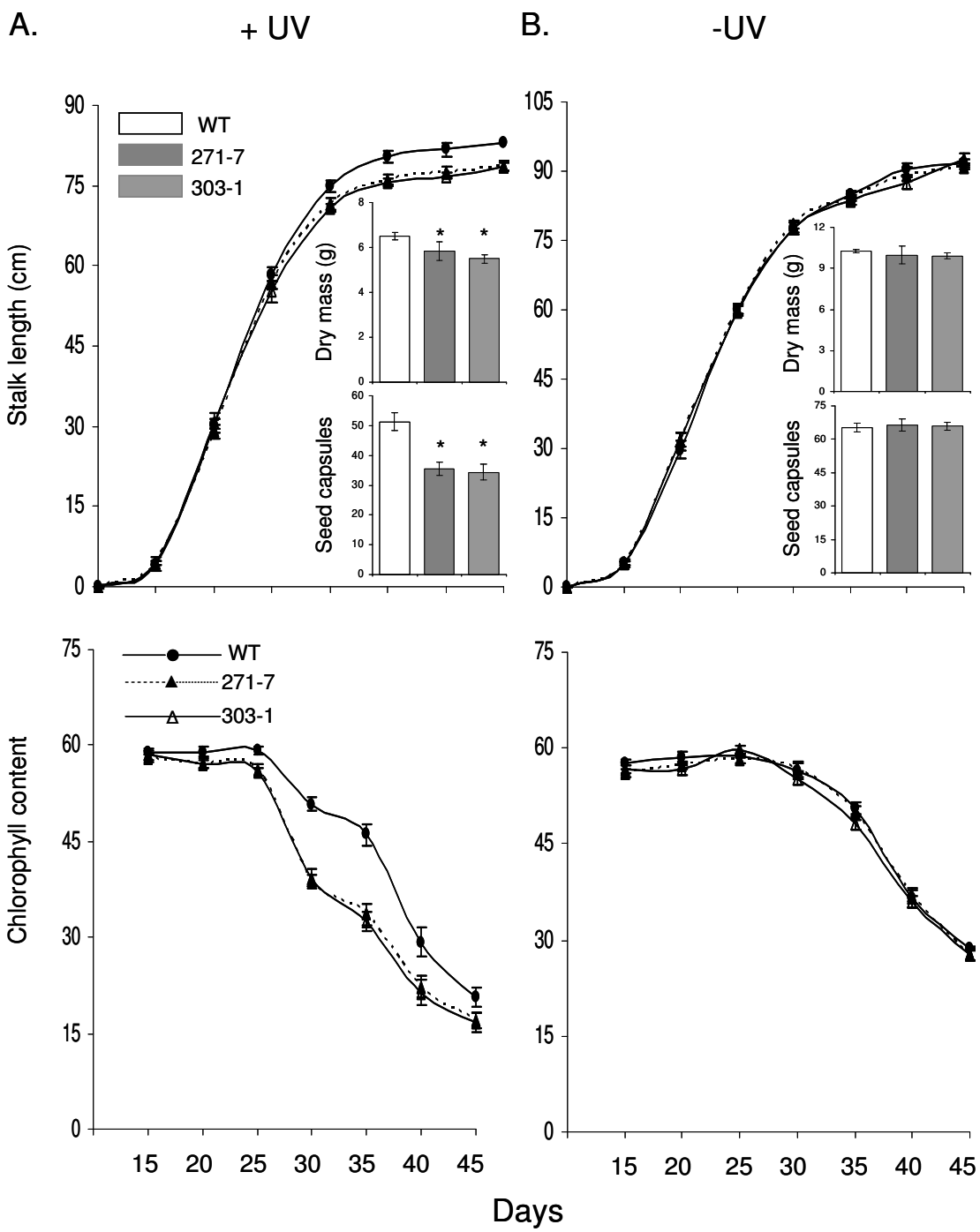


Figure 6. irRdR2 lines were highly susceptible to enhanced UV-B.
Left (**A**) and right (**B**) panels show the performance of irRdR2 and WT plants when grown with (+UV) and without (-UV) supplemental UV-B. Upper panel shows stalk length, above-ground dry mass, and seed capsule production, and the lower panel shows the degradation of chlorophyll in the UV-treated and untreated plants. When grown with supplemental UV-B, irRdR2 plants were shorter (A; Repeated Measures ANOVA, $P<0.0005$) and their chlorophyll content decreased rapidly (A; measured, in arbitrary units, by a SPAD-502 chlorophyll meter; Repeated Measures ANOVA, $P<0.0001$). Plants were transferred to York Chambers (day 0) where they were allowed to acclimatize for seven days after which the UV treatment was started. The first observation of stalk length and chlorophyll content was recorded 15 days after transfer to York Chamber. Subsequent observations were recorded at 5-day intervals. Total above-ground plant dry mass and seed capsule production

were measured at the end of the study, 48 days after plants were placed in the York chamber. * significantly different at P<0.05.

Finally, no differences were observed in the mass gain of *M. sexta* caterpillars on both irRdR2 genotypes or WT plants in glasshouse experiments (Figure S5; Repeated Measures ANOVA; $F_{2,182}$=0.45, P>0.05), suggesting that defense responses were unaffected by *RdR2* silencing, an inference consistent with the phytohormone analysis and analysis of herbivory in the field (Figs. S3 and 4).

Analysis of phenolics

Reduced performance in the field and in the UV-enhanced environment in the glasshouse, and reduced levels of phenolic secondary metabolites in field-grown irRdR2 plants suggested that irRdR2 plants were less protected from UV-B radiation. We measured total phenolics in WT and irRdR2 plants (Figure 7A) with the Folin-Ciocalteau assay (Imeh and Khokhar, 2002). UV-treated WT plants accumulated 25% more phenolics (ANOVA, $F_{3,20}$=5.42, P< 0.01) than either the untreated WT or irRdR2 plants, or the UV-treated irRdR2 plants (Fisher's PLSD < 0.005). UV-treated irRdR2 plants failed to accumulate more than constitutive levels. Additionally, we used HPLC to analyze the levels of rutin, which were reduced by >30% in irRdR2 plants (Figure 7B; n=4, paired t-test, t=8.48, P<0.005), and 4' chlorogenic acid which was reduced by >25% (Figure 7B; n=4, paired t-test, t=4.37, P<0.05). In summary, the analysis of total and specific phenolics revealed that irRdR2 plants produced fewer phenolics which likely function as "sunscreens," reducing the penetration of UV-B to leaves (Stratmann, 2003).

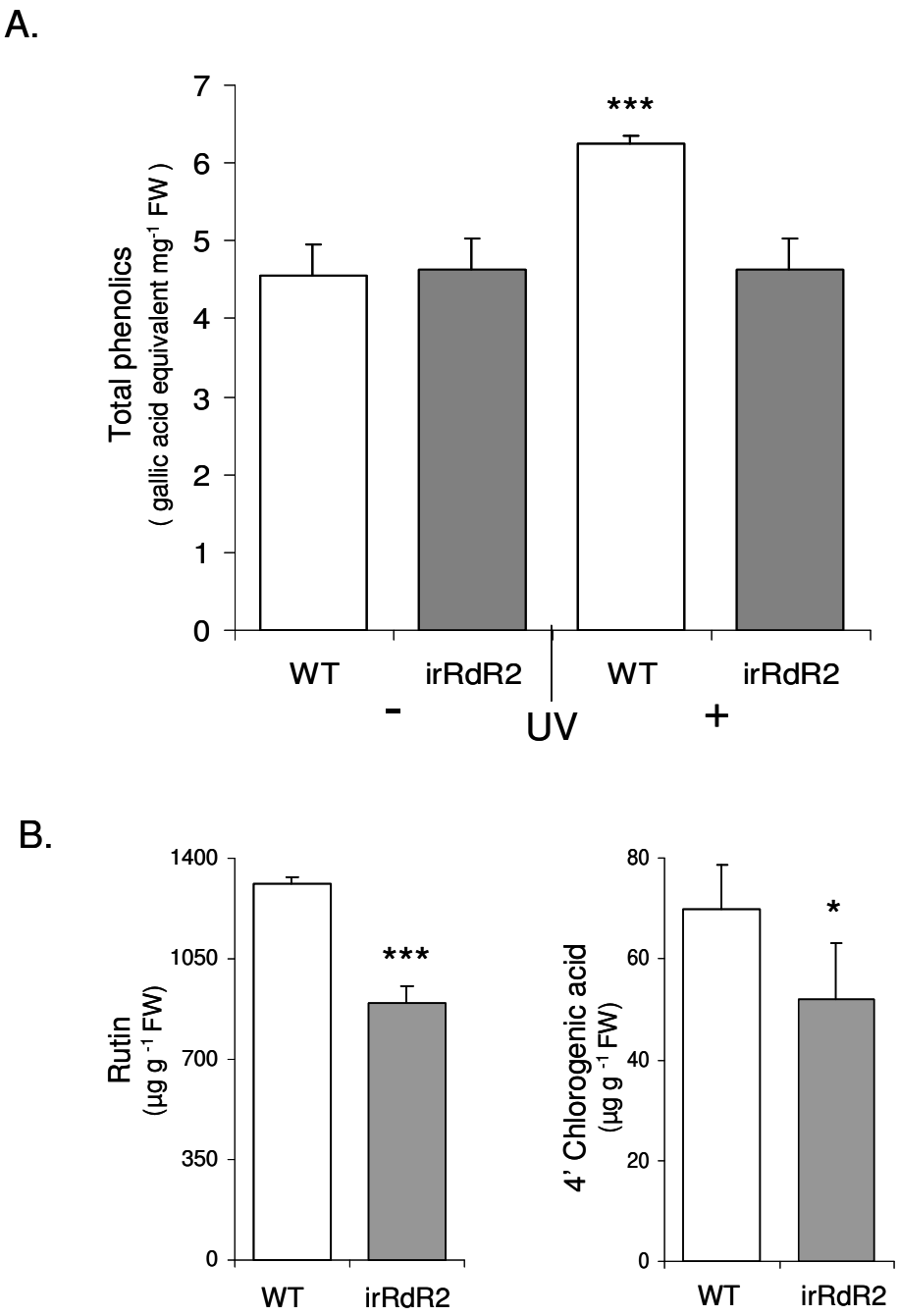


Figure 7. Silencing *RdR2* reduces elicited phenolic production after UV-B exposure.
(A) Total phenolics were measured with the Folin-Ciocaulteu assay and levels are represented as gallic acid equivalents per mg of plant tissue. (B) Analysis of two abundant phenolic compounds, rutin and chlorogenic acid, in plants exposed to UV-B in the glasshouse: levels of both the metabolites are reduced in irRdR2 plants, just as they are in irRdR2 plants grown in their natural habitat. *** significantly different at $P<0.005$; * significantly different at $P<0.05$.

Proteomic analysis of* RdR2-*silenced plants

We performed 2-dimensional gel electrophoresis to profile the effects of UV-B exposure on the proteome of WT and irRdR2 plants. MALDI and/or LC-MS/MS of 27 excised spots revealed that they were down-regulated in irRdR2 plants compared to their WT counterparts (Figure 8).

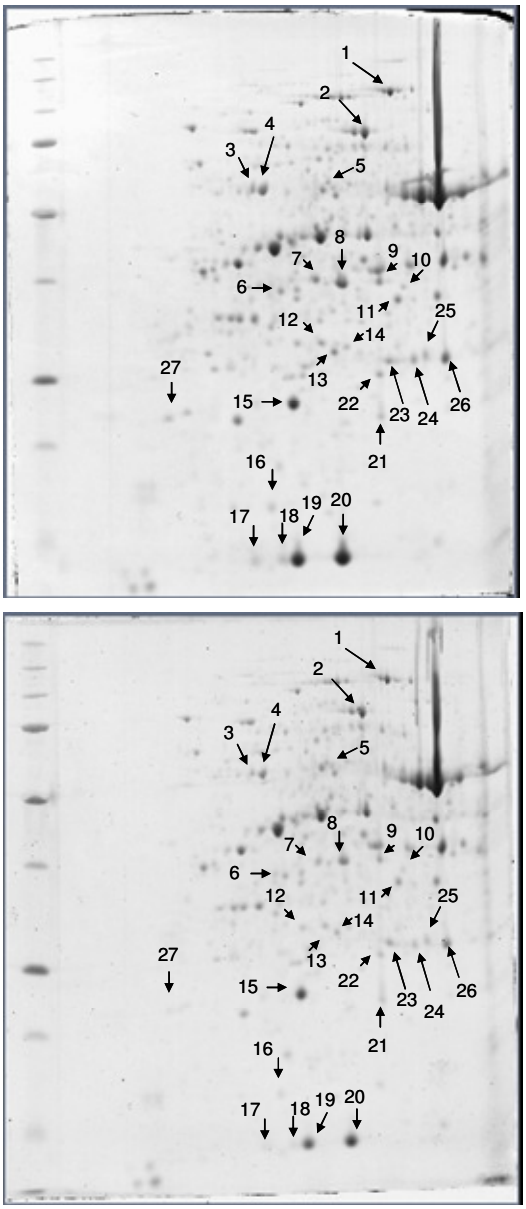


Figure 8. Profiling proteomic differences between WT and irRdR2 plants during UV-B exposure.
Upper and lower gels represent 2-dimensionally separated plant proteins of WT and irRdR2 plants, respectively. 250 µg of total plant protein was resolved on IPG strips of pH range 3 to 7 (isoelectric focusing), and further resolved on 12% SDS-PAGE gels and stained with Bio-Safe Coomassie G-250 stain. Arrows and numbers indicate protein spots on gels as

Many proteins were related to pathways essential for protecting plants from high UV and oxidative stress (Table 1). These 27 spots can be grouped into six broad and functionally related classes: (I) phenylpropanoid, oxidative stress/UV-B and photoprotection [MYB

Table 1. Identification of differentially expressed protein spots with MALDI-TOF and/or LC-MS/MS analyses

Spot No.	Name of identified protein	MALDI (Score / No. of peptides)	LC-MS/MS (Score / No. of peptides)	Sequence	Function/Pathway
1	Glycine dehydrogenase /Glycine decarboxylase complex subunit P	11.08 / 7	5.57 / 7	(R)IIGVSVDSSGK(Q) (K)IVAVGTDAK(G) (K)GNINIEELR(N) (K)IAILNANYMAK(R) (K)NTAGIEPEDVAK(R) (R)FCDALISIR(E) (R)VDNVYGDR(N)	Oxidative stress
2	Transketolase (TK)	12.18 / 3	12.16 / 12	(K)YPEEAAELK(S) (R)NLSQQNLNALAK(V) (K)ESVLPSSVTAR(V)	Phenylpropanoid
3	ATP synthase F1 sector	9.41 / 6	10.81 /8	(R)ADEISNIIR(E) (R)IEQYNR(E) (R)IAQIPVSEAYLGR(V) (R)GEISASEFR(L) (R)LIESAAPGIISR(R) (K)ATENQLAR(G) (R)ADEISNIIR(E) (R)IEQYNR(E)	Primary metabolism; physiological stress
4	ATP synthase subunit beta	12.18 / 5	12.12 / 19	(R)AVAMSATDGLTR(G) (K)LSIFETGIK(V) (R)EGNDLYMEMK(D) (K)DSGVINEENIAESK(V) (K)VALVYGQMNEPPGAR(M)	Primary metabolism; physiological stress
5	S adenosyl L homocysteine hydrolase AdoHcyase	12.18 / 2	10.93 / 11	(R)SEFGPSQPFK(G) (K)FDNLYGCR(H)	DNA methylation
6	p32 1 annexin	12.18 / 3	12.16 / 10	(R)ELTNDFEK(L) (K)AYSDNEVIR(I) (K)TIADEYQK(R)	Cell cycle and maturation
7	Fructose bisphosphate aldolase	12.17 / 7		(R)GILAMDESNATCGK(R) (R)LASIGLENTEVNQ(Q) (R)SAAYYQQGAR(F) (R)TVVSIPDGPSALAVK(E) (K)EAAWGLAR(Y) (R)TFEVAQK(V) (K)TWGGLPENVK(A)	Primary metabolism
8	Chloroplast aldolase	12.18 / 5	9.47 / 5	(-)SAAYYQQGAR(F) (-)SAAYYQQGAR(F) (R)TVVSIPDGPSALAVK(E) (K)EAAWGLAR(Y) (R)ALQNTCLK(T)	Primary metabolism
9	Fructose	12.18 / 4	7.1 / 4	(R)LASIGLENTEANR(Q)	Primary

	bisphosphate aldolase			(K)EAAWGLAR(Y) (R)ALQNTCLK(T) (K)YTGEGESEEAK(E)	metabolism
10	Phenylcoumaran benzylic ether reductase	12.18 / 2		(K)FVVEASAK(A) (R)ESTVSDPVK(R)	Phenylpropanoid
11.	Ferredoxin NADP reductase leaf type isozyme chloroplast precursor FNR	12.18 / 5	7.89 / 9	(K)TVSLCVK(R) (R)LVYTNDK(G) (R)LDFAVSR(E) (K)MYIQTR(M) (R)DGIVWADYK(K)	Oxidative stress (NADH/$NADP^+$ homeostasis)
12	Cytosolic ascorbate peroxidase 1	12.18 / 2	11.84 / 7	(K)NCAPLMLR(L) (R)DGLLQLPSDK(A)	UV-B and photoprotection
13	MYB transcription factor		7.64 / 4	(K)IAEFFPER(T) (K)NHWNSSLKK(K) (K)DIADGDRDSK(Q) (K)DSDSLTQTSSGNTDSNEVGR(D)	Phenylpropanoid
14	Ascorbate peroxidase	12.18 / 1	9.73 / 5	(K)NCAPIMLR(L) (K)NCAPIMLR(L) (R)LAWHSAGTYDVCSK(T) (K)TGGPFGTMR(F) (K)TGGPFGTMR(F)	UV-B and photoprotection
15	DNA binding NAD ADP ribosyltransferase	11.23 / 1	5.57 / 5	(K)SVDDVEGIESLR(W) (R)EMLEVNEQSTR(G) (K)VEEMSKSDAVHEFK(R) (K)MLFDVETYR(S) (K)KVPQDSEFAK(W)	Oxidative damage
16	RNA polymerase beta chain		8.13 / 4	(K)EWKPKYK(M) (R)NNSIIGVDTR(I) (K)RVEGWNER(I) (R)ALDEAICYR(A)	Transcription
17	Ribulose bisphosphate carboxylase (RuBisCO)		10.13 / 4	(K)NNNVDITSLASNGGRVR(C) (R)YWTMWK(L) (K)AYPQAWIR(I) (R)IIGFDNVR(Q)	Primary metabolism
18	RuBisCO small chain A	12.18 / 1	6.68 / 4	(R)IKCMQVWPIEGIK(K) (K)WVPCLEFSK(V) (R)SPGYYDGR(Y) (R)IIGFDNVR(Q)	Primary metabolism
19	RuBisCO small subunit 3	12.18 / 1	9.96 / 5	(K)NNNVDITSLASNGGRVR(C) (K)SPGYYDGR(Y) (R)YWTMWK(L) (K)AYPQAWIR(I) (R)IIGFDNVR(Q)	Primary metabolism
20	RuBisCO small subunit S41	12.18 / 1	10.23 / 4	(K)QIEYLLR(S) (R)SPGYYDGR(Y)	Primary metabolism

				(R)YWTMWK(L) (R)IIGFDNVR(Q)	
21	bZIP transcription factor bZIP78	12.18 / 1	5.64 / 4	(K)SSGNTDQGLMKK(L) (R)KQAETEELAR(K) (K)MRVENATLR(G) (R)ATPVSTENLLSR(V)	Transcription; UV-B response
22	Signal recognition particle 54 kDa protein	12.04 / 1	5.78 / 7	(R)HSSVPALHLR(A) (K)DNIAEPMR(D) (R)RALLEADVSLPVVR(S) (K)KTDVIIVDTAGR(L) (R)IIGMIPGMNK(V) (K)NERQVSQLVAQLFR(M) (R)QVSQLVAQLFRMR(A)	Signal transduction; light perception
23	Chromatin remodeling complex SWI SNF component DNA RNA helicase superfamily ISS	12.18 / 1	5.08 / 4	(R)GGRALIGDEMGLGK(T) (K)LDEGRMYNIVPYSLCVK(L) (K)RLLNELFLASAK(A) (K)LAVVDVTQR(T)	Transcription; chromatin remodeling; DNA repair
24	Carbonic anhydrase dehydratase	12.18 / 1	9.71 / 10	(K)YEKNPALYGELSK(G) (K)NPALYGELSK(G) (K)FMVFACSDSR(V) (R)NIANMVPAYDK(T) (R)YSGVGAAIEYAVLHLK(V) (K)VENIVVIGHSACGGIK(G) (K)GLMSLPADGSESTAFIEDWVK(I) (K)VQGEHVDK(C) (K)CFADQCTACEK(E) (K)EAVNVSLGNLLTYPFVR(E)	Primary metabolism
25	Endo beta 1 4 D glucanase		7.01 / 4	(K)QLFDFADK(Y) (R)GRYDNSITVAR(N) (R)YDNSITVAR(N) (R)GTSYMVGYGAVYPR(Q)	Physiological stress
26	MYB like protein		9.17 / 4	(K)IAEFFPER(T) (K)NHWNSSLKK(K) (K)DIADGDRDSK(Q) (K)DSDSLTQTSSGNTDSNEVGR(D)	Phenylpropanoid
27	Signal transducer		6.57 / 4	(-)MDINPSIFHLVTR(F) (R)HRDLIESEK(M) (R)IVMQVLFVSQMQIR(D) (K)TSVWREVK(R)	Signal transduction

transcription factors (Galis *et al.,* 2006), phenylcoumaran benzyl reductase (Gang *et al.,* 1999; Vander Mijnsbrugge *et al.,* 2000), transketolase, TK (Henkes *et al.* 2001; Harding *et al.,* 2005), ascorbate peroxidase (Rao *et al.,* 1996; Smirnoff, 2000), glycine decarboxylase (Taylor *et al.,* 2002), ferredoxin NADP reductase, FNR (Palatnik *et al.,* 1997), and ADP

ribosyltransferase (De Block *et al.,* 2005)]; (II) physiological stress [ATP synthase (Zhang *et al.,* 2006), and endo β-1,4 glucanase (Akiyama *et al.,* 1996)]; (III) chromatin remodeling and DNA methylation [S adenosyl-L-homocysteine hydrolase (Rocha *et al.,* 2005; Mull *et al.,* 2006), and SWI SNF complex (Dirscherl and Krebs, 2004)]; (IV) transcription and cell cycle [p32 annexin (Proust *et al.,* 1999), RNA polymerase beta chain, and pZIP transcription factors (Jakoby *et al.,* 2002; Siberil *et al.,* 2001)]; (V) primary metabolism [fructose bisphosphate aldolase (Marsh and Lebherz, 1992), RuBPCase, and carbonic anhydrase dehydratase (Tripp *et al.,* 2001); and (VI) signal transduction (signal recognition particle 54 kDa protein, signal transducer protein). pZIP transcription factors are also essential for the UV-B response (Ulm *et al.,* 2004). Interestingly, none of the proteins specific to herbivore resistance was found to be differentially regulated.

Transcripts in* RdR2-*silenced plants

Products of phenylpropanoid pathways are known to be essential for providing protection from UV radiation, and the biosynthesis of many phenolics begins by activating phenylalanine ammonia-lyase (*PAL*) genes (Stracke *et al.,* 2007). Two *PAL* genes are present in *N. attenuata* and transcript levels of *PAL1* and *2* in irRdR2 plants growing their natural habitat were found by qPCR to be significantly lower than those of paired WT plants (Figure 9A). Further, our proteomic analysis revealed the down-regulation of two MYB proteins in irRdR2 plants (Table 1). An R2R3 *MYB* transcription factor is reported to regulate phenylpropanoid biosynthesis by regulating *PAL* genes in tobacco (Galis *et al.,* 2006). We quantified the transcript levels of this R2R3 *MYB* homolog in field-grown irRdR2 and WT *N. attenuata* plants, and found them to be significantly reduced (Figure 9A).

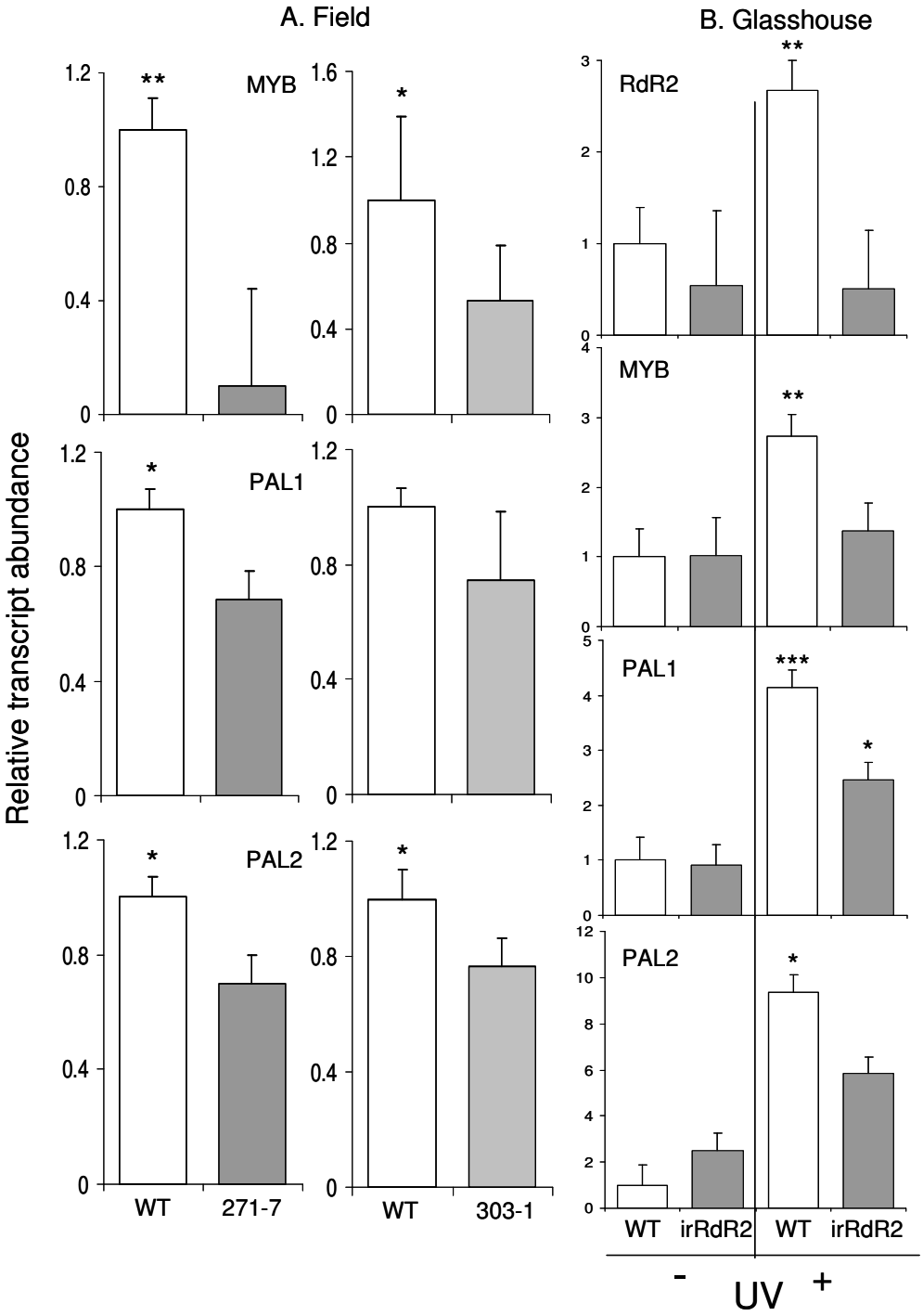


Figure 9. qPCR analysis of transcript accumulation in plants grown in nature and in the glasshouse.
(A) Analysis of transcript levels of *MYB*, *PAL1* and *PAL2* genes shows their reduced levels in irRdR2 lines compared to WT counterparts in plants growing in their native habitat. Plants in their natural habitat are exposed to high UV-B environments throughout their life. Relative values of transcripts in irRdR2 plants were calculated with respect to their values in WT counterparts (set to 1). (B) qPCR reveals that *RdR2* transcript levels are enhanced when WT plants are exposed to UV-B in the glasshouse (showing that *RdR2* is indeed a UV-responsive gene), but the *RdR2* transcripts fail to accumulate in irRdR2 plants. Silencing *RdR2* reduces the UV-B elicited increases in transcripts of *MYB*, *PAL1,* and *PAL2* genes. In the glasshouse study, relative transcript levels were calculated with respect to the constitutive levels in the unexposed WT, which was fixed to 1. Inserts represent the

gene names. *** significantly different at P<0.005; ** significantly different at P<0.05; * significantly different at P<0.05.

We verified the above transcriptional patterns in the glasshouse-grown plants by analyzing the transcripts of 5 genes. WT plants exposed to UV-B had three times the levels of *RdR2* transcripts compared to the levels in untreated WT plants. *RdR2* transcripts did not accumulate further after UV-treatment in the irRdR2 plants, confirming the silencing of *RdR2* gene expression (Figure 9B). Unlike in WT plants, where a 4-fold increase after UV-treatment was observed, in ir*RdR1* plants transcripts of the *PAL1* gene accumulated only 2.5-fold over the untreated controls (Figure 9B). In other words, UV-induced *PAL1* transcripts were suppressed by 38% in the irRdR2 plants. Similarly, the *PAL2* transcript levels were 39 % lower in UV-treated irRdR2 plants compared to UV-treated WT plants (Figure 9B).

An R2R3 *MYB* transcription factor regulates *PAL* gene expression in tobacco cell cultures (Galis *et al.,* 2006), and R2R3-*MYB* transcription factors in *Arabidopsis* regulate flavonoid biosynthesis (Stracke *et al.,* 2007). Although *MYB* transcript levels in WT plants increased nearly 3-fold after UV treatment in WT plants, levels were unaffected by UV exposure in irRdR2 plants (Figure 9B).

In *Lycopersicon peruvianum* cells, UV-B activates components of systemin signaling (Yalamanchili and Stratmann, 2002) and therefore, we measured levels of systemin (*SYS*) transcripts in both genotypes. No differences were found between irRdR2 and WT plants (Figure S6).

Silencing* MYB *reduces levels of* PAL *transcripts and phenolics.

Transcriptional and proteomic analyses indicated that the reduced accumulation of *PAL* transcripts and phenolics in field- and glasshouse-grown UV-supplemented irRdR2 plants may be due to the reduced accumulation of *MYB* transcripts. We tested this hypothesis by silencing the expression of the R2R3 *MYB* homolog in *N. attenuata* using virus-induced gene silencing (VIGS; Figure 10A). We measured transcript levels of the two *PAL* genes by qPCR and found both to be significantly reduced in *MYB*-silenced plants (Figure 10A). We also analyzed the accumulation of rutin, which was significantly reduced by 42% (Figure 10B; ANOVA; $F_{1,14}$=12.29, P<0.005), as well as of chlorogenic acid, which was significantly reduced by 33.5% (Figure 10B; ANOVA; $F_{1,14}$=5.59, P<0.05).

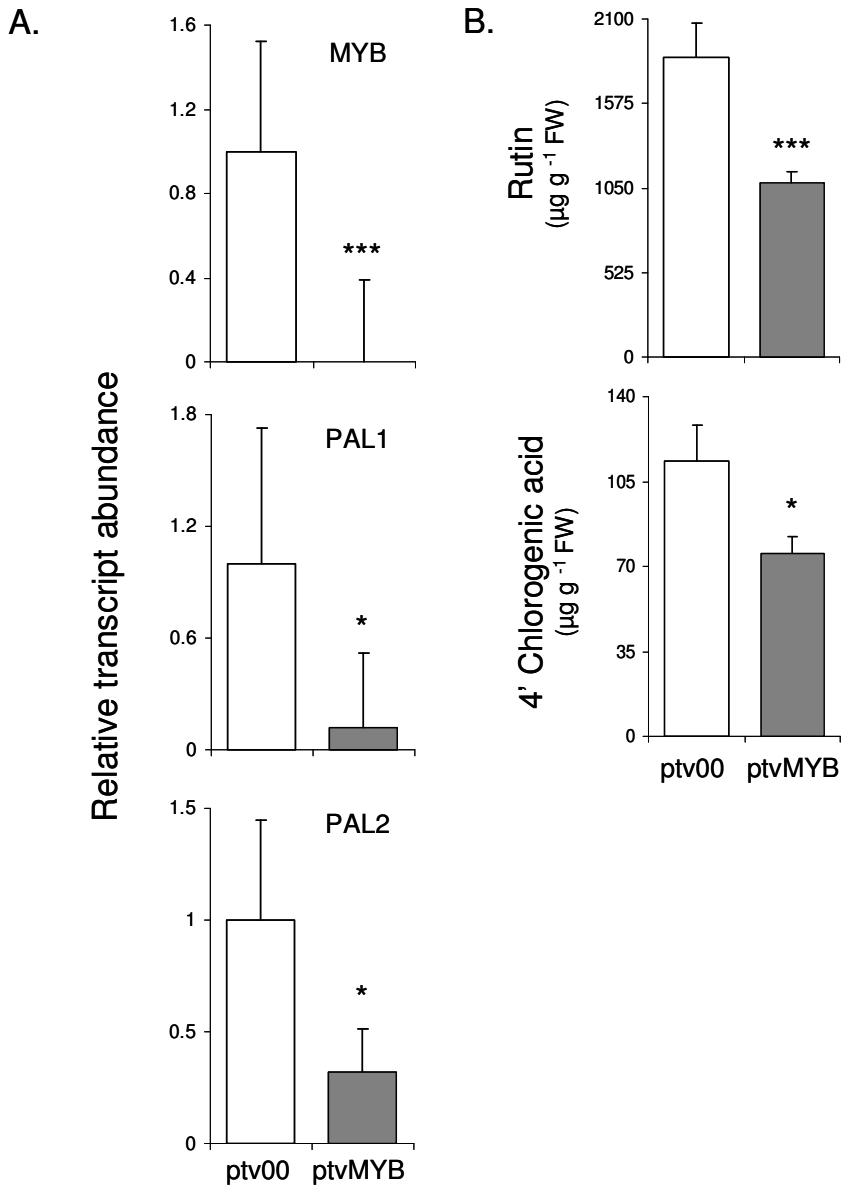
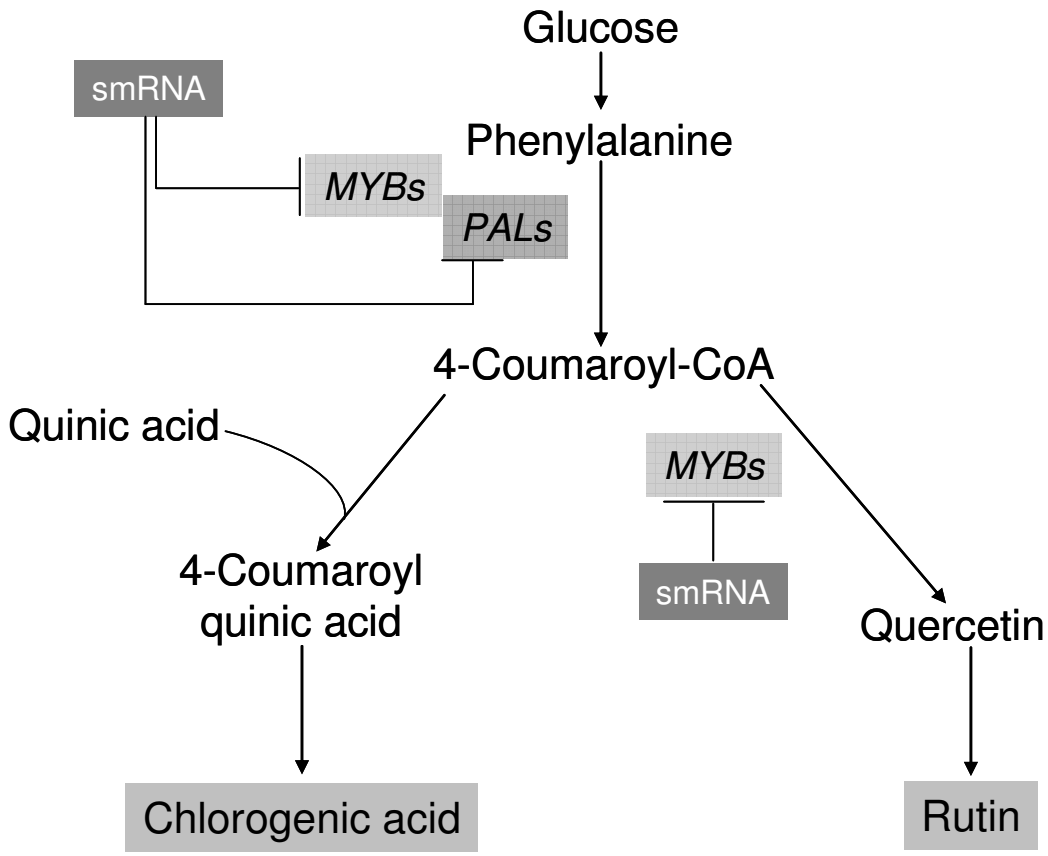


Figure 10. Silencing the R2R3 *MYB* transcription factor with virus-induced gene silencing (VIGS).
(A) qPCR analysis shows that silencing *MYB* reduces levels of *PAL1* and *PAL2* transcripts in *N. attenuata.* Relative transcript levels in *MYB*-silenced plants (ptvMYB) were calculated with respect to values in empty vector (EV; ptv00) controls, fixed to 1. (B) Silencing *MYB* with VIGS reduces accumulation of phenolics (rutin and chlorogenic acid), as it does in the field-grown irRdR2 plants and those grown under UV-B supplemented glasshouse conditions. *** significantly different at P<0.005; * significantly different at P<0.05.

Bioinformatic analysis of smRNA binding sites

The miRNA-mediated regulation of the target is initiated by the interaction of the miRNAs and their target genes through the 5'-end of the miRNA, which is referred to as the "seed" sequence (Lewis, *et al.,* 2005; Brenneck *et al.,* 2005). Mutating the seed of miRNAs renders the interaction ineffective (e.g. Doench and Sharp, 2004), though in *Caenorhabditis*

elegans G-U base pairing may be tolerated (Didiano and Hobert, 2006). In general, the longer the seed sequence (and thus the greater the perfect complementation), the higher the chances of an effective miRNA-target interaction. miRNAs that have dominant 5' sites with sufficient miRNA 5′-end-complementarity to the target can function with little or no support from pairing to the miRNA 3′ end (Brenneck *et al.,* 2005). Informed by these considerations (although these are lenient criteria for predictions), we performed a bioinformatic analysis of smRNAs that could bind to the *MYB* and *PAL* genes which we had found to be differentially regulated in UV-elicited WT and irRdR2 plants. The biosynthesis of many phenolics is well studied (Levy and Zucker, 1960) and its close mechanistic association with the *MYB* transcription factors has been established in *Arabisopsis* and tobacco (Figure 11; Stracke *et al.,* 2007; Galis *et al.,* 2006). Therefore, we not only searched for smRNA-binding motifs in the differentially regulated *MYB*, *PAL1* and *PAL2* genes in *N. attenauta*, but also in *MYB*111 from *Arabidopsis* (Figure 11). Our analysis revealed that key components of the rutin and chlorogenic acid biosynthetic pathway (Figure 11) have a large number of smRNA bindings sites, indicating the mechanistic relations between the smRNAs, *MYB*s, *PAL*s, and reduced generation of phenols which act as sunscreens. Given that a single miRNA can target more than 100 genes (Brenneck *et al.,* 2005; Lim *et al.,* 2005) and that a single gene can be targeted by several miRNAs, substantially more work will be required to understand which of the identified smRNA are actually responsible for UV-B susceptible phenotype. A detailed hit-map is presented in Supplementary Table 1.



Number of smRNAs (normalised over 100) having target motives for *MYB*- and *PAL*-genes

	MYB/JS1	**MYB111**	NaPAL1	NaPAL2
irRdR2	14	**15**	26	27
WT	14	**5**	26	27

Figure 11. Proposed scheme for the smRNA-mediated regulation of chlorogenic acid and rutin biosynthesis.
A simplified scheme highlighting the major regulatory events in the biosynthesis of flavonols/phenolics (chlorogenic acid and rutin) [from Levy and Zucker (1960), Moriguchi *et al.* (2002), Galis *et al.* (2006) and Stracke *et al.* (2006)]. The lower panel shows the number of smRNAs (normalized over 100 smRNAs) that potentially target *MYB*- and *PAL*-transcripts, identified from the sequencing of smRNAs from WT and irRdR2 plants. The identity of the smRNAs is given in Supp Table 1.

Discussion

Here we provide a second example of a "bottom-up" approach toward defining the whole-plant function of an *RdR*. In our first example, we discovered that *RdR1* mediates herbivore resistance responses by planting *RdR1*-silenced plants into their natural habitat and

noticed that they were practically defenseless in comparison to WT plants (Pandey and Baldwin, 2007). Here we used a similar approach with *RdR2*-silenced plants and report that Na*RdR2* is essential for growth in UV-B-enriched environments. We propose that *RdR2* modulates the phenylpropanoid pathway, essential for generating phenolic "sunscreens," by regulating the expression of *PAL* genes through *MYB* transcription factor(s). In irRdR2 plants, smRNAs with strong base-pair matches for genes regulating the phenylpropanoid pathway (e.g. *MYB*s) are amplified, thus providing a potential mechanism for the reduced accumulation of transcripts of these genes; the down-regulation of the phenylpropanoid pathway results in reduced accumulation of rutin and chlorogenic acid that likely act as phenolic sunscreens, which finally contribute to greater UV susceptibility of *RdR2*-silenced plants. Transcriptional responses of *Nicotiana* plants to UV-B stress and herbivore attack are known to overlap and we further try to locate where these convergent responses subsequently diverge.

RdR2 has been associated with processes related to genomic stability such as in *de novo* DNA methylation (May *et al.,* 2005), using siRNAs to methylate direct repeats (Chen *et al.,* 2004), and paramutation (Alleman *et al.,* 2006), although investigators did not detect repeat-specific siRNAs during paramutation. Also in yeast, an *RdR* is an essential component of the heterochromatin-formation machinery at centromeric repeats through a self-enforcing loop assisted by siRNAs (Sugiyama *et al.,* 2005). On the other hand, in *N. attenuata*, *RdR2* (Na*RdR2*) is elicited by herbivore-specific signals. This indicates that *RdR2* is involved in defenses against herbivores. However, herbivory is not known to elicit methylation of direct repeats, paramutation, etc., which suggests a disconnect between the factors that elicit the accumulation of *RdR2* transcripts and the gene's known function. Such a discrepancy motivated the need to conduct an unbiased analysis of the gene's function at an organismic level in the real world.

RdR2-silenced *N. attenuata* plants introduced into natural habitats were as able as their WT counterparts to defend themselves against insect herbivores. On the other hand, irRdR2 plants failed to grow well and had reduced reproductive output, which could be attributed to their susceptibility to the high UV-B fluence of the light regimes of the Great Basin Desert. Effects of UV radiation on plant growth and performance depend strongly on the fluence of photosynthetically active radiation (PAR; 400-700 nm): when plants are grown under low PAR levels, the effects of UV-B tend to be more pronounced than they are when plants are grown under high PAR levels (Krizek 2004). While we cannot rule out the possibility that the

relative low PAR levels of our growth chamber (~800 μmoles $m^{-2}s^{-1}$) may have exaggerated the UV-B sensitivity of irRdR2 plants, the results from the growth chamber experiments are consistent with the results from the field, where PAR levels were close to 2000 μmoles $m^{-2}s^{-1}$ for every day of the growing season. Future field research will be focused on understanding the role of variation in PAR and UV-A fluence on the sensitivity of irRdR2 plants to UV-B radiation.

The reduced fitness of irRdR2 plants may have been an indirect effect of "perturbed physiology" but this seems unlikely for two reasons: irRdR2 plants grown without supplemental UV-B were as fit as WT plants; and phytohormone analysis, herbivore performance (both in the field and in the glasshouse), and competition assays revealed no differences between WT and irRdR2 plants. Moreover, the proteomics analysis detected the differential regulation of proteins related to UV and genomic stress.

In response to UV-B exposure, plants deploy a multi-layered UV-protection response. Flavonoids, phenolics and esters are deposited in epidermal layers to absorb UV-B so as to minimize cellular damage (Stratmann, 2003). These sunscreens are synthesized by the phenylpropanoid pathway, where the committed steps are regulated by *PAL* genes (Takeda *et al.,* 2002) and the *MYB* transcription factors (Galis *et. al.*, 2006; Stracke *et al.*, 2007). If the sunscreens are not sufficient, plants try to repair the oxidative damage, deploy antioxidants to scavenge and detoxify ROS and regenerate tocopherols. Some of the enzymes involved in these repair processes include glutathione reductase, superoxide dismutase, catalase, and ascorbate peroxidase (Rao *et al.,* 1996; Smirnoff, 2000; Stratmann, 2003). irRdR2 plants selectively accumulated fewer of these "sunscreen" barriers, e.g. rutin and chlorogenic acid, and had lower levels of MYB and *PAL*s as well as of the enzymes necessary to scavenge ROS and combat oxidative damage. Ascorbate peroxidase**,** phenylcoumarin benzyl reductase, and transketolase (Gang *et al.,* 1999; Vander Mijnsbrugge *et al.,* 2000; Henkes *et al.,* 2001; Harding *et al.,* 2005), glycine decarboxylase (Taylor *et al.,* 2002), FNR (Palatnik *et al.,* 1997), and pZIP transcription factors (Ulm *et al.,* 2004) among others – were down-regulated in irRdR2 plants. Plants also deploy responses to repair DNA under dark conditions, which include excision repair and homologous recombination events (Sinha and Hader, 2002); these require chromatin remodeling and irRdR2 plants had lower levels of components of DNA methylation and chromatin remodeling [adenosyl cysteine hydrolase (Rocha *et al.,* 2005; Mull *et al.,* 2006) as well as the SWI/SNF complex (ATP-dependent chromatin remodeling

complex; Dirscherl and Krebs, 2004)]. In addition, irRdR2 plants also showed down-regulation of some components of primary metabolism (e.g RuBisCo), which may result from the rapid degradation of chlorophyll due to the lack of sunscreens in the irRdR2 plants. The decrease in chlorophyll content and photosynthesis has been correlated with UV-B susceptibility in other plant species (Fujibe *et al.,* 2004; Yao and Liu, 2007).

There are two possible mechanisms for *RdR2* action: (i) a siRNA-mediated regulation as in yeast and *Arabidopsis* (Chen *et al.,* 2004; Sugiyama *et al.,* 2005); or (ii) another RNA-directed mechanism that requires *RdR* but not Dicer or Drosha, thus not involving siRNAs, as in *C. elegans* (Maine *et al.,* 2005). Our data is more consistent with the former hypothesis, as we observed strong changes in 22-24 nt and 19-21 nt smRNAs in irRdR2 plants. Bioinformatic analysis of smRNA-target interactions revealed that components of phenylpropanoid pathway had large number of smRNA binding sites. *RdR2*-silenced plants accumulated new smRNAs, not present in WT plants. Elicitation by stress exposure is expected to further enrich many of these smRNAs. Such smRNAs may target the transcription factors (e.g. *MYB*s) or even directly regulate the transcripts of genes identified as being differentially regulated in our transcriptional and proteomic analysis. Limited overlap was observed between the smRNAs populations from WT and irRdR2 genotypes. Similar observations have been reported in *Arabidopsis*, where *RdR2* mutants were enriched in many smRNAs and generated new ones as well (Lu *et al.,* 2006); their eco-physiological roles, however, remained unstudied. An analysis similar to that which has been done in *Arabidopsis* (Lu *et al.,* 2006; Kasschau *et al.,* 2007) was not possible in the present study due to the lack of a genome sequence for *N. attenuata*; therefore genome matches and annotations will require additional research.

Survival in highly variable stressful environments requires the capacity to modulate and allocate resources according to the severity of the stress. The need to economize may have led to the evolution of a tiered strategy, involving certain common (and preferably rapid) preliminary responses, followed by the elicitation of stress-specific responses. *RdR2* elicitation after a diversity of stresses (treatment of wounds with *M. sexta* OS or JA, and exposure to UV-B) may result from a similar tiered system of responses whereby *RdR2*-responses afford stress-specific resistance. Perhaps the phenolics that function as “sunscreens” also function as anti-herbivore for some plants and insects (Rehill *et al.*, 2005; Izaguirre *et al.*, 2007). The

RdR2 may thereby be the junction where responses to herbivory diverge from the responses to UV-B.

In conclusion, this study uncovers the importance of *RdR2* in the *N. attenuata* genome for protecting plants from the threat of genotoxic stresses that may result from oxidative damage and DNA cross-linking in high UV-B environments, particularly in absence of sufficient sunscreens. As such, it sets the groundwork for a detailed analysis of different components of the silencing machinery that function at different PAR and UV levels so as to better understand the molecular mechanisms responsible for UV-B protection. Further research is needed to dissect the changes in the smRNA transcriptome and work out the molecular mechanisms regulating *MYB* transcripts during UV stress. This study also highlights the advantage of conducting field trials early in the discovery process: due to the multitude of stresses a plant faces under natural conditions, a single field trial can allow a researcher with an intimate understanding of a plant's natural history to rapidly identify a difference in a phenotype.

Experimental procedures

Plant and insect materials

Plant and insect materials are described in the Supplementary Experimental Procedures.

Isolation of the N. attenuata RdR2 *gene*

A previously described PCR-based strategy was used to isolate the *RdR2* from *N. attenuata* (Pandey and Baldwin, 2007), as described in the Supplementary Experimental Procedures.

DNA gel blots and quantitative real-time PCR analysis

Southern blot analysis to determine the copy number of the endogenous *RdR2* gene or transgene was done as described earlier (Bubner *et al.,* 2004) and detailed in Supplementary Experimental Procedures. Na*RdR2* expression analyses after different treatments were performed with quantitative real-time PCR, as described in detail in the Supplementary Experimental Procedures.

Generation and characterization of* RdR2*-silenced transgenic plants

Plants stably silenced for *RdR2* gene expression were produced by cloning an *RdR2* gene fragment in an inverted-repeat orientation in pRESC5 transformation vector, performing *Agrobacterium tumifaciens*–mediated transformation, and a transgenic screening as described previously (Bubner *et al.,* 2006; Kruegel *et al.,* 2002) and further detailed in Supplementary Experimental Procedures.

Performance under field conditions

The planting of plants silenced for the expression of *RdR2* (irRdR2) into the natural habitats of *N. attenuata*, in the Great Basin Desert in southwestern Utah, USA, under APHIS notification number 06-003-08, was as described in Pandey and Baldwin (2007). 10 days after germination, seedlings were transferred to borax-soaked Jiffy 703 pots (AlwaysGrows) and transferred to field plots 3-4 weeks later. Ten irRdR2-WT pairs of same-size adapted seedlings from both 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 days. 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 readings at 5-day intervals, stalk length was recorded and the leaves of irRdR2-WT 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).

Performance assays under different stress conditions

Insect performance- and competition- assays were as described in Supplementary Experimental Procedures

A UV-B screen assay similar to that described in Izaguirre *et al.,* (2003 and 2007) was conducted. Plants were assigned to two treatment groups: plants with supplemental UV-B (to have an environment with enhanced UV-B) and plants without supplemental UV (non-UV conditions, control). Approximately three weeks after germinating, the plants were placed in a York Chamber with controlled light and temperature conditions (32/27 ^{0}C, 16/8 h light/dark at 65% relative humidity and high light levels of 400-1000 μmoles $m^{-2}s^{-1}$). The chamber was separated into two parts with an opaque partition that prevented UV exposure to the other half.

In the first half, two UV-B lamps were installed (F40 UVB 40W; Phillips, Eindhoven, the Netherlands; Casati *et al.,* 2006). Other than the supplemental UV in one half, both parts of the York Chamber had the same light source and experienced the same temperature regulation. PAR in both halves of the chamber was 300-800 μmoles $m^{-2}s^{-1}$. Plants were germinated as described by Kruegel *et al.* (2002). The plants were allowed to adapt for 7 days to the chamber conditions before starting the UV treatment. Initially the plants were subjected to only 2 h of UV treatment per day for 7 days, which was increased to 8-10 h per day approximately, with the irradiation period occurring at solar noon for rest of the experiment. Plants were subjected to radiation daily for the rest of the study period. The study was terminated 45 days after transplanting.

Metabolite analysis

Tissues derived from the field study were analyzed for plant phytohormones as previously described in Schmidt and Baldwin (2006) and detailed in Supplementary Experimental Procedures.

Total phenolics were analyzed with the Folin-Ciocaulteu assay (Imeh and Khokhar, 2002). Samples were collected from plants 26 days after transfer into a York Chamber. Around 200 mg of plant tissue, from three biological replicates of both genotypes and both treatment groups (UV-treated and untreated) was heated with 10 ml of 1.2M HCL in 50% aqueous methanol for 2h at 90 ^{0}C. 20 μl of samples (or standards) were mixed with 1.58 ml water and 100 μl Folin-Ciocaulteu reagent (Sigma), allowed to stand for 2 min, then mixed with 300 μl 1.9 M sodium carbonate and incubated at 40 ^{0}C for 30 min. Absorbance was measured at 765 nm in a spectrophotometer and amounts were calculated as gallic acid equivalents from a standard curve with 6 concentrations of gallic acid. The entire experiment was replicated.

Secondary metabolites (rutin and 4' chlorogenic acid) were analyzed using the HPLC as described earlier (Steppuhn *et al.,* 2004). Briefly, around 100 mg leaf material was extracted with 2:3 methanol: 0.05% acetic acid (v/v) and injected into the HPLC system. A standard curve was generated using a dilution series of rutin and chlorogenic acid, and levels of both metabolites were quantified.

Proteomics analysis

The 2-D electrophoresis of the proteins extracted from WT and irRdR2 plants was carried out as previously described by Giri *et al.* (2006) and described in Supplementary Experimental Procedure.

***Virus-induced gene silencing (VIGS) of* MYB**

As described earlier (Ratcliff *et al.,* 2001; Saedler and Baldwin, 2004) and detailed in Supplementary Experimental Procedure, VIGS, based on tobacco rattle virus (TRV), was used to silence the R2R3-*MYB* homolog of *N. tabacum* (Galis *et al.,* 2006) in *N. attenuata*.

Analysis of smRNA portion of transcriptome

The smRNA portion of the transcriptome of the WT and irRdR2 genotypes was sequenced by 454 sequencing (Lu *et al.,* 2006; Kasschau *et al.,* 2007; Molnar *et al.,* 2007). Leaf material was ground under liquid nitrogen, RNA species <200 bp were enriched with the mirVana miRNA isolation kit (Ambion, Austin, TX, USA). Small-RNAs were separated on a denaturing 12.5% polyacrylamide gel and stained with SYBERgreen II, and size fractions of 15-30 nt were isolated, precipitated with ethanol, and dissolved in water. RNA were first poly(A)-tailed with poly(A) polymerase, RNA adapter were ligated to 5'-phosphate, and first-strand cDNA synthesis was performed using oligo(dT) primer linker, and PCR amplified to about 30ng/µl with 22 cycles using high fidelity Taq DNA polymerase. The bar codes, attached to a 5' flanking sequence, for the two genotypes were ACTA (WT) and CACA (irRdR2). 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 (Freising, Germany).

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 faithful 5' and 3' flanking sequences. Adapter sequences, 5' and 3' flanking sequences were identified and removed from each bin, and sequences shorter than 15 nt were discarded. Because the technology is not able to distinguish more than 8 identical nucleotides in a stretch, according to manufacturer's instructions, all the A's from the 3' end (or any continuous run of single

nucleotide at the 5' end) were removed. This may cause under representation of sequences by 1 nucleotide at the 5' or 3' end in some cases. Total frequency and number of distinct sequences were determined in each case. All the analyses were performed with custom-written programs in Perl.

Bioinformatic analysis of smRNAs targeted at sites in* PAL- and MYB-*transcripts

In order to determine if the smRNAs generated in the irRdR2-silenced plants targeted phenylpropanoid biosynthetic genes responsible for the production of phenolic sunscreens, we used the concept of "seed-pairing" (Lewis *et al.,* 2005; Brenneck *et al.,* 2005). Complete coding sequences were used for *N. attenuata PAL1* and *2* genes, as well as the MYBs from *N. tabacum* (*MYBJS1*; AB236951.1; Galis *et al.,* 2006) and *Arabidopsis* (*MYB111*; NM_124310.2; Stracke *et al.,* 2007). Using Perl script, "seed-pair analysis" of smRNAs isolated from WT and irRdR2 plants was conducted for the above-mentioned target genes. We compared sequences from the 3' ends with smRNA sequences from the 5' ends after generating 7–13 nucleotide "seeds" starting from the 1st as well as the 2nd nucleotide at the 5' end of the smRNA. These seeds were mapped to the sequences of the target genes for "Watson-Crick" base-pairing and a hit-map was generated (Supplementary Table 1).

Statistical analysis

Statistical analysis was performed as stated in Supplementary Experimental Procedure.

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Supplementary Materials

Supplementary Experimental Procedures

Supplementary Tables

Supplementary Table 1. Analysis of smRNA-transcriptome of *N. attenauta*, targeting the differentially regulated genes of phenylpropanoid biogenesis during UV-exposure, shows that large number of smRNAs-binding motifs exist in these genes. Tables S1a and b show the smRNAs from irRdR2 and WT genotypes respectively.

Supplementary Figures

Figure S1. DNA gel blot analysis of the two independently transformed irRdR2 lines showing single insertions.

Figure S2. Characterization of irRdR2 plants in the glasshouse.

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Figure S3. Phytohormone analysis of the WT and irRdR2 plants.

Figure S4. Silencing *RdR2* did not affect plant performance, even when irRdR2 plants competed with WT neighbors in the same pot.

Figure S5. *M. sexta* performance on the WT and two independently transformed irRdR2 lines.

Figure S6. Silencing *RdR2* did not affect *systemin* (*SYS*) transcript levels (ANOVA; $F_{3,18}$=2.85; P>0.05).

Table S1. smRNAs from (a) *RdR2* –silenced- and (b) wild type plants having target-motifs in the phenylpropanoid biosynthesis genes

References

Akiyama, T., Kaku, H. and Shibuya, N. (1996) Purification and properties of a basic endo-1,3-beta-glucanase from rice (*Oryza sativa* L.). *Plant Cell Physiol*, **37**, 702-705.

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

Brennecke, J., Stark, A., Russell, R.B. and Cohen, S.M. (2005) Principles of microRNA-target recognition. *PLoS Biology*, **3**, e85.

Bubner, B., Gase, K. and Baldwin, I.T. (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *BMC Biotechnology*, **4**, http://www.biomedcentral.com/1472-6750/1474/1414.

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

Callegari, A.J. and Kelly, T.J. (2006) UV irradiation induces a postreplication DNA damage checkpoint. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 15877-15882.

Casati, P. and Walbot, V. (2004) Rapid transcriptome responses of maize (*Zea mays*) to UV-B in irradiated and shielded tissues. *Genome Biology*, **5**.

Casati, P., Stapleton, A.E., Blum, J.E. and Walbot, V. (2006) Genome-wide analysis of high-altitude maize and gene knockdown stocks implicates chromatin remodeling proteins in response to UV-B. *Plant Journal*, **46**, 613-627.

Chen, X.M. (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. *Science*, **303**, 2022-2025.

Cogoni, C. and 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. and Baulcombe, D.C. (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell*, **101**, 543-553.

De Block, M., Verduyn, C., De Brouwer, D. and Cornelissen, M. (2005) Poly (ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *Plant Journal*, **41**, 95-106.

De Gruijl, F.R. and Van der Leun, J.C. (1994) Estimate of the wavelength dependency of ultraviolet carcinogenesis in humans and its relevance to the risk assessment of a stratospheric ozone depletion. *Health Phys*, **67**, 319-325.

Didiano, D. and Hobert, O. (2006) Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. *Nat Struct Mol Biol*, **13**, 849-851.

Dirscherl, S.S. and Krebs, J.E. (2004) Functional diversity of ISWI complexes. *Biochem Cell Biol*, **82**, 482-489.

Fujibe, T., Saji, H., Arakawa, K., Yabe, N., Takeuchi, Y. and Yamamoto, K.T. (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive rcd1, is tolerant to supplemental ultraviolet-B irradiation. *Plant Physiol*, **134**, 275-285.

Galis, I., Simek, P., Narisawa, T., Sasaki, M., Horiguchi, T., Fukuda, H. and Matsuoka, K. (2006) A novel R2R3 MYB transcription factor NtMYBJS1 is a methyl jasmonate-dependent regulator of phenylpropanoid-conjugate biosynthesis in tobacco. *Plant Journal*, **46**, 573-592.

Gang, D.R., Kasahara, H., Xia, Z.Q., Vander Mijnsbrugge, K., Bauw, G., Boerjan, W., Van Montagu, M., Davin, L.B. and Lewis, N.G. (1999) Evolution of plant defense mechanisms - relationships of phenylcoumaran benzylic ether reductases to pinoresinol-lariciresinol and isoflavone reductases. *Journal of Biological Chemistry*, **274**, 7516-7527.

Giri, A.P., Wunsche, H., Mitra, S., Zavala, J.A., Muck, A., Svatos, A. and Baldwin, I.T. (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., Schittko, U., Pohnert, G., Boland, W. and Baldwin, I.T. (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 Physiology*, **125**, 711-717.

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

Harding, S.A., Jiang, H., Jeong, M.L., Casado, F.L., Lin, H.W. and Tsai, C.J. (2005) Functional genomics analysis of foliar condensed tannin and phenolic glycoside regulation in natural cottonwood hybrids. *Tree Physiol*, **25**, 1475-1486.

Henkes, S., Sonnewald, U., Badur, R., Flachmann, R. and Stitt, M. (2001) A small decrease of plastid transketolase activity in antisense tobacco transformants has dramatic effects on photosynthesis and phenylpropanoid metabolism. *Plant Cell*, **13**, 535-551.

Imeh, U. and Khokhar, S. (2002) Distribution of conjugated and free phenols in fruits: antioxidant activity and cultivar variations. *J Agric Food Chem*, **50**, 6301-6306.

Izaguirre, M.M., Scopel, A.L., Baldwin, I.T. and Ballare, C.L. (2003) Convergent responses to stress. Solar ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. *Plant Physiology*, **132**, 1755-1767.

Izaguirre, M.M., Mazza, C.A., Svatos, A., Baldwin, I.T. and Ballare, C.L. (2007) Solar ultraviolet-B radiation and insect herbivory trigger partially overlapping phenolic responses in *Nicotiana attenuata* and *Nicotiana longiflora*. *Annals of Botany*, **99**, 103-109.

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. and Parcy, F. (2002) bZIP transcription factors in *Arabidopsis*. *Trends Plant Sci*, **7**, 106-111.

Kasschau, K.D., Fahlgren, N., Chapman, E.J., Sullivan, C.M., Cumbie, J.S., Givan, S.A. and Carrington, J.C. (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. *PLoS Biology*, **5**, e57.

Kessler, A., Halitschke, R. and Baldwin, I.T. (2004) Silencing the jasmonate cascade: induced plant defenses and insect populations. *Science*, **305**, 665-668.

Krizek, D.T. (2004) Influence of PAR and UV-A in determining plant sensitivity and photomorphogenic responses to UV-B radiation. *Photochemistry and Photobiology*, **79**, 307-315.

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

Landry, L.G., Stapleton, A.E., Lim, J., Hoffman, P., Hays, J.B., Walbot, V. and Last, R.L. (1997) An *Arabidopsis* photolyase mutant is hypersensitive to ultraviolet-B radiation.

Proceedings of the National Academy of Sciences of the United States of America, **94**, 328-332.

Levy, C.C. and Zucker, M. (1960) Cinnamyl and p-coumaryl esters as intermediates in the biosynthesis of chlorogenic acid. *J Biol Chem*, **235**, 2418-2425.

Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, **120**, 15-20.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S. and Johnson, J.M. (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769-773.

Logemann, E., Tavernaro, A., Schulz, W.G., Somssich, I.E. and Hahlbrock, K. (2000) UV light selectively coinduces supply pathways from primary metabolism and flavonoid secondary product formation in parsley. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 1903-1907.

Lu, C., Kulkarni, K., Souret, F.F., MuthuValliappan, R., Tej, S.S., Poethig, R.S., Henderson, I.R., Jacobsen, S.E., Wang, W., Green, P.J. and Meyers, B.C. (2006) MicroRNAs and other small RNAs enriched in the *Arabidopsis* RNA-dependent RNA polymerase-2 mutant. *Genome Res*, **16**, 1276-1288.

Madronich, S., McKenzie, R.L., Caldwell, M. and Bjorn, L.O. (1995) Changes in ultraviolet-radiation reaching the earths surface. *Ambio*, **24**, 143-152.

Maine, E.M., Hauth, J., Ratliff, T., Vought, V.E., She, X.Y. and Kelly, W.G. (2005) EGO-1, a putative RNA-dependent RNA polymerase, is required for heterochromatin assembly on unpaired DNA during *C. elegans* meiosis. *Current Biology*, **15**, 1972-1978.

Marsh, J.J. and Lebherz, H.G. (1992) Fructose-bisphosphate aldolases - an evolutionary history. *Trends in Biochemical Sciences*, **17**, 110-113.

May, B.P., Lippman, Z.B., Fang, Y., Spector, D.L. and Martienssen, R.A. (2005) Differential regulation of strand-specific transcripts from *Arabidopsis* centromeric satellite repeats. *PLoS Genetics*, **1**, e79.

Molnar, A., Schwach, F., Studholme, D.J., Thuenemann, E.C. and Baulcombe, D.C. (2007) miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. *Nature*, **477**, 1126-1129

Moriguchi, T., Kita, M., Ogawa, K., Tomono, Y., Endo, T. and Omura, M. (2002) Flavonol synthase gene expression during citrus fruit development. *Physiol Plant*, **114**, 251-258.

Mourrain, P., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Jouette, D., Lacombe, A.M., Nikic, S., Picault, N., Remoue, K., Sanial, M., Vo, T.A. and Vaucheret, H. (2000) *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell*, **101**, 533-542.

Mull, L., Ebbs, M.L. and Bender, J. (2006) A histone methylation-dependent DNA methylation pathway is uniquely impaired by deficiency in *Arabidopsis* S-adenosylhomocysteine hydrolase. *Genetics*, **174**, 1161-1171.

Palatnik, J.F., Valle, E.M. and Carrillo, N. (1997) Oxidative stress causes ferredoxin NADP(+) reductase solubilization from the thylakoid membranes in methyl viologen treated plants. *Plant Physiology*, **115**, 1721-1727.

Pandey, S.P. and Baldwin, I.T. (2007) RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. *Plant J*, **50**, 40-53.

Pickford, A.S. and Cogoni, C. (2003) RNA-mediated gene silencing. *Cellular and Molecular Life Sciences*, **60**, 871-882.

Preston, C.A. and Baldwin, I.T. (1999) Positive and negative signals regulate germination in the post-fire annual, *Nicotiana attenuata*. *Ecology*, **80**, 481-494.

Proust, J., Houlne, G., Schantz, M.L., Shen, W.H. and Schantz, R. (1999) Regulation of biosynthesis and cellular localization of Sp32 annexins in tobacco BY2 cells. *Plant Mol Biol*, **39**, 361-372.

Rao, M.V., Paliyath, C. and Ormrod, D.P. (1996) Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiology*, **110**, 125-136.

Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant Journal*, **25**, 237-245.

Rehill, B., Clauss, A., Wieczorek, L., Whitham, T. and Lindroth, R. (2005) Foliar phenolic glycosides from *Populus fremontii*, *Populus angustifolia*, and their hybrids. *Biochemical Systematics and Ecology*, **33**, 125-131.

Rocha, P.S., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B., Wagner, C., Vaucheret, H. and Furner, I. (2005) The *Arabidopsis* HOMOLOGY-DEPENDENT GENE SILENCING1 gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. *Plant Cell*, **17**, 404-417.

Saedler, R. and Baldwin, I.T. (2004) Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *Journal of Experimental Botany*, **55**, 151-157.

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

Schmidt, S. and Baldwin, I.T. (2006) Systemin in *Solanum nigrum*. The tomato-homologous polypeptide does not mediate direct defense responses. *Plant Physiol*, **142**, 1751-1758.

Searles, P.S., Flint, S.D. and Caldwell, M.M. (2001) A meta analysis of plant field studies simulating stratospheric ozone depletion. *Oecologia*, **127**, 1-10.

Siberil, Y., Doireau, P. and Gantet, P. (2001) Plant bZIP G-box binding factors. Modular structure and activation mechanisms. *Eur J Biochem*, **268**, 5655-5666.

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

Sinha, R.P. and Hader, D.P. (2002) UV-induced DNA damage and repair: a review. *Photochemical & Photobiological Sciences*, **1**, 225-236.

Smirnoff, N. (2000) Ascorbate biosynthesis and function in photoprotection. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, **355**, 1455-1464.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R. and Baldwin, I.T. (2004) Nicotine's defensive function in nature. *PLoS Biology*, **2**, 1074-1080.

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K. and Weisshaar, B. (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *Plant J*, **50**, 660-677.

Stratmann, J. (2003) Ultraviolet-B radiation co-opts defense signaling pathways. *Trends in Plant Science*, **8**, 526-533.

Sugiyama, T., Cam, H., Verdel, A., Moazed, D. and Grewal, S.I.S. (2005) RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling

heterochromatin assembly to siRNA production. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 152-157.

Takeda, J., Ito, Y., Maeda, K. and Ozeki, Y. (2002) Assignment of UVB-responsive cis-element and protoplastization-(dilution-) and elicitor-responsive ones in the promoter region of a carrot phenylalanine ammonia-lyase gene (gDcPAL1). *Photochem Photobiol*, **76**, 232-238.

Taylor, N.L., Day, D.A. and Millar, A.H. (2002) Environmental stress causes oxidative damage to plant mitochondria leading to inhibition of glycine decarboxylase. *Journal of Biological Chemistry*, **277**, 42663-42668.

Teramura, A.H., Biggs, R.H. and Kossuth, S. (1980) Effects of ultraviolet-B irradiances on soybean. 2. Interaction between ultraviolet-B and photosynthetically active radiation on net photosynthesis, dark respiration, and transpiration. *Plant Physiology*, **65**, 483-488.

Tiwari, S., Ramachandran, S., Bhattacharya, A., Bhattacharya, S. and Ramaswamy, R. (1997) Prediction of probable genes by Fourier analysis of genomic sequences. *Computer Applications in the Biosciences*, **13**, 263-270.

Tripp, B.C., Smith, K. and Ferry, J.G. (2001) Carbonic anhydrase: new insights for an ancient enzyme. *Journal of Biological Chemistry*, **276**, 48615-48618.

Ulm, R., Baumann, A., Oravecz, A., Mate, Z., Adam, E., Oakeley, E.J., Schafer, E. and Nagy, F. (2004) Genome-wide analysis of gene expression reveals function of the bZIP transcription factor HY5 in the UV-B response of *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 1397-1402.

van Dam, N.M., Horn, M., Mares, M. and Baldwin, I.T. (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. *Journal of Chemical Ecology*, **27**, 547-568.

Vander Mijnsbrugge, K., Beeckman, H., De Rycke, R., Van Montagu, M., Engler, G. and Boerjan, W. (2000) Phenylcoumaran benzylic ether reductase, a prominent poplar xylem protein, is strongly associated with phenylpropanoid biosynthesis in lignifying cells. *Planta*, **211**, 502-509.

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

Woodhouse, M.R., Freeling, M. and Lisch, D. (2006) Initiation, establishment, and maintenance of heritable MuDR transposon silencing in maize are mediated by distinct factors. *PLoS Biology*, **4**, e339.

Yalamanchili, R.D. and Stratmann, J.W. (2002) Ultraviolet-B activates components of the systemin signaling pathway in *Lycopersicon peruvianum* suspension-cultured cells. *Journal of Biological Chemistry*, **277**, 28424-28430.

Yang, S.J., Carter, S.A., Cole, A.B., Cheng, N.H. and Nelson, R.S. (2004) A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 6297-6302.

Yao, X.Q. and Liu, Q. (2007) Changes in photosynthesis and antioxidant defenses of *Picea asperata* seedlings to enhanced ultraviolet-B and to nitrogen supply. *Physiologia Plantarum*, **129**, 364-374.

Yu, D.Q., Fan, B.F., MacFarlane, S.A. and Chen, Z.X. (2003) Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense. *Molecular Plant-Microbe Interactions*, **16**, 206-216.

Zavala, J.A., Patankar, A.G., Gase, K. and Baldwin, I.T. (2004) Constitutive and inducible trypsin proteinase inhibitor production incurs large fitness costs in *Nicotiana attenuata*.

Proceedings of the National Academy of Sciences of the United States of America, **101**, 1607-1612.

Zhang, X., Takano, T. and Liu, S. (2006) Identification of a mitochondrial ATP synthase small subunit gene (RMtATP6) expressed in response to salts and osmotic stresses in rice (*Oryza sativa* L.). *J Exp Bot*, **57**, 193-200.

2.4 Manuscript IV

Plant Physiology (2008) In Review

Functional characterization of RNA-directed RNA polymerase (RdR) 3 from *Nicotiana attenuata*

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Summary

SDE1/*SGS2*/*RdR6*, a putative RNA-directed RNA polymerase maintains plant defense against viruses in *Arabidopsis* and *Nicotiana benthamiana*. But its function has not been tested in plant species which do not encounter viruses as their primary biotic stress. We evaluated the organismic-level function of this gene (Na*RdR3*) in such a species, *Nicotiana attenuata*. We stably silenced *RdR3* by transforming *N. attenuata* with an inverted-repeat *RdR3* constructs (irRdR3). Minor phenotypes, typical to *RdR6*-silencing were observed. But irRdR3 plants had normal growth of WT plants and were able to defend themselves as well as the WT plants when challenged with insect herbivores. 454-sequence analysis of irRdR3's smRNA transcriptome showed that abundance of smRNAs of 22-23 nt length was specifically reduced, whereas 21 and 24 nt smRNAs were not affected. Other size ranges showed an increased abundance. When WT - irRdR3 pairs were planted into *N. attenuata*'s natural habitat in the Great Desert Basin (USA), no differences in plant defenses between WT and irRdR3 plants were observed. On the other hand, irRdR3 lines had severely reduced growth, indicating that silencing *RdR3* reduced their ability to compete with WT neighbors. We tested this hypothesis in glasshouse by planting irRdR3 and WT pairs in close competition: irRdR3 plants had severely reduced plant growth and fitness. This reduced competitive ability of irRdR3 plants was attributed to altered phytohormone homeostasis. irRdR3 plants were obstructed in auxin transport. This shows that *RdR3* plays an important role in regulating hormone balance during adaptation of plants to highly competing natural environments.

Keywords: competition, *N. attenuata,* RNA-directed RNA polymerase, RdR6

Introduction

The genetic information is coded as "central dogma", according to which DNA is replicated from DNA and is then transcribed into RNA to make proteins, which ultimately regulate the cellular processes. The pathway that has gained importance outside this central scheme is the replication of RNA from itself. This mode of self-replication is important for viruses and is mediated by RNA-dependent and -directed RNA polymerases. The viral *RdRPs* (to distinguish from their plant counterparts) are different from the mechanistically similar sequences in plants which participate in RNA-mediated silencing. RNA silencing regulates processes affecting many layers of endogenous gene expression (Voinnet, 2002). All of the RNA-silencing pathways involve cleaving double-stranded (ds) RNA into short, 21-26 nucleotide RNAs (Baulcombe, 2004). The dsRNA molecules are produced by the plant RNA-directed RNA polymerases [*RdRs*; (Wassenegger and Krczal, 2006)]. *RdRs* 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 *RdRs* have been elusive; based on earlier observation, they have been mainly associated with antiviral defenses. In *Arabidopsis*, six *RdR* sequences have been identified, but until now, only three functionally distinct *RdRs* have been identified (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 post transcriptional gene silencing (PTGS) and antiviral defense (Dalmay et al., 2000; Mourrain et al., 2000). This *RdR* is probably the most studied one in different plant species, and is implicated in virus resistance and PTGS.

In *Arabidopsis*, *RdR6* mutants were found to be susceptible to cucumber mosaic virus (Mourrain et al., 2000). In *Nicotiana benthamiana*, *RdR6* has gained importance as gene that can make plants resistant to viruses. Its mechanistic details have been somewhat elucidated: the action of RdR6, which acts in a broad spectrum manner against viruses, is temperature-dependent (Qu et al., 2005). The transitivity of the RNA-silencing signal depends on the activity of the *RdRs* (Himber et al., 2003). The activity of *RdR6* in "transitive gene silencing" in *N. benthamiana* could be primed or unprimed (Petersen and Albrechtsen, 2005). In *N.*

benthamiana, the *RdR6* homolog, during virus resistance, 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).

Although intensively studied with respect to *RdR6*'s antiviral role under controlled laboratory conditions, the association with plant defense against viruses in native habitats has not been tested. Hardly anything is known about the ecological relevance of *RdR6* in species where viruses are not the main ecological challenge. One such highly studied ecological model system is *Nicotiana attenuata*. *N. attenuata* is a native of the southwestern United States and grows in the immediate post-fire environment. Its peculiar germination behavior in post-fire environments – it germinates from long-lived seed banks immediately fires (Preston and Baldwin, 1999) - governs its association with other organisms. Because nutrient-rich environments are more concentrated in burnt patches and soil resources vanish fast with rising temperatures, plants are compelled to grow in close to each other. Under such ephemeral post-fire environments, the herbivore community that forms the main biotic stress for *N. attenuata* in the Great Basin Deserts of United States (Baldwin, 2001) is constantly being reestablished. *N. attenuata* produces self-compatible flowers, which mature into seed capsules: the limiting factor in seed production is not the pollen load but the amount of resources that are available. Plants have to manage their resources, especially the rapidly depleting supply of nitrogen (N), allocating them between plant growth and defense (Lynds and Baldwin, 1998), as they deal with an unpredictable herbivore community. To our knowledge, viruses do not pose a challenge to this plant community, and viral symptoms have not been detected in natural *N. attenuata* populations in the Great Basin Desert in the past seventeen years (Baldwin, unpublished observations).

In this study, we try to decipher 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 an essential way to determine the function of the gene; in this case, the customary role of antiviral defense is not important. Traditionally, functions have been assigned based on elicitor studies or sequence similarity, or by silencing the gene and challenging mutants with known lab-based stresses. Relying only on any of these approaches may be misleading in *N. attenuata,* due to the ecologically different 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 (USA), to study the role of Na*RdR3* gene in *N. attenuata*. The study was further extended in the glasshouse with competition experiments.

Results

Isolation and characterization of RdR3 *from* N. attenuata

The complete Na*RdR3* gene was isolated using a PCR-based approach. By comparing sequences from amplified genomic DNA and cDNA, and Fourier transformation, we showed that the Na*RdR3* gene contains a single intron (Figure 1). *RdR3* from *N. attenuata* had high levels of sequence similarity (>90%; Figure 1) with its corresponding homologues from *N. benthamiana* and *Arabidopsis,* but an extremely low level of sequence similarity was found in the other *RdRs* of *N. attenuata* or from closely related species.

The expression patterns of the genes after application of different stresses or elicitors that mimic them have been used as reliable marker for function determination. In order 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 the transcripts was recorded (Figure 1C) when the leaves were elicited with mechanical wounding followed by the immediate application of *Manduca sexta* oral secretions (OS elicitation). OS elicitation has been proved 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). Applying salicylic acid (SA) had little effect on the accumulation of Na*RdR3* transcripts (Figure 1C). SA accumulates after herbivore and pathogen attacks, and orchestrates induced plant defense responses (Rayapuram and Baldwin, 2007). Also, no variations in the *RdR3* transcripts were detected during the diurnal change of the plant (Supplementary figure 1).

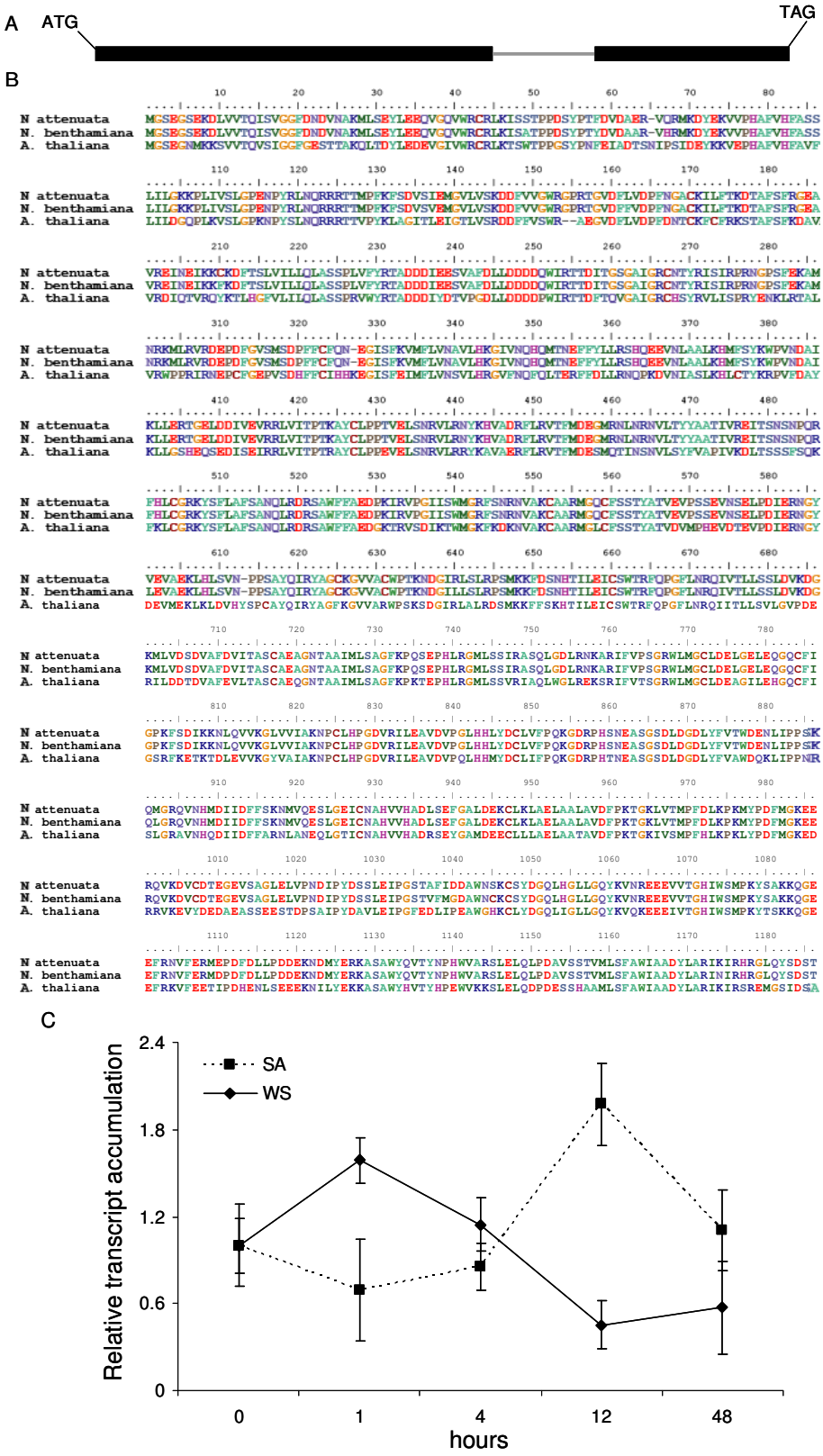

Figure 1. Characterization of *RdR3* from *N. attenuata*. **(A)** shows the *RdR3* gene structure **(B)** shows that the Na*RdR3* is highly similar to other *RdR6* homologs **(C)** Time-course analysis of *RdR3* transcript induction after 2

mM salicylic acid (SA) (dotted line, solid squares) treatment and simulated herbivory (W+OS: wounding with a fabric pattern wheel and immediate treatment with 20 mL *M. sexta* oral secretions) (solid line, solid diamonds).

Minor phenotypic changes after stably silencing* RdR3 *do not affect plant growth or defense

Plants stably silenced for *RdR3* expression (irRdR3) were produced by transforming *N. attenuata* with an *RdR3*-specific gene fragment in an inverted-repeat orientation (Supplementary Figure 2), using *Agrobacterium*-mediated transformation (Kruegel et al., 2002). After a high-throughput phenotypic screening (Kruegel et al., 2002) and verification by Southern analysis that a single-copy of transgene had been incorporated, two independently transformed lines harboring single inserts (244-4 and 282-3; Supplementary Figure 2) were analyzed for relative transcript accumulation of the *RdR3* gene after OS elicitation: neither had accumulated any *RdR3* transcripts (Figure 2A). Because, *RdR6* homologs in *N. benthamiana* and *Arabidopsis* have been traditionally 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 further confirm that *RdR3* had been silenced at a functional level. Both lines were severely affected by tobamo-viruses, resulting in highly impaired growth, rapid senescence and death (Figure 2B).

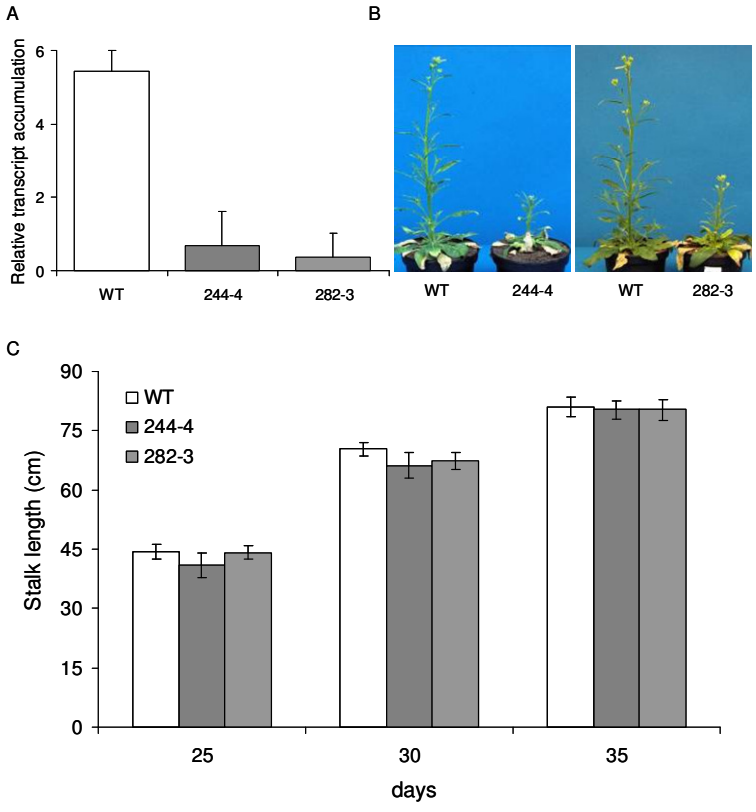

Figure 2. Silencing *RdR3* does not affect normal plant growth. **(A)** *RdR3* transcript accumulation in two independently transformed homozygous lines harboringi a single copy of a fragment of Na*RdR3* in an inverted-repeat construct. Both lines (244-4 and 282-3) failed to accumulate *RdR3* trancripts after OS elicitation. **(B)** Susceptibility of irRdR3 lines to viruses. When rosette leaves were inoculated with tobamo-virus, growth of the two irRdR3 lines was severely impaired and plants of both rapidly senesced and died, confirming that the *RdR3*-transcripts levels were severely reduced. **(C)** Shows that silencing *RdR3* transcript accumulation did not affect plant growth. The stalk (cm) of irRdR3 (gray bars) and WT (white bars) plants in single pots was measured 25, 30 and 35 days after transplanting (Ps > 0.05).

Single mutants of *RdR6* in *Arabidopsis* display minor phenotypes, which typically include elongated leaves, and reduced leaf numbers (Peragine et al., 2004) (Li et al., 2005). They have been associated with leaf development but silencing *RdR6* only does not alter plant physiology very much. We observed similar phenotypes of elongated leaves due to increased petiole length, as well as reduction in one-two leaf nodes (Supplementary Figure 3). But these alterations in plant morphology did not affect plant growth (repeated measures ANOVA, $F_{2.51}$=0.5, P>0.05; Figure 2C).

Because herbivores instead of viruses are the main biotic stress, as well as the accumulation of *RdR3* transcripts is increased upon OS elicitation, we investigated the possible role of *RdR3* in plant defense against insect herbivores. We challenged WT and irRdR3 plants with *M. sexta* larvae and compared insect performances: no differences were found (ANOVA, F= 0.02, P>0.05; Supplementary Figure 4). This indicated that silencing *RdR3* had no effect on plant defense against insect herbivores.

Changes in smRNA transcriptome after* RdR3 *silencing

We next investigated how the small-RNA profiles change after silencing *RdR3* in *N. attenuata*. *Arabidopsis RdR6* homolog is associated with trans-acting 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) smRNAs in the range of 22-23 nucleotides were specifically reduced upon *RdR3* silencing in *N. attenuata* (Supplementary Figure 5). Out of 31256 unique smRNAs from irRdR3 plants, only 5% overlapped with the WT smRNA sequences (Figure 3A). This may be due to the increased abundance of smRNAs in size classes <21 nt and >24 nt (Supplementary Figure 5). Analysis of the commonly regulated 1529 smRNAs between WT and irRdR3 plants revealed that 315 smRNAs were down-regulated, whereas 580 smRNAs were enriched in irRdR3 plants (Figure 3B). We annotated these sequences against the non-redundant nucleotide database (NR-DB) of NCBI and the miRBase Sequence Database (Tables S1 and 2). Out of 31,256 unique smRNAs from irRdR3 plants, 12,792 sequences matched different categories of RNA sequences (Figure 3C), including 21 miRNAs.

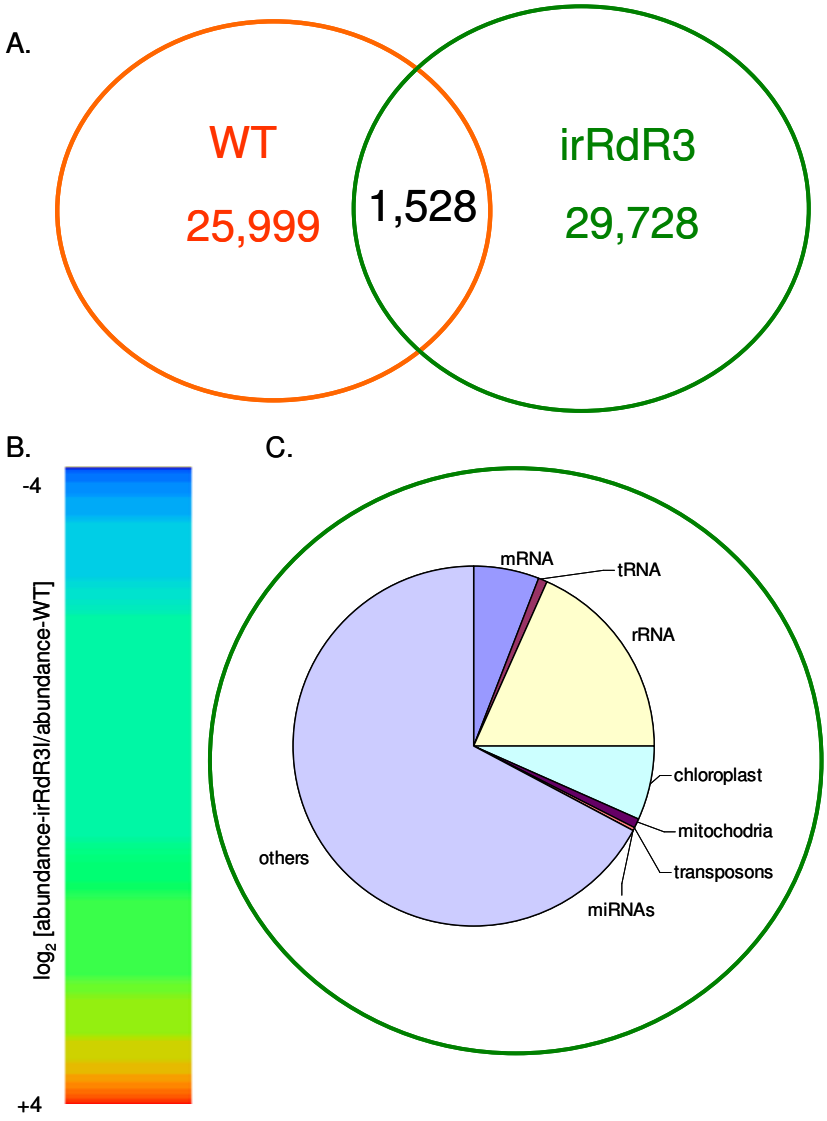

Figure 3. Changes in small-RNA transcriptome after *RdR3* silencing. Changes in smRNA profiles after RdR3 silencing were measured and compared with the WT profiles using 454 sequencing. **(A)** Venn diagram of the overlapping and non-overlapping smRNA sequences between irRdR3 and WT plants. Out of 31256 unique smRNAs from irRdR3 plants, only 5% overlapped with the WT profile. **(B)** Behaviour of the commonly regulated smRNAs between WT and irRdR3 plants profiles. The ratio of their abundance in irRdR3 plants compared to WT was *log*(2) transformed and plotted as a color gradient. 315 smRNAs were down-regulated in irRdR3 plants and 580 smRNAs were enriched. **(C)** Unique smRNAs from irRdR3 were annotated against the non-redundant nucleotide database (NR-DB) of NCBI and the miRBase Sequence Database (Tables S1 and S2). 12792 sequence matches were classified into to 8 different sequence categories.

Silencing RdR3 *reduces plant performance in native habitats but does not affect plant defense against herbivores*

Silencing *RdR3* had no effect on plant growth and defense under glasshouse conditions. In order 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

eco-physiology of plants, we tested the performance of irRdR3 lines in their native habitat. In nature, *N. attenauta* plants face two major stresses, an unpredictable herbivore population and high intra-species competition. To simulate the latter, we planted WT and irRdR3 plants in pairs in close proximity (~20 cm apart) (Supplementary Figure 6). In natural environments, irRdR3 plants were able to defend both themselves as well as the WT plants against natural herbivores (Figure 4).

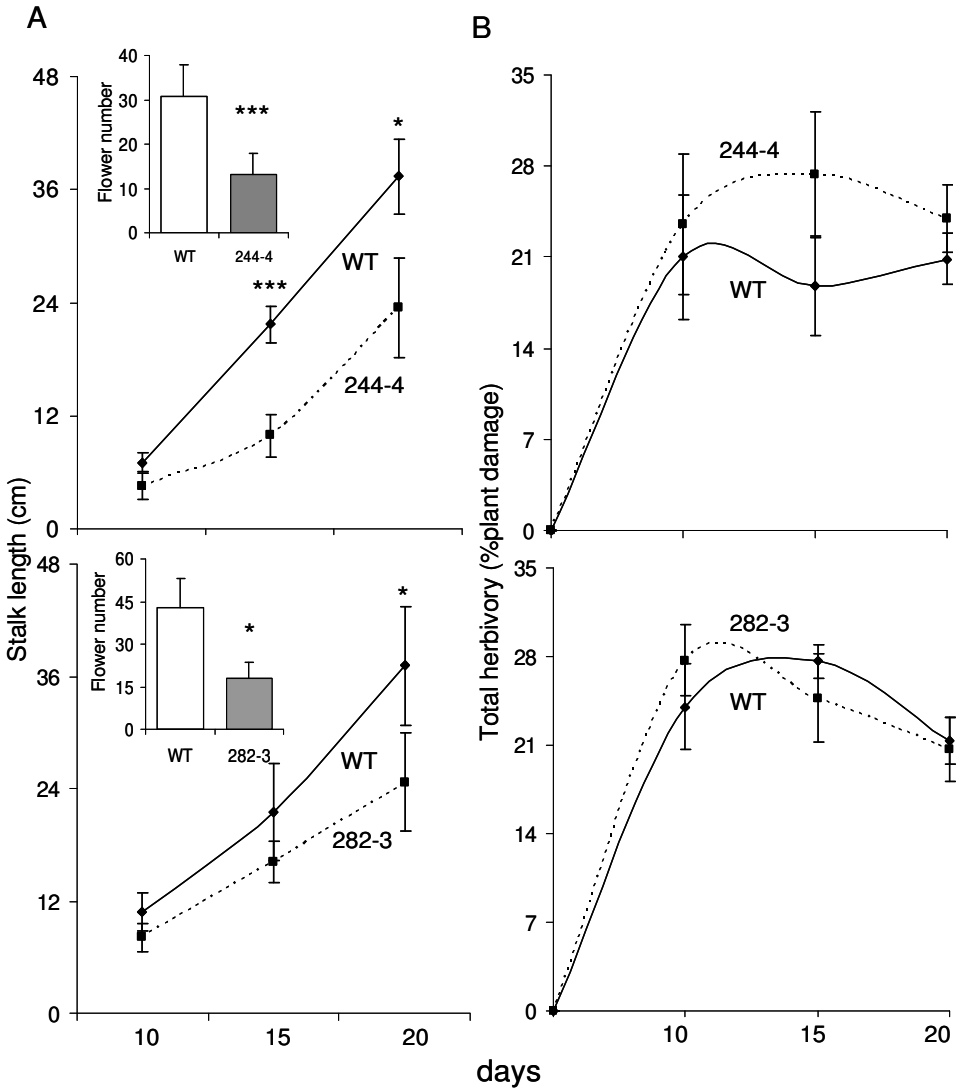

Figure 4. Silencing *RdR3* reduces performance in plants' native habitat but does not affect plant defense against herbivores. Every irRdR3 plant from each line (244-4 and 282-3) was individually paired with a WT plant in close proximity (~20 cm) in the plant's native habitat. **(A)** Plants were transplanted to the field at day 0 and were undamaged. The stalk lengths (cm) of irRdR3 (dotted lines) and WT (solid lines) plants were measured 10, 15, and 20 days after transplanting to the field. Total numbers of flowers produced during the duration of the study are presented as inserts. (**B**) The cumulative damage to irRdR3 plants (dotted lines) and WT plants (solid lines) was monitored with a similar frequency. *** significantly different at P<0.005, *significantly different at P<0.05.

Because the plants were highly susceptible to tobamo-viruses when tested in the glasshouse (Figure 2), we expected the irRdR3 plants to act as biosensors, to demonstrate viral infection symptoms even if only the slightest inoculum was present in nature. Yet, no viral infections were recorded on WT or irRdR3 plants. Instead, the irRdR3 plants could not grow as well as their WT counterparts. Total plant canopy area damaged and plant heights were recorded 10 days after release of WT and irRdR3 plants in the field. No differences were observed in herbivory (WT-244-4 pairs; n=10 pairs; paired t-test; $P>0.05$) or plant performance (WT-244-4 pairs; n=10 pairs; paired t-test, $P>0.05$) between the WT and irRdR3 plants. In a subsequent reading after 5 days, herbivory remained unaltered (Figure 4; n=10 pairs; paired t-test; $P>0.05$), but irRdR3 plants grew more slowly than the WT plants (Figure 4; n=10 pairs, paired t-test, $t=6.16$, $P<0.005$). A third and final reading was recorded 5 days after the second one. The slow growth of irRdR3 plants continued (n=10 pairs; paired t-test; $t=2.51$; $P<0.05$), with similar amounts of damage on irRdR3 and WT controls (Figure 4; n=10 pairs; pairedt-test, $P>0.05$). Similarly decreased plant performance (Figure 4; paired-test, $t=2.69$; $P<0.05$) and unaltered plant defense (Figure 4; paired t-test; $P>0.05$) were observed in second transformant (282-3; Figure 4).

Although we were not able to measure fitness parameters completely, silencing irRdR3 had deleterious effects on plant fitness. The total numbers of flowers produced during the duration of the study was significantly less in 244-4 (Figure 4; paired t-test, $t=4.45$, $P<0.005$) as well as 282-3 (Figure 4; paired t-test, $t=2.55$, $P<0.05$) lines compared to WT counterparts. This indicated that irRdR3-silenced plants were reduced in their ability to compete with their WT neighbors.

Silencing* RdR3 *reduces plants' ability to compete with their neighbors

In order to verify the reduced plant performance observed in nature, we conducted extensive assays under controlled glasshouse conditions (I) without resource competition (single pots); and (II) with resource competition (plants were grown in close proximity ---~7-10 cm apart -- in 2 L pots). In competition experiments, the irRdR3 and WT plants were combined as follows: WT – WT, 244-4 – 244-4, WT – 244-4, 282-3 – 282-3, and WT – 282-3. No differences in plant performance were recorded between the WT and irRdR3 plants when grown in single pots (described above). In contrast to their performance in single pots, WT plants out-competed the irRdR3 plants when these genotypes were tested in competition

(Figure 5; repeated measures ANOVA; WT-244-4 combination; $F_{1,118}$=256.1; P<0.0001; WT-282-3 combination; $F_{1,138}$=89.0; P<0.0001).

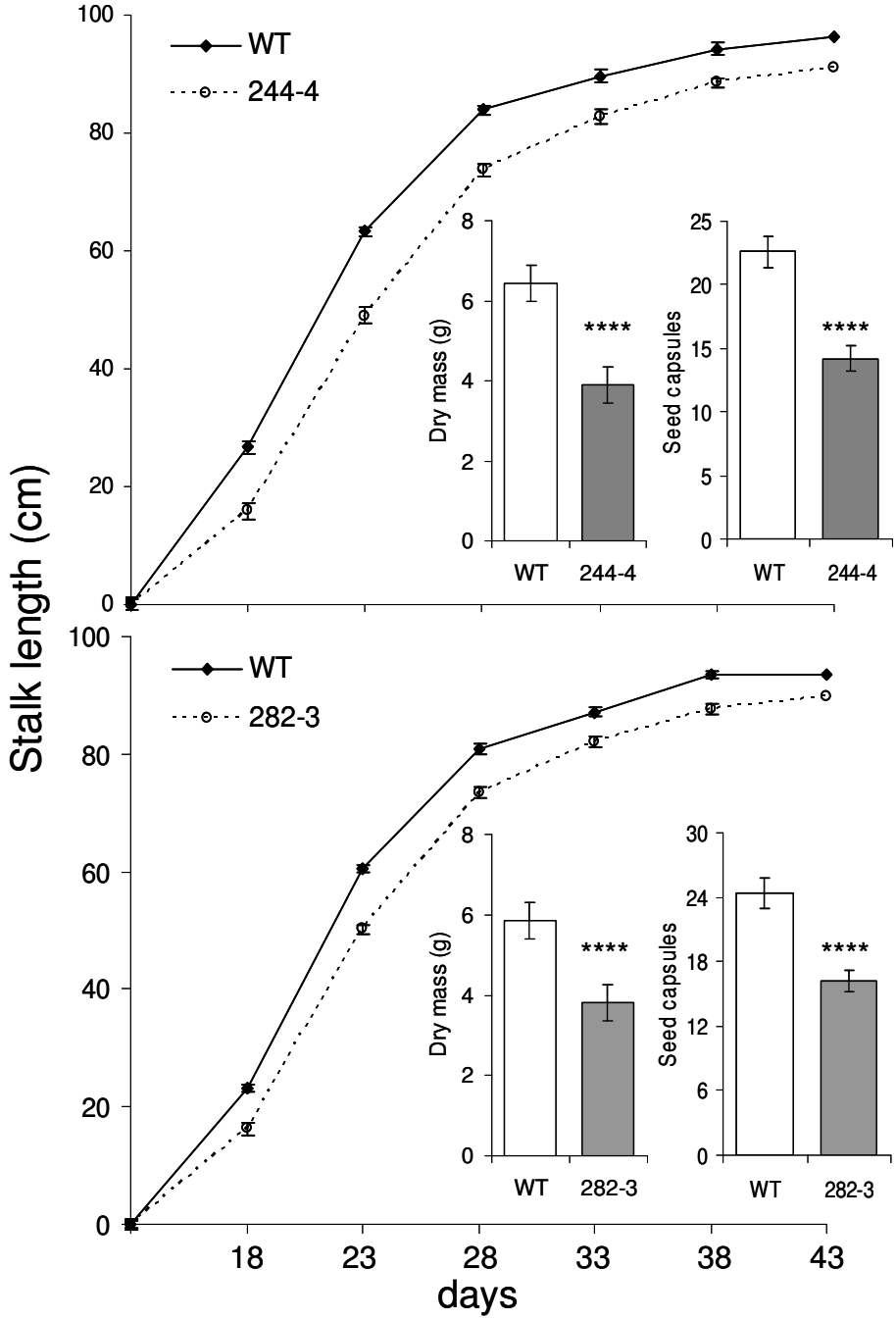

Figure 5. Silencing *RdR3* reduces plants' ability to compete with neighbours. Every irRdR3 plant from each line and WT plants were grown in close proximity (7-10 cm) in 2 L pots. The stalk lengths (cm) of irRdR3 (dotted lines) and WT (solid lines) plants were measured 18, 23, 28, 33, 38, and 43 days after transplanting. Plant dry mass (g) and (lifetime) seed capsule numbers produced during the duration of the study are presented as inserts. **** significantly different at P<0.0005.

No differences in plant performance was observed when the plants from the same genotype competed with each other (Supplementary Figure 7; repeated measures ANOVA; Ps>0.05). The statistically significant reduction in plant height during competition would be biologically significant only if the fitness of irRdR3 plants was affected. Confirming this hypothesis, there was a strong reduction in the lifetime seed capsule numbers (Figure 5; paired t-test; for WT-244-4, t=6.42; P<0.0001; for WT-282-3; t=10.70, P<0.0001) of irRdR3 plants as well as in plant dry weight (Figure 5; paired t-test; for WT-24404, t=8.01; P<0.0001; for WT-282-3; t=15.48, P<0.0001.). There were no differences in either seed capsule numbers (Supplementary Figure 7; paired t-tests; t Ps>0.05) or dry weight (Supplementary Figure 7; paired t- tests; Ps>0.05) when plants from the same genotype competed with each other.

Transcriptional responses of* RdR3-*silenced plants when these compete with WT plants

In order to understand how transcriptional responses are altered in *RdR3*-silenced plants and to predict the potential targets of smRNAs, we performed microarray analysis with an unbiased potato 10K-cDNA microarray (TIGR), previously proven suitable for *N. attenuata* (Schmidt et al., 2005). Using RNA extracted from irRdR3 plants, we hybridized arrays against the corresponding WT plants; both genotypes had been growing in competition prior to leaf tissue harvest. Three replicate chips were made with biologically replicated RNA samples. Only 97genes were differentially regulated (Table S3) at a cut-off of 1.5-fold or greater; 90 genes were down-regulated and 7 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 down-regulated, as were some transport proteins. Many of the regulated genes were of unknown function. In *Arabidopsis*, when the transcript profiles of WT and *RdR6*-mutated plants were compared with Affymetrix ATH1 arrays with 22800 genes, at a cut-off as low as 1.3-fold, only 17 genes were differentially regulated, most of which were false-positive (Peragine et al., 2004). This indicates that the *RdR6*-dependent siRNAs may not act on the transcripts but may affect the end-product, possibly by regulating protein biosynthesis.

Phytohormone analysis

In order to understand the mechanistic bases 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 potentiated by natural herbivores and OS elicited, we tested the accumulation of 4 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 isoleucine/leucine conjugate (JA-Ile/Leu). Whereas ABA may be regarded as a general abiotic stress marker, JA, JA-Ile/Leu, and SA are involved in plant defense against natural herbivores in *N. attenuata* (Halitschke and Baldwin, 2003; Kang et al., 2006; Paschold et al., 2007; Rayapuram and Baldwin, 2007). Constitutive ABA levels were slightly higher in irRdR3 plants than in their WT counterparts (Supplementary Figure 8). JA and JA-Ile/Leu levels in irRdR3 were similar to those in WT plants (Supplementary Figure 8; paired t-test, $P>0.05$), indicating that irRdR3 plants had intact defense signaling. Compared to WT plants, irRdR3 plants had enhanced SA levels after 120 min of OS elicitation (Supplementary Figure 8; ANOVA, $F_{1,6}=5.68$, $P<0.05$). High SA and ABA levels may contribute to reduced growth of irRdR3 plants when grown in competitive stress conditions.

In order to confirm this hypothesis, we monitored the profiles of phytohormones in controlled glasshouse conditions, when irRdR3 and WT plants were subjected only to competitive stress. 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 between WT and irRdR3 plants (Supplementary Figure 9). 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 increased over their levels in WT plants.

The phytohormone most associated with plant growth (Teale et al., 2006) and response to competition-related stresses is auxin (Morelli and Ruberti, 2000; Hoecker et al., 2004). We tested auxin levels in the first stem leaves of the competing irRdR3 and WT plants at different stages of plant growth, starting just after the stem began to elongate and lasting until the plants entered the reproductive phase and started to produce seed capsules. Overall irRdR3 plants had reduced auxin levels (Figure 6A), with maximum differences occurring during the early vegetative phase, 18 days after transplanting (repeated measures ANOVA, $F_{1,22}=13.62$, $P<0.01$).

There are two possible explanations for the reduction in auxin levels: either the transport of auxin from the apical meristem downwards may be hampered, or its biosynthesis may be altered. In order to test whether the reduced auxin levels, and hence the growth of irRdR3 plants, was due to obstructed auxin transport, we performed auxin-complementation assays using lanolin paste as previously described in Reinhardt et al. (Reinhardt et al., 2000);

there auxin was exogenously supplied to the apical meristem and plant performance was measured . Such complementation assays are commonly used to study hampered transport. Exogenous supply of auxin (1 mM) could not restore the growth of irRdR3 plants to WT levels (Figure 6B; repeated measures ANOVA, $F_{1,34}$=41.64, P<0.0001); rather the additional auxin slowed growth. In order to confirm that the biosynthesis of auxin was not affected in irRdR3 plants, we measured auxin levels in the apical meristem of competing irRdR3 and WT plants: no differences were found (Figure 6C; paired t-test, P>0.05).

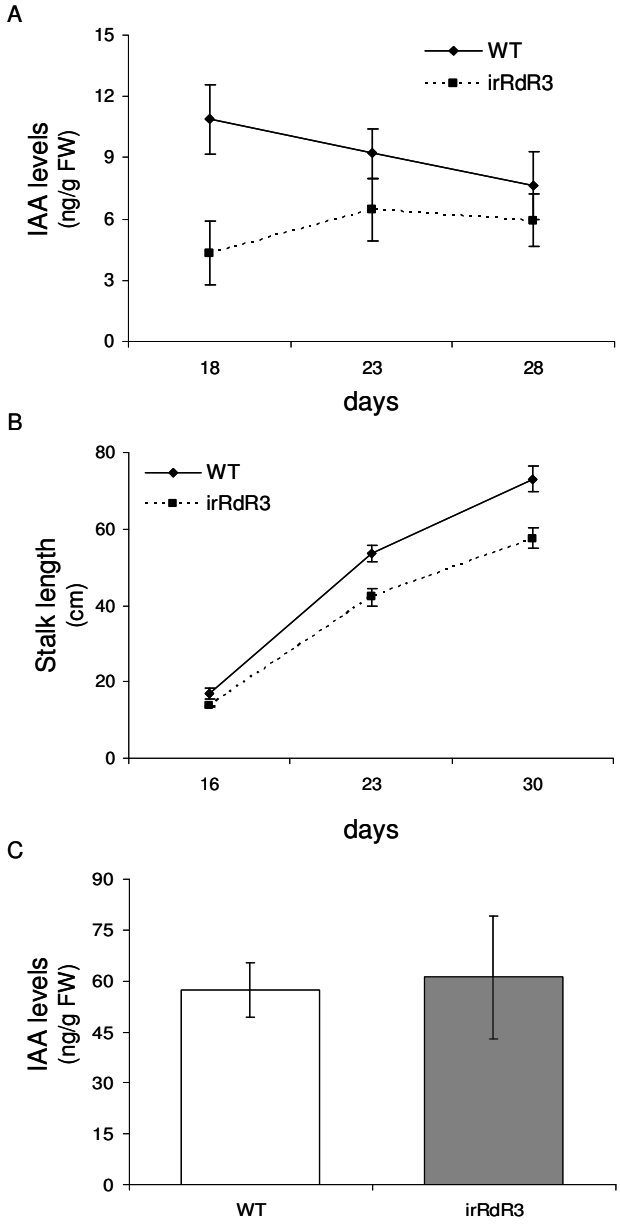

Figure 6. The transport rather than biosynthesis of IAA is altered in irRdR3 plants competing with WT plants. **(A)** IAA concentration was measured in the first stem leaves of the competing irRdR3 (dotted lines) and WT (solid lines) plants from the early vegetative phase till the plants started to produce seed capsules (18, 23, and 30

days after transplanting). IAA levels were highly significantly lower in irRdR3 plants than WT plants (repeated measures ANOVA, P<0.001) **(B)** Exogenous supply of IAA did not restore growth of irRdR3 plants when they competed with WT (repeated measures ANOVA, P<0.0001). IAA in lanolin paste (1 mM) was exogenously supplied to the apical meristem of competing irRdR3 (dotted lines) and WT (solid lines) plants at the late rosette and early elongation stages, and their stalk lengths (cm) were measured after 16, 23, and 30 days. **(C)** IAA concentration was measured in the apical meristem of competing irRdR3 (white bar) and WT (black bar) plants. No differences were observed (Paired t-test, P>0.05).

Discussion

In this study, we tried to decipher the ecological role of *RdR6* homolog in *N. attenuata* (NaRdR3) and to show that it is essential for plants' acclimatization in highly competing environments. *N. attenuata* plants whose *RdR3* gene was silenced, were unable to grow well and had severely reduced fitness in their natural habitats as well as in the glasshouse, where they were planted in close competition with WT neighbors. This reduced adaptive plasticity of *RdR3*-silenced plants in highly competing environments was due to altered homeostasis of phytohormones. Traditionally, virus resistance has been functionally associated with RdR6, but its relevance in natural habitats has not been tested; neither has its function been evaluated in environments where viruses are not a selective force. In this regard, the current study demonstrates the real-world relevance of *RdR3* phenotypes, which is significant from mechanistic as well as educational viewpoints, a point largely neglected till now.

RdRs forms an important component of RNA-silencing/PTGS machinery by synthesizing dsRNAs which are progenitors of siRNAs. Six *RdRs* 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 a previous study we demonstrated that *RdR1* mediates plant resistance to insect herbivores (Pandey and Baldwin, 2007). *N. attenuata* plants silenced for their *RdR1* expression were as able to grow and acclimatize in natural habitat as their WT counterparts, but were susceptible to natural herbivores, the most important biotic stress for this species.

The other most important (abiotic) stress that *N. attenuata* plants encounter in their natural habitat is intra-species competition. *N. attenuata*, the founding species in post-fire environments, grows 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 capacity to grow fast and reproduce in environments with initially unlimited resources which are then quickly depleted. The plants have to allocate their resources, especially their rapidly depleting nitrogen (N) supply, between plant growth and defense (Lynds and Baldwin, 1998). Initial differences in

the 'vegetative fitness' of plants (its ability to successfully grow, increase biomass, maximize and defend its photosynthetic area by avoiding light competition) severely affect their reproductive fitness (number of seed capsules), and thus their representation in seed banks. In this investigation we have demonstrated that the *RdR3* gene provides the regulatory basis for this part of phenotypic plasticity in *N. attenuata*.

Phenotypic plasticity can be defined simply as environmentally dependent phenotypic expression, i.e. the ability of an individual to alter its physiology, morphology, and/or behavior in response to environment (Sultan, 2000). *N. attenuata* engineers its physiology in response to an unpredictable herbivore community and the surrounding plant population; it must use resources as efficiently as possible to achieve high vegetative fitness and r transform it into high reproductive fitness. Altering the expression of *RdR3* transcripts altered the vegetative and reproductive fitness of plants in nature. This was confirmed in glasshouse experiments where the plants were grown in close proximity in single pots.

In order to understand the mechanistic basis underlying the reduced competitive ability of the irRdR3 plants, we adopted a two-tiered strategy. At first we performed an unbiased comparative analysis of the transcriptomes of irRdR3 and WT plants grown in competition. This analysis was not very helpful in obtaining mechanistic details. Similar observations were made in *Arabidopsis*, where microarray studies with a much larger array (of 22,800 clones) and low cut-offs of 1.3-fold showed negligible changes in transcript (just 17, most of which were false positive) when RNA from *RdR6*-mutated plants was hybridized against RNA from WT plants (Peragine et al., 2004). It may be that *RdR3*-dependent siRNAs are regulating not the transcript accumulation of their targets, rather, protein levels.

Second, we profiled a set of phytohormones known to relay signaling networks that fine-tune plant growth and chemical adaptative changes. The levels of JA and its amino-acid JA-Ile/Leu, the two well-described regulators of most of *Nicotiana attenuata* responses to herbivory (Halitschke and Baldwin, 2003; Kang et al., 2006), were unchanged in irRdR3 plants, which clearly supports the data ruling out the involvement of *RdR3* in anti-herbivore defense. On the other hand, we showed that under competing conditions, irRdR3 plants produced more SA and contained lower local IAA amounts than WT did. In contrast to the well-documented role of IAA during shade avoidance (Morelli and Ruberti, 2000; Hoecker et al., 2004), the influence of SA during light competition is rather scarce (Genoud et al., 2002). Nevertheless, it is noteworthy that plants accumulating SA frequently display morphological

phenotypes that are reminiscent of IAA-deficient or IAA-insensitive mutants, like reduced apical dominance and stunted growth (Bowling et al., 1997; Clarke et al., 1998; Li et al., 2001). Recently published data have linked this phenotype to 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 alteration of IAA's transport rather than of its biosynthesis could be one element responsible for the decreased vegetative fitness of irRdR3 plants. Forming and maintaining IAA gradients *in planta* are well-known to depend on a specific polar auxin-transport system. Carrier proteins involved in such a process have been recently identified in *A. thaliana* (Galweiler et al., 1998; Chen et al., 1999); however, how they drive developmental plasticity in the real world is still under-investigated. Like our results, results from glasshouse-based studies of the shade-avoidance phenomenon have shown that IAA transport more than production plays an essential coordinating function (Morelli and Ruberti, 2000). Treatment with napthylphtalamic acid, an IAA transport inhibitor, significantly reduced hypocotyl elongation of wild-type seedlings in response to FR-rich light (Steindler et al., 1999).

In conclusion, we have identified some of *RdR3*'s roles in plant adaptation to highly competing habitats. 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 regulating IAA signaling during competition.

Experimental procedures

Plant and insect material

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

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*, *N. benthamiana,* and *N. tabaccum*. PCR was made of 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 a gel band purification kit (Amersham Biosciences, Freiburg, Germany) 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. tabaccum*. Fragments giving a positive alignment were considered to be exons. Sequences that did not match the corresponding homologues were tentative introns and subjected to Fourier analysis to determine if they were non-coding (Tiwari et al., 1997). Gene sequences from different *RdRs* (accession numbers given below) were aligned with MegAlign (DNASTAR, Madison, WI, USA).

Expression analysis by quantitative real-time PCR (qPCR)

RdR3 homologs (*RdR6*) in *Arabidopsis* and *N. benthamiana* are elicited by salicylic acid treatments and virus attack, and their role in viral defense 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 20µl (diluted 1:1 with distilled water) *M. sexta* oral secretions (OS), which are known to activate the herbivore-specific responses in *N. attenuata* (Halitschke et al., 2001). We also determined the effect of SA on *RdR3* transcript levels by spraying plants with a 2mM SA solution until runoff (Yang et al., 2004; Pandey and Baldwin, 2007). OS- or SA- treated +2 leaves were harvested from 3-4 replicate plants at 0, 1, 4, 12, and 48 h after each treatment. To determine diurnal fluctuations in *RdR3* transcripts, we harvested leaves from untreated +2 nodes of four replicate plants at 4:00h, 8:00h, 12:00h, 16:00h, 20:00h, and 24:00h.

Total RNA was extracted following the TRIZOL method and reverse-transcribed to prepare first-strand cDNA with the SuperScript first-strand synthesis system for RT-PCR, with oligo(dT) as primers (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol.

"SYBR Green" assays were developed (qPCR core kit for SYBR Green I, Eurogentec, Seraing, Belgium, following the manufacturer's protocol); all the qPCR assays were performed with cDNA corresponding to 100ng 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 0h treatment) were fixed to 1 and relative expression levels were calculated with respect to this reference value. *N. attenuata* sulfite reductase (*ECI*), a house-keeping gene involved in sulfur metabolism, whose transcript abundance is unchanged from constitutive levels upon OS elicitation, was used as 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.

***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 tobamo-viruses, tomato mosaic virus and bell pepper mosaic viruses were inoculated into +1, 2, and 3 leaves of the WT and transgenic plants. Leaves of 3 replicate plants of WT and both transgenic lines in the rosette stage (28 days after germination) were rubbed with corborundem powder, and 50µl 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 corborundem powder and treated with 50µl of phosphate buffer without any virus. Plants were monitored for the development of symptoms for 12-14 days.

Insect performance and competition assays

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

In order to compare the competitive ability of irRdR3 and WT 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 days after germination, sized-matched seedling pairs were transplanted, ~7 cm apart in 2 L pots in following combinations: WT – WT, 244-4 – 244-4, 282-3 – 282-3, WT – 244-4 and WT – 282-3. Plant growth was recorded 18 days after transplanting, when all the plants had started to elongate. 6 subsequent readings were taken at 7-day intervals.

Performance under native conditions

The planting of RdR3-silenced plants (irRdR2) into the natural habitats of *N. attenuata*, in the Great Basin Desert in southwestern Utah, USA, under APHIS notification number 06-003-08, was as described in Pandey and Baldwin (2007). 10 days after germination, seedlings were transferred to borax-soaked Jiffy 703 pots (AlwaysGrows) and 3-4 weeks later they were transferred to field plots. Ten irRdR3-WT pairs of same-size adapted seedlings from both the 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 days. 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 readings at 5-day intervals, stalk length was recorded and the leaves of irRdR3-WT 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 10K-cDNA microarray chips (TIGR) initially tested for *N. attenuata* (Schmidt et al., 2005). First stem leaves were harvested from three biological

replicates of WT-irRdR3 pairs, grown in competition for 18 days. Total RNA was extracted following 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 WT counterparts were 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 package, and differential regulation was determined at a cut-off of 1.5-fold.

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, ~ 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 re-extracted 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 hr 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 hr 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. Eluates were combined and then 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 (RP-HPLC/ESI-MS/MS) as described earlier (Wu et al., 2007). Multiple reactions monitoring (MRM) was conduced on a 1200L MS/MS system (Varian, Palo Alto, CA, USA), 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), 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 x (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 (Larodan Fine Chemicals, Malmö, Sweden) 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 (Edlund et al., 1995; Kasschau et al., 2007; Molnar et al., 2007) along with the other genotypes described elsewhere (Pandey et al., 2007). The 454 sequencing was performed by Vertis Biotechnologie AG (Freising, Germany). Briefly, leaf material was ground under liquid nitrogen, RNA species <200 bp were enriched (mirVana miRNA isolation kit, Ambion, Austin, TX, USA), small-RNAs were separated on a denaturing 12.5% polyacrylamide gel and stained with SYBERgreen II, and size fractions of 15-30 nt 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'-phosphate, and first-strand cDNA synthesis was performed using oligo(dT) primer linker, and PCR amplified to about 30ng/µl with 22 cycles using high fidelity Taq DNA polymerase. The bar codes, attached to a 5' flanking sequence, for the two genotypes were ACTA (WT) 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 (Freising, Germany).

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 nt were discarded. According to manufacturer's instructions, the technology is not able to distinguish more than 8 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 1 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., Berkeley, CA, USA). Assays in the glasshouse were analyzed for the analysis of variance (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-test because all the field and competition experiments were conducted in pairs. A transgenic line and WT control plant was planted as single pair.

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Supplemental figures

Figure S1. qPCR analysis of *RdR3* transcript accumulation as a function of diurnal rhythm shows no differences.

Figure S2. Southern analysis of the two independently transformed irRdR1 lines showing single insertion. Genomic DNA (10 µg) from individual plants was digested with ECoR1 and blotted onto a nylon membrane. The blot was hybridized with a PCR fragment of the hygromycin phosphotransferase II gene, specific for the selective marker on the T-DNA.

Figure S3. Minor phenotypic changes after stably silencing *RdR3*. *RdR3*-silenced plants display typical phenotypes: elongated leaves and reduced leaf number (Peragine et al., 2004).

Figure S4. *M. sexta* performance in *N. attenuata* WT 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 days. No differences were observed ($P>0.05$), indicating that irRdR3 plants had intact direct defenses.

Figure S5. Qualitative and quantitative smRNAs profiles obtained using 454 sequencing. Abundance of smRNAs in the range of 22-23 nucleotides were specifically decreased in irRdR3 plants (gray bars) compared to WT plants (white bars).

Figure S6. Morphology of irRdR3 lines and WT plants competing in their natural habitat. Left picture shows 244-4 in combination with a WT plant. Right picture shows 282-3 in combination with a WT plant. Both irRdR3 lines were severely reduced in their growth ($P<0.05$) but had similar herbivore damage ($P>0.05$).

Figure S7. irRdR3 and WT plants were grown in close proximity (7-10 cm) in 2 L pots in the following combination: WT – WT, 244-4 – 244-4, 282-3 – 282-3. The stalk length (cm) of irRdR3 and WT plants was measured 18, 23, 28, 33, 38 and 43 days after transplanting. No differences were observed ($P>0.05$). Plant dry mass (g) and seed capsule numbers produced during the duration of the study are presented as inserts.

Figure S8. Phytohormone analysis in the field. (A) Analysis of jasmonic acid (JA), its isoleucine/leucine conjugate (JA-Ile/Leu), and salicylic acid (SA) accumulation in first fully expanded leaves during simulated herbivory (W+OS: wounding with a fabric pattern wheel and immediate treatment with 20 mL *M. sexta* oral secretions) shows that JA and JA-Ile/Leu levels were similar, whereas SA levels were enhanced in irRdR3 plants. **(B)** Constitutive ABA levels were more in irRDR3 (gray bars) than those in WT plants (white bars).

Figure S9. Phytohormone analysis in glasshouse conditions during competition. Analysis of the constitutive concentrations of jasmonic acid (JA), its isoleucine/leucine conjugate (JA-Ile/Leu), salicylic acid (SA) and abscissic acid (ABA) in first fully expanded leaves in irRdR3 (gray bars) and WT (white bars) plants confirms results from the field.

References

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 Physiology **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 Biotechnology **4:** http://www.biomedcentral.com/1472-6750/1474/1414

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 Reports **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 posttranscriptional 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 Physiology **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 Journal **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 Physiology **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 Journal **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, Meyers BC (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 Physiology **122:** 621-626

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (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, Shahi P, Gase K, Baldwin IT (2007) Herbivory-induced changes in the Small-RNA Transcriptome and Phytohormone Signaling in Nicotiana attenuata. Proc Natl Acad Sci U S A **In Review**

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 Journal ***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 Molecular Biology **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. Molecular Ecology **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 Physiology **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 Physiology **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 Biology **2:** 1074-1080

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends in Plant Science **5:** 537-542

Teale WD, Paponov IA, Palme K (2006) Auxin in action: signalling, transport and the control of plant growth and development. Nature Reviews Molecular Cell Biology **7:** 847-859

Tiwari S, Ramachandran S, Bhattacharya A, Bhattacharya S, Ramaswamy R (1997) Prediction of probable genes by Fourier analysis of genomic sequences. Computer Applications in the Biosciences **13:** 263-270

Van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in Nicotiana attenuata. Journal of Chemical Ecology **27:** 547-568

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. Current Opinion in Plant Biology **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 in Plant Science **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. Proceedings of the National Academy of Sciences of the United States of America **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 Biology **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. Proceedings of the National Academy of Sciences of the United States of America **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. Molecular Plant-Microbe Interactions **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 U S A **101:** 1607-1612

Accession numbers

The gene sequences of the *N. attenuata* RdR3 has been submitted to the NCBI under accession 1042781 The accession numbers for the other RdRs used in this study are: AY722008 NbSDE1; AF239718 AtRdR6/SGS2.

3. Discussion

The specificity of RNA silencing is conferred by smRNAs. Much is understood about their biogenesis, and different components of the biosynthetic pathways have been worked out in model organism such as nematodes, yeast, drosophila, mammals, and *Arabidopsis*. But the exploration of functions of smRNAs in regulating different (transcriptional) network has just begun. Identifying functional roles of smRNA-based regulation at the level of specific tissues and whole organisms is crucial for a more integrative understanding of how RNA silencing functions in cellular, developmental (Chapman and Carrington, 2007) and ecological contexts. In the current study, we have done exactly this. We have explored the roles of smRNA biosynthesis pathways in the context of whole-organism functions: defense and adaptation in natural habitats in the ecological model, *N. attenuata*.

3.1 Small-RNA pathways: diversification and specialization

The discovery of (sm)RNAs as guides in processes of sequence-specific recognition and in processing other RNAs, and not just as carriers of genetic information, has radically changed our understanding of gene regulation. We have gone from a state of collective ignorance about the regulatory roles of (sm) RNA (and RNA silencing pathways) to an understanding which puts them at center stage in molecular and developmental biology research. Although smRNA-mediated regulations have emerged as topic of general interest in the past 8-9 years, the first RNA silencing paper, as pointed out by Baulcombe (2004), may have been published as long ago as 1928, and could be regarded as sthe tarting point for our current state of understanding of RNA silencing processes. Gaining attention because of their roles in plant defense against viruses, components for smRNA biogenesis have specialized in eukaryotic lineages, resulting in diversified pathways that control transposons, regulation of gene expression during development and differentiation, and viral defense.

Functionally, there are several kinds of smRNAs in plants and animals (Chapman and Carrington, 2007), such as miRNAs, siRNAs from inverted and direct repeats, smRNAs from bidirectional transcripts, TAS loci, piRNAs, and siRNAs from exogenous agents. We can group these smRNAs into three broad categories according to the type of loci that generated them: some form precursor transcripts that fold into secondary structures, having DICER substrate activity, which do not require RNA-directed RNA polymerases (*RdRs*); types which

form smRNAs after primary transcripts are processed through dsRNA-forming mechanisms that require *RdR* activity; and types which produce smRNA through non-RdR-dependent amplification mechanisms. miRNAs and *RdRs* interact when generating trans-acting siRNAs in plants (Allen et al., 2005). More broadly, smRNAs may be put into two groups based on their biogenesis: miRNAs (originating for miRNA genes) and siRNAs (originating from dsRNA generated by *RdRs*). It may be safely concluded that smRNA biogenesis pathways are more diverse in plants than in animals (especially mammals). According to one estimate, there may be more than 1.5 million siRNAs in Arabidopsis (Lu et al., 2005). This places *RdRs* in a central spot. In terms of biochemical processes, *RdRs* are involved maintaining PTGS and virus-induced gene silencing (VIGS), silencing endogenous genes, nuclear RNAi (e.g. methylation and heterochromatin formation), long-distance spreading of silencing signal, and preventing accumulation of viruses (Figure. 1).

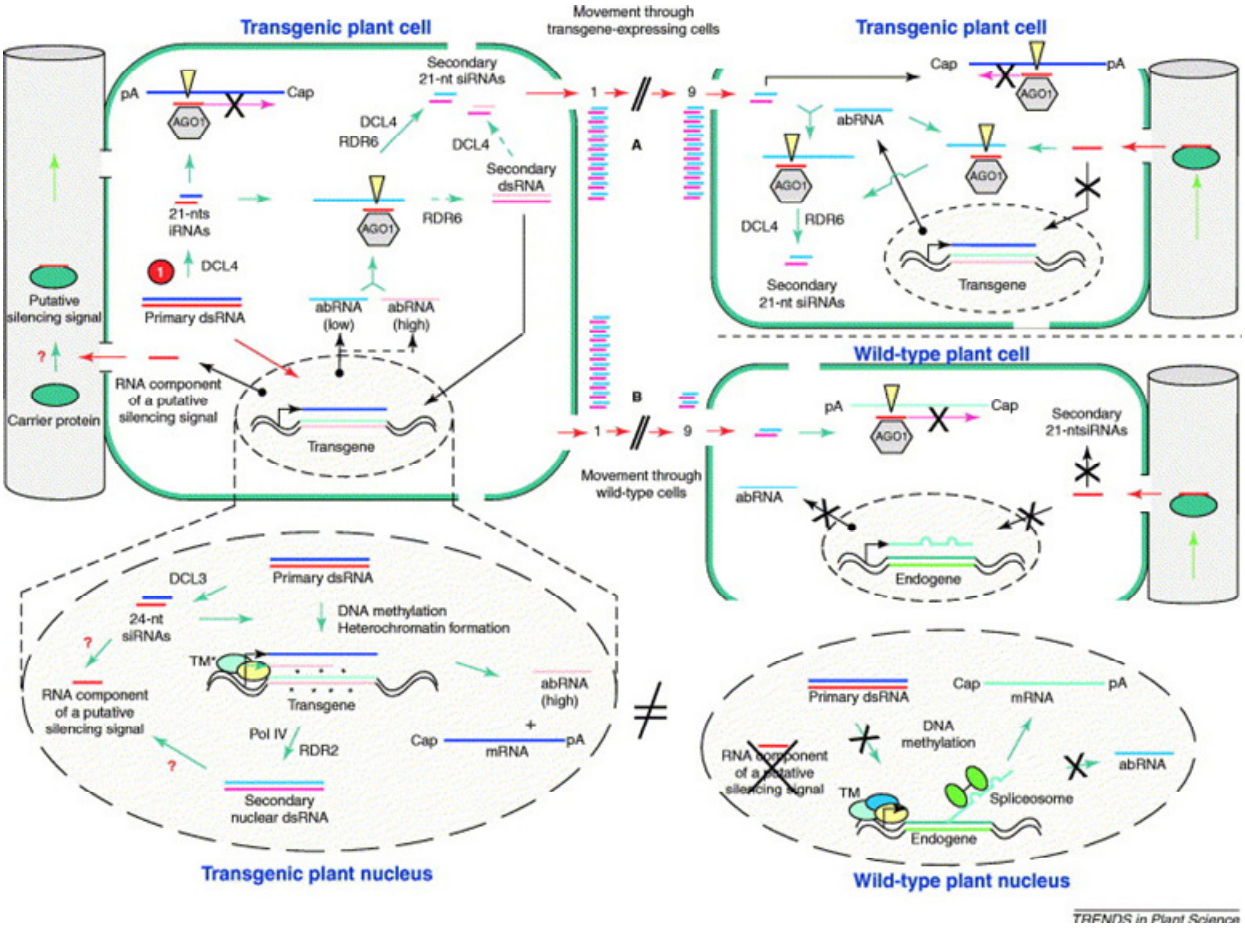

Figure 1. Model of the plant post-transcriptional gene silencing (PTGS) mechanism with a focus on the involvement of RNA-directed RNA polymerases (*RDRs*). Primary dsRNA initiates PTGS (indicated by '1' highlighted in red circle) and most of it is processed into 21-nt siRNAs by DCL4. The 21-nt siRNAs are loaded onto AGO1 to target complementary mRNA for cleavage. Targeting of mature mRNA would not recruit RDR6 activity. Thus, mRNA cleavage does not contribute to maintaining PTGS. Maintenance of PTGS requires the production of abRNA [abRNA (low)] to generate secondary siRNAs. Transgene transcription might be associated with abRNA production because transgenes usually lack introns and are regulated by artificial regulatory elements. Artificial promoters might recruit incomplete transcription machinery (TM*). Alternatively, lack of introns might prevent the elimination of abRNA by the spliceosome. If RDR6 only used abRNA as a template, the accumulation of abRNA but not of steady-state mRNA, would be the most crucial step for maintaining PTGS. A portion of the primary dsRNA enters the nucleus to initiate nuclear RNAi. The trigger could be the dsRNA itself or the 24-nt siRNAs that are produced from nuclear dsRNA by DCL3. The targeting of coding regions by nuclear RNAi could affect the accuracy of Pol II, leading to frequent premature termination of transcription and thereby to enhanced generation of abRNA. Nuclear RNAi involves dsRNA amplification that requires Pol IV and RDR2. The resulting secondary nuclear dsRNA reinforces nuclear RNAi and probably provides the RNA component of a putative silencing signal. The signal RNA could be bound to a carrier protein, enabling long-distance movement of the signal throughout the plant. Unloading the signal RNA into accompanying cells initiates PTGS. The RNA could be either processed into siRNAs or directly loaded onto AGO1. Upon targeting the abRNA, secondary siRNAs would be synthesized to maintain PTGS. The 21-nt siRNAs move through the plasmodesmata into neighbouring cells. In a transgene-expressing cell, they associate with abRNA to produce secondary siRNA. As a result of this amplification step, the siRNA concentration would stay constant in transgenic cells surrounding the 'silencing inducer cell' (A, 1 to 9). In wild-type cells, no abRNA and no secondary siRNA would be produced. Thus, the siRNA concentration would decline with the distance to the 'silencing inducer cell' (B, 1 to 9). Likewise, unloaded signal RNAs cannot mediate secondary siRNA production. RdDM is only initiated in the 'silencing inducer cell'. However, RdDM can be efficiently established in transgenic plants that have the potential to undergo spontaneous silencing (S-PTGS). This might indicate that in S-PTGS plants, a second abRNA threshold was reached [abRNA (high)]. A high abRNA concentration would be required to initiate RDR6-mediated secondary dsRNA production (broken arrow). The dsRNA would enter the nucleus to induce nuclear RNAi. Because nuclear RNAi is essential for generating the signal RNA, only S-PTGS-competent cells could re-initiate silencing signal production (Wassenegger and Krczal, 2006).

Thus, *RdRs* are involved in transcriptional, post-transcriptional, and translational processes, acting in cytoplasm as well as in nucleus; extensive genetic and biochemical analysis is necessary to further elucidate the mechanistic function of this class of RNA-producing enzyme (Wassenegger and Krczel, 2006). Such studies would further help in exploring the eco-physiological functions of smRNAs derived from *RdRs* in regulating different transcriptional network. This has remained a daunting challenge and is the subject of presented manuscripts.

3.2 Determining the role of small-RNAs

Determining the role of small-RNAs in eco-physiological adaptations of N. attenuata

We have just begun to explore the functional relevance of smRNAs in controlling transcriptional networks. Whole-organism-level studies of smRNA-controlled pathways are crucial for an integrative understanding of how RNA-silencing functions in broader contexts of development, defense and adaptation. *N. attenuata* is a suitable model for exploring this due to its well-characterized responses to its ecological challenges. Because *RdRs* are central to the

biogenesis of siRNAs, they are appropriate targets for studying the role of smRNAs in induced responses to natural stresses. The three known members of the *RdR* family were isolated and characterized in relation to different transcriptional elicitors. The expression of the three different *RdRs* was manipulated and organismic-level responses were characterized.

In order to determine the role of different *RdRs* in stress responses in *N. attenuata*, we inverted the sequence of steps followed generally in assigning function to an unknown gene. The most popular way to determine gene function is to silence its expression and observe the phenotype or challenge the silenced plants with favorite stresses under controlled laboratory conditions, preceded by molecular and biochemical characterization of the manipulated plants. This widely adapted strategy has a large drawback: the test of assigned function in a 'real-world' setting is postponed and often never conducted. Postponing proof of function in an organism's real world may lead to interesting situations. One such example of PR1 is cited in Manuscript I. Therefore, this commonly adopted approach was inverted, and the test of function in the real world done first. Manuscripts I, III and IV show the value of such an approach. Studies carried out in the natural habitat were followed with a detailed biochemical and molecular characterization of the plants; analysis and confirmation of traits observed in natural habitat were carried out in glasshouse studies.

Silencing the three *RdR*s and introducing *RdR*-silenced plants into the natural habitat revealed that different *RdR*-mediated smRNA pathways were functionally specialized. Whereas *RdR1* was identified as conferring resistance on natural herbivores, *RdR2* and *3* did not affect plant defense against herbivores, although they were induced by herbivory-specific elicitors. *RdR2* and *3* specialized in regulating plant responses against high UV-B and competition-induced stresses.

Herbivore attack reconfigures the plant's transcriptome: the changes in the mRNA profile are tightly coupled with rearrangement in the smRNA portion of the transcriptome. This reconfiguration of the smRNA transcriptome is correlative to the rapid elicitation of *RdR1* transcripts when plants are challenged with herbivore-specific elicitors. Silencing *RdR1* makes *N. attenuata* susceptible to a suite of herbivores in nature, whereas silencing *RdR2* and *3* had no effect on herbivory. This susceptibility of *RdR1*-silenced plants could be attributed to alterations in defense-related phytohormone signaling, which was specifically altered only in *RdR1*-silenced plants and not in *RdR2*- or *3*- silenced plants. Jasmonic acid (JA), which acts as positive regulator of defense responses (Halitschke et al., 2003), was down-regulated, whereas

ethylene, which acts as negative regulator (Kahl et al., 2000), was up regulated. Together they presumably reduced the accumulation of the (induced) direct defense compound, nicotine.

Nicotine is a proven effective direct defense against natural herbivores in *N. attenuata* (Steppuhn et al., 2004). At the same time nicotine's speed or amount of production and accumulation can be regarded as a measure of plant's "immunological memory" (Baldwin and Schmelz, 1996). The induction of nicotine production is signaled to roots via JA and synthesized in the damaged leaves. JA signaling can be faithfully mimicked by applying its methyl ester (MJA). Just like responses in animals, nicotine production is highly altered by plants, depending upon the plant's prior exposure to MJA (Baldwin and Schmelz, 1996). MJA seems to signal the learned response. But when the plants are silenced in their RdR1 expression, they fail to elicit and accumulate sufficient nicotine (and nicotine biosynthesis enzymes) even in complex natural environments, where there has been sufficient prior exposure to herbivore attack, before samples were collected for nicotine measurements. This indicates that *RdR1*-silenced plants failed to remember that they were under attack, strongly suggesting that plant memory is under smRNA control.

Contrary to the belief that *RdR2* is involved only in processes of paramutation, heterochromatin formation, and prevention of retro-transposons, it responded to physiological stress (Manuscript II). Upon being challenged with herbivory-related elicitors or with UV-B radiation, *N. attenuata* increased its accumulation of *RdR2* transcripts. Their role in the physiological adaptation of *N. attenuata* to high UV-B was apparent only when the RdR2-silenced plants were introduced into natural habitats. Broadly, UV-B protection mechanisms may be classified into three categories: protection by phenolic sunscreens, protection for reactive oxygen and free radical species and dark excision repair. RdR2-silenced plants were altered in several components of all three mechanistic pathways. The accumulation of phenolic sunscreens was studied in detail. MYB-transcription factors, which regulated production of phenolics in wide variety of plants, were identified as *RdR2*-dependent-smRNA targets. If *RdR2*-silenced plants had not been introduced into their natural habitats, *RdR2*'s involvement in protecting plants from ambient and high UV-B might never been discovered.

A previously undiscovered function of *RdR3* was discovered similarly: in *N. attenuata*, *RdR3* regulates phytohormone homeostasis during competition, auxin transport and presumably shade avoidance. Rapid growth as well as shade avoidance during extreme competition is necessary for plants to maximize their fitness. *RdR3* homologs in other species

have been traditionally associated with virus defense. But in its natural habitat *N. attenuata* is often not challenged with viruses. Interestingly, as discussed for PR1 in Manuscript 1, *RdR6*-silenced plants have not been tested for their susceptibility in natural habitats. When we evaluated such plants in nature, instead of showing (complex) viral susceptibility symptoms, and susceptibility to herbivores, they were reduced in growth due the presence of out-competing WT neighbors. This also indicated that natural herbivore community of *N. attenuata* may not be acting as viral vectors and warrants further investigation.

A subtext of this study is the indication that miRNAs may not actually be as widely conserved in plants as has been originally thought. smRNA-sequencing of *N. attenuata* transcriptome and comparisons of sequenced smRNAs with publicly available information could identify only 41 miRNA which belonged to 17 families. Our sequencing efforts showed that silencing one *RdR* enriches several smRNAs and at the same time gives rise to several new ones. This indicates the importance of smRNA pathways that are triggered by silencing different *RdR*s and needs further research.

Based on observation in ManuscriptsI-IV and the available literature, a simplified model showing a possible mode of smRNAs in *N. attenuata* is presented in Figure 2.

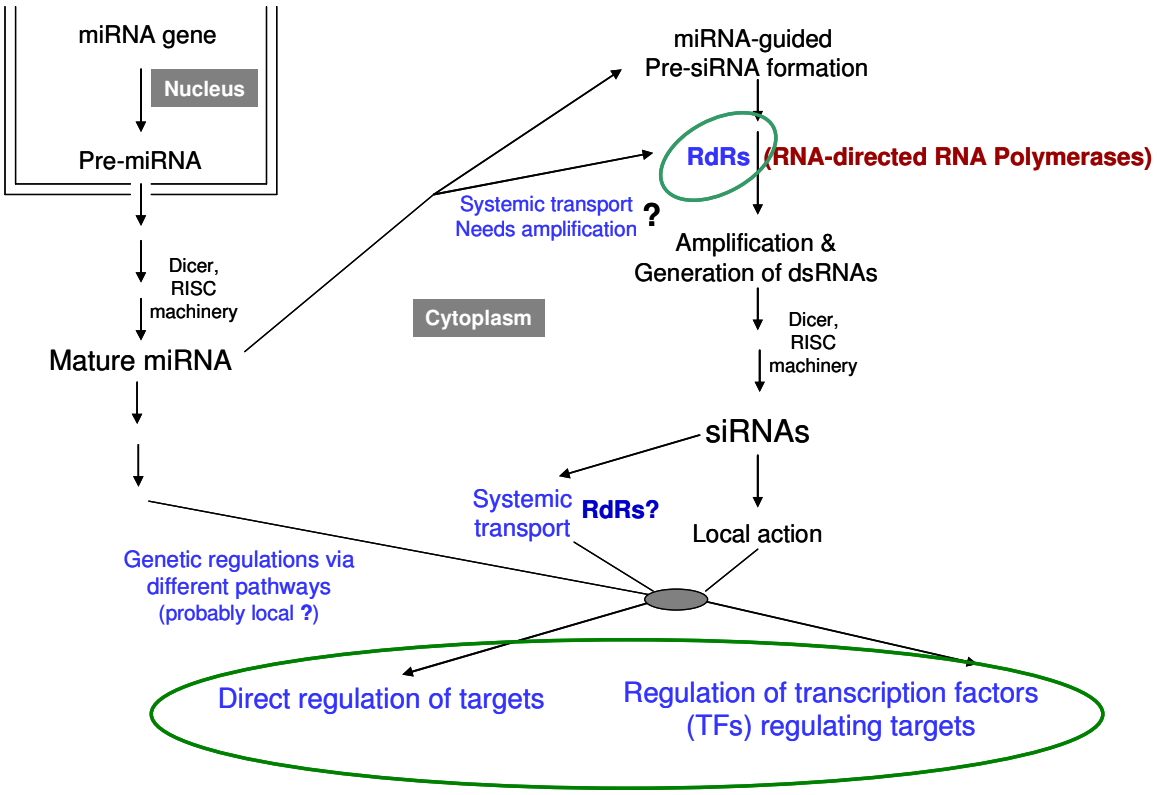

Figure 2. Modeling RdR-dependent action of smRNAs in regulating stress-induced defense responses.

RdR-dependent generation of smRNAs fulfills all the properties of molecular regulators of phenotypic plasticity: (a) they are rapidly elicited specially by herbivore-specific elicitors. These elicited smRNAs may regulate the transcriptome at the local site of action. (b) These rapidly elicited smRNAs are amplifiable by *RdR*s and are also capable of getting systemically transported, an ability which is essential for executing induced defense responses, as well as tolerance and escape responses. The differential regulation of nicotine between WT and irRdR1 plants is an example. (c) Its action is not masked by the environment as is evident from Manuscripts 1, 3 and 4. Not only are *RdR*-specific phenotypes expressed in nature, but their severity is further increased. (d) Most importantly, they are able to coordinate different pathways, because in nature, plants encounter many simultaneous stresses. JA- and ethylene-signaling seems under control of *RdR1*-dependent smRNAs, whereas *MYB*-regulated phenylpropanoid pathways and various DNA-repair mechanisms are under the control *RdR2*. At the same time, hormone homeostasis involving SA, auxin, and ABA signaling during competition is regulated by *RdR3*. smRNAs may act directly on various genes or may regulate transcriptional networks by regulating the transcription factors, activators and/or repressors.

3.3 Conclusions

In the current investigation, using *N. attenuata* as model organism, we explored the possibility, if small-RNAs (smRNAs) may be involved in regulating induced stress responses in plants. Because RNA-directed RNA polymerases (*RdR*s) are central to smRNA-biogenesis and amplification pathways, we characterized them against different stresses. We also sequenced the smRNA-transcriptome of *N. attenuata* and mapped the changes during herbivory.

We identified that *RdR1* mediates herbivore resistance, indicating that smRNAs produced by *RdR1* are likely involved in orchestrating some of the rapid metabolic adjustments required for plants to survive herbivore attack in their natural habitats. Using 454-sequencing we characterized *N. attenuata*'s transcriptome before and after insect-specific elicitation in wild type and *RdR1*-silenced plants. Comparisons of *N. attenuata* smRNAs with publicly available information on plant miRNA could identify 41 miRNAs. Several of them were differentially regulated between the uninduced and herbivory-induced genotypes.

Computational predictions indicated that large number of *N. attenuata* smRNAs could target phytohormone (JA, JA-Ile, and ethylene) signaling related genes. Phytohormones mediate the perception of insect-specific signals and the elicitation of defenses during insect attack. Transcriptional profiling and phytohormone measurements in glasshouse and field showed that phytohormone signaling was under control of *RdR*-dependent smRNAs, the susceptibility of *RdR1*-silenced plants to herbivores was due to altered phytohormone signaling, and that smRNAs play a central role in coordinating the large-scale changes that occur after herbivore attack. Given that dsRNAs synthesized in the host plants may trigger RNAi in the insect midgut, *N. attenuata* may employ similar strategies where *RdR1*-dependent smRNAs or their dsRNA precursors may help protect plants by targeting genes in the insect midgut.

Transcripts of *RdR2* accumulated when *N. attenuata* plants were treated with herbivore-specific elicitors or with UV-B. This indicated that *RdR2* may be involved in plant protection processes. Silencing of *RdR2* transcript accumulation did not alter plant defenses. Further characterization of *RdR2*-silenced plants showed that *RdR2* regulates the responses that have been tailored to provide protection from UV-B. *RdR2*-silencing reduced the accumulation of phenolics that act as sunscreens. This uncovers the importance of *RdR2* in *N. attenuata* genome for protecting plants from genotoxic stresses that may result from oxidative damage and DNA cross-linking in high UV-B environments, particularly in absence of sufficient sunscreens.

Further, the third *RdR*, RdR6/SGS2/SDE1 (in *Arabidopsis* and *N. benthamiana*, homolog of which we call as Na*RdR3*, as functionally there are only three distinct *RdR*s) has been regarded as indispensable for virus resistance. N. attenuata does not often encounter virus threat in nature. We explored the physiological importance of this gene. Na*RdR3* fine-tunes hormone homeostasis during adaptation of plants to highly competing natural environments. Imbalance in phytohormone levels (e.g. IAA and SA) reduces competitive ability, which severely affects plant's fitness. *RdR3* probably regulates this hormonal balance and transport.

We deduce that plant's phenotypic plasticity during multiple stresses, in complex natural habitats, is regulated by smRNAs and *RdRs* central to their biogenesis and spread. *RdRs* are functionally specialized and different *RdRs* have specific regulatory roles in different stresses. The studies presented here highlight the value of carrying out real-world tests of gene function early in the discovery process. Due to multitude of stresses a plant faces under natural

conditions, a single field trail can allow a researcher with an intimate understanding of a plants's natural history to rapidly identify a difference in a phenotype.

4. Summary

For the successful completion of their life cycle and further adequate representation in the seed banks, plants need strategies for successful growth and defense. This need for phenotypic plasticity requires the reconfiguration of plants' transcriptome, proteome, and hence the metabolome to optimize resource use. The induced responses to various stresses in nature, which spread systemically from the site of production, require efficient regulators. The speed and magnitude of responses during stresses (such as herbivory or UV-B exposure) suggest that these regulators may not be only transcription factors or phytohormones. Moreover, how the phytohormones and transcription factors are regulated remains largely unknown. We explored the possibility of small-RNAs (smRNAs) as regulators of plants' induced stress responses.

Because RNA-directed RNA polymerases (*RdR*s) are central to smRNA biogenesis, especially the biogenesis of siRNAs, they can be manipulated in order to study the smRNA-response of the whole organism. Combining large-scale pyro-sequencing (454 sequencing); computational predictions; *RdR*-silencing (transient, using VIGS and stable using inverted-repeat constructs); field studies; and assays that are molecular, biochemical, and glasshouse-based, we determined whether the induced physiological responses of plants in response to stress are under smRNA control. Specifically, three *RdR*s were isolated, characterized, and silenced in the ecological model *N. attenuata*. Using 454 sequencing, the smRNA transcriptome of *N. attenuata* was elucidated when the plants' *RdR*s were intact (that is, in wild-type – WT plants) as well as when the expression of the *RdR*s was reduced in stably transformed plants. The smRNA transcriptomes of uninduced and herbivory-induced WT and *RdR1*-silenced plants were also elucidated and differences were mapped.

Evaluations of three *RdR*-silenced genotypes in nature as well as in the glasshouse showed that each specialized in regulating different plant processes. Whereas herbivory-specific elicitors induced the expression of all the three *RdR*s, silencing only *RdR1* made *N. attenuata* plants susceptible to natural herbivores (Manuscript 1). This susceptibility of *RdR1*-silenced plants (irRdR1) was due to their altered direct defenses rather than indirect defenses (Manuscript I).

Large-scale transcriptional reconfigurations are known to occur when *N. attenuata* is attacked by herbivores. Similar to the reconfiguration of the mRNA-transcriptome, a large-scale change in the smRNA transcriptome was also elucidated when plants were treated with herbivore-specific elicitors (Manuscript II). When uninduced and induced WT and irRdR1 plants were compared, little overlap was found. When *N. attenuata* smRNA transcriptome was compared to publicly available information, 40% smRNAs were found to have similar sequences of various structural and cellular RNAs. When smRNAs were compared to publicly available miRNAs, only 41 miRNAs could be identified (Manuscript II). Computational predictions showed that a large number of smRNAs could target transcripts related to phytohormone signaling. Transcriptional analysis in time-course experiments showed that the biosynthesis of jasmonic acid (JA) could be reduced and ethylene could be increased. This was verified by measuring the accumulation of JA in plants grown in the field as well as the glasshouse. Also, OS-elicited ethylene accumulation was increased (Manuscript II). It was confirmed that the susceptibility of irRdR1 plants was due to altered direct defenses rather than to changes in primary metabolism (Manuscripts I and II).

RdR2 transcripts were also elicited with herbivore-specific signals as well as with UV-B (Manuscript III). But silencing *RdR2* did not change the defense ability of plants against insect herbivores. Instead, *RdR2*-silenced plants were unable to adapt in ambient and high UV-B environments (Manuscript III). This susceptibility of irRdR2 plants to high UV-B was due to reduced phenolic sunscreens and altered repair mechanism (Manuscript III). Initially convergent responses to herbivory and UV-B may diverge down-stream at smRNA levels, as discussed in Manuscript 3.

RdR3-silencing specifically reduced the abundance of ~22nt smRNAs (Manuscript IV). *RdR3*-silenced plants were not able to compete well with their WT neighbors, which severely reduced plant fitness. This reduction in competitive ability was due to altered phytohormone homeostasis (Manuscript IV). Auxin transport was altered in irRdR3 plants when they competed with WT neighbors. On the other hand, plant defenses against herbivores and JA signaling were intact in *RdR3*-silenced plants (Manuscript IV).

In conclusion, the functions of different *RdR*s were specific to particular stresses, and in nature different smRNA pathways co-ordinate to optimize plant phenotypic responses. Thus, plants' phenotypic plasticity in order to circumvent stress and adapt to complex natural habitats highly depends on smRNAs.

5. Zussammenfassung

Um ihren Lebenszyklus erfolgreich zu komplettieren und ihre Samen in angemessener Weise in den Samenbanken zu repräsentieren, müssen Pflanzen Strategien entwickeln, um zum Einen erfolgreich zu wachsen und sich zum Anderen gegen verschiedenste Arten von Streß zu wehren. Diese enorme phänotypische Plastizität bedarf der Rekonfiguration des pflanzlichen Transkriptoms, Proteoms und folglich des Metaboloms, um die verfügbaren Ressourcen möglichst optimal für Wachstum und Verteidigung zu nutzen. Induzierte Antworten gegen verschiedenste Arten von Streß sollten sich ausgehend vom Induktionsort systemisch ausbreiten. Dies bedarf der Mithilfe effizienter Regulatoren, welche höchst wahrscheinlich nicht nur durch Transkriptionsfaktoren oder Phytohormone repräsentiert sind. Darüber hinaus ist nahezu unbekannt, wie Transkriptionsfaktoren und Phytohormone in Streßsituationen reguliert sind. Wir untersuchten die Hypothese, dass 'small-RNAs' (smRNAs) als Regulatoren pflanzlicher induzierter Antworten von Bedeutung sind.

Nachdem RNA-gerichtete RNA Polymerasen (*RdRs*) eine zentrale Rolle in der Produktion von small-RNAs (im Speziellen von small interfering RNAs (siRNAs)) spielen, stellen sie ein geeignetes Ziel manipulativer Studien zur Erforschung der Gesamtorganismus-basierten smRNA-Antworten dar. In einem multidisziplinären Ansatz wurden large-scale pyro-Sequenzierung (454-Sequenzierung), computergestützte Vorhersagen, *RdR*-Silencing (transient unter Verwendung von VIGS und stabil unter Verwendung von 'inverted-repeat'-Konstrukten), molekularbiologische und biochemische Untersuchungen und Freiland- und Gewächshausexperimente kombiniert. Ziel war es, herauszufinden, ob die induzierten physiologischen Streßantworten der Kontrolle von smRNAs unterliegen. Im Speziellen wurden drei *RdRs* isoliert, charakterisiert und in der Modellpflanze *Nicotiana attenuata* gentechnisch ausgeschalten ('*RdR*-silencing'). Unter Verwendung der 454-Sequenzierung wurde das smRNA Transkriptom von *N. attenuata* Wildtyp (WT) Pflanzen mit dem von stabilen Transformanden verglichen. Weiterhin wurde das smRNA Transkriptom uninduzierter und von Herbivoren befallener Pflanzen (WT und 'RdR-gesilenct') untersucht, um etwaige Unterschiede herauszuarbeiten.

Der Vergleich verschiedener RdR-gesilencter Genotypen sowohl im Freiland als auch im Gewächshaus ergab, dass verschiedene *RdRs* auf die Regulation bestimmter pflanzlicher Prozesse spezialisiert sind. Während alle drei *RdRs* durch herbivorenspezifische Elizitoren

induziert wurden, führte allein das Ausschalten von *RdR1* dazu, dass *N. attenuata* anfälliger für natürliche Herbivoren wurde (Manuskript I). Diese Anfälligkeit 'RdR1-gesilencter' Pflanzen (irRdR1) konnte auf deren veränderte direkte Herbivorenantwort zurückgeführt werden (Manuskript I).

Es ist bekannt, dass *N. attenuata* nach Herbivorenbefall mit einer drastischen transkriptionellen Umorganisierung reagiert. Entsprechend wurden enorme Änderungen im smRNA Transkriptom beobachtet, wenn die Pflanzen mit herbivorenspezifischen Elizitoren behandelt wurden (Manuskript II). Vergleiche zwischen uninduzierten und induzierten WT und irRdR1-Pflanzen ergaben wenige Gemeinsamkeiten. Ein Abgleich des *N. attenuata* smRNA Transkriptoms mit öffentlich zugänglichen Informationen ergab, dass 40% der smRNAs verschiedenen strukturellen und zelluläen RNAs sehr ähnelten. Über einen Vergleich von smRNAs mit öffentlich zugänglichen miRNAs konnten 41 miRNAs identifiziert werden (Manuskript II). Computergestützte Vorhersagen zeigten, dass eine große Anzahl der smRNAs Transkripte, die mit Phytohormonsignalen in Verbindung stehen, als Ziel haben könnten. Transkriptionelle Analysen von Zeitreihenexperimenten legten nahe, dass die Biosynthese von Jasmonsäure reduziert und die von Ethylen erhöht sein könnte. Die Quantifizierung von Jasmonsäure in Pflanzen, die im Freiland und im Gewächshaus wuchsen, bestätigte diese Vorhersage. Ebenso war die Ethylenbiosynthese erhöht (Manuskript II). Es konnte gezeigt werden, dass die Anfälligkeit von irRdR1-Pflanzen auf eine geänderte direkte Abwehr und nicht auf eine Störung des Primärstoffwechsels zurückzuführen war (Manuskript I und II).

RdR2-Transkripte waren durch Herbivorie und UV-B induziert. Allerdings änderte das Ausschalten von *RdR2* nichts an der Verteidigungsfähigkeit der Pflanzen gegen folivore Insekten. Stattdessen waren 'RdR2-gesilencte' Pflanzen nicht mehr in der Lage sich an verschiedene UV-B-Bedingungen anzupassen (Manuskript III). Diese reduzierte Anpassungsfähigkeit von irRdR2-Pflanzen an hohe UV-B-Dosen konnte auf einen Mangel an phenolischen Verbindungen sowie auf veränderte Reparaturmechanismen zurückgeführt werden (Manuskript III). Ehemals parallel laufende Antworten auf Herbivorie und UV-B haben sich möglicherweise auf der Ebene der smRNAs auseinander entwickelt.

irRdR3-Pflanzen waren dramatisch in ihrer Konkurrenzfähigkeit gegenüber WT-Pflanzen eingeschränkt, was zu einer schwerwiegenden Fitnessreduktion führte. Diese Reduktion der Konkurrenzfähigkeit beruhte auf einer Veränderung des Phytohormongleichgewichts (Manuskript IV). In Konkurrenzsituationen war der

Auxintransport der irRdR3-Pflanzen verändert. Im Gegensatz dazu war die Verteidigung gegen Herbivoren und die Jasmonsäuresignalkette in irRdR3-Pflanzen intakt (Manuskript IV).

Zusammenfassend lässt sich sagen, dass die verschiedenen *RdRs* spezifisch für verschiedene Streßsituationen sind und dass unterschiedliche smRNA-Wege in der Natur koordiniert werden, um die phänotypischen Antworten der Pflanzen zu optimieren. Folglich sind sowohl die phänotypische Plastizität zur Umgehung von Streßsituationen als auch die Anpassungsfähigkeit an komplexe Habitate abhängig von smRNAs.

6. References

Alborn T, Turlings TCJ, Jones TH, Stenhagen G, Loughrin JH, Tumlinson JH (1997) An elicitor of plant volatiles from beet armyworm oral secretion. Science **276:** 945-949

Allen E, Xie ZX, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell **121:** 207-221

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

Baldwin IT, Schmelz EA (1996) Immunological "memory" in the induced accumulation of nicotine in wild tobacco. Ecology **77:** 236-246

Baldwin IT, Staszakkozinski L, Davidson R (1994) Up in Smoke .1. Smoke-Derived Germination Cues for Postfire Annual, Nicotiana-Attenuata Torr Ex Watson. Journal of Chemical Ecology **20:** 2345-2371

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

Braam J (2005) In touch: plant responses to mechanical stimuli. New Phytologist **165:** 373-389

Bray EA (1997) Plant responses to water deficit. Trends in Plant Science **2:** 48-54

Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. Nat Rev Genet **8:** 884-896

Gershenzon J (2007) Plant volatiles carry both public and private messages. Proceedings of the National Academy of Sciences of the United States of America **104:** 5257-5258

Halitschke R, Gase K, Hui DQ, Schmidt DD, Baldwin IT (2003) Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid-amino acid conjugates. Plant Physiology **131:** 1894-1902

He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet **5:** 522-531

Kahl J, Siemens DH, Aerts RJ, Gabler R, Kuhnemann F, Preston CA, Baldwin IT (2000) Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. Planta **210:** 336-342

Lu C, Tej SS, Luo SJ, Haudenschild CD, Meyers BC, Green PJ (2005) Elucidation of the small RNA component of the transcriptome. Science **309:** 1567-1569

Schmelz EA, Carroll MJ, LeClere S, Phipps SM, Meredith J, Chourey PS, Alborn HT, Teal PEA (2006) Fragments of ATP synthase mediate plant perception of insect attack. Proceedings of the National Academy of Sciences of the United States of America **103:** 8894-8899

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

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends in Plant Science **5:** 537-542

Sultan SE (2003) Phenotypic plasticity in plants: a case study in ecological development. Evolution & Development **5:** 25-33

Tang GL, Reinhart BJ, Bartel DP, Zamore PD (2003) A biochemical framework for RNA silencing in plants. Genes & Development **17:** 49-63

Voinnet O (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. Current Opinion in Plant Biology **5:** 444-451

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

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8. Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as coauthors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University Jena or to any other University.

Jena, December 6th 2007 ……

Shree P. Pandey

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Publications

1. Pandey SP and Baldwin IT (2007): RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature. *The Plant Journal*, 50 (1), 40–53.
2. Pandey SP and Baldwin IT (2007): Silencing RNA-directed RNA polymerase 2 (RdR2) increases *Nicotiana attenuata*'s susceptibility UV in the field and in the glasshouse. *The Plant Journal*, (Under Revision).
3. Pandey SP, Shahi P, Gase K and Baldwin IT (2007): Herbivory-induced changes in the Small-RNA Transcriptome and Phytohormone Signaling in *Nicotiana attenuata. Proceeding of National Academy of Sciences (2007*) In Review
4. Pandey SP, Gaquerel E, Gase K and Baldwin IT (2007):Characterization of RNA-directed RNA polymerase (RdR) 3 from *Nicotiana attenuata*. In Preparation
5. Pandey SP, Sharma S., Chand R, Shahi P, and Joshi AK (2007): Clonal variability and its relevance in generation of new pathotypes in the spot blotch pathogen, *Bipolaris sorokiniana*. *Current Microbiology*, (PMID: 17909887; Oct 2; [Epub ahead of print]).
6. Pandey SP and Krishnamachari A (2006): Computational analysis of plant RNA Pol-II promoters. *Biosystems*, 83, 38-50.
7. Pandey SP, Kumar S, Kumar U, Chand R, Joshi AK (2005): Sources of inoculum and reappearance of spot blotch of wheat in rice–wheat cropping systems in eastern India. *European Journal of Plant Pathology*, 111, 47-55.
8. Jaiswal SK, Sweta, Prasad LC, Sharma S, Kumar S, Prasad R, Pandey SP, Chand R, Joshi AK (2007): Identification of molecular marker and aggressiveness for different groups of *Bipolaris sorokiniana* isolates causing spot blotch disease in wheat (*Triticum aestivum* L.). *Current Microbiolgy*, 55(2), 135-141.
9. Singh RK, Mishra RPN, Jaiswal HK, Kumar V, Pandey SP, Rao SB, Annapurna K (2006): Isolation and identification of natural endophytic rhizobia from rice (*Oryza sativa* L.) through rDNA PCR-RFLP and sequence analysis. *Current Microbiology*, 52(5):345-349.
10. Chand R, Pandey SP, Singh HV, Kumar S, and Joshi AK (2003): Diversity in the populations of Bipolaris sorokiniana and probable cause of its emergence in spring wheat in India. *J. Plant Disease and Protection*, 110 (1), 27-35.

Presentations

Pandey SP: RNA-directed RNA polymerase 1 (RdR1) regulates defence responses after herbivore attack in *Nicotiana attenuata* (*Talk*; 6th Biannual IMPRS Symposium, Altes Schloss Dornburg, Germany, March 2007)

Pandey SP and Baldwin IT: RNA-directed RNA Polymerase 1 mediates plant defence against insect herbivores (*Poster*; 5th Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany, November 2006)

Pandey SP and Baldwin IT: Isolation and characterization of RNA-directed RNA polymerases (RdRp) from *N. attenuata* (*Poster*; ICE Symposium, MPI for Chemical Ecology, Jena,

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Pandey SP: Exploring how smallRNAs regulate herbivore-induced responses: developing a tool-kit for *N. attenuata* (*Talk*, 4th Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany, March 2006)

Pandey SP: Using microarays to study microRNAs (*Invited Talk*; BIORHIZ Workshop: Rhizosphere processes and induced defence, Jena, Germany, January 2006)

Pandey SP and Baldwin IT: Resistance of *Nicotiana attenuata* to herbivore attack is mediated by RNA dependent RNA polymerases (*Poster*; 3rd Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany, November 2005)

Pandey SP: Molecular basis of ecological sophistication: herbivore attack and plant defence (*Invited talk*; Centre for Comp. Biol and Bioinformatics, Jawaharlal Nehru University, N. Delhi, India, October, 2005)

Pandey SP: Are Plant-Herbivore Interactions driven by Micro RNAs? (*Talk*; ICE Symposium, MPI for Chemical Ecology, Jena, Germany, July 2005)

Pandey SP and Baldwin IT: The role of mi-RNAs in regulating herbivore induced responses in *N. attenuata* (*Poster*; 2nd Biannual IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany, March 2005)

Pandey SP, Kumar S, Joshi AK, and Chand R: Survival of spot blotch pathogen of wheat under rice – wheat cropping system in eastern India (*Poster*; 2nd International Group Meeting on "Wheat Technologies for Warmer Areas", Agharkar Research Institute, Pune, India, September, 2002).

10. Appendix

The supplementary materials, methods and data for manuscripts I – IV are provided in the attached CD-ROM.

10.1 Supporting materials for Manuscript I

Supporting Method Figure 1. Characteristic damage symptoms of different herbivore species that attack *N. attenuata* plants in Utah.

Figure S1. Partial sequences of the three *N. attenuata* RdRs used to make silencing constructs

Figure S2. No morphological abnormalities were seen after silencing the three RdRs with VIGS during rosette- and flowering-stage growth.

Figure S3. Relative expression of RdR1 in WT *N. attenuata.*

Figure S4. Southern analysis of the two independently transformed irRdR1 lines showing single insertion.

Figure S5. Growth phenotypes of the two independently transformed lines.

Figure S6. irRdR1 silenced lines are highly susceptible to viruses.

Figure S7. Microarray analysis of significantly differentially OS-regulated genes in irRdR1 compared to WT plants.

Table S1. Analysis of Variance (ANOVA) for growth phenotypes of WT and EV – transformed plants used in this study.

10.2 Supporting materials for Manuscript II

Materials and Methods

References in the supporting material

Table S1. Small-RNA populations in *N. attenuata*

Table S2. Annotations of the *N. attenuata* smRNAs from NR-database at NCBI.

Table S3. Identification of *N. attenuata* miRNAs

Table S4. Expression analysis of smRNAs common to OS-elicited and unelicited WT plants

Table S5. Expression analysis of smRNAs common to unelicited WT and irRdR1 plants

Table S6. Expression analysis of smRNAs common to OS-elicited and unelicited irRdR1 plants

Table S7. Expression analysis of smRNAs common to OS-elicited WT and irRdR1 plants

Table S8. *N. attenuata* miRNAs targeting the phytohormone signaling/biosynthesis-related genes

Table S9. *N. attenuata* specific smRNAs targeting the phytohormone signaling/biosynthesis-related genes when WT and irRdR1 plants are OS-elicited

Table S10. Primers and probes used for q-PCR assays.

S Fig. 8. Simplified schematic representation of the procedure for the elucidation of smRNA transcriptome of *N. attenuata*.

S Fig. 9. Phylogenetic analysis of 41 miRNAs distributed in 17 families, identified on the basis of sequence conservation across different plant species.

S Fig. 10. Time course analysis of elicitation dynamics of *HPL* (which regulates GLV production) shows no difference in its transcript accumulation in WT and irRdR1 plants.

S Fig. 11. Time course analysis of accumulation of transcripts of the genes responsible for JA-Ile biosynthesis

S Fig. 12. Silencing *RdR1* did not affect ethylene perception.

S Fig. 13. Silencing *RdR1* did not affect plant growth and performance.

S Fig. 14. qPCR analysis of the transcript accumulation of RuBPCase Activase (*RCA)* in WT and irRdr1 plants did not show any differences after OS elicitation.

S Fig. 15. JA supplementation restores the lack of resistance of irRdR1 plants to *M. sexta* larvae attack to the WT levels.

10.3 Supporting materials for Manuscript III

Supplementary Table 1. Analysis of smRNA-transcriptome of *N. attenauta*

Figure S1. DNA gel blot analysis of the two independently transformed irRdR2 lines showing single insertions.

Figure S2. Characterization of irRdR2 plants in the glasshouse.

Figure S3. Phytohormone analysis of the WT and irRdR2 plants.

Figure S4. Silencing *RdR2* did not affect plant performance, even when irRdR2 plants competed with WT neighbors in the same pot.

Figure S5. *M. sexta* performance on the WT and two independently transformed irRdR2 lines.

Figure S6. Silencing *RdR2* did not affect *systemin* (*SYS*) transcript levels (ANOVA; $F_{3,18}$=2.85; P>0.05).

Table S1. smRNAs from (a) *RdR2* –silenced- and (b) wild type plants having target-motifs in the phenylpropanoid biosynthesis genes

10.4 Supporting materials for Manuscript IV

Figure S1. qPCR analysis of RdR3 transcript accumulation as a function of diurnal rhythm shows no differences.

Figure S2. Southern analysis of the two independently transformed irRdR1 lines showing single insertion.

Figure S3. Minor phenotypic changes after stably silencing *RdR3*.

Figure S4. *M. sexta* performance in *N. attenuata* WT and irRdR3 plants.

Figure S5. Qualitative and quantitative smRNAs profiles obtained using 454 sequencing.

Figure S6. Morphology of irRdR3 lines and WT plants competing in their natural habitat.

Figure S7. irRdR3 and WT plants grown in close proximity (7-10 cm) in 2 L pots in: WT – WT, 244-4 – 244-4, 282-3 – 282-3.

Figure S8. Phytohormone analysis in the field.

Figure S9. Phytohormone analysis in glasshouse conditions during competition.