The role of small RNAs in plant and insect interactions

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Chapter 1

General introduction

Tohir A. Bozorov

GENERAL INTRODUCTION

As sessile organisms, plants have evolved responses to diverse environmental cues in order to maximize their fitness in the face of often unpredictable changes (Howe and Jander 2008). Environmental stresses vary widely in space and in time, and plants are constantly adapting to their environment and adjusting their physiology to the particular environmental conditions. Furthermore, each plant does not exist in isolation but co-exists in interaction together with other organisms such as other plants, bacteria, fungi, and animals. This required having efficient strategy to interact with other organisms.

The research performed in this dissertation is dedicated to the understanding of how small RNAs (smRNAs) in plants play important roles in the orchestrating transcriptome in response to herbivore attack, which is a major stress faced by plants in nature. In this dissertation I used the ecological model plant *Nicotiana attenuata* (wild coyote tobacco) with its transformants silenced in Dicer-like proteins involved in smRNA generation to investigate the roles of these smRNAs during the plant's interaction with herbivores.

N. attenuata as an ecological model system for studying plant-insect interactions

The wild tobacco, *N. attenuata* (Torr. ex Watson) is a largely selfing, annual plant native to the Great Basin Desert in Southwestern USA (Figure 1). *N. attenuata* seeds germinate into nitrogen-rich soils in post-fire environments triggered by chemical cues derived from smoke, and the absence of inhibitory chemicals from other vegetation (Figure 1) (Baldwin and Morse 1994; Preston and Baldwin 1999; Schwachtje and Baldwin 2004). This germination behavior creates large post-fire monocultures and strong intraspecific competition. In nature, more

than twenty different mammalian and insect herbivorous species attack this plant (Baldwin 2001). Among them, the Lepidopteran specialist herbivore *Manduca sexta* dramatically affects plant physiology. *N. attenuata* can rapidly recognize this herbivore by elicitors from its regurgitant or oral secretions (OS), introduced into plant wounds during feeding. The fast and specific response is one aspect of the plant's plasticity, and regulates defense against and tolerance of specialist herbivores (Baldwin 1998).

Figure 1. *Nicotiana attenuata* plants in their natural habitat in the Great Basin desert, USA. Upper left, a naturally occurring wildfire; upper right, plants growing in a post-fire environment; lower panel, *Manduca sexta* attacking *N. attenuata.* Photographs by D. Kessler.

N. attenuata has emerged as a model plant for understanding the molecular basis of smoke-induced germination, and of the associated morphological and chemical phenotypic plasticity that allow plants to adapt to a stressful environment (Preston and Baldwin 1999; Baldwin 2001). To deepen our understanding of the ecological interaction of model plant systems such as *N. attenuata* with other organisms, it is necessary to apply both reverse genetics and molecular biology approaches. The functional characterization of biotic-stress related genes is achieved through the reverse genetics approach, in which the plant genome is modified by suppressing or enhancing expression of a gene of interest. In this case, application of stable plant transformation has proven a powerful tool in ecological research to investigate roles of genes of interest and their ecological relevance. In the **Manuscript I**, we developed and optimized the strategy for efficient screening of transformants for ecological studies.

Defense responses to herbivory

During evolution, flowering plants and insects emerged independently of each other, but they largely interacted with each other and evolved together. Attracting a number of insect pollinators, plants at the same time suffer from herbivory by many insects, especially their larval stages (Baldwin 1998, 1999, 2001; Allmann and Baldwin 2010). Plant developed two ways of defending against herbivore attack: either directly or indirectly by producing secondary metabolites which not directly involved in plant development, growth and reproduction. Direct defenses can be mechanical or chemical. Plants have many structural defenses such as sharp prickles, spines, thorns, or trichomes- hairs on the leaf (Fernandes, 1994). Chemical defense include a wide range of defense metabolites which make plants distasteful or toxic to insects such as toxic metabolites, anti-digestive compounds, anti-nutritive proteins or peptides that negatively influence to herbivory physiology (Howe and Jander 2008). Thousands of secondary metabolites have been identified, and their true number is so large that it is still not all of them are

known, and many chemists are still identifying new metabolites. In indirect defense, plant being attacked by herbivores produces herbivore induced volatiles that attract predators (Heil 2008; Dicke 2009; Allmann and Baldwin 2010).

It is well known that elicitors in herbivore oral secretion (OS) can induce anti-herbivore response in plants (McCloud and Baldwin 1997). Some of the best-known examples are β-glucosidase, which was isolated from *Pieris brassicae.* The best-studied herbivore elicitors in insect OS are the fatty acid-amino acid conjugates (FACs), which have been isolated from different chewing insect herbivore species and have been found to be specific for insect species (Mattiacci et al. 1995; Alborn et al. 1997; Halitschke et al. 2001; Diezel et al. 2009; Yoshinaga et al. 2010). Among FACs: *N*-(17-hydroxylinolenoyl)-L-glutamine or volicitin isolated from *Spodoptera exigua*, *N*-linolenoyl-L-glutamine rather than volicitin from *Spodoptera litura*, *N*-linolenoyl-L-glutamate (18:3- Glu), *N*-linoleoyl-L-glutamate (18:2-Glu) and *N*-linolenoyl-L-glutamine (18:3-Gln) isolated from *M. sexta* (Mattiacci et al. 1995; Alborn et al. 1997; Halitschke et al. 2001; Diezel et al. 2009; Yoshinaga et al. 2010). Plants recognize specialist and generalist herbivores by the qualitative and quantitative content of their OS (Schmelz et al. 2007; Wu et al. 2008; Diezel et al. 2009).

In the early stages of herbiovry attack in plants, the activated mitogen activated protein kinase (MAPK) cascade modulates the metabolism of the jasmonate hormones, in particular jasmonic acid (JA) (Yamakawa et al. 2004; Kandoth et al. 2007; Wu et al. 2007) and its active form, the derivative JA-Isoleucine (JA-Ile), which together play key roles in direct defense responses (Kang et al. 2006; Howe and Jander 2008). The concentration of jasmonates rapidly increases in the early stage of herbivore attack (Bell et al. 1995; Halitschke and Baldwin 2003; Kang et al. 2006; Paschold et al. 2007). The mode of action of jasmonate signaling and perception has been characterized in detail (Devoto and Turner 2005; Paschold et al. 2007; Galis et al. 2009). Jasmonate signaling leads to the release of transcription factors involved in the activation of defense-related

genes during herbivore attack (Chini et al. 2007; Howe and Jander 2008; Galis et al. 2009). As a result, direct and indirect defense metabolites accumulate, including e.g. 17- hydroxygeranyl-linalool diterpene glycosides, nicotine, phenolics, oxidative enzymes, phenylpropanoid-polyamine conjugates: (caffeoylputrescine and dicaffeoyl spermidine), and trypsin proteinase inhibitors (Saedler and Baldwin 2004; Shi et al. 2006; Heiling et al. 2010; Kaur et al. 2010) and herbivory induced volatiles (Dicke 2009; Allmann and Baldwin 2010) in *N. attenuata*.

Transcriptional regulation for defense during herbivore attack

Large-scale reconfiguration of the transcriptome, proteome, and metabolome occur during abiotic and biotic stresses including herbivore attack (Hermsmeier et al. 2001; Schittko et al. 2001; Halitschke et al. 2003; Hui et al. 2003; De Vos et al. 2005; Giri et al. 2006; Phillips et al. 2007; Gaquerel et al. 2009; Ruiz-Ferrer and Voinnet 2009; Gilardoni et al. 2010; Kim et al. 2011). *Arapidopsis* exposed to pathogenic leaf bacteria (*Pseudomonas syringae pv. tomato*) or fungi (*Alternaria brassicicola*), tissue-chewing caterpillars (*Pieris rapae*), cell-content-feeding thrips (*Frankliniella occidentalis*), or phloem-feeding aphids (*Myzus persicae*) demonstrated complex sets of transcriptional alterations in which, in all cases, stress-related genes were overrepresented (De Vos et al. 2005). Notably, although these four attackers all stimulated JA biosynthesis, the majority of the changes in JA-responsive gene expression was attacker-specific (De Vos et al. 2005).

Applying OS to damaged leaves generally changes plant transcriptome by up- and down-regulation of transcripts and secondary metabolites (Halitschke et al. 2001; Hermsmeier et al. 2001; Winz and Baldwin 2001; Schmidt et al., 2005; Schwachtje and Baldwin 2008; Gaquerel et al. 2009). Recent study by our group has been shown that OS-elicitation changed around ten thousands of transcripts in *N. attenuata* (Gulati et al, unpublished data). Transcriptional changes require rapidly elicited and transportable regulators, and small RNAs (smRNAs) are good

candidates for this role. Several studies have shown that smRNAs are involved in abiotic and biotic stress responses (Choi and Sano 2007; Phillips et al. 2007; Ruiz-Ferrer and Voinnet 2009; Zhang et al. 2010; Kulcheski et al. 2011; Yan et al. 2011; Khraiwesh et al. 2012). Moreover, silencing of individual smRNA-silencing pathways demonstrates their involvement in stress responses (Blevins et al. 2006; Liu et al. 2009b; Ziebell and Carr 2009; Boyko et al. 2010; Yang et al. 2011). Plants benefit from the transcriptional control which allows them to quickly and plasticly respond to stresses (Schmidt et al. 2005).

Regulation of the transcriptome during abiotic and biotic stress responses often depends on histone and DNA modifications (Chinnusamy and Zhu 2009; Verhoeven et al. 2010; Karan et al. 2012) which are known as transcriptional regulators of gene expression. Several studies have reported that genome rearrangements play a key role in gene expression and plant development under stress (Chinnusamy and Zhu 2009). Under continuous stress responses such genome rearrangements may become transgenerationally heritable, increasing the fitness of following generations in the stressed environment (Boyko et al. 2010; Boyko and Kovalchuk 2010; Verhoeven and van Gurp 2012). It was shown that herbivore stress and pathogen defenses triggered considerable methylation throughout the genome (Verhoeven et al. 2010; Rasmann et al. 2012). Genome modifications such as methylation and acetylation can cause altered regulation of gene activity. These changes are associated with silencing of transposons, imprinting, and silencing transgenes and endogenous genes (Kooter et al. 1999; Zilberman et al. 2007). The transmission of such genome modifications to offspring (Johannes et al. 2009; Verhoeven et al. 2010; Rasmann et al. 2012) is a mechanism of plant plasticity. Recent studies demonstrated that transgenerational inheritance of DNA modifications mediates phenotypic plasticity in *Arapidopsis* and tomato plants during herbivory (Rasmann et al. 2012). *Arapidopsis* mutants that are deficient in jasmonate perception (*coronatine insensitive 1*) or in the biogenesis of small interfering RNA (*dicer-like2 dicer-like3 dicer-like4* and nuclear *RNA*

polymerase d2a nuclear RNA polymerase d2b) do not exhibit this type of inherited resistance (Rasmann et al. 2012).

Applying the reverse genetics approach to individually characterize functions of the components of RNAi demonstrated a key role in the regulation of stress responsive genes, including genes responsive to herbivory (Pandey and Baldwin 2007, 2008; Pandey et al. 2008a; Pandey et al. 2008b; Liu et al. 2009b; Ruiz-Ferrer and Voinnet 2009; Yang et al. 2011; Bozorov et al. 2012). Silencing RNA-dependent RNA polymerase 1 (RDR1) in *N. attenuata* impairs plants' ability to activate JA-mediated responses and makes plant highly susceptible to insects (Pandey and Baldwin 2007). In **Manuscript II**, we isolated a component of RNA interference (RNAi), *Dicer-like* (*DCL*) genes and individually silenced them. This help to understand whether smRNA pathways plays role in anti-herbiovre defense. We found that they play important roles in plant during herbivory.

Role of small RNAs in biotic stress responses

SmRNA have been intensively investigated recently because of their important regulatory role in gene expression. 18-24 nt size class of small RNAs such as microRNA (miRNA) and small interfering RNAs (siRNA) control gene expression at the transcriptional and posttranscriptional levels (Ruiz-Ferrer and Voinnet 2009; Chellappan et al. 2010; Chen et al. 2011). Aside from their role in developmental patterning and maintaining genome integrity by modification chromatin and DNA methylation (Selvi et al. 2010; van Wolfswinkel and Ketting 2010), they also play key roles in plant responses to environmental stresses (Reinhart et al. 2002; Ruiz-Ferrer and Voinnet 2009).

SmRNAs are classified into miRNAs, and endogenous siRNAs which are in turn classified into chromatin-associated siRNAs (hcsiRNA), natural antisense siRNAs (natsiRNA) and trans-acting siRNAs (tasiRNA) (Reinhart et al. 2002; Bartel 2004). Biogenesis of all these smRNAs requires specific components of the RNAi

pathway (Figure 2) (Bartel 2004; Vaucheret 2006). Different members of the RNAi pathways: DCL, RDR and Argonaut (AGO) protein families are implicated in the biogenesis of each smRNA (Figure 2) (Vaucheret 2006; Chapman and Carrington 2007; Ruiz-Ferrer and Voinnet 2009). Primary miRNA genes (*MIR*) are transcribed in the nucleus as primary transcripts which are processed into precursor stem and loop structures (pre-miRNA) and transported into the cytoplasm, where they are "diced" by DCL1 into miRNA-duplexes (Bartel 2004; Chen 2005). In contrast to miRNAs, biogenesis of siRNAs begins from primary non-protein coding transcripts processed into double stranded RNA (dsRNA) which are processed by RDRs (RDR2, RDR6).

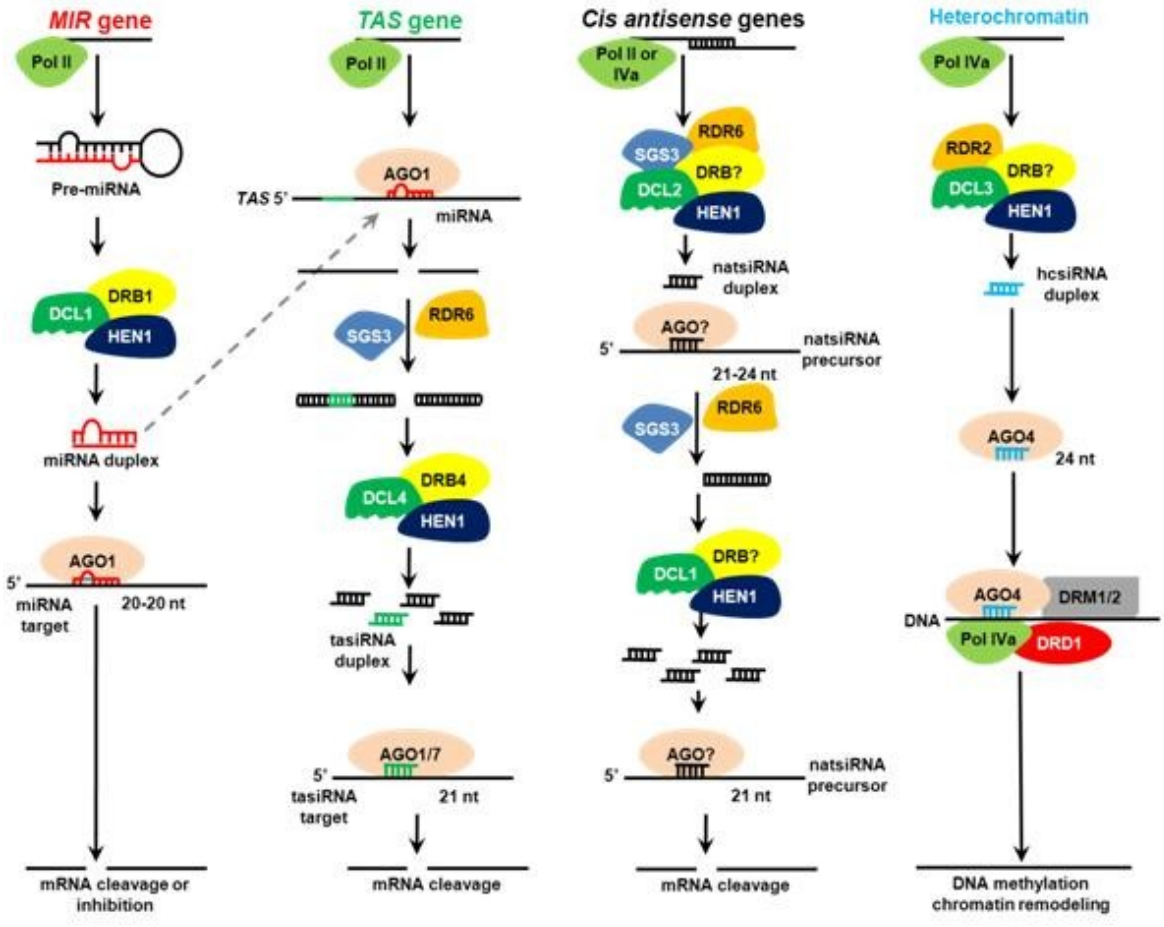


Figure 2. miRNA, ta-siRNA, nat-siRNA, and hc-siRNA pathways in plants.

These dsRNAs are also “diced” by DCLs (DCL2, 3 and 4) into 21-24 nt pieces depending on the DCL (Gasciolli et al. 2005) (Table 1). Both smRNAs bind to mRNA targets in a perfect or imperfect complementary manner, resulting in inhibition or degradation of the target mRNA. However, siRNAs have also been implicated in DNA methylation of gene regulatory elements, resulting in transcriptional inhibition of their targets (Pontes et al. 2009; Baev et al. 2010; Chellappan et al. 2010; Chen et al. 2011).

Abiotic and biotic stress change smRNA levels in plants as well as in animals (Bhattacharyya et al. 2006; Mallory and Vaucheret 2006; Zhao et al. 2007; Zhou et al. 2007; Li et al. 2008; Jia et al. 2009; Zhou et al. 2010; Tang et al. 2012). When plants are exposed to stress, they must switch off or reduce the expression level of genes which are involved in growth and developmental processes, and activate stress-responsive genes (Sunkar 2010). It would be costly for plant to maintain the expression of all genes under non-stress conditions. This could be costly in two ways: maintaining synthesis of proteins which cannot be used, and the potential loss of photosynthesis and primary metabolites to attackers.

Table I. Genetic requirements of endogenous small RNAs in plants. (Vaucheret 2006; Ruiz-Ferrer and Voinnet 2009)

SmRNA	Size	DCL1	DCL2	DCL3	DCL4	RDR2	RDR6
miRNA	18-21	+	-	-	-	-	-
tasiRNA	20-21	+	-	-	+	-	+
natsiRNA	21-22	+	+	-	-	-	+
hcRNA	22-24	-	-	+	-	+	-

A plus sign (+) indicates that the enzyme is required. A minus sign (−) indicates that the enzyme is dispensable.

In previous studies, it has been shown that herbivory induces changes in the smRNA transcriptome and phytohormone signaling in *N. attenuata* during herbivory in nature and in glasshouse conditions (Pandey and Baldwin 2008; Pandey et al. 2008a; Pandey et al. 2008b). In **Manuscript III**, I computationally identified several conserved miRNAs and profiled their expression patterns during herbivory. Moreover, I predicted the expression of their predicted targets. I found that miRNAs play a role in the response to herbivory in both jasmonate-dependent and-independent manners.

Chapter 2

Manuscript overview

Manuscript I

Efficient screening of transgenic plant lines for ecological research.

Klaus Gase, Arne Weinhold, **Tohir Bozorov**, Stefan Schuck and Ian T. Baldwin.

Published in Molecular Ecology Resources 2011 (11), 890–902.

In Manuscript I, we developed a flow chart protocol that allows for the efficient production and selection of transgenic plants for ecological research.

KG designed most of the binary vectors and wrote the paper; I isolated plant DNA/RNA, performed diagnostic PCRs and qPCRs, and contributed to creating flow chart; AW constructed overexpression vectors and screened the transgenic lines; SS screened part of the transgenic *N. attenuata* lines; and ITB designed research and wrote the paper.

Manuscript II

Dicer-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata*.

Tohir A. Bozorov, Shree P. Pandey, Son T. Dinh, Sang-Gyu Kim, Maria Heinrich, Klaus Gase and Ian T. Baldwin

Published in Journal of Integrative Plant Biology 2012, (54:3), 189–206

In Manuscript II, I explored the ecological relevance of *Dicer-like 1, 2, 3* and *4* in the wild tobacco, *N. attenuata*, to elucidate their regulatory roles in the defense pathways elicited by herbivore attack.

I carried out all experiments, analyzed data and drafted the manuscript; SPP, SGK, and ITB participated in the design and coordination of the study; STD and M H helped to extract nucleic acids and phytohormones, and participated in plant-treatment experiments; ITB conceived the study and wrote the paper.

Manuscript III

Identification and profiling of miRNAs during herbivory reveals jasmonate-dependent and -independent patterns of accumulation in *Nicotiana attenuata*.

Tohir A. Bozorov, Ian T. Baldwin and Sang-Gyu Kim

Submitted to BMC Plant Biology, (Submitted)

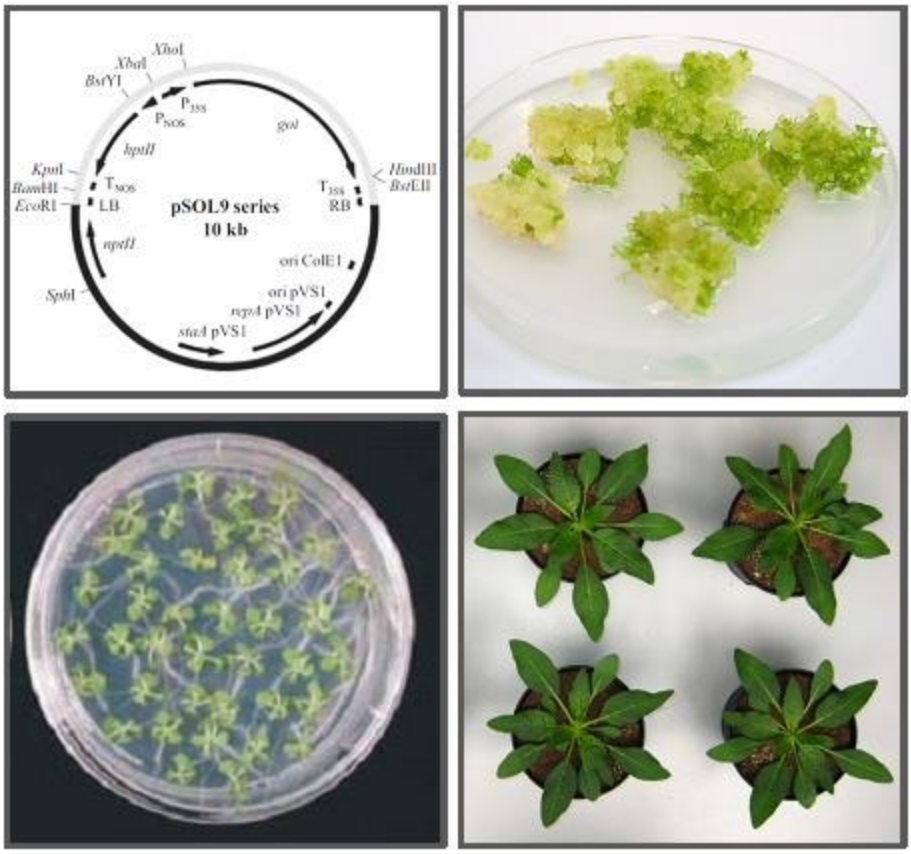
In Manuscript III, I computationally identified conserved microRNAs (miRNA) and endogenous *trans* acting small interfering RNAs (tasiRNA). SmRNA regulation during herbivory reveals jasmonate-dependent and -independent patterns of accumulation. Also I predicted targets of smRNAs, the expression of which co-related with the abundance of the respective smRNAs.

I performed all experiments, analyzed the data, and drafted the manuscript; I, ITB, and SGK participated in the design of the study; ITB and SGK conceived the study and edited the manuscript.

Chapter 3

Efficient screening of transgenic plant lines for ecological research

Klaus Gase, Arne Weinhold, Tohir Bozorov, Stefan Schuck and Ian T. Baldwin.



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Efficient screening of transgenic plant lines for ecological research

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Abstract

Plants stably transformed to manipulate the expression of genes mediating ecological performance have profoundly altered research in plant ecology. *Agrobacterium*-mediated transformation remains the most effective method of creating plants harbouring a limited number of transgene integrations of low complexity. For ecological/physiological research, the following requirements must be met: (i) the regenerated plants should have the same ploidy level as the corresponding wild-type plant and (ii) contain a single transgene copy in a homozygous state; (iii) the T-DNA must be completely inserted without vector backbone sequence and all its elements functional; and (iv) the integration should not change the phenotype of the plant by interrupting chromosomal genes or by mutations occurring during the regeneration procedure. The screening process to obtain transformed plants that meet the above criteria is costly and time-consuming, and an optimized screening procedure is presented. We developed a flow chart that optimizes the screening process to efficiently select transformed plants for ecological research. It consists of segregational analyses, which select transgenic T_1 and T_2 generation plants with single T-DNA copies that are homozygous. Indispensable molecular genetic tests (flow cytometry, diagnostic PCRs and Southern blotting) are performed at the earliest and most effective times in the screening process. qPCR to quantify changes in transcript accumulation to confirm gene silencing or overexpression is the last step in the selection process. Because we routinely transform the wild tobacco, *Nicotiana attenuata*, with constructs that silence or ectopically overexpress ecologically relevant genes, the proposed protocol is supported by examples from this system.

Keywords: ecological genomics, field releases, plant transformation, ploidy, reverse genetics, screening

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Introduction

Stably transformed plants have proven a powerful tool in ecological research to investigate the ecological relevance of particular genes. For this, the inserted sequence elements are designed either to silence intrinsic or to overexpress intrinsic or heterologous genes of interest. This reverse genetics approach allows for the creation of transgenic lines with either abnormally low or high levels of transcripts for a particular gene in an otherwise isogenic background. These isogenic lines provide a particularly efficient means of studying the fitness consequences of a given gene's expression (Steppuhn *et al.* 2004; Zavala *et al.* 2004; Kang *et al.* 2006; Schwachtje *et al.* 2008).

Two general strategies are used for plant transformation: *Agrobacterium*-based transformation and a group of unrelated techniques collectively referred to as 'direct DNA transfer' (Kohli *et al.* 2003). Direct DNA transfer methods such as particle bombardment (Christou 1992) often result in transgenic loci with a high transgene copy number (often more than 40) (Kohli *et al.* 2003; Latham *et al.* 2006). *Agrobacterium*-based transformation procedures produce lines with less complex transgenic loci, but still, the integration of multiple T-DNA copies into a limited number of loci is common (De Buck *et al.* 2009; Bhat & Srinivasan 2002). The transformation mediated by *Agrobacterium* involves the transfer of the T-DNA molecule to the eukaryotic host cell and its integration into the host genome. The machinery required for this process comprises proteins encoded by bacterial chromosomal genes and Ti-plasmid virulence genes as well as the host intracellular transport and DNA repair machinery (Tzfira & Citovsky 2006; Gelvin 2009; Lacroix & Citovsky 2009). The T-DNA region is defined by its left and right border sequences, two 25-bp inverted repeats, originally present on the Ti plasmid (Tzfira *et al.* 2004). To allow the creation of transgenic plant lines with desired insertions, the

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T-DNA borders were transferred to a binary vector, and the sequences that should be integrated into the plant genome are cloned between these border sequences (Hoekema *et al.* 1983).

T-DNA integration occurs randomly throughout the plant genome (Gelvin & Kim 2007; Kim *et al.* 2007) by non-homologous end-joining (Gheysen *et al.* 1991) and is accompanied by deletions and rearrangements of the T-DNA flanks and of the target DNA near the integration site (Latham *et al.* 2006; Muller *et al.* 2007; Gambino *et al.* 2009). The integration of DNA from binary plant transformation vectors is not always limited to the region between the T-DNA borders. 'Read-through' events can occur and result in the unwanted cotransfer of vector backbone sequences, such as bacterial resistance genes. After *Agrobacterium*-mediated T-DNA transfer, binary vector backbone sequences can be detected in up to 75% of the transgenic plants (Kononov *et al.* 1997; Wenck *et al.* 1997; McCormac *et al.* 2001; Kohli *et al.* 2003; Lange *et al.* 2006; Gambino *et al.* 2009). Regulatory agencies that govern the release of transgenic organisms do normally not permit the release of plants carrying such sequences. *Agrobacterium* infection and, in most cases, the regeneration process necessary to regenerate a transgenic plant via cell culture and callus formation from a single transformed plant cell can lead to somoclonal variations (Bhat & Srinivasan 2002), including genome-wide mutations (Latham *et al.* 2006) and polyploidization (Bubner *et al.* 2006).

Agrobacterium-based transformation has proven to be the best-suited approach to produce transgenic plant lines with single-copy T-DNA insertions (De Buck *et al.* 2009; Bhat & Srinivasan 2002; O'Malley & Ecker 2010, Meza *et al.* 2002; Sallaud *et al.* 2003; Olhoft *et al.* 2004; Yu *et al.* 2010). Transformed plant lines may contain two or more independent transgenic loci, but sufficient lines with single T-DNA insertions in a single locus can be expected (De Buck *et al.* 2004). This makes *Agrobacterium*-based transformation the preferred method to create transgenic plants for ecological research.

If the transgenic plants are to be used to answer questions about the organismic-level consequences of a particular gene's expression, then the transformants must fulfil a number of strict criteria: The transgenic line should have the same ploidy level as the plant that was transformed (Schwachtje & Baldwin 2008). The insert should comprise the complete T-DNA originating from the binary vector used for transformation without deletions, rearrangements or other mutations. In each line, only one transgenic locus with a single T-DNA copy should be present, and each line should be homozygous with respect to this locus. The integration site of the transgenic DNA should not disrupt other functional genes, which could confound the analysis of phenotypes associated with the gene of interest. To exclude mutations arising from T-DNA insertion or regeneration, at least two independent lines created with the same T-DNA should be evaluated, and both should exhibit the same phenotype, as the chances that the T-DNA inserted twice into the same functional gene are vanishingly small (Schwachtje *et al.* 2008).

For many plant scientists interested in the function of genes at a whole plant level, the utilization of genetically modified plants silencing or overexpressing a particular gene of interest is the most powerful means of answering functional questions. One important challenge for this approach is the lack of transformation and selection procedures for many plant species of ecological interest. This protocol paper will help ecological researchers to create and select transgenic lines that fulfil the requirements of their research questions. We have developed an *Agrobacterium*-mediated transformation system for the wild tobacco *Nicotiana attenuata* Torrey ex Watson, an ecological model plant (Baldwin 2001). Based on our experiences with this method in producing transgenic lines for use in ecological research, we describe a protocol that optimizes the efficiency of the transformation and selection system and can be applied to other plants of ecological interest.

The transformation procedure is laborious and comprises the construction of appropriate binary plant transformation vectors, *Agrobacterium*-based transformation and the selection and regeneration of the first transgenic generation (T_0 plants). Because the T_0 generation originates from regenerated calli, growth regulators used during regeneration may have a lasting effect on plant performance (Bhat & Srinivasan 2002). It also cannot be excluded that T_0 plants are chimeras regenerated from two or more transformed plant cells, or have increased ploidy levels (Bubner *et al.* 2006). Because of these properties, T_0 plants should not be used for experiments. The first non-chimeric transgenic plant generation that did not undergo the regeneration process (T_1) is produced after self-pollination of the T_0 flowers, and the resulting T_1 seeds should be germinated on medium containing the selective antibiotic. This allows the identification of individuals carrying T-DNA insertions and indicates possible silencing problems because of promoter methylation that may interfere with the transcription of the transgene (Stam *et al.* 1997).

The T_1 generation represents an important intermediate stage in the selection of transgenic lines that fulfil the criteria for ecological research. In most cases, this generation contains homozygous lines with a single T-DNA insertion locus. The ploidy level, the arrangement of the T-DNA insertion and often the level of transcription of the transgene will—if no further chromosomal rearrangements occur—remain unchanged in subsequent inbred generations.

Further inheritance studies with the T_2 generation, obtained by self-pollination of the T_1 generation and germination, are necessary to identify candidate transgenic T_1 lines that harbour single transgene insertions in a homozygous state. Each of the selected lines should be evaluated by a number of molecular genetic analyses to confirm that the above criteria are met. These analyses include flow cytometry to determine the ploidy level of the transgenic lines, appropriate diagnostic polymerase chain reactions (PCR) to ensure the insertion of the complete T-DNA into the plant chromosome and to exclude vector backbone integrations, Southern blotting to confirm single T-DNA insertions into the plant chromosome and qPCR to evaluate silencing efficiency or the level of transcript accumulation of the transgene.

The transformation procedure and the inheritance studies necessary to produce and identify the required transgenic lines consume an enormous amount of human and material resources. The time to grow one generation from germination to seed ripening can take, depending on the species, several months, in the case of *N. attenuata* 3–4 months. It is therefore important to limit the screening effort to what is absolutely necessary. In this study, we present a workflow protocol that enables researchers to produce and select transgenic lines in an optimized screening process. We analyse each step of the selection process using examples from the screening of *N. attenuata* lines and discuss possible variations.

Materials and methods

Construction of plasmids for the transformation of N. attenuata

The initial vector for the construction of the pSOL8/pSOL9 plasmid series was pSOL3LOX (10.6 kb) (Bubner *et al.* 2006). After replacing the *nptIII* (aminoglycoside phosphotransferase class III) gene with *nptII* from pAcGFP1-1 (http://www.clontech.com) and replacing the inverted repeat gene fragments with *N. attenuata DCL2* cDNA (HQ698849) fragments, thereby providing optimized cloning sites, cloning vector pSOL8DCL2 (10.2 kb; HQ698851) was created.

The pSOL8 series inverted repeat gene silencing plasmids (Fig. 1a) were created by replacing the *Xho*I-*Sac*I and *Pst*I-*Hin*dIII inverted repeat fragments of pSOL8DCL2 with inverted repeat PCR fragments (0.3–0.6 kb) of the following *N. attenuata* genes: pSOL8DC3 [10.8 kb; RNA-dependent RNA polymerase 1 gene (DQ988990) combined with WRKY6 gene (AY456272)], pSOL8PNRP [10.2 kb; gene similar to *Arabidopsis thaliana* putative nematode resistance protein mRNA (AY080778)], pSOL8AEP65 [10.2 kb; gene similar to *N. tabacum* Avr9/Cf-9 rapidly elicited protein 65 mRNA

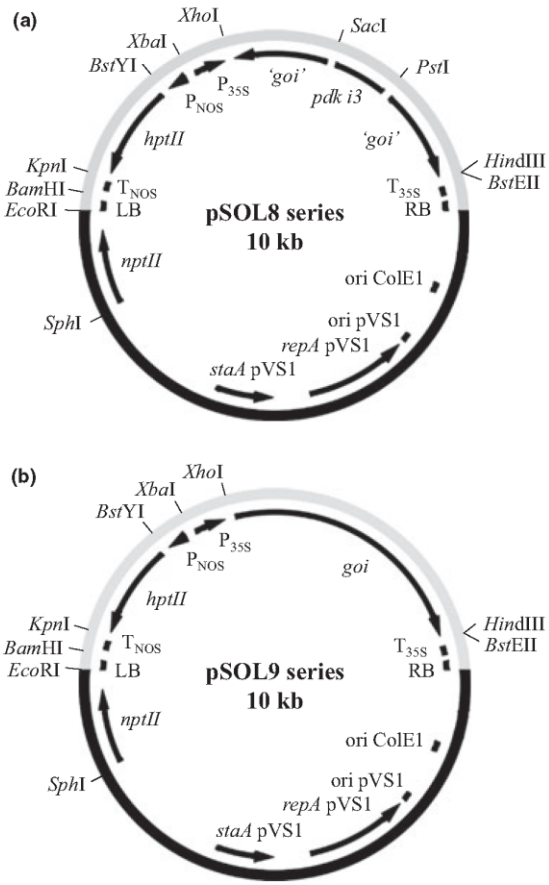

Fig. 1 The pSOL8 series (a) and pSOL9 series (b) binary plant transformation vectors. Abbreviations: LB/RB, left/right border of T-DNA; P_{NOS}/T_{NOS}, promoter/terminator of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*; P_{35S}/T_{35S}, 35S promoter/terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); *goi*, gene of interest; i, intron 3 of *Flaveria trinervia pdk* gene for pyruvate, orthophosphate dikinase; *nptII*, aminoglycoside phosphotransferase class II; ori, origin of replication.

(AF211539)] and pSOL8AEP150 [10.1 kb; gene similar to *N. tabacum* Avr9/Cf-9 rapidly elicited protein 150 mRNA (AY775041)].

The following pSOL9 series gene expression plasmids (Fig. 1b) were obtained by replacing the 1.4 kb *Xho*I-*Hin*dIII-fragment of pSOL8DCL2 with appropriate PCR fragments (0.2–0.4 kb) allowing the overexpression of the following antimicrobial protein genes: pSOL9CAP [9.2 kb; *Capsella bursa-pastoris* antimicrobial peptide mRNA (HQ698850)], pSOL9ESC [9.0 kb; synthetic gene similar to *Rana plancyi fukienensis* mRNA for esculentin-1P precursor protein (AJ968397)], pSOL9FAB [9.0 kb; synthetic gene similar to *Vicia faba* fabatin precursor

mRNA (EU920043)], pSOL9ICE [9.0 kb; *Mesembryanthemum crystallinum* antimicrobial peptide 1 precursor mRNA (AF069321)], pSOL9LEA [9.1 kb; synthetic gene similar to *Leonurus japonicus* antimicrobial protein mRNA; (AY971513)], pSOL9PNA [9.1 kb; synthetic gene similar to *Ipomoea nil* antifungal protein mRNA (U40076)], pSOL9SSP [9.0 kb; synthetic gene for *Aptenodytes patagonicus* Spheniscin-2 (P83430)] and pSOL9VRD [9.0 kb; synthetic gene similar to *Vigna nakashimae* defensin-like protein gene (AY856095)]. As reference examples, the sequences of pSOL8DC3 and pSOL9CAP have been submitted to GenBank (HQ698853 and HQ698852).

Plant transformation, regeneration and cultivation

Transformation of *N. attenuata* was performed as described in Kruegel *et al.* 2002. In brief, hypocotyls from 8- to 10-day-old seedlings were cut into 3-mm-long pieces with a scalpel that previously had been dipped into a culture of *A. tumefaciens* LBA4404 (Invitrogen, http://www.invitrogen.com) carrying the binary plant transformation vector. After 3 days of cocultivation with *Agrobacterium*, the transgenic tissue went through the following regeneration steps on specific phytagel-based media, containing the selective antibiotic hygromycin B (20 g/l) from Duchefa, http://www.duchefa.com (H0192) and the antibacterial antibiotic ticarcillin disodium/clavulanate potassium (125 mg/l) (Duchefa T0190): callus induction (14–21 days), shoot regeneration (14–21 days) and shoot maturation (14–21 days). Subsequently, plantlets were cultured for at least 21 days on rooting medium without both antibiotics. After root regeneration, plants were grown on soil, first in Magenta boxes (http://www.bio-world.com), and finally in 2-litre pots for flowering, self-pollination and seed production in the glasshouse.

Flow cytometry

Flow cytometry was performed with leaf material from *N. attenuata* on a flow cytometer CCA-II (Partec, http://www.partec.com) as described in Bubner *et al.* 2006.

Germination of N. attenuata *and screening for individuals with T-DNA insertions*

Germination of *N. attenuata* was performed as described in Kruegel *et al.* 2002 with the exception that 60 seeds were germinated per plate. If screening for individuals with T-DNA insertions should be performed, the selective antibiotic hygromycin B (Duchefa H0192) was added at a concentration of 35 mg/l to the germination medium. After 10 days, the ratio of seedlings surviving the antibiotic selection was determined.

Diagnostic PCRs for integrity of T-DNA insertions

Genomic DNA (gDNA) was isolated from leaves or seedlings of *N. attenuata* by a modified cetyltrimethylammonium bromide method (Bubner *et al.* 2004). PCR was performed with DreamTaq™ DNA Polymerase (Fermentas, http://www.fermentas.com) according to the instructions of the manufacturer with 1–100 ng of gDNA per sample. The following primer pairs were used: PROM FOR/INT REV and INT FOR/TER REV for the inverted repeat gene silencing constructs; PROM FOR/TER REV for the gene overexpression constructs; and DCL2GF1/DCL2GR1 or GGPP22-22/GGPP23-21 as positive controls amplifying a 334-bp or a 241-bp fragment of *DCL2* (GenBank GU479998) or *ggpps* (GenBank EF382626). Cycles were 5 min 95 °C (30s 95 °C, 30s 55–60 °C, 1 min 72 °C), repeated 30 times, 5 min 72 °C, hold 20 °C. Primer sequences are given in Table S1 (Supporting Information).

Southern blotting

Southern blotting was performed as described in Jassbi *et al.* (2008), with the exception that a 287-bp hygromycin phosphotransferase gene (*hptII*) probe obtained by PCR with primer pair HYG1-18/HYG2-18 (Table S1, Supporting Information) was used. Labelling was performed with the GE Healthcare (http://www.gehealthcare.com) Readyprime DNA labelling system and ProbeQuant g-50 microcolumns according to the instructions of the manufacturer; 7 μg of genomic DNA was digested with restriction enzymes from New England Biolabs (http://www.neb.com) and blotted onto a nylon membrane (GeneScreenPlus; PerkinElmer, http://www.perkinelmer.com) according to the manufacturer's protocol.

qPCR

Plant material was ground in liquid nitrogen with mortar and pestle. Total RNA was extracted with TRI reagent™ (SIGMA, http://www.sigmaaldrich.com) according to the manufacturer's instructions. RNA quality was checked on a 1% agarose gel, and concentration was measured spectrophotometrically at 260 nm.

For qPCR analysis, at least three replicated biological samples were used. One microgram of total RNA obtained from each sample was reverse transcribed using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen) for a total volume of 20 μl according to the instructions of the manufacturer. cDNA samples were diluted 1:10 and used for SYBR®Green-based qPCR, carried out on a Stratagene MX3005P™ using qPCR™ Core Kits for SYBR®Green No ROX (Eurogentec, http://www.eurogentec.com) according to the instructions of

the manufacturer. Analysis of data was carried out according to the comparative Ct ($2^{-\Delta\Delta Ct}$) (Bubner & Baldwin 2004) method or by standard curves (Jassbi *et al.* 2008). The actin cDNA was amplified with primer pair Actin-F1/Actin-R1 (Table S1, Supporting Information) and used as an internal standard for normalizing cDNA concentration variations. For the determination of transcript abundances of the genes of interest, appropriate gene-specific primers were used.

Results and discussion

Here, we discuss the most efficient protocol to produce genetically modified plants utilizable in ecological research; the workflow that we describe is summarized in Fig. 2.

Construction of binary plant transformation vectors

Binary plant transformation vectors consist of two general regions—one representing the T-DNA, defined by the left and right border repeats, and one with the regions and genes necessary for replication in *Escherichia coli* and *Agrobacterium tumefaciens*. To avoid unwanted side effects and to achieve a high stability of the T-DNA inserts in the plant genome, the T-DNA should only contain the elements necessary for the intended function of the transgene. These elements are the two expression cassettes for

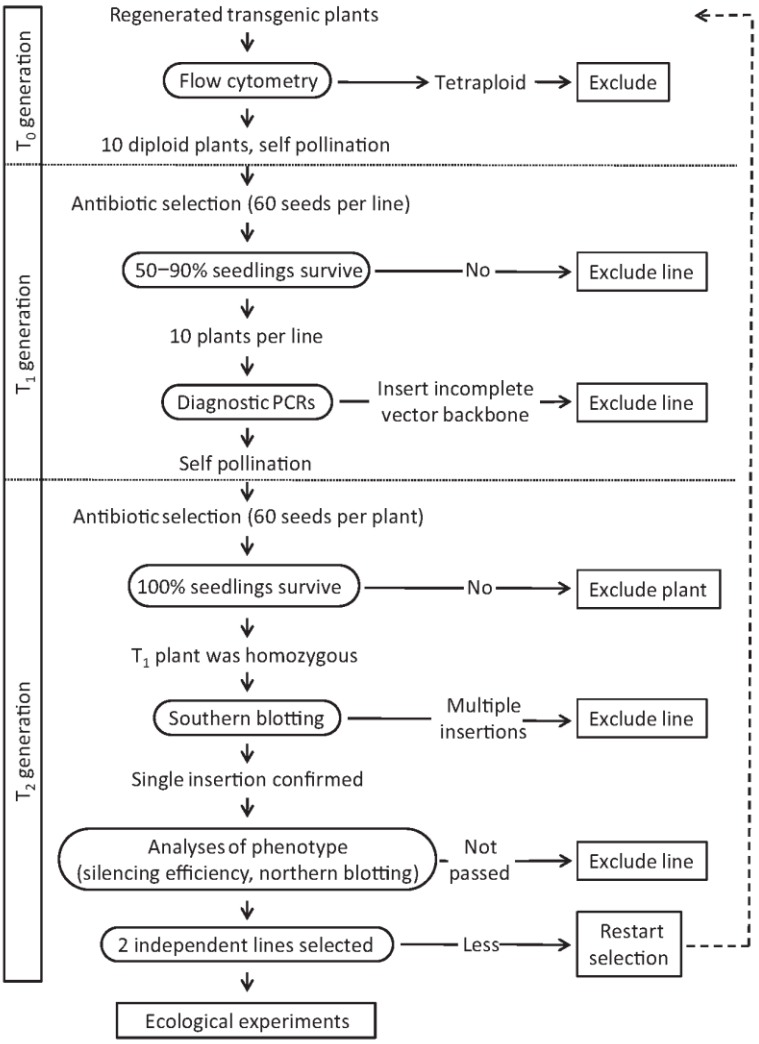

Fig. 2 Workflow for efficient screening of transgenic lines for ecological research. Each test is performed as early as possible during plant screening to obtain a fast and reliable selection.

the transgene and the plant selectable marker gene, both containing promoter, transgene sequence and terminator. In gene silencing vectors, the transgene region should consist of an inverted repeat of a partial sequence of the gene to be silenced, separated by a functional intron larger than 100 bp. The presence of this non-repeated sequence allows replication of the plasmid in bacteria despite the long inverted repeat (Warren & Green 1985). Splicing of the intron from the mRNA in the host plant greatly increases the probability that a dsRNA molecule is formed, which in turn efficiently initiates the silencing of the target gene. In our experience, target gene fragments with sizes ranging from 150 bp to about 1 kb can be used for efficient gene silencing. Routinely, our silencing constructs carry inverted repeat fragments of about 300 bp. If a member of a gene family or a certain allele should be silenced, the choice of the gene sequence requires special consideration. Nucleic acid homology of 23 nt is sufficient to direct post-transcriptional silencing of a gene (Thomas *et al.* 2001). To silence a single gene, sequence homology of more than 22 nt should be avoided. To silence a gene family, a sequence with homologies of more than 22 nt should be chosen. To enhance transgene stability, the use of different promoters and terminators, e.g. promoter/terminator of the nopaline synthase gene from the Ti plasmid of *A. tumefaciens* and 35S promoter/terminator from the cauliflower mosaic virus, on the same T-DNA is advisable.

The bacterial part of the binary transformation vector should contain an origin of replication functional in *E. coli*, e.g. from the ColE1 plasmid (construction of the binary vectors is performed in *E. coli*), an origin of replication and the genes that are necessary for plasmid replication in *A. tumefaciens*, e.g. from plasmid pVS1 and an antibiotic resistance marker both selectable in *E. coli* and *A. tumefaciens*. Because T-DNA border 'read-through' events are quite common during T-DNA integration, the use of a bacterial resistance gene that is already widely spread in nature, e.g. the *nptII* kanamycin resistance gene, is advisable.

Examples for binary plant transformation vectors constructed according to the principles described above are the pSOL8 gene silencing series (Fig. 1a) and the pSOL9 gene overexpression series (Fig. 1b), both extensively used to transform *N. attenuata*.

Transformation and regeneration

Agrobacterium-based transformation is the preferred method to create transgenic plants for ecological research. After transformation, the regeneration of differentiated plants from the transformed cells is necessary. Establishing transformation and regeneration procedures is probably the most challenging step in the utilization of transgenic approaches for non-model plants. Published transformation and regeneration procedures [as examples see *N. tabacum* (Horsch *et al.* 1985; Gallois & Marinho 1995), *Beta vulgaris* (Lindsey & Gallois 1990), *A. thaliana* (Valvekens *et al.* 1988; Clough & Bent 1998) and *Hordeum vulgare* (Tingay *et al.* 1997)] suggest that protocols specifically adapted for each species, and sometimes for each cultivar of each species (Valvekens *et al.* 1988) to be transformed, need to be worked out. With considerable effort, including as many as 10 people-years, we developed a transformation and regeneration procedure for *N. attenuata* (described in the 'Materials and methods' section). Because of different media and hormone requirements of this organism, established procedures from other closely related species like *N. tabacum* could not be applied.

Determination of ploidy level

One of the heritable somoclonal variations that may occur during tissue culture is autopolyploidy (Bubner *et al.* 2006). The extent of polyploidization can be substantial: In diploid tomato, 24.5–80% of transformants were found to be tetraploid [depending on cultivar and method; (Ellul *et al.* 2003)], and up to 92% of originally triploid bermuda grass *Cynodon dactylon* × *transvaalensis* cv. Tif Eagle transformants were found to be hexaploid (Goldman *et al.* 2004). To ensure the comparability and the relevance of the results obtained from experiments with transgenic plants and control plants, it is essential that only transgenic plants with the same ploidy level as the plant that has been transformed are selected. The ploidy level of the first transgenic generation (T_0) is preserved in all following generations produced by self-pollination. Ploidy-level determination of the lines of this generation should be the first step in selecting transgenic lines. This approach allows the early elimination of unwanted lines and saves resources.

The most efficient and conclusive method of determining ploidy levels is by flow cytometry. Because T_0 plants may be chimeric, material from leaves close to the flowers is determined to obtain results indicative of the ploidy level of the T_1 generation. Ideally, 10 independent transgenic lines with the correct ploidy level should be identified from this step in the screening process. According to our experience with *N. attenuata*, plants with doubled sets of chromosomes can often be visually distinguished from plants with the original set of chromosomes, as these plants commonly have 10% broader leaves and larger trichomes, stomates, flowers and seeds. They produce fewer seeds (20–40 vs. 100–200 seeds per capsule in diploid individuals) and have abnormal growth forms. Critical assessment of the phenotypes of the T_0 plants with respect to measured ploidy values has

in our experience greatly facilitated reliable elimination of tetraploid plants.

The ploidy analyses performed with transgenic *N. attenuata* lines from transformation experiments with more than 200 different constructs document that the frequency of occurrence of tetraploid plants substantially varies depending on the construct used for transformation. This effect occurred with all inbred lines (6th to the 30th inbred generation) used as starting material for the transformation. An example for the frequency of occurrence of tetraploid plants is shown in Fig. 3. Plants from the 30th inbred generation of a genotype collected from Utah (Glawe *et al.* 2003) were transformed with a series of binary vectors (pSOL9CAP, pSOL9ESC, pSOL9FAB, pSOL9ICE, pSOL9LEA, pSOL9PNA, pSOL9SSP and pSOL9VRD), all ectopically overexpressing heterologous genes coding for proteins with antimicrobial activities. The ploidy levels of plants from 11 to 25 independent T_0 lines per construct were measured using flow cytometry. Depending on the transgene, 0–57% of the transgenic plants were tetraploid. All together, 159 plants were analysed, 37% were tetraploid and 63% diploid. The increase in the portion of tetraploid plant lines occurring after transformation with particular constructs may require that a larger number of transgenic lines need to be tested to obtain the desired number of diploid lines.

Screening for homozygous lines with single-copy T-DNA

Transgenic plant lines used for ecological research should carry a single T-DNA copy in a single transgenic

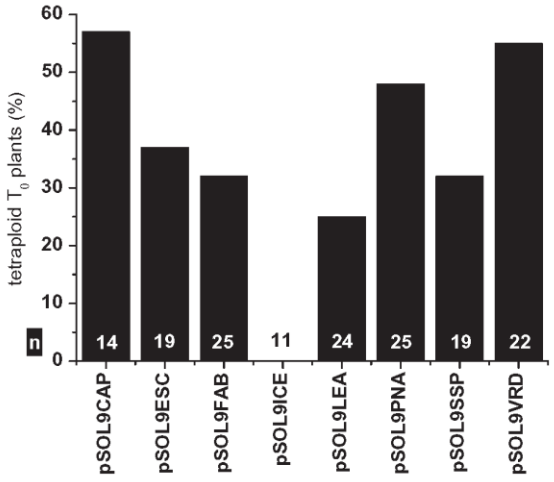

Fig. 3 The fraction of tetraploid *Nicotiana attenuata* plants after transformation of the 30th Utah inbred line with pSOL9 vectors. The numbers of tested plants are indicated.

locus. We have found that a single copy is sufficient for the expected phenotypic effect (gene silencing or overexpression). To avoid segregation, these lines should be homozygous with respect to the T-DNA insertion. Multiple independent transgenic loci would dramatically increase the effort required to generate and identify non-segregating, homozygous lines. In the case of overexpression, multiple T-DNA copies or even strong promoters of a single T-DNA copy may lead to transgene mRNA concentrations above a critical level and elicitation of silencing of the overexpressed gene (Lechtenberg *et al.* 2003; Eamens *et al.* 2008; Hirai *et al.* 2010).

Transgenic plants with two or more independent transgenic loci can generally be identified by inheritance studies (Vain & Thole 2009). Non-independent transgenic loci (T-DNA insertions at a distance smaller than 50 cM on the same chromosome) and complex insertions into one locus need to be identified by Southern analysis (protocol section 7) at later stages in the screening process. Any T-DNA insertion into T_0 plants will usually be hemizygous, because the probability of simultaneous insertions into the same locus of two homologous chromosomes is very low.

To produce homozygous lines with respect to a transgenic locus, self-pollination of T_0 plants is required. Screening for T-DNA insertions can be performed most efficiently by growing seedlings on medium containing the selective antibiotic. Only plants carrying the T-DNA and expressing the resistance marker gene will grow. The expected Mendelian inheritance ratio for a single transgenic locus in the T_1 offspring should be 1 (homozygous) to 2 (hemizygous) to 1 (wild type); thus, 75% of the offspring would carry the T-DNA. A second independent transgenic locus would lead to a ratio of 15 (any transgenic locus) to 1 (wild type), and 93.75% of the offspring would carry the T-DNA. Additional transgenic loci would further increase this portion.

If the ratio of transgenic vs. non-transgenic seedlings is lower than expected (<50%), silencing of the selectable marker gene may be the reason. In this case, and only if a simultaneous silencing of the transgene can be excluded, screening for the selectable marker gene could be performed by PCR genotyping of seedlings grown on non-selective medium. To assess the extent to which silencing of the selectable marker gene occurs during the screening process, the T_1, T_2 and T_3 inbred generations of the *N. attenuata* pSOL9 overexpression lines were analysed (Fig. 4). The initial number of T_1 lines was 10–12 depending on the construct. Because according to our observations (data not shown) silencing of the selectable marker gene is associated with silencing of the transgene and will, once initiated, reoccur in the following generations, all plant lines exhibiting signs of silencing were excluded from further screening. Silencing of the resistance gene

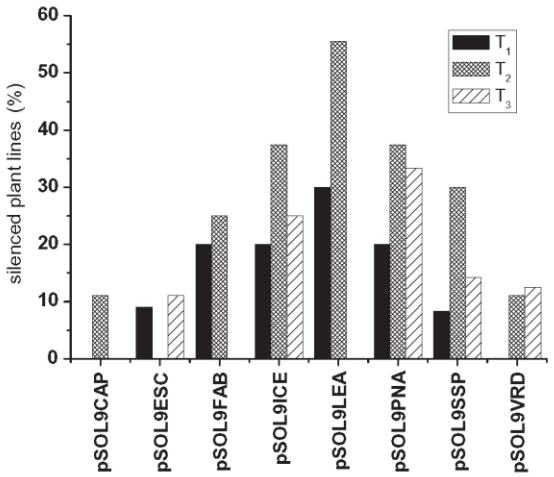

Fig. 4 Portion of lines in which silencing of the *hpt*II gene occurred during different stages of screening (T_1, T_2 or T_3 generation) as detected by hygromycin sensitivity. The initial number of T_1 lines was 10–12 depending on the construct. Plants in T_1 and T_2 stage were considered as silenced if more than 50% of seedlings were hygromycin sensitive. Homozygous T_3 plants were considered as silenced if segregation occured.

occurred unpredictably and irregularly. The mean portion of silenced lines was 13.4% for T_1, 26.0% for T_2 and 12.0% for T_3 generations. Gene silencing in sense overexpression lines is most likely caused by promoter methylation (Weber *et al.* 1990; Stam *et al.* 1997). This form of epigenetic regulation can occur in each offspring generation of a previously unsilenced line. To guarantee a high expression level of the transgene, the functionality of the resistance marker gene should be examined for each new generation by germination on medium containing the selective antibiotic.

T-DNA insertions may interrupt genes essential for embryo or gametophyte development, thus leading to embryo- (Errampalli *et al.* 1991) or gametophyte-lethal lines (Feldmann *et al.* 1997; Howden *et al.* 1998). This will result in exceptional segregation, characterized by progeny segregating for fewer T-DNA carrying seedlings than predicted by Mendelian principles. Exceptional segregation may occur in about 9% of the transgenic lines (Feldmann *et al.* 1997) and should be accounted for in the segregation analyses.

The inheritance-based screening of transgenic plant lines is always performed via self-pollination and germination of 60 seedlings per plant. In the first step, the T_1 generation seeds from 10 independent T_0 lines with the correct ploidy level are germinated. A T_0 plant is considered an appropriate candidate line for carrying a single T-DNA insertion if 50–90% (75% calculated) of the T_1 offspring are transgenic. More transgenic seedlings point to multiple T-DNA integrations, while fewer indicate gene silencing or exceptional segregation.

In the second screening step, T_2 seeds from 10 T_1 plants of each single T-DNA insertion candidate line are germinated. If all T_2 seedlings from a T_1 plant carry the T-DNA, this plant represents the desired homozygous genotype of the respective line and can be used for further screening (Fig. 2). Plants with extreme, unexpected phenotypes should not be used for seed production.

At this point, it should be mentioned that because of extremely long generation times or self-incompatibility in some ecologically interesting systems (e.g. trees), self-pollination cannot be applied in the screening process. Each system may present its specific challenges, which have to be overcome in a specific way, for instance, by deeper genetic analyses of the T_0 generation.

Confirmation of complete T-DNA integrations using PCR

Strand breaks followed by deletions, inversions and rearrangements at the T-DNA flanking regions are some of the events during *Agrobacterium*-mediated T-DNA integration that may result in non-functional transgenes (Latham *et al.* 2006; Muller *et al.* 2007; Gambino *et al.* 2009). Selection for non-functional integrations of the transgene occurs when a plant is transformed with a transgene the product of which interferes with plant regeneration. This process leads to a dramatically reduced transformation efficiency. When this occurs, the regenerated lines often carry large deletions of the T-DNA flank containing the transgene, in our experience with *N. attenuata*. In contrast, lines that carry no functional resistance marker are eliminated by selection for antibiotic resistance during tissue culture.

It is essential to demonstrate that each candidate line that should be used in further screening carries a complete, functional T-DNA. Antibiotic selection guarantees the intactness of the T-DNA flank harbouring the selectable marker gene, but the integrity of the T-DNA transgene flank still has to be demonstrated. This is efficiently carried out with a diagnostic PCR-based analysis of the T_1 genome from which selected fragments of the transgene are amplified. The presence of a PCR product at the expected size provides strong evidence of a complete T-DNA insertion. Lines that do not yield the expected PCR product carry an incorrect transgene, most likely a deletion of the region to be amplified, and should be excluded from further screening. The results of this analysis are only informative for plants with single T-DNA insertions, because in plants with multiple insertions, a positive result does not exclude the presence of an additional defective transgene. If appropriate, this test can already be performed on T_0 plants to exclude lines

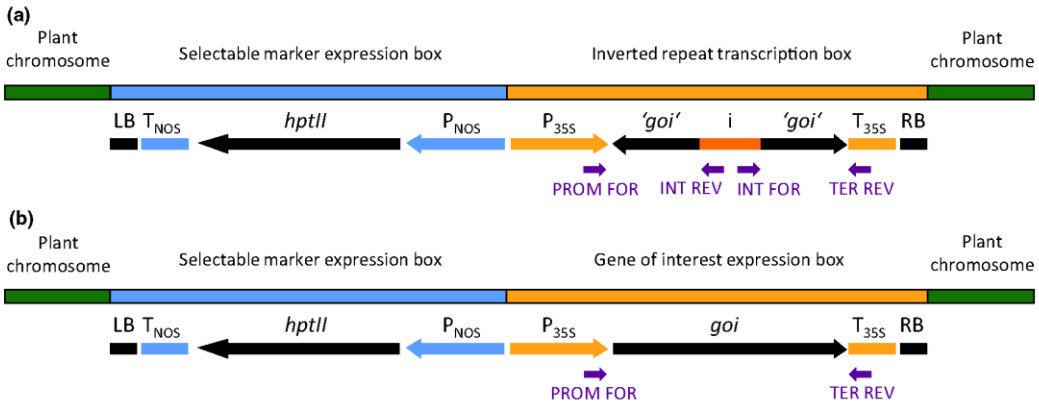

Fig. 5 The positions of annealing sites of the diagnostic PCR primers at the T-DNA integrated into the plant genome: (a) inverted repeat silencing vectors, (b) overexpression vectors. Abbreviations: see Fig. 1.

without functional T-DNA insertions or on plants from later generations to confirm the presence of a correctly inserted transgene.

To test whether the transgene expression cassettes of different *N. attenuata* lines transformed with pSOL8 or pSOL9 constructs were integrated completely, we designed four universal primers that bind to the cassettes' functional elements (Fig. 5): PROM FOR (35S promoter), INT REV (*pdk*-intron 5′-end) and INT FOR (*pdk*-intron 3′-end) TER REV (35S terminator). The *N. attenuata* silencing lines were analysed with primer pairs PROM FOR/INT REV (amplifying the transgene 5′ copy) and INT FOR/TER REV (amplifying the transgene 3′ copy adjacent to the right T-DNA border). These allow for the amplification of separate fragments for each transgene copy, thus avoiding low PCR efficiency because of amplification of an inverted repeat. For the analyses of the transgenic *N. attenuata* lines that were transformed with pSOL9 overexpression constructs, we used primer pair PROM FOR/TER REV, amplifying the DNA between promoter and terminator of the transgene expression box.

Examples for diagnostic PCR are shown in Fig. 6. The results of all diagnostic PCR performed with 24 transgenic *N. attenuata* lines transformed with the inverted repeat silencing constructs pSOL8DC3, pSOL8PNRP, pSOL8AEP65 and pSOL8AEP150 are summarized in Table S2 (Supporting Information). The rate of incomplete insertions varied between 0 and 60% depending on the transgene construct. Any transgenic line yielding a negative PCR result for the amplification of the 5′ transgene copy always yielded the same result for the 3′ transgene copy, whereas lines with the combination negative PCR result for the 3′ transgene copy and positive result for the 5′ transgene copy were found. These results demonstrate that deletions at the left border T-DNA flank

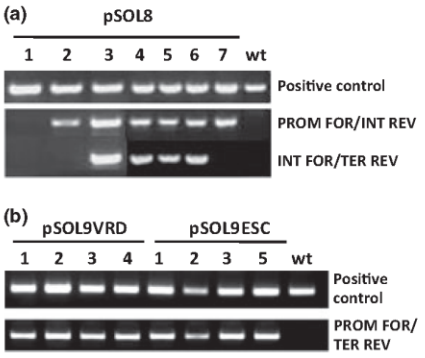

Fig. 6 Diagnostic PCRs with chromosomal DNA from transgenic *Nicotiana attenuata* plants transformed with (a) pSOL8 inverted repeat vectors (1–3: pSOL8DC3; 4: pSOL8PNRP; 5: pSOL8AEP65; 6: pSOL8AEP150: 7 pSOL8PNRP; wt: wild type) and (b) pSOL9 overexpression vectors (lines indicated). Primer pairs are shown.

are common in *N. attenuata*. The highest rates of incomplete insertions were found in plants transformed with pSOL8PNRP and pSOL8DC3 for the silencing of the PNRP gene and for the combined silencing of the RdR1 and WRKY3 genes. We interpret this result as being consistent with selection for incomplete insertions when the product of a transgene is detrimental to the plant.

Confirming the lack of vector backbone

The absence of binary vector backbone sequences in the genome is an important quality criterion established by many regulatory agencies for plants that are to be used in field releases. Demonstrating that a transgenic line does not contain vector sequences outside the T-DNA can be done by a PCR-based approach or—parallel to the

determination of T-DNA copy number described in the following chapter—by Southern analysis. Because vector backbone integration often occurs after a T-DNA border 'read-through', the lack of PCR products from primer pairs designed for the amplification of the vector backbone adjacent to the T-DNA borders demonstrates the absence of vector backbone. For Southern analysis, these primer pairs are used to amplify probes from the transformation vector. Genomic DNA of plants, which does not allow the detection of a DNA fragment with these probes, is considered to contain no vector backbone. If the regulatory agencies require that the absence of critical vector regions, such as the bacterial antibiotic resistance gene or plasmid origins of replication, is to be explicitly demonstrated, primers for these regions can be designed and used in the PCR analyses accordingly. All transgenic lines that contain unwanted vector sequences should be eliminated.

A recent study (Oltmanns *et al.* 2010) demonstrated that starting plant transformation from the *A. tumefaciens* chromosome could be a way to reduce the portion of transgenic plant lines with vector backbone integrations dramatically, but so far, this procedure to produce *Agrobacterium* plant transformation strains is laborious and not applicable to the widely used strain *A. tumefaciens* LBA4404.

Determination of T-DNA copy number by Southern analysis

Multiple transgenic loci and complex T-DNA insertions into one locus present in the genome of transgenic plant lines can, in most cases, be reliably detected by Southern analysis. To confirm single T-DNA insertions in lines selected so far, the probe should be identical to a part of the antibiotic resistance marker gene. This gene does not exist in the wild-type plant genome, but should be present in the genome of all transgenic plants as a consequence of antibiotic selection during regeneration. Moreover, this allows the same probe for the screening of different transgenic plant lines generated with T-DNA from different binary vectors to be used, as long as the selectable marker gene is the same.

For Southern analysis, chromosomal DNA from two or more homozygous T_2 generation individuals of each transgenic line that passed all previous screening tests is completely digested in separate reactions with at least two different restriction enzymes. These enzymes must not cut the T-DNA on both sides or inside the probe sequence, but in order to detect multiple insertions into a single transgenic locus and to reduce the expected size of the genomic fragments carrying this sequence, the T-DNA should be cut once. Under these conditions, all transgenic lines that yield in the Southern analysis multiple bands with any of the restriction enzymes harbour multiple transgenic loci or multiple T-DNA insertions at one locus. Incomplete T-DNA insertions or unwanted sequence rearrangements of the transgenic locus are indicated when fragments smaller than the minimal possible T-DNA size calculated from T-DNA borders and the restriction sites on it are detected. Single bands equal to or larger than the calculated minimal size obtained with all used restriction enzymes are indicative of a single T-DNA insertion. Transgenic lines yielding this pattern are chosen for further screening. An example for a Southern blot of each four independent lines from three different constructs is shown in Fig. 7. However, the existence of additional T-DNA fragments in the genome of the chosen lines, not detectable with the selected probe, cannot be definitively excluded.

Confirmation of transgene function

Before a transgenic line that has been demonstrated to harbour a correct single T-DNA insertion in homozygous stage can be used in ecological experiments, the function of the transgene should be confirmed on the level of RNA. For overexpression lines, the mRNA abundance of the transgene is quantified by qPCR. Choosing an amplicon comprising the stop codon will provide an additional control that the full-length gene is expressed. For gene silencing lines, the silencing efficiency is determined. The

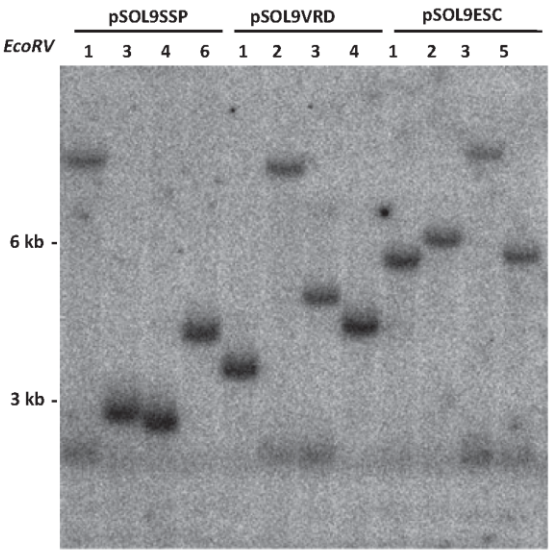

Fig. 7 Southern blotting with nucleic acid from each four independent transgenic *Nicotiana attenuata* T_2 lines transformed with vectors pSOL9SSP, pSOL9VRD or pSOL9ESC. The DNA for the Southern blot was digested with *Eco*RV. A fragment of the marker gene (*hpt*II) served as probe.

relative transcript abundance of the gene of interest both for the transgenic line and wild type plants is measured by qPCR using an amplicon originating from the target gene outside the inverted repeat. Silencing efficiency is then calculated as 100% minus the ratio (in percentage) of relative transcript abundance in the transgenic line and in wild type plants grown under the same conditions and treated in the same way. In our experience with *N. attenuata*, only lines that allow efficient silencing of the target gene (>20%) are valuable for ecological experiments.

qPCR is a very robust means of determining how efficiently an endogenous plant gene is silenced. Nevertheless, the choice of the intrinsic plant gene that shows constant transcript abundance in all the experiments performed and serves as standard gene for normalization is essential for the reliability of the results obtained with this method (Czechowski *et al.* 2005; Gutierrez *et al.* 2008).

If appropriate, transgene function should be confirmed on a phenotypic level by studying the phenotypes associated with silencing or overexpression of the targeted gene. Depending on the insertion site, the strength of expression of the transgene can vary considerably because of 'position effects' (Prols & Meyer 1992; Matzke & Matzke 1998; *Qin et al.* 2003). Different lines with the same T-DNA insertion may thus have modulated phenotypes with different strengths. This titration of phenotypes can be a powerful means of examining the fitness consequences of a gene. In certain cases, the functional transgene will lead to morphological changes, which may allow a pre-selection during the screening process.

Conclusions

Reverse genetics is a powerful tool in plant ecology. To take advantage of this tool, transgenic plants that fulfil the requirements for the structure and stable inheritance of the transgene need to be created and selected. The screening of transgenic plants is a costly and time-consuming procedure. We developed a flow chart protocol (Fig. 2) that allows for the efficient production and selection of transgenic plants for ecological research. We encourage groups working in the field of ecology to make use of the resources described in this study, and the authors will be happy to share plasmids, plant lines and experience with all interested groups.

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References

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

Bhat SR, Srinivasan S (2002) Molecular and genetic analyses of transgenic plants: considerations and approaches. *Plant Science*, **163**, 673–681.

Bubner B, Baldwin IT (2004) Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Reports*, **23**, 263–271.

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**, 14.

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

Christou P (1992) Genetic-transformation of crop plants using microprojectile bombardment. *Plant Journal*, **2**, 275–281.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of Arabidopsis thaliana. *Plant Journal*, **16**, 735–743.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology*, **139**, 5–17.

De Buck S, Windels P, De Loose M, Depicker A (2004) Single-copy T-DNAs integrated at different positions in the *Arabidopsis* genome display uniform and comparable beta-glucuronidase accumulation levels. *Cellular and Molecular Life Sciences*, **61**, 2632–2645.

De Buck S, Podevin N, Nolf J, Jacobs A, Depicker A (2009) The T-DNA integration pattern in Arabidopsis transformants is highly determined by the transformed target cell. *Plant Journal*, **60**, 134–145.

Eamens A, Wang MB, Smith NA, Waterhouse PM (2008) RNA silencing in plants: yesterday, today, and tomorrow. *Plant Physiology*, **147**, 456–468.

Ellul P, Garcia-Sogo B, Pineda B *et al.* (2003) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L.Mill.) is genotype and procedure dependent. *Theoretical and Applied Genetics*, **106**, 231–238.

Errampalli D, Patton D, Castle L *et al.* (1991) Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. *Plant Cell*, **3**, 149–157.

Feldmann KA, Coury DA, Christianson ML (1997) Exceptional segregation of a selectable marker (Kan(R)) in Arabidopsis identifies genes important for gametophytic growth and development. *Genetics*, **147**, 1411–1422.

Gallois P, Marinho P (1995) Leaf disk transformation using *Agrobacterium tumefaciens*: expression of heterologous genes in tobacco. *Methods in Molecular Biology*, **49**, 39–48.

Gambino G, Chitarra W, Maghuly F *et al.* (2009) Characterization of T-DNA insertions in transgenic grapevines obtained by *Agrobacterium*-mediated transformation. *Molecular Breeding*, **24**, 305–320.

Gelvin SB (2009) *Agrobacterium* in the Genomics Age. *Plant Physiology*, **150**, 1665–1676.

Gelvin SB, Kim SI (2007) Effect of chromatin upon Agrobacterium T-DNA integration and transgene expression. *Biochimica Et Biophysica Acta-Gene Structure and Expression*, **1769**, 410–421.

Gheysen G, Villarroel R, Vanmontagu M (1991) Illegitimate recombination in plants—a model for T-DNA integration. *Genes & Development*, **5**, 287–297.

Glawe GA, Zavala JA, Kessler A, Van Dam NM, Baldwin IT (2003) Ecological costs and benefits correlated with trypsin protease inhibitor production in *Nicotiana attenuata*. *Ecology*, **84**, 79–90.

Goldman JJ, Hanna WW, Fleming GH, Ozias-Akins P (2004) Ploidy variation among herbicide-resistant bermudagrass plants of cv. TifEagle transformed with the bar gene. *Plant Cell Reports*, **22**, 553–560.

Gutierrez L, Mauriat M, Pelloux J, Bellini C, van Wuytswinkel O (2008) Towards a systematic validation of references in real-time RT-PCR. *Plant Cell*, **20**, 1734–1735.

Hirai S, Takahashi K, Abiko T, Kodama H (2010) Loss of sense transgene-induced post-transcriptional gene silencing by sequential introduction of the same transgene sequences in tobacco. *FEBS Journal*, **277**, 1695–1703.

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature*, **303**, 179–180.

Horsch RB, Fry JE, Hoffmann NL *et al.* (1985) A simple and general-method for transferring genes into plants. *Science*, **227**, 1229–1231.

Howden R, Park SK, Moore JM *et al.* (1998) Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in *Arabidopsis*. *Genetics*, **149**, 621–631.

Jassbi AR, Gase K, Hettenhausen C, Schmidt A, Baldwin IT (2008) Silencing geranylgeranyl diphosphate synthase in *Nicotiana attenuata* dramatically impairs resistance to tobacco hornworm. *Plant Physiology*, **146**, 974–986.

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.

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of Agrobacterium T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. *Plant Journal*, **51**, 779–791.

Kohli A, Twyman RM, Abranches R *et al.* (2003) Transgene integration, organization and interaction in plants. *Plant Molecular Biology*, **52**, 247–258.

Kononov ME, Bassuner B, Gelvin SB (1997) Integration of T-DNA binary vector 'backbone' sequences into the tobacco genome: evidence for multiple complex patterns of integration. *Plant Journal*, **11**, 945–957.

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.

Lacroix B, Citovsky V (2009) Agrobacterium aiming for the host chromatin. *Communicative & Integrative Biology*, **2**, 42–45.

Lange M, Vincze E, Moller MG, Holm PB (2006) Molecular analysis of transgene and vector backbone integration into the barley genome following *Agrobacterium*-mediated transformation. *Plant Cell Reports*, **25**, 815–820.

Latham JR, Wilson AK, Steinbrecher RA (2006) The mutational consequences of plant transformation. *Journal of Biomedicine and Biotechnology*, **2006**, 25376.1–25376.7.

Lechtenberg B, Schubert D, Forsbach A, Gils M, Schmidt R (2003) Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing. *Plant Journal*, **34**, 507–517.

Lindsey K, Gallois P (1990) Transformation of sugar-beet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. *Journal of Experimental Botany*, **41**, 529–536.

Matzke AJM, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. *Current Opinion in Plant Biology*, **1**, 142–148.

McCormac AC, Fowler MR, Chen DF, Elliott MC (2001) Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. *Transgenic Research*, **10**, 143–155.

Meza TJ, Stangeland B, Mercy IS *et al.* (2002) Analyses of single-copy Arabidopsis T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. *Nucleic Acids Research*, **30**, 4556–4566.

Muller AE, Atkinson RG, Sandoval RB, Jorgensen RA (2007) Microhomologies between T-DNA ends and target sites often occur in inverted orientation and may be responsible for the high frequency of T-DNA-associated inversions. *Plant Cell Reports*, **26**, 617–630.

Olhoft PM, Flagel LE, Somers DA (2004) T-DNA locus structure in a large population of soybean plants transformed using the Agrobacterium-mediated cotyledonary-node method. *Plant Biotechnology Journal*, **2**, 289–300.

Oltmanns H, Frame B, Lee LY *et al.* (2010) Generation of backbone-free, low transgene copy plants by launching T-DNA from the *Agrobacterium* chromosome. *Plant Physiology*, **152**, 1158–1166.

O'Malley RC, Ecker JR (2010) Linking genotype to phenotype using the Arabidopsis unimutant collection. *Plant Journal*, **61**, 928–940.

Prols F, Meyer P (1992) The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in *Petunia hybrida*. *Plant Journal*, **2**, 465–475.

Qin HX, Dong YZ, von Arnim AG (2003) Epigenetic interactions between Arabidopsis transgenes: characterization in light of transgene integration sites. *Plant Molecular Biology*, **52**, 217–231.

Sallaud C, Meynard D, van Boxtel J *et al.* (2003) Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. *Theoretical and Applied Genetics*, **106**, 1396–1408.

Schwachtje J, Baldwin IT (2008) Why does herbivore attack reconfigure primary metabolism? *Plant Physiology*, **146**, 845–851.

Schwachtje J, Kutschbach S, Baldwin IT (2008) Reverse genetics in ecological research. *PLoS ONE*, **3**, e1543.

Stam M, Mol JNM, Kooter JM (1997) The silence of genes in transgenic plants. *Annals of Botany*, **79**, 3–12.

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

Thomas CL, Jones L, Baulcombe DC, Maule AJ (2001) Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector. *Plant Journal*, **25**, 417–425.

Tingay S, McElroy D, Kalla R *et al.* (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant Journal*, **11**, 1369–1376.

Tzfira T, Citovsky V (2006) Agrobacterium-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology*, **17**, 147–154.

Tzfira T, Li JX, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics*, **20**, 375–383.

Vain P, Thole V (2009) Gene Insertion Patterns and Sites. In: *Methods in Molecular Biology, Transgenic Wheat, Barley and Oats* (eds Jones HD & Shewry PR), vol. 478, pp. 203–226. © Humana Press, a part of Springer Science + Business Media, LLC, New York.

Valvekens D, van Montagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 5536–5540.

Warren GJ, Green RL (1985) Comparison of physical and genetic properties of palindromic DNA-sequences. *Journal of Bacteriology*, **161**, 1103–1111.

Weber H, Ziechmann C, Graessmann A (1990) *In vitro* DNA methylation inhibits gene-expression in transgenic tobacco. *EMBO Journal*, **9**, 4409–4415.

Wenck A, Czako M, Kanevski I, Marton L (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during Agrobacterium-mediated transformation. *Plant Molecular Biology*, **34**, 913–922.

Yu JG, Lee GH, Kim JS, Shim EJ, Park YD (2010) An insertional mutagenesis system for analyzing the Chinese cabbage genome using *Agrobacterium* T-DNA. *Molecules and Cells*, **29**, 267–275.

Zavala JA, Patankar AG, Gase K, Baldwin IT (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.

Data accessibility

Tables S1 and S2 (Supporting Information): Dryad repository http://dx.doi.org/10.5061/dryad.8951.

DNA sequences: GenBank accessions GU479998; HQ698849-HQ698853.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Sequences of the primers used for diagnostic PCRs, Southern blotting, and qPCR.

Table S2 Results of diagnostic PCRs with 24 different transgenic *N. attenuata* lines.

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Supplemental Table S1 Sequences of the primers used for diagnostic PCRs, Southern blotting, and qPCR.

Primer Name	Sequence
Actin-F1	5'-GGTCGTACCACCGGTATTGTG-3'
Actin-R1	5'-GTCAAGACGGAGAATGGCATG-3'
DCL2GF1	5'-AAGGATGGCTCATTCCTGGTG-3'
DCL2GR1	5'-AGAGCTTCAACAAGCAGAGAAGG-3'
GGPP22-22	5'-GAAGATTCGCGAGGTGTATTGG-3'
GGPP23-21	5'-CAAGGCAACCAACGGAGCAGC-3'
HYG1-18	5'-CCGGATCGGACGATTGCG-3'
HYG2-18	5'-CTGACGGACAATGGCCGC-3'
INT FOR	5'-GGTAACATGATAGATCATGTC-3'
INT REV	5'-CATACTAATTAACATCACTTAAC-3'
PROM FOR	5'-GGAAGTTCATTTCATTTGGAG-3'
TER REV	5'-GCGAAACCCTATAGGAACCC-3'

Supplemental Table S2 Results of diagnostic PCRs with 24 different transgenic *N. attenuata* lines.

Construct	Type	Generation	Number of lines tested	PCR1 negative	PCR2 negative	PCR1 + PCR2 negative*	% Incomplete insertions
pSOL8DC3	ir	T_1	9	2	5	2	56
pSOL8PNRP	ir	T_3	10	2	6	2	60
pSOL8AEP65	ir	T_3	2	0	0	0	0
pSOL8AEP150	ir	T_3	3	0	0	0	0

PCR1: PROM FOR/INT REV (35S promoter to 5' intron)

PCR2: INT FOR/TER REV (3' intron to 35S terminator)

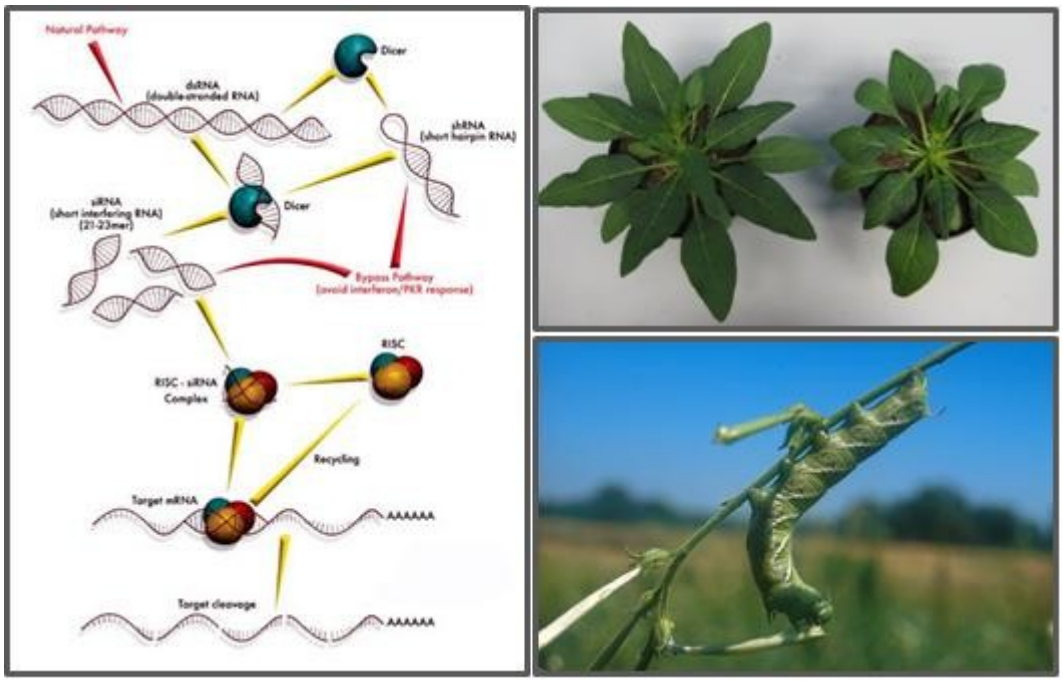
*a negative PCR1 occurred only in lines with negative PCR2

ir: inverted repeat silencing

Chapter 4

Dicer-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata*

Tohir A. Bozorov, Shree P. Pandey, Son T. Dinh, Sang-Gyu Kim, Maria Heinrich, Klaus Gase and Ian T. Baldwin



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Research Article

DICER-like Proteins and Their Role in Plant-herbivore Interactions in *Nicotiana attenuata*

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Abstract

DICER-like (DCL) proteins produce small RNAs that silence genes involved in development and defenses against viruses and pathogens. Which DCLs participate in plant-herbivore interactions remains unstudied. We identified and stably silenced four distinct *DCL* genes by RNAi in *Nicotiana attenuata* (Torrey ex. Watson), a model for the study of plant-herbivore interactions. Silencing *DCL1* expression was lethal. *Manduca sexta* larvae performed significantly better on ir-*dcl3* and ir-*dcl4* plants, but not on ir-*dcl2* plants compared to wild type plants. Phytohormones, defense metabolites and microarray analyses revealed that when *DCL3* and *DCL4* were silenced separately, herbivore resistance traits were regulated in distinctly different ways. Crossing of the lines revealed complex interactions in the patterns of regulation. Single ir-*dcl4* and double ir-*dcl2* ir-*dcl3* plants were impaired in JA accumulation, while JA-Ile was increased in ir-*dcl3* plants. Ir-*dcl3* and ir-*dcl4* plants were impaired in nicotine accumulation; silencing *DCL2* in combination with either *DCL3* or *DCL4* restored nicotine levels to those of WT. Trypsin proteinase inhibitor activity and transcripts were only silenced in ir-*dcl3* plants. We conclude that DCL2/3/4 interact in a complex manner to regulate anti-herbivore defenses and that these interactions significantly complicate the already challenging task of understanding smRNA function in the regulation of biotic interactions.

Keywords: DICER-like proteins; anti-herbivore defense; phytohormone signaling; *Manduca sexta*; *Nicotiana attenuata*.

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Introduction

Plants have evolved to grow under different environmental conditions and in response to various abiotic and biotic stresses. They have developed strategies to increase their Darwinian fitness by producing viable offspring. Herbivore attack dramatically affects all aspects of a plant's physiology (Schwachtje and Baldwin 2008). In the wild tobacco, *N. attenuata,* herbivore resistance is known to be activated by herbivore-specific elicitors, which induce phytohormone dependent signaling resulting in large-scale transcriptional and posttranscriptional changes (Halitschke et al. 2001; Kessler and Baldwin 2004; Voelckel and Baldwin 2004; Giri et al. 2006). As a result of such transcriptional changes, secondary metabolites are synthesized such as neurotoxins, anti-feedants, anti-digestive compounds and volatiles, which directly or indirectly affect the performance of herbivores (Halitschke et al. 2001; Kessler and Baldwin 2004; Voelckel and Baldwin 2004; Giri et al. 2006). Transcriptional changes during herbivore attack result in part from RNA interference (RNAi) which in turn is orchestrated by the action of small RNAs (Pandey et al. 2008b), whose regulation remains unclear.

RNAi is an epigenetic process which regulates gene expression at transcriptional and posttranscriptional levels.

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Gene silencing is triggered by endogenous 21–24 nucleotide (nt) smRNAs (Bartel 2004; Sunkar and Zhu 2007). These smRNAs are produced from long double-stranded RNA or hairpin-loop-structured RNAs by DICER-like (DCL) proteins of the RNase III family (Dunoyer et al. 2005). DCL proteins process precursors of various types of smRNAs: microRNA (miRNA), heterochromatin-associated small-interfering RNAs (hc-siRNA), *trans*-acting siRNA (tasi-RNA), natural antisense siRNA (natsi-RNA) and secondary siRNAs, so as to reduce transcript accumulations (Papp et al. 2003; Vaucheret 2006). Four DCL proteins with partialy redundant functions have been identified in *Arabidopsis thaliana* (Hiraguri et al. 2005; Liu et al. 2009a). DCL1 plays a role in 21 – 24 nt miRNA biogenesis and triggers translational inhibition through imperfect base pairing (Park et al. 2002; Reinhart et al. 2002). DCL2 is responsible for processing 22 – 24 nt natsi-RNAs from the double-stranded RNA of two overlapping reverse-complement mRNA transcripts and from viral RNA (Xie et al. 2004; Bouche et al. 2006). DCL3 is involved in producing 22 – 24 nt hc-siRNAs that function at the transcriptional level. In this process, RNA-directed DNA methylation modifies *cis*- and *trans*-elements of the gene, DNA repeats and transposon loci (Xie et al. 2004; Bouche et al. 2006). DCL4 processes the biogenesis of some 21 nt miRNAs, tasi-RNAs, secondary viral smRNAs and siRNAs from transgenes (Bouche et al. 2006; Rajagopalan et al. 2006). Moreover, DCL4 is required for cell-to-cell movement of silencing signals that result in systemic silencing (Dunoyer et al. 2005; Dunoyer et al. 2010b). All types of smRNAs are potential regulators at different stages of plant development (Chen 2009).

DCL proteins play central roles in antiviral and antibacterial responses (Bouche et al. 2006; Navarro et al. 2006; Ruiz-Ferrer and Voinnet 2009). They dice the double stranded RNA precursors of smRNAs that are critical to plants' ability to respond to the abiotic and biotic stresses that orchestrate changes in corresponding transcripts involved in resistance (Phillips et al. 2007; Pandey et al. 2008b; Ruiz-Ferrer and Voinnet 2009). When plants are stressed, they alter their smRNA populations, which in turn may alter gene expression: when rosette leaves of *N. attenuata* were wounded and oral secretions (OS) of *M. sexta* were applied (W+OS treatment), the smRNA populations changed dramatically (Pandey et al. 2008b). OS from larvae can mimic herbivore attack in *N. attenuata* (McCloud and Baldwin 1997; Halitschke et al. 2001). Double-stranded precursors of these smRNAs are produced by RNA-directed RNA polymerases (RDRs), which are key enzymes of the RNAi machinery in the production of siRNA (Bouche et al. 2006; Garcia-Ruiz et al. 2010). Moreover, RDRs are reported to be involved in defense responses to viruses, pathogen and herbivore attack (Pandey and Baldwin 2007; Qu et al. 2008). Previous studies have shown the ecological relevance of RDRs in adapting wild tobacco plants to biotic stresses, including herbivory (Pandey and Baldwin 2007; Pandey et al. 2008a). Their importance in responding to abiotic stress has also been shown (Liu et al. 2009b).

In this study, we explored the ecological relevance of *NaDCL2, NaDCL3* and *NaDCL4* in the wild tobacco, *N. attenuata,* to elucidate their regulatory roles in the defense pathways elicited by herbivore attack. We generated stably silenced lines of three *DCL*s (ir-*dcl2,* ir-*dcl3,* and ir-*dcl4)* by RNAi. Our attempts to obtain stably silenced ir-*dcl1* lines were not successful since the transformation was lethal and no callus was formed. It has been shown that DCL1-mediated miRNAs play key role in regulating the timing of embryogenesis in *Arabidopsis* and in moss (Khraiwesh et al. 2010; Nodine and Bartel 2010). We characterized the three ir-*dcl* plants' responses to insect herbivory under glasshouse conditions and found that ir-*dcl3* and ir-*dcl4* plants were highly susceptible to *M. sexta* larvae which grew much better compared to larvae feeding on wild-type (WT) plants. To better understand the role of *NaDCL*s during *M. sexta* herbivory, we analyzed the accumulation of phytohormones that are known to mediate resistance traits, secondary metabolites that function as direct defenses and, by oligo nucleotide microarray analyses for evaluating transcriptomic responses.

Results

Identification and characterization of *DCL*s in *N. attenuata*

We obtained full-length coding sequences of *N. attenuata DCL2* (*NaDCL2*; 4212 nt) and *DCL4* (*NaDCL4;* 4869 nt) and partial coding sequences of *DCL1* (5074 nt) and *DCL3* (1792 nt) by using a PCR-based cloning approach (see Material and Methods for details). Each translated putative DCL protein sequence was compared to homologous DCL sequences from other plant species such as *A. thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Physcomitrella patens* and *Ricinus communis* and a phylogenetic tree was constructed (**Figure 1A**). Putative NaDCL1 showed a similarity of 70–82%, NaDCL2 has 70–73% similarity, whereas NaDCL3 and NaDCL4 has 64–66% and 64–74% similarity respectively to other plant DCL orthologs (Supporting Figure S1). DNA gel blot analysis showed that all 4 endogenous *DCL*s occur as single copies in the *N. attenuata* genome (**Figure 1B**). Next, we determined the transcript abundance of all four *DCL* genes in different tissues (flowers, source leaf, cauline leaf and stem) of *N. attenuata.* Quantitative real time PCR assays (qPCR) indicated that the expression of all four *DCLs* was significantly different among tissues. *NaDCL1* showed maximum accumulation in cauline leaves, whereas no differences in *NaDCL1* accumulation were seen among the other tissues. Expression patterns of *NaDCL2* and *NaDCL4* were similar; the transcripts of both genes were found at the highest levels in stems, followed by leaves (**Figure 2A**). *NaDCL3*

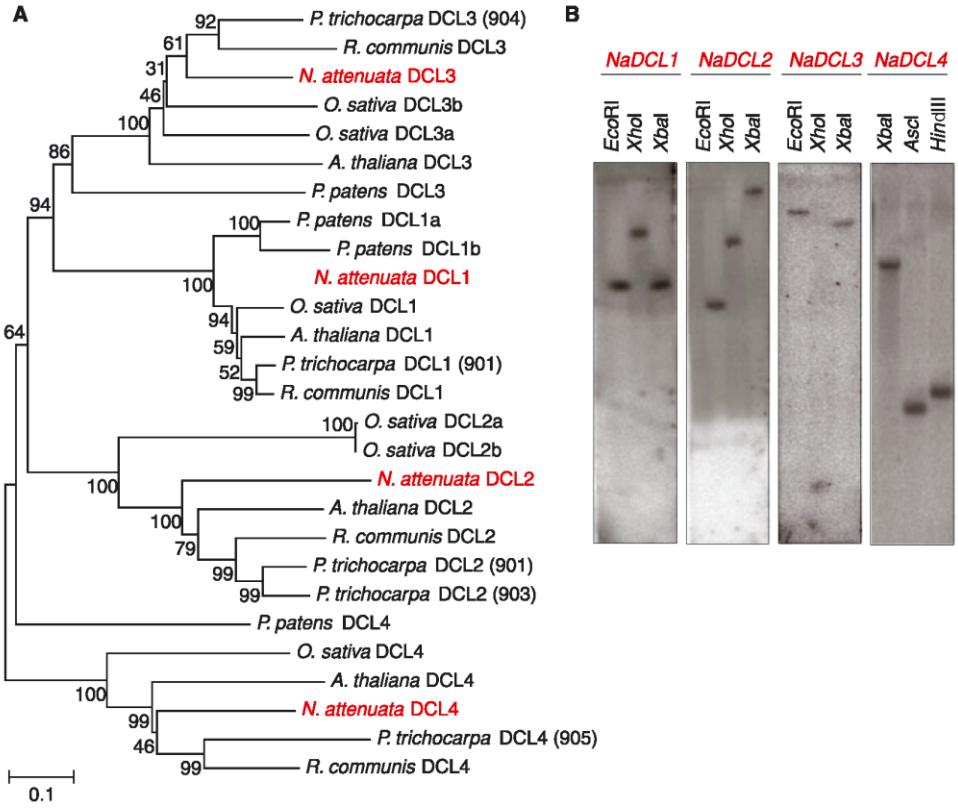

Figure 1. Characterization of the *DCL* genes in *Nicotiana attenuata*.

(A) Phylogenetic analysis of the four encoded DCLs in *Nicotiana attenuata*. Amino acid sequences of four NaDCLs were aligned with their orthologous DCL proteins of *Arabidopsis thaliana, Populus trichocarpa, Oryza sativa, Physcomitrella patens* and *Ricinus communis*. Distance values were calculated with the neighbor-joining method with 1 000 bootstrap replicates.
(B) Southern blot analysis revealed that all four members of *DCL* genes occur as single copies in the *N. attenuata* genome. Genomic DNA was digested with restriction endonucleases as indicated.

transcripts were most abundant in cauline and source leaves as compared to flower and stem tissues (**Figure 2A**).

An unpredicted herbivore population and competition with conspecifics are the major ecological challenges for *N. attenuata*; viruses have not yet been reported as a threat to plants in their natural habitats of the Great Basin Desert of the USA (Pandey et al. 2008a). To investigate the ecological relevance of the DCLs in defense responses to herbivore attack, we began by determining the changes in gene expression using qPCR in control, wounding followed by treatment of water (W+W) and W+OS treated rosette leaves in time-course analyses. To simulate herbivory, rosette leaves were wounded and treated with OS, and then the leaf material was harvested at different time points (Halitschke et al. 2001). Complex, biphasic patterns of changes in gene expression were observed for the different *DCL*s in response to wounding and OS elicitors. Both elicitors reduced *NaDCL1* transcript abundance 6 h post-treatment but increased its expression 14 h after elicitation. Whereas both elicitors reduced *NaDCL2* and *NaDCL3* transcript accumulations within 4 h, an increase in expression for both the genes after 12 h compared to untreated control plants was observed (**Figure 2B**). *NaDCL4* expressions were not different after OS-elicitation compared to control plants (**Figure 2B**). Of the four *DCL*s, only the expression pattern of *NaDCL3* and *NaDCL4* displayed a diurnal rhythm (**Figure 2B**).

Morphological phenotypes of ir-*dcl* plants

To further investigate the functions of the four *NaDCL*s, we generated RNAi constructs to silence their expression individually (Supporting Figure S2A and S3). All transformation experiments of *N. attenuata* with the ir-*dcl1* construct led to lethality

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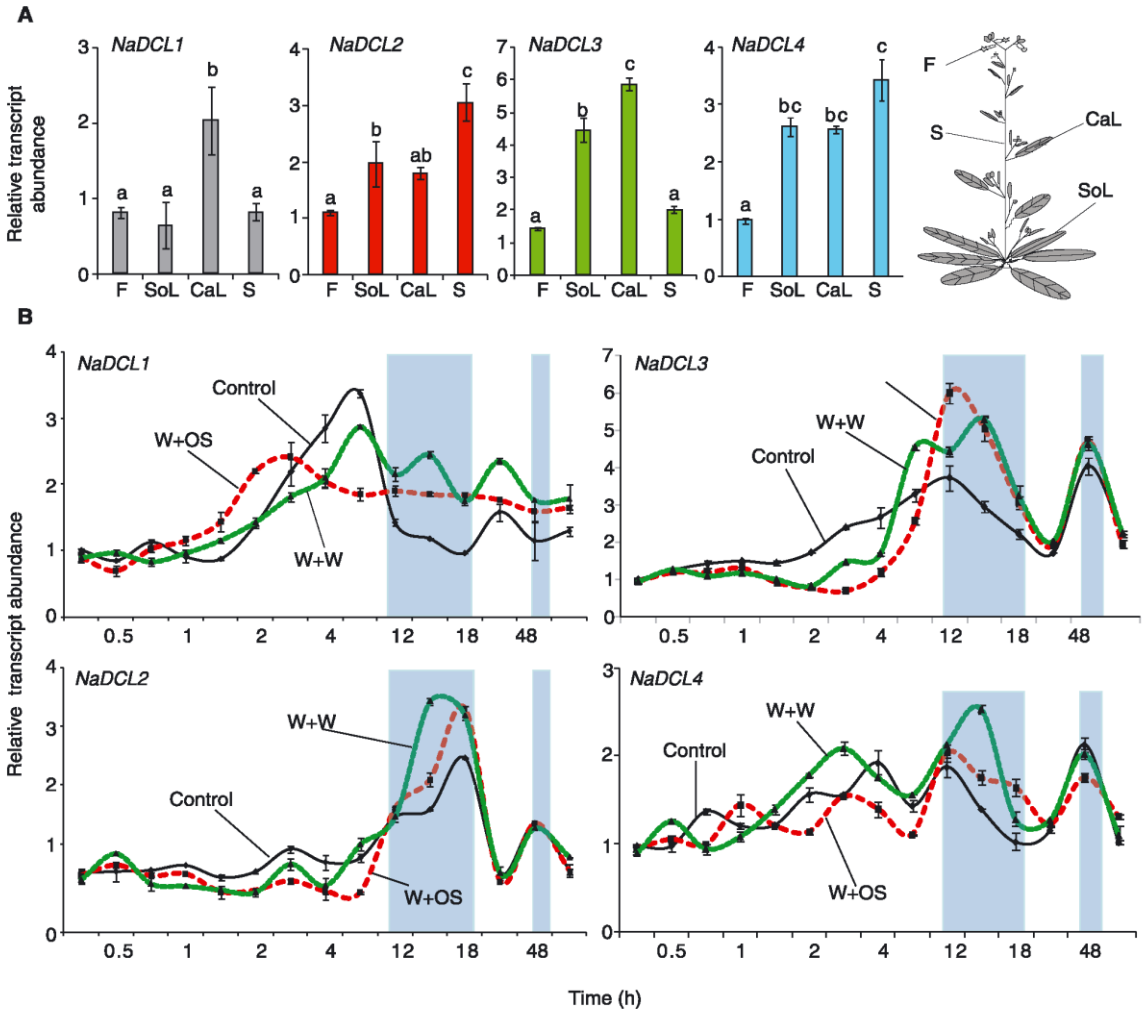

Figure 2. *NaDCL* genes expression.

(A) Accumulation of *NaDCL* transcripts in different tissues: flower (F), source leaf (SoL), cauline leaf (CaL) and stem (S) of *N. attenuata* relative to actin. Mean (±*SE*) of five replicates. Different letters indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA.

(B) Time-course expression of the *NaDCL* transcripts with different treatments in wild-type *N.attenuata*. For elicitation experiments, +1 node rosette leaves from 22-day-old plants were wounded and treated either with 20 μL of water (W+W) or oral secretions (W+OS, diluted 5 times) from *M. sexta* larvae. Untreated plants served as control. Mean (±*SE*) of five replicates. Induction experiments were started at 10:00 am. Shaded blocks reflect dark periods.

of transformants and no callus was formed, thus no transgenic somatic embryos were obtained. Two stably transformed homozygous IR lines with single T-DNA insertions showing efficient knockdowns of *NaDCL2, NaDCL3* and *NaDCL4* transcript accumulations were selected (**Figure 3A**; Supporting Figure S2B). Efficient *DCL* silencing was also observed in double silenced plants (Supporting Figure S2C). Minor morphological changes were observed in ir-*dcl2* and ir-*dcl3* plants compared to WT, whereas ir-*dcl4* plants displayed clear morphological alterations (**Figure 3B,C**). However, double silenced *NaDCL* plants showed different morphological alterations compared to WT plants. Ir-*dcl3* lines showed slightly increased rosette leaf diameters, whereas ir-*dcl4* and ir-*dcl4* x ir-*dcl3* plants showed decreased rosette leaf diameters

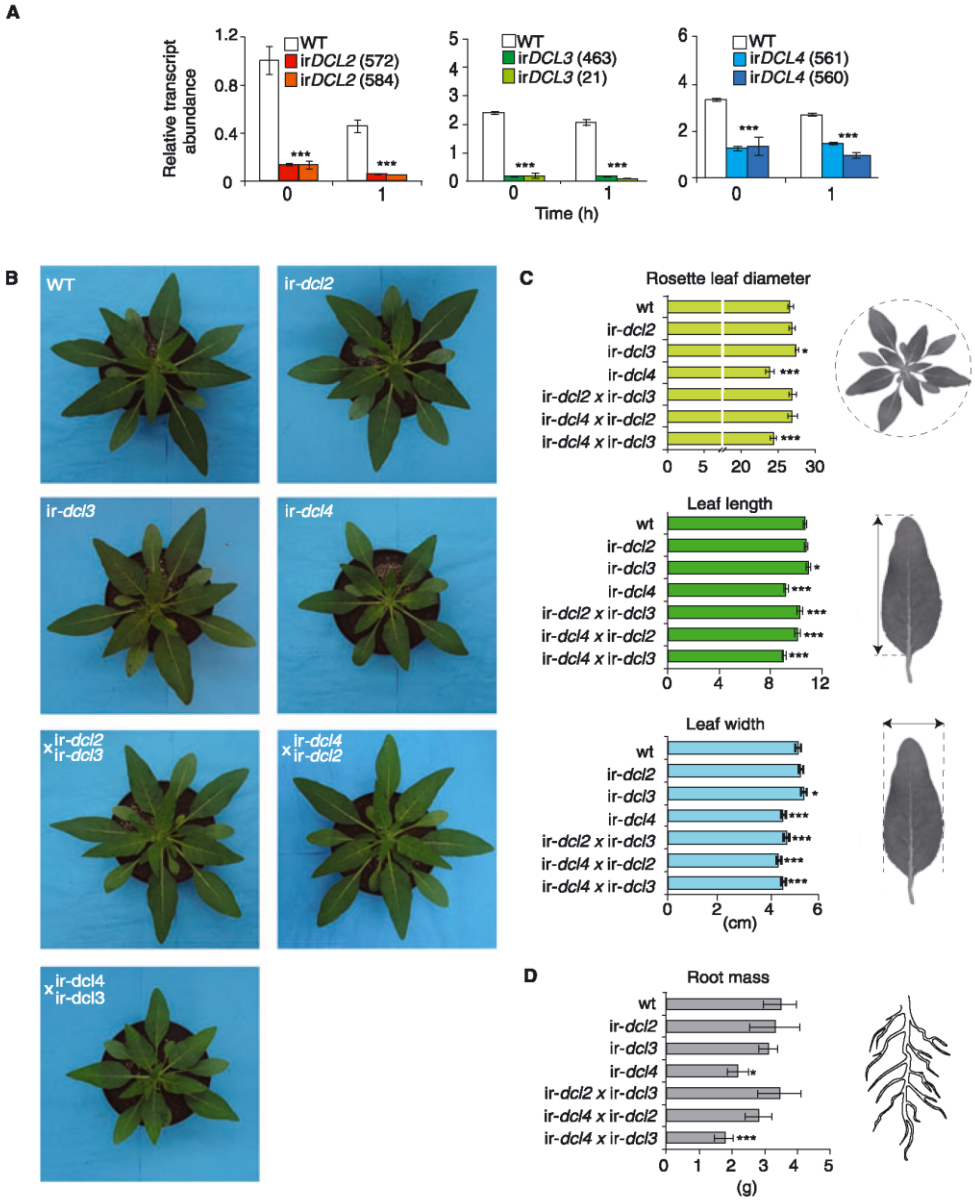

Figure 3. Silencing *NaDCL*s gene expression results in morphological changes.

(A) Silencing efficiency of *NaDCL2*, *3* and *4* genes transcripts in stably transformed ir-*dcl* lines. Transcript accumulation was determined in OS-elicited plants. Asterisks indicate significant differences (***$P < 0.001$) in Fisher's PLSD test following an ANOVA.

(B) The morphological phenotype of stably transformed ir-*dcl*s. Rosette-stage ir-*dcl4* and ir-*dcl4* x ir-*dcl3* plants were significantly smaller compared to WT.

(C) Quantitative data of 32-day-old WT and ir-*dcl*s rosette leaf diameter, leaf length and width. Mean (±*SE*) of ten replicates per line. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) in Fisher's PLSD test following an ANOVA.

(D) Fresh root mass of *NaDCL* silenced plants. Mean (±*SE)* of six replicates per line. Asterisks indicate significant differences (*$P < 0.05$) in Fisher's PLSD test following an ANOVA.

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compared to WT (ANOVA, Fisher's PLSD test, $P = 0.05$ and $P < 0.001$; Figure 3B,C). No difference was observed in ir-*dcl2* x ir-*dcl3* and ir-*dcl4* x ir-*dcl2* double silenced plants. Compared to WT, leaves were significantly longer in ir-*dcl3* but smaller in ir-*dcl4* and double silenced ir-*dcl2* x ir-*dcl3*, ir-*dcl4* x ir-*dcl2* and ir-*dcl4* x ir-*dcl3* plants (ANOVA, Fisher's PLSD test, $P < 0.05$, $P < 0.001$; Figure 3B); similarly, leaf width was significantly increased in ir-*dcl3* and decreased in ir-*dcl4* and double silenced ir-*dcl2* x ir-*dcl3*, ir-*dcl4* x ir-*dcl2* and ir-*dcl4* x ir-*dcl3* plants (ANOVA, Fisher's PLSD test, $P < 0.05$, $P < 0.001$; Figure 3B). Root development was significantly impaired in ir-*dcl4* and ir-*dcl4* x ir-*dcl3* plants. These plants had reduced root mass compared to WT (Figure 3D). Ir-*dcl4* and ir-*dcl4* x ir-*dcl3* showed fewer lateral roots developed and the stems of theses genotypes tended to tip over. Further, comparisons of our ir-*dcl* lines showed clear similarities to *Arabidopsis dcl*-mutants (Gasciolli et al. 2005; Xie et al. 2005; Adenot et al. 2006; Marin et al. 2010) thus confirming the loss of NaDCLs.

M. sexta caterpillars performed better on ir-*dcl3* and ir-*dcl4* but not on ir-*dcl2*

In *Arabidopsis*, all the four DCLs have been implicated in antiviral and antibacterial defense (Ruiz-Ferrer and Voinnet 2009). Due to the importance of herbivory on *N. attenuata* plants, we challenged the three ir-*dcl* genotypes with the lepidopteran specialist tobacco hornworm, *M. sexta,* whose larvae commonly feed on *N. attenuata* in its native habitats (Steppuhn et al. 2004). Caterpillars were weighed 6, 9, and 11 days after hatching and significant differences were observed after 11 days. Caterpillars performed similarly to WT on the two independently silenced ir-*dcl2* lines (Figure 4A). *M. sexta* larvae grew significantly larger on the *ir-dcl3* and *ir-dcl4* plants than on WT (ANOVA, Fisher's PLSD, $P < 0.01$, $P < 0.001$; Figure 4B,C).

Silencing *NaDCL3* and *NaDCL4* resulted in different phytohormone responses to OS-elicitation

To understand the observed changes in the anti-herbivory defense responses, we measured the phytohormones, jasmonic acid (JA) and its isoleucine conjugate (JA-Ile), salicylic acid (SA) and abscisic acid (ABA); these are important for mediating resistance to insects (Bell et al. 1995; Halitschke and Baldwin 2003; Kang et al. 2006; Paschold et al. 2007; Diezel et al. 2009). For phytohormone extraction, rosette-stage leaves were OS-elicited and harvested at different times. JA is a key signaling component that accumulates in wounded *N. attenuata* rosette leaf tissue within 1 h of OS-elicitation (Halitschke and Baldwin 2003; Wang et al. 2008). Significantly less JA and JA-Ile accumulated in the two independently transformed ir-*dcl4* lines (ANOVA, Fisher's PLSD, $P < 0.001$

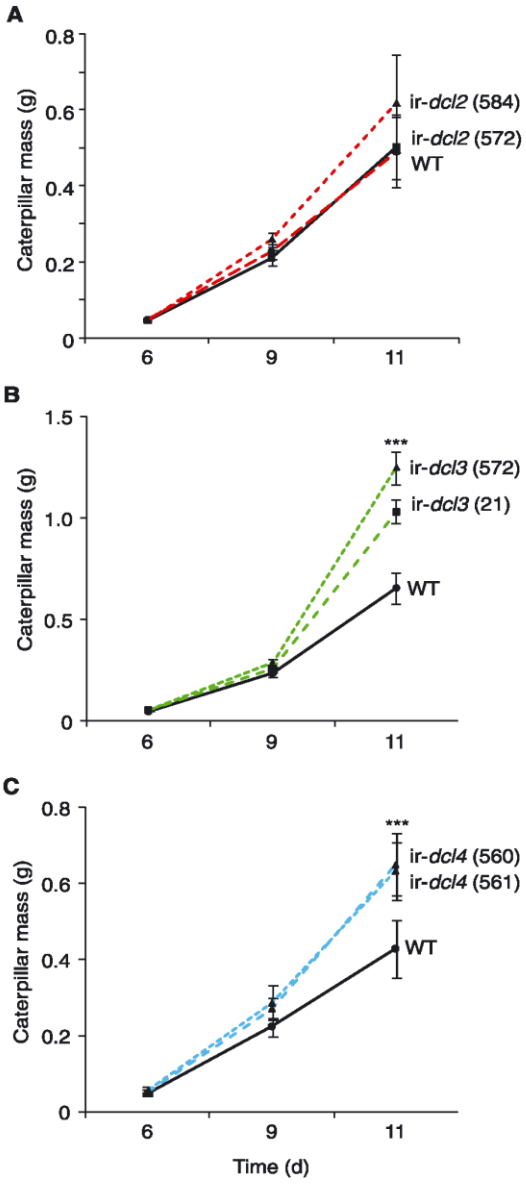

Figure 4. Herbivores grew significantly larger on ir-*dcl3* and ir-*dcl4* plants than on WT plants.

M. sexta performance on **(A)** ir-*dcl2* plants, **(B)** ir-*dcl3* plants and **(C)** ir-*dcl4* plants compared to WT. *M. sexta* growth did not differ on ir-*dcl2* compared to WT plants. Mean (±*SE*) of larval mass after 6, 9, and 11 days of feeding on 25 replicates WT and stably transformed ir-*dcl2*, ir-*dcl3* and ir-*dcl4* lines. Asterisks indicate significant differences between two independently transformed lines and WT plants (***$P < 0.001$) in Fisher's PLSD test following an ANOVA.

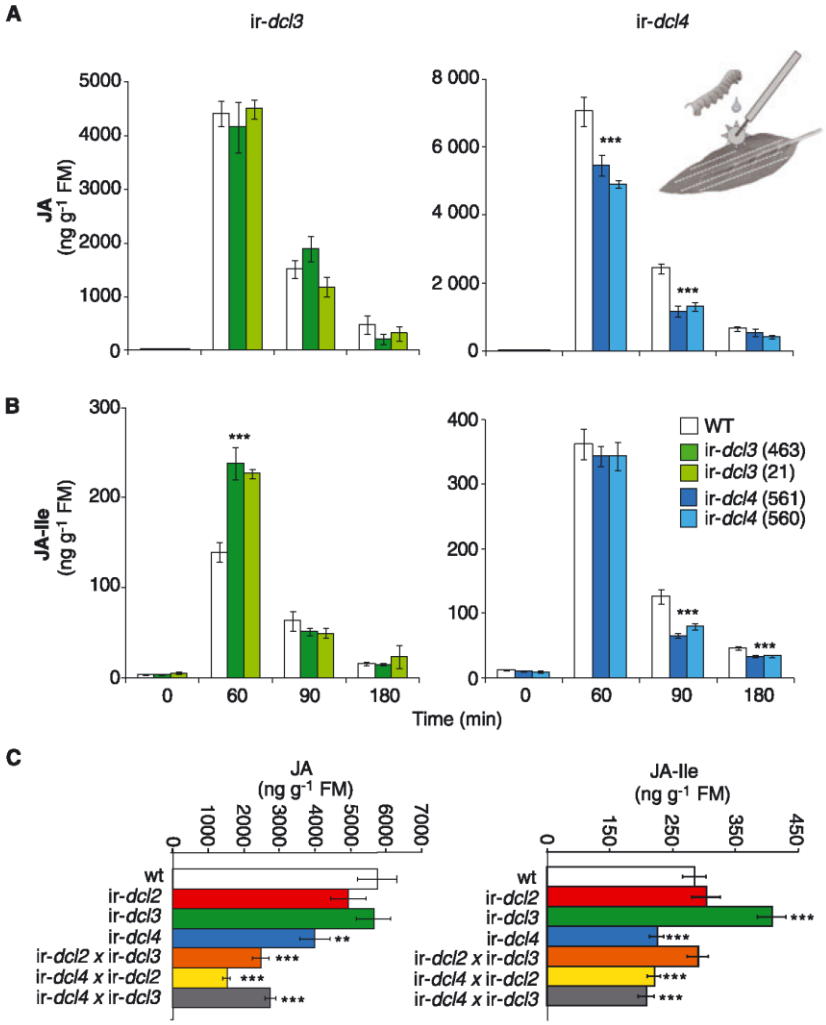

Figure 5. Phytohormone levels in WT, ir-*dcl3* and ir-*dcl4* plants after OS-elicitation.

(A) Jasmonic acid levels were significantly reduced in two independently transformed ir-*dcl4* lines but not in the ir-*dcl3* lines after OS-elicitation. **(B)** The level of the JA conjugate, JA-Ile, was significantly increased in two ir-*dcl3* lines but not changed in ir-*dcl4* plants after OS-elicitation. **(C)** JA and JA-Ile levels in single and double silenced *NaDCL*s plants 1h after OS elicitation. Mean (±*SE*) of six replicates per treatment. Asterisks indicate significant differences between lines and WT plants (**$P < 0.01$; ***$P < 0.001$) in Fisher's PLSD test following an ANOVA.

Figure 5A). In contrast, no difference in JA levels were seen in the two independently transformed ir-*dcl3* lines (Figure 5A), but significantly increased levels of JA-Ile were observed in ir-*dcl3* compared to WT plants 1 h after OS-elicitation (ANOVA, Fisher's PLSD, $P < 0.001$; Figure 5B). However, double silenced plants had different patterns of jasmonate accumulation (Figure 5C). Double silencing *NaDCL*s (ir-*dcl2* x ir-*dcl3*, ir-*dcl4* x ir-*dcl2* and ir-*dcl4* x ir-*dcl3*) reduced JA levels, whereas the ir-*dcl2* x ir-*dcl3* cross combination restored WT JA-Ile level (Figure 5C). The ABA and SA levels were not significantly changed after OS-elicitation in either ir-*dcl2*, ir-*dcl3* or ir-*dcl4* genotypes and their cross combinations compared to WT (Supporting Figure S4). Moreover, W+W treatment did not change JA, JA-Ile, SA and ABA levels in ir-*dcl3* and ir-*dcl4* plants compared to WT (Supporting Figure S5 and S6). These results indicate that the differences in JA/JA-Ile levels in ir-dcl

lines and WT are induced and are specific to herbivore-specific elicitors.

Silencing *NaDCL*s differentially affected defense metabolite levels after OS-elicitation

Plant secondary metabolites such as nicotine, caffeoylputrescine, dicaffeoyl spermidine, proteinase inhibitors and various antifeedants under the control of phytohormone signaling, act as direct defenses against insects and negatively affect their physiology (Steppuhn and Baldwin 2007; Wang et al. 2007; Kaur et al. 2010). We therefore determined the role of *NaDCL*s in the regulation of these defensive secondary metabolites. The alkaloid, nicotine, starts to accumulate 24 h after OS-elicitation and reaches maximal levels after 72 h (Steppuhn et al. 2004). There is significantly less nicotine accumulation in OS-elicited leaves of ir-*dcl3* and ir-*dcl4* plants and their double silenced cross combination ir-*dcl4* x ir-*dcl3* 72 h post-elicitation compared to WT plants (ANOVA, Fisher's PLSD, $P < 0.01$, $P < 0.001$; **Figure 6**). No effect of *NaDCL2* was observed on nicotine accumulation after OS elicitation (**Figure 6**). However, silencing *NaDCL2* either together with *NaDCL3* or *NaDCL4* restored WT nicotine level. Similar nicotine accumulation patterns were also observed in different treatments when rosette leaves were treated either with MeJA in lanolin paste, wounding and application of JA-Ile to the wounds or continuous attack by *M. sexta* larvae (**Figure 6**).

Plants' trypsin proteinase inhibitors (TPIs) inhibit insect midgut proteases activity and thus defend *N. attenuata* against chewing insects (Steppuhn and Baldwin 2007; Zavala et al. 2008). We compared TPI activity in OS-elicited leaves of ir-*dcl* and WT plants. The TPI activity was reduced significantly in the rosette leaves of ir-*dcl3,* but not in ir-*dcl4* plants 120 h post-elicitation compared to the leaves of WT (ANOVA, Fisher's PLSD, $P < 0.001$; **Figure 7A**). Silencing *NaDCL3* either with *NaDCL2* or *NaDCL4* reduced TPI level after OS elicitation, but not after JA-Ile treatment. No difference was observed in double silenced ir-*dcl4* x ir-*dcl2* plants compared to WT plants (**Figure 7A**). *NaTPI* transcript abundance was significantly reduced in ir-*dcl3* plants compared to WT (**Figure 7B**). Levels of two phenylpropanoid-polyamine conjugates, caffeoylputrescine and dicaffeoyl spermidine, are known to increase in local and systemic leaves after herbivore attack (Kaur et al. 2010). Significantly reduced levels of caffeoylputrescine were observed in both ir-*dcl3* and ir-*dcl4* plants 72 h after OS-elicitation (Supporting Figure S7). Dicaffeoyl spermidine was accumulated significantly lower in ir-*dcl3* line and higher in ir-*dcl4* plants compared to WT (ANOVA, Fisher's PLSD, $P < 0.01$; Supporting Figure S7). Levels of chlorogenic acid and rutin did not differ significantly in ir-*dcl* silenced plants and in their crosses compared to WT (Supporting Figure S8).

OS-elicited ir-*dcl3* and ir-*dcl4* plants showed different transcriptional patterns

To further explore the roles of *NaDCL3* and *NaDCL4* in defense responses to insect herbivore attack, and to understand alterations in gene expression patterns, we performed microarray analyses using a *N. attenuata* specific 4 × 44k Agilent custom microarray (GEO Platform: GPL13527, GEO Accession: GSE30124). Microarrays were hybridized using RNA isolated 1 h post-elicitation from OS-elicited rosette leaves of ir-*dcl3*, ir-*dcl4* and WT plants.

Microarray data analysis indicated that 379 probe-sets in ir-*dcl3* plants and 352 probe-sets in ir-*dcl4* plants were up-regulated compared to WT and only 97 probe-sets were co-up-regulated (**Figure 8A**). 116 probe-sets in the ir-*dcl3* line and 369 probe-sets in the ir-*dcl4* line were down-regulated compared to WT; of these, only 24 probe-sets were co-down-regulated (**Figure 8A**; Supporting Table S1). Heat map analysis provides a convenient visualization of the different transcriptional profiles in ir-*dcl3* and ir-*dcl4* compared to WT plants (**Figure 8B**). A total of 1 095 significantly altered transcripts were used for BLASTX (http://www.ncbi.nih.gov) analysis for gene annotations. Among these, 284 transcripts were annotated as non-protein coding transcripts or were not found in GenBank database.

To understand the biological relevance of altered transcripts in ir-*dcl3* and ir-*dcl4* lines, we performed gene ontology (GO) annotations for the biological processes and molecular functions of the regulated probes. Due to insufficient annotations for *Nicotiana* species, we used GO annotations and categorization from AmiGO (http://www.geneontology.org) and TAIR (http://www.arabidopsis.org) databases. GO BLAST results suggested that ir-*dcl4* plants, compared to WT, may have been strongly impaired in various biological processes such as metabolic and developmental processes, responses to abiotic and biotic stimuli and stresses, nucleic acid and protein metabolism (Supporting Figure S9).

Several stressrelated genes were altered in ir-*dcl3* and ir-*dcl4* plants (**Table 1**). Mitogen activated protein kinases (MAP kinases) play an important role in the early steps of the signal transduction during herbivore attack and modulate transcription factors of the JA-mediated defense pathway (Wu et al. 2007; Meldau et al. 2009). Members of MAP kinase kinase (MAPKK) and MAP kinase kinase kinase (*MAPKKK*)-like genes of the MAPK signaling cascades were significantly down-regulated in the ir-*dcl4* line, whereas MAPKK3-like transcripts were significantly down-regulated in the ir-*dcl3* line (**Table 1**). Another protein of the MAPK signaling cascades, the MAPK phosphatase, involved in phosphorylation processes of defense-related MAPKs in *Arabidopsis* and tobacco plants (Ulm et al. 2002; Yamakawa et al. 2004), was significantly down regulated in ir-*dcl3* plants. Other stress-related transcripts,

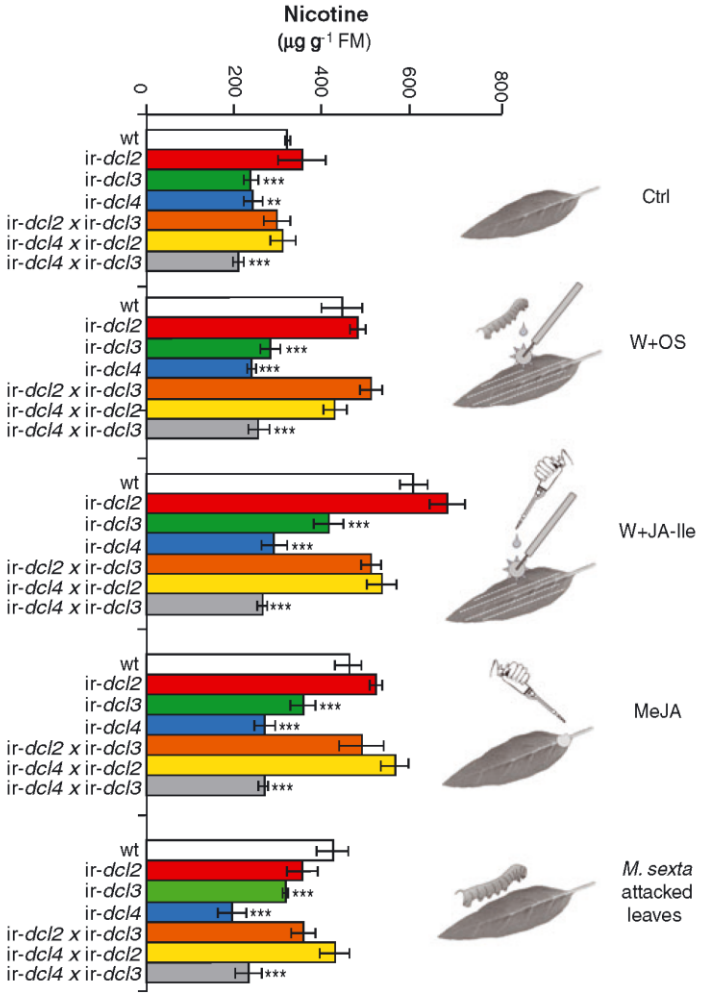

Figure 6. Nicotine levels of *NaDCLs* plants differed from WT plants after different treatments.

Double silencing *NaDCL2* either with *NaDCL3* or *4* restored nicotine WT levels 72 h after OS, wounding and JA-Ile elicitations and MeJA treatment and after 11 days of continuous *M. sexta* attack. Mean (±*SE*) of six replicates per treatment. Asterisks indicate significant differences between lines and WT (***P* <0.01; ****P* <0.001) in Fisher's PLSD test following an ANOVA.

12-oxophytodienoate reductase 1 and 2 like transcript, that are rapidly activated during wounding (Biesgen and Weiler 1999) were significantly down-regulated in ir-*dcl3* plants (**Table 1**). Phospholipase 2a, which is involved in oxylipin biosynthesis and is rapidly activated during pathogen and viral attack in *Arabidopsis* and tobacco plants (Dhondt et al. 2000; La Camera et al. 2009), was also significantly down-regulated in ir-*dcl3* plants (**Table 1**). On the other hand, *jasmonate-resistance 1 like* (*JAR1*) transcripts was up-regulated in ir-*dcl3* plants: JAR1 protein catalyzes the conjugation of JA with Ile to form JA-Ile that binds to the F-box protein coronatine insensitive 1 (COI1), to activate jasmonate signaling. These may be responsible for the elevated JA-Ile levels observed in the ir-*dcl3* line after OS-elicitation.

Discussion

Attack from herbivorous insects rapidly activates transcriptional reprogramming in plants (Kessler and Baldwin 2004; Voelckel and Baldwin 2004). These transcriptional changes need to be controlled and adjusted to fine tune defense responses. In our previous study, we observed large-scale changes in smRNA population of *N. attenuata* plants when they were subjected

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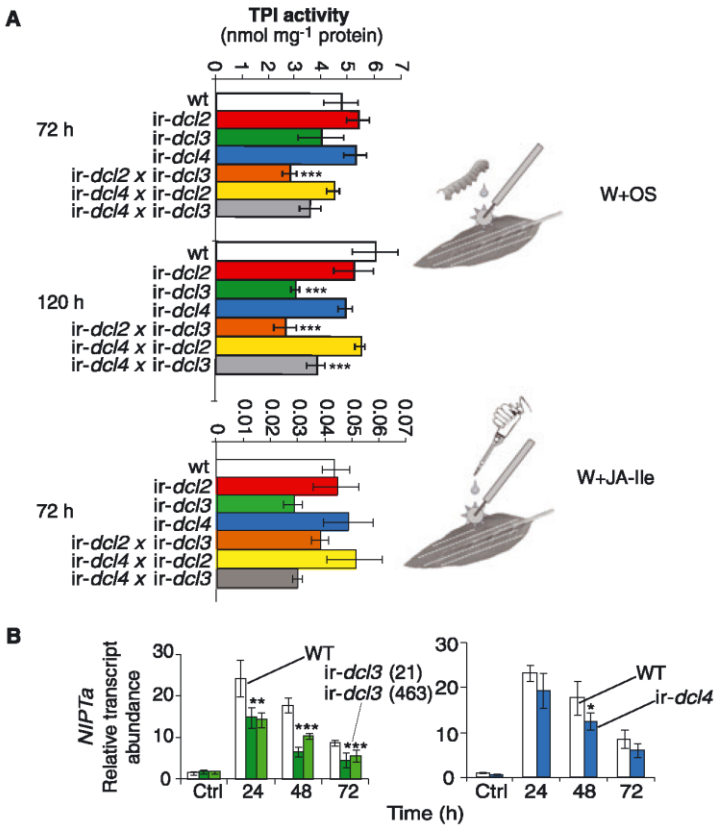

Figure 7. TPI activity is specifically regulated in ir-*dcl3* compared to WT and other ir-*dcl* plants after OS elicitation.

(A) TPI activity in WT and ir-*dcl*s rosette leaves after 72 h and 120 h of OS elicitation.
(B) *NaTPI* transcript abundance in ir-*dcl3* and ir-*dcl4* after OS elicitation. Mean (±*SE*) of six replicates per treatment. Asterisks indicate significant differences between lines and WT (**P* <0.05; ***P* <0.01; ****P* <0.001) in Fisher's PLSD test following an ANOVA.

to simulated-herbivory (Pandey et al. 2008b). In this study, we isolated four *DCL* genes from the ecological model plant *N. attenuata* and stably silenced the expression of three individual *DCL*s (*NaDCL2-4)* to examine their relevance to plant-insect interactions. Our results suggest that DCL3 and 4 are the main DCLs that orchestrate plant defense responses and that the modules of the defense cascade regulated by individual DCLs are largely non-overlapping yet synergistic.

Due to unavailability of knock-out mutants in the *N. attenuata* system, our interpretations of DCL function rely on loss-of-function analyses using gene silencing in genetically manipulated plants expressing inverted-repeat constructs, in which residual DCL function cannot be ruled out. Despite this caveat, these lines displayed clear phenotypes of *dcl*-knockdown plants and did not constitutively express components of the plant's complicated defense system. Silencing *NaDCL3* and *NaDCL4* increased the susceptibility of *N. attenuata* to *M. sexta* herbivory while silencing *NaDCL2* did not have any detectable influence (**Figure 4**). Moreover, the molecular response signature of ir-*dcl3* and ir-*dcl4* genotypes only partially overlapped. This suggests different functions of individual *DCL*s in mediating responses to herbivory. Complex interactions among NaDCL2, NaDCL3 and NaDCL4 regulate different components of the plant's direct defenses by differentially regulating particular events in the signal transduction pathways during herbivory.

It may be argued that we are trying to silence genes which may themselves play a role in silencing strategy. Certainly, such strategy may prevent 100% knockdown of DCL mRNAs; still we expect little effects on the overall qualitative outcome of this study. Nevertheless it is worth noting that similar RNAi strategies have been previously used to silence *DCL1* and *AGO1* genes in *Arabidopsis* allotetraploid plants: a 23% reduction in *DCL1* level reported by Lackey et al. (2010)

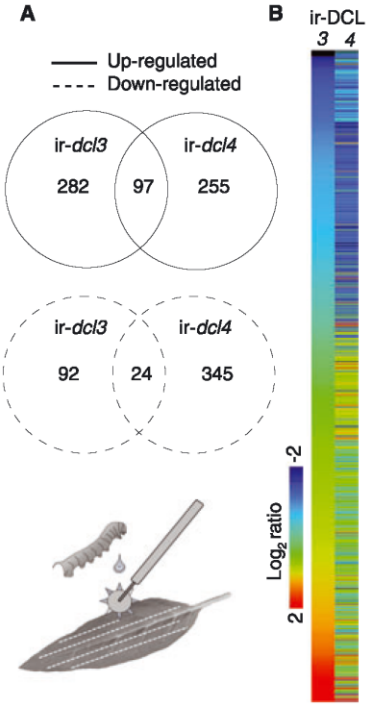

Figure 8. Microarray analysis of ir-*dcl3* and ir-*dcl4* lines compared to WT after OS-elicitation.

(A) Venn diagram summarizes up- and down-regulated transcripts which are specifically regulated in plants silenced in *NaDCL3* and *NaDCL4* expression.

(B) Heat map analysis displaying comparison of the logarithmic (log2) fold changes in expression between ir-*dcl3* and ir-*dcl4* lines compared to WT. Log_2-transformed fold changes in expression values of ir-*dcl4* were sorted from highest to lowest value. Transcript abundance ratios with two fold change ≥ 2 for up-regulated probes and ≤ 0.5 down-regulated probes (*t* test, *P* value < 0.05).

demonstrated that such low silencing efficiencies could still be used to study plant development. Our objective in this study is to characterize the DCL genes in this unique ecological model plant system and to further test which branch of the DCL-dependent RNAi pathway may be involved in herbivory.

The induced phytohormones and defensive secondary metabolites were differently regulated in ir-*dcl2*, ir-*dcl3*, and ir-*dcl4* plants suggesting that the all three *DCL*s could have potentially different roles. Oral secretions from insect herbivores activate multiple steps in the herbivore-induced pathways (McCloud and Baldwin, 1997; Kallenbach et al. 2010). In *N. attenuata,* herbivore resistance is activated by fatty acid conjugates (FAC) in the OS of larvae which elicit the JA-mediated defense pathways (Halitschke et al. 2001; Kallenbach et al. 2010). In the early steps of defense signaling, several components of the MAP signaling cascades act upstream of JA biosynthesis (Heinrich et al. 2011; Wu et al. 2007). Down-regulated MAPK signaling which was not observed in ir-*dcl3* plants may contribute to the attenuated JA responses in ir-*dcl4* plants (**Figure 5A**). Hence, MAPK signaling may be under the control of a NaDCL4-dependent smRNA pathway. Interestingly, silencing *NaDCL2* or *NaDCL3* separately does not affect JA accumulation, however silencing both *NaDCL2* and *NaDCL3* reduce OS-elicited JA levels (**Figure 5C**). This indicates that these two DCLs are involved in JA biosynthesis. In addition, silencing *NaDCL4* either together with *NaDCL2* or *NaDCL3* resulted in significantly reduced levels of JA (**Figure 5C**), what suggests that *NaDCL4* acts upstream of JA biosynthesis or signaling during herbivory and functions independently from *NaDCL2* or *NaDCL3*.

JA is conjugated to JA-Ile, which in turn, activates the biosynthesis of defense metabolites (Kang et al. 2006). High JA-Ile levels in ir-*dcl3* plants correlated with the up-regulation of *JAR1* transcript (**Table 1**), which code for proteins with adenylyltransferase activity required for the conjugation of JA with Ile to form JA-Ile (Staswick and Tiryaki 2004; Wang et al. 2008). However, the high JA-Ile levels did not correlate with the levels of the nicotine in ir-*dcl3* plants (**Figure 6**). Treatment with MeJA or JA-Ile did not restore nicotine levels in ir-*dcl3* and ir-*dcl4* plants and also not in double DCL-silenced plants compared to WT plants (**Figure 6**). It was shown that JA treatment could not recover WT defense levels in plants silenced in the expression of *NaCOI1* (*Coronatine Insensitive 1*), the receptor of jasmonate in JA signaling pathway in *N. attenuata* (Paschold et al. 2007).This suggests that NaDCL3 and 4 may interact with or affect JA signaling pathway at the level of a perception protein such as *NaCOI1*.

One of the most important direct defense metabolites in *N. attenuata* is nicotine, which is synthesized in roots and transported to leaves in response to herbivore attack and functions as a neurotoxin in all organisms (Steppuhn et al. 2004). Nicotine levels were significantly lower in ir-*dcl3* and ir-*dcl4* plants and their cross compared to WT (**Figure 6**). This indicates that *NaDCL3* and *NaDCL4* may control nicotine biosynthesis or nicotine transport from root to shoot. In contrast, WT nicotine levels were observed in ir-*dcl2* plants (**Figure 6**). However, silencing *NaDCL2* in combination with either *NaDCL3* or *NaDCL4* restored WT nicotine level (**Figure 6**). *NaDCL2* may negatively regulate nicotine accumulation by affecting nicotine signaling, transport or biosynthesis. This suggests a complex interplay of *NaDCL2* with *NaDCL3* and *NaDCL4* in regulating the many steps involved in nicotine accumulation (**Figure 6**). An analysis of root phenotypes demonstrated that ir-*dcl4* and ir-*dcl4* x ir-*dcl3* plants had fewer lateral roots and less mass compared to ir-*dcl3,* ir-*dcl2 x* ir-*dcl3,* ir-*dcl4 x* ir-*dcl3* and WT plants (**Figure 3D**). Due to their impaired root systems, ir-*dcl4* and ir-*dcl4* x ir-*dcl3* plants tended to tip over during the early

Table 1. Fold change in the expression of defense-related genes in OS-elicited ir-*dcl3* and ir-*dcl4* lines compared to WT plants

Probe ID	Protein	Biological process	Fold change		GenBank number
			ir-*dcl3*	ir-*dcl4*	
Na_454_43260	Ethylene-insensitive 3	ethylene signaling	−1.29	**−2.08**	AAR08677
Na_454_26824	Long chain acyl-CoA synthetase like	fatty acid biosynthesis	**−2.00**	**−3.82**	AT1G77590
Na_454_11600	MAPKKK-like	protein phosphorylation	−1.88	**−2.70**	AT3G58640
Na_454_30081	MKK3-like	JA and ethylene signaling	**−2.43**	−1.29	AT5G40440
Na_454_28706	MAP Phsophatase 1	response to UV-C, response to salt stress	**−2.25**	**−1.67**	AT3G55270
Na_454_34570	Major facilitator superfamily protein	response to JA and wounding	**−2.13**	**−2.97**	AT2G26690
Na_454_36521	Jasmonate resistance 1 like	JA signaling and metabolism	**3.07**	1.05	AT2G46370
Na_454_00163	Chalcone synthase	flavonoid biosynthesis, response to JA and wounding	**2.04**	**2.28**	AT5G13930
Na_454_03027	Terpene synthase 03	sesquiterpenoid biosynthesis, response to insect and wounding,	**2.02**	**2.63**	AT3G25830
Na_454_24100	Inositol 1,3,4-trisphosphate 5/6-kinase family protein	response to wounding	**−2.17**	−1.28	AT4G08170
Na_454_40011	12-oxophytodienoate reductase 1 and 2	response to wounding, JA biosynthesis	**−3.15**	1.05	AT1G76680
Na_454_27057	Nicotine N-demethylase	nicotine metabolism	1.28	**2.00**	ADP65809
Na_454_39179	Phospholipase a 2a, patatin like protein 2	oxylipin biosynthetic process	**−2.68**	−1.21	AT2G26560
Na_454_21104	Myb-DNA binding protein	gene silencing	1.10	**−3.20**	AT2G03500
Na_454_22274	Myb-like DNA binding protein	regulation of transcription	**−2.30**	1.32	AAU90342

Three replicates from WT, ir-*dcl3* and ir-*dcl4* were used to perform oligo microarray. "-" indicates down-regulated transcripts. Numbers in bold indicate significant differences in gene expression compared to WT ($P < 0.05$).

stages of flowering growth. A similar phenotype has been observed in *dcl4* mutants in *Arabidopsis* (Marin et al. 2010). It has been shown that lateral root development in *Arabidopsis* is inhibited by DCL4-dependent tasi-RNA3 (Marin et al. 2010). Since nicotine biosynthesis is produced in growing root tips, the low levels of nicotine in DCL4-silenced plants are likely due to impaired root growth. In contrast, ir-*dcl3* plants had normally developed root systems with significantly lower nicotine levels after OS-elicitation. This suggests that DCL3 regulates nicotine production independently of root growth.

TPI is an important defense protein that directly acts in the insect midgut (Steppuhn and Baldwin 2007; Zavala et al. 2008). We found that among the ir-*dcl* genotypes, only ir-*dcl3* showed reduced TPI activity after OS-elictation (**Figure 7A**). Expression of *NaTPI* was significantly reduced in ir-*dcl3* plants, and silencing *NaDCL3* together with *NaDCL2* or *NaDCL4* also reduced TPI levels after OS-elicitation similarly to that observed in ir-*dcl3* plants. This suggests that only *NaDCL3*, but not *NaDCL2* and *NaDCL4,* is involved in the OS-elicited transcriptional regulation of TPI (**Figure 7B**). However, higher JA-Ile did not correlate with the levels of TPIs in ir-*dcl3* plants or plants co-silenced in *NaDCL3* and either *NaDCL2* or *NaDCL4*. Treatment of*JAR*-silenced plants with JA-Ile restored TPI activity in *N. attenuata* (Kang et al. 2006). However, treatment with JA-Ile did not restore TPI acitivity (**Figure 7**), demonstrating a phenotype similar to ir-*coi1* plants (Paschold et al. 2007). This suggests that NaDCL3 may be involved in the regulation of the *NaCOI1* receptor complex or of downstream genes of the NaCOI1-mediated defense pathway.

Transcript abundance of all *NaDCL*s varied by tissue type (**Figure 2A**). The accumulation of *NaDCL4* transcripts in stem tissues may relate to its role in the systemic distribution of smRNAs as has been shown in *Arabidopsis* (Dunoyer et al. 2005; Kehr and Buhtz 2008; Dunoyer et al. 2010a, 2010b). *NaDCL3* and *NaDCL4* transcripts appeared to be diurnally regulated and responded to OS-elicitation in rosette leaves (**Figure 2B**). In addition, the homologous *PHYB* transcript of *N. attenuata* was significantly down-regulated in both ir-*dcl3* and ir-*dcl4* lines (Table S1). Light receptors entrain endogenous diurnal rhythms in plants and also control the plant's sensitivity to jasmonates during herbivore attack in *Arabidopsis* (Chen and Ni 2006). Interestingly, herbivores perform better on plants exposed to far-red light, and total phenolics are lower in *phyb* mutants than in wild type plants (Moreno et al. 2009).

A similar chain of events may contribute to the decreased levels of the defensive phenylpropanoid-polyamine conjugates, caffeoylputrescine and dicaffeoyl spermidine, observed in the ir-*dcl3* and ir-*dcl4* plants (Supporting Figure S7).

In conclusion, we found that NaDCL2, NaDCL3 and NaDCL4 are involved in the regulation of a number of different genes, signaling and defense metabolites in response to herbivory (as summarized in Supporting Figure S10). Furthermore, our current study provides us with the needed genetic tools to better understand the role of smRNAs in plant-herbivore interactions. The next step would be to decipher the identity and regulation of DCL2, DCL3 and DCL4 dependent smRNAs and smRNA-target relationships during plant-insect interactions. Completion of the on-going genome-sequencing project of *N. attenuata* will make this task feasible.

Materials and Methods

Isolation and characterization of *DICER-LIKE* cDNAs from *Nicotiana attenuata*

A PCR-based approach with cDNA from *Nicotiana attenuata* (Torrey ex. Watson) was used for sequencing the coding regions of the DCLs. Ortholog DCL mRNA sequences of *N. benthamiana*: *NbDCL1* (239 nt), *NbDCL2* (675 nt), *NbDCL3* (297 nt), *NbDCL4* (356 nt) were used to design primers for 3' and 5' RACE cloning (Table S2). Next, total RNA was extracted from *N. attenuata* rosette leaf following the TRIZOL method as recommended by the manufacturer (Invitrogen; http://www.invitrogen.com) and reverse-transcribed to first-strand cDNA following manufacturer's protocol (SuperScript, Invitrogen). Hot start PCR reactions were performed to provide sufficient sequence for 3'/5' RACE cloning (3'/5' RACE cloning, 2nd Generation Kit, Roche). PCR products were separated on a 1.5% agarose gel; single bands were gel-purified using the Nucleo Spin Extract II Kit (Macherey-Nagel, http://www.mn-net.com) following the manufacturer's instructions. Sequence reactions were prepared and purified using the Big Dye Purification Kit (DyeEx 2.0 Spin KIT, Qiagen http://www.qiagen.com) for sequencing. Sequences were aligned using SeqMan Software (DNA STAR Lasergene 8, http://www.dnastar.com). Partially sequenced *DCL* cDNA fragments were blasted against non-solanaceous *DCL* ortholog mRNA sequences. Degenerate primers were designed and gaps between contigs were closed by sequencing PCR-products. *DCL* sequences from other species were aligned using MegAlign (DNA STAR Lasergene 8). Using the *NaDCL* contig sequences, BLASTX analyses were performed to determine NaDCL amino acid identities to other plant DCLs orthologs. A neighbor-joining tree was built using MEGA4 with groups' evaluation using 1 000 bootstrap replicates (Molecular Evolutionary Genetic Analysis; Tamura et al. 2007).

Generation and characterization of transgenic lines

Partial cDNA fragments of *NaDCL2*, *NaDCL3* and *NaDCL4* were inserted into pSOL8 (Gase et al. 2011) or pRESC5 binary transformation vectors as inverted-repeats (Bubner et al. 2006). Wild-type *N. attenuata* plants from the twenty-third inbred generation were used for *Agrobacterium*-mediated plant transformation as described by Krügel et al. (2002). Seed germination and plant growth were conducted as described by Krügel et al. (2002). Screening procedure of transgenic lines was done as described by Gase et al. (2011). Briefly, complete single T-DNA insertions in transgenic lines were determined by diagnostic PCR using primers which were designed from the flanking regions of the IR-cassettes and plasmid DNA. Lines with complete T-DNA insertions were chosen and screened for homozygosity through the T_2 generation. Transgenic seeds were germinated on agar plates containing 35 mg L^{-1} hygromycin B. Ten seeds of each transgenic T_1 line were randomly selected and inbred through the T_2 generation to obtain homozygous lines. Southern blot hybridization was performed to determine lines harboring a single insertion using a ^{32}P-labeled fragment of the *hptII* gene as probe. Homozygous T_2 plants harboring single insertions were used for further characterization. Plants were grown in a glasshouse under 16/8 h (26–28°C) supplemental light from Master Suns-T PIA Agro 400-W sodium light.

Quantitative real-time PCR

TRIZOL method was used to extract total RNA from leaf material ad reverse-transcribed to first-strand cDNA with SuperScript II following manufacturer protocol (Invitrogen). 20 ng of cDNA were used to for quantitative analysis with SYBR Green I assay (Eurogentec) following manufacturer protocol. Five biological replicates were pooled and triplicates were used for qPCR analysis. A house-keeping gene transcript, *ACTIN* was used as an endogenous reference. The $2^{-\Delta\Delta CT}$ method was used for transcript evaluation (Bubner et al. 2004). Primer pairs used in this study are listed in Table S2.

Herbivore performance assays

Eggs of *Manduca sexta* were obtained from North Carolina State University (Raleigh, NC) and kept in a growth chamber at 26°C under 16/8 h (light/dark) photoperiod until larvae hatched. Neonates were placed on *N. attenuata* rosette-staged plants under glasshouse conditions. Twenty five to thirty biological replicates were used for the assay. Larval mass measurements were performed after 6, 9, and 11 days.

Phytohormone extraction and analysis

Induced and uninduced rosette leaves from WT and transgenic plants were used for JA, JA-Ile, SA and ABA phytohormone

extraction following Wang et al. (2007). About 150–200 mg of lamina tissue were placed in 2 mL Eppendorf tube containing 900 mg FastPrep Matrix (BIO 101) and 1 mL of ethyl acetate spiked with 200 ng of D_2-JA, 40 ng of D_4-SA, 40 ng of D_6 ABA, and 40 ng of $^{13}C_6$-JA-Ile (as internal standards) were homogenized by reciprocal shaking in GenoGrinder (http://www.spexcsp.com) for 1.5 min at 250 strokes min^{-1}. After samples were centrifuged at 16 000 *g* for 20 min at 4 °C, the supernatant was transferred into new 2 mL tubes. Pellets were re-extracted by adding 500 μL ethyl acetate without internal standards. Subsequently, the supernatants were combined and evaporated at 30°C in a vacuum concentrator (Eppendorf) until dryness and the remaining dried pellet was dissolved in 500 μL of 70% methanol (v/v). Samples were vortexed for 5 min and centrifuged at 16 000 g for 10 min. 15 μL of the supernatant were analyzed on Varian 1200L Triple-Quadrapol-MS with a ProntoSIL column (C18; 5 μm, 50 × 2 mm).

Secondary metabolite analysis

The secondary metabolites analysis was performed by using high-performance liquid chromatography (HPLC) as described earlier (Keinanen et al. 2001). Approx. 100 mg of leaf material were aliquot into 2 mL safe-lock tube containing 900 mg FastPrep matrix (Sili GmbH) with 1 mL of extraction solvent (40% MeOH (v/v) prepared with 0.5% acetic acid in deonized water (v/v), pH 4.8). Leaf material was homogenized with GenoGrinder (http://www.spexcsp.com) at 250 strokes per min for 30 sec. The supernatant was transferred into a new tube and centrifuged at 16 000 g for 12 min at + 4 °C, transferred into a glass vial and analyzed with Agilent-HPLC 1100 series (http://www.chem.agilent.com). Serial dilution of external nicotine, rutin and chlorogenic acid were used to quantify metabolites. Caffeoylputrescine and dicaffeoyl spermidine were quantified by using chlorogenic acid calibration curve.

Proteinase inhibitor extraction and determination

Trypsin proteinase inhibitor assay was carried out as previously described (Jongsma et al. 1993; Zavala et al. 2008). Briefly, leaf samples were harvested 1, 2 or 3 days after OS-elicitation. For total protein extraction, leaf material was frozen in liquid nitrogen and ground in 1.5 mL Eppendorf tubes containing FastPrep Matrix (Sili GmbH), after which 300 μl extraction buffer (0.1M Tris-Cl, 5% PVPP, 0.2% phenylthiourea, 0.5% diethyldithiocarbonate, 1.86% disodium EDTA (w/v), pH7.6) was added and vortexed. Samples were centrifuged at 13 400 g for 20 min at 4 °C. 100 μL of supernatant was transferred to new tubes. For protein determination, serial dilutions of immunoglobulin G (Sigma-Aldrich, http://www.sigmaaldrich.com) and triplicates of each sample were loaded into a 96 well plate and measured in a TECAN ELISA Reader (http://www.tecan.de). A solution of 1.8% agar (0.1 M Tris Buffer (pH 7.5) containing trypsin (Sigma-Aldrich) was poured into a 25×25 cm square Petri dish). Holes with 4 mm diameter were punched out with a vacuum pump. Samples were loaded into these holes and kept at +4°C for 16 h. Five serial dilutions of soybean trypsin proteinase inhibitor (Sigma-Aldrich) were used as external standard for quantification.

Microarray experiment

Total RNA was extracted as described in Kistner and Matamoros (2005) from W+OS treated rosette leaves of WT, ir-*dcl3* and ir-*dcl4* plants 1 h post-elicitation. Three biological replicates were used for each genotype. Agilent's Low Input Quick Amp Labeling Kit (Agilent Technologies) was used for RNA labeling, cleaning, cDNA/cRNA and hybridization to an *N. attenuata* transcriptome specific microarray (GEO GPL13527) according to the manufacturer's instructions. Microarray data have been deposited in the Gene Expression Omnibus (GEO) database as GEO Series Entry (NCBI GEO platform number GSE30124).

For data analyses, raw data normalization was performed with a 75 percentile scale and transformed to $\log_2$ scale. The expression values of the probes were analyzed using *t* test and *P*-values. Probes with fold changes ≥2 and *P* value ≤ 0.05 were selected for further analyses.

In order to annotate transcripts for GO terms, we used manual GO annotation using BLAST search for each transcript on AmiGO (http://www.geneontology.org) and TAIR (http://www.arabidopsis.org) databases. BLASTX and BLASTP searches for each gene were used to confirm best hits to plant orthologous genes manually. GO functional categorization was used according to *Arabidopsis* TAIR GO annotation.

Statistical analysis

Data were analyzed with the StatView Software using the ANOVA algorithm (SAS Institute Inc., Cary, NC, USA).

GenBank accession numbers

Gene bank accession numbers of genes and proteins used in this study (mRNA) sequences: JN032013 (*NaDCL1*), JN032014 (*NaDCL2*), JN032015 (*NaDCL3*), JN032016 (*NaDCL4*), FM986780 (*NbDCL1*), FM986781 (*NbDCL2*), FM986782 (*NbDCL3*), FM986783 (*NbDCL4*), NM_099986 (*AtDCL1*), NM_111200 (*AtDCL2*), NM_001161190 (*AtDCL3*), NM_122039 (*AtDCL4*), XM_002302643 (*PtDCL902*), XM_002315083 (*PtDCL901*), XM_002324168 (*PtDCL904*), XM_002308348 (*PtDCL905*), XM_002515051 (*RcDCL1*), XM_002514764 (*RcDCL2*), XM_002516114 (*RcDCL3*), XM_002523486 (*RcDCL4*).

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References

Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouche N, Gasciolli V, Vaucheret H (2006) DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Curr. Biol.* **16,** 927–932.

Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–297.

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **92,** 8675–8679.

Biesgen C, Weiler EW (1999) Structure and regulation of OPR1 and OPR2, two closely related genes encoding 12-oxophytodienoic acid-10,11-reductases from *Arabidopsis thaliana*. *Planta* **208,** 155–165.

Bouche N, Lauressergues D, Gasciolli V, Vaucheret H (2006) An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J.* **25,** 3347–3356.

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

Chen M, Ni M (2006) RED AND FAR-RED INSENSITIVE 2, a RING-domain zinc finger protein, mediates phytochrome-controlled seedling deetiolation responses. *Plant Physiol.* **140,** 457–465.

Chen X (2009) Small RNAs and their roles in plant development. *Annu. Rev. Cell Dev. Biol.* **25,** 21–44.

Dhondt S, Geoffroy P, Stelmach BA, Legrand M, Heitz T (2000) Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. *Plant J.* **23,** 431–440.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiol.* **150,** 1576–1586.

Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010a) An endogenous, systemic RNAi pathway in plants. *EMBO J.* **29,** 1699–1712.

Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat. Genet.* **37,** 1356–1360.

Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O (2010b) Small RNA duplexes function as mobile silencing signals between plant cells. *Science* **328,** 912–916.

Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Brempelis KJ, Carrington JC (2010) *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during Turnip Mosaic Virus infection. *Plant Cell* **22,** 481–496.

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr. Biol.* **15,** 1494–1500.

Gase K, Weinhold A, Bozorov T, Schuck S, Baldwin IT (2011) Efficient screening of transgenic plant lines for ecological research. *Mol. Ecol. Resour*. **11,** 890–902.

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 proteome. *Plant Physiol.* **142,** 1621–1641.

Heinrich M, Baldwin IT, Wu J (2011) Three MAPK kinases, MEK1, SIPKK and NPK2, are not involved in activation of SIPK after wounding and herbivore feeding but important for accumulation of trypsin proteinase inhibitors. *Plant Mol. Rep.* DOI: 10.1007/s11105-011-0388-0.

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

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

Hiraguri A, Itoh R, Kondo N, Nomura Y, Aizawa D, Murai Y, Koiwa H, Seki M, Shinozaki K, Fukuhara T (2005) Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in *Arabidopsis thaliana*. *Plant Mol. Biol.* **57,** 173–188.

Jongsma MA, Bakker PL, Stiekema WJ (1993) Quantitative determination of serine proteinase inhibitor activity using a radial diffusion assay. *Anal. Biochem.* **212,** 79–84.

Kallenbach M, Alagna F, Baldwin IT, Bonaventure G (2010) *Nicotiana attenuata* SIPK, WIPK, NPR1, and fatty acid-amino acid conjugates participate in the induction of jasmonic acid biosynthesis by affecting early enzymatic steps in the pathway. *Plant Physiol.* **152,** 96–106.

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.

Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis I (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiol.* **152,** 1731–1747.

Kehr J, Buhtz A (2008) Long distance transport and movement of RNA through the phloem. *J. Exp. Bot.* **59,** 85–92.

Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. *J. Agric. Food Chem.* **49,** 3553–3558.

Kessler A, Baldwin IT (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 J.* **38,** 639–649.

Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W (2010) Transcriptional control of gene expression by microRNAs. *Cell* **140,** 111–122.

Kistner C, Matamoros M (2005) RNA isolation using phase extraction and LiCl precipitation. In: AJ Marquez, ed. *Lotus japonicus Handbook*. Springer Verlag, Dordrecht, The Netherlands, pp. 123–124.

Krügel 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.

La Camera S, Balague C, Gobel C, Geoffroy P, Legrand M, Feussner I, Roby D, Heitz T (2009) The *Arabidopsis* patatin-like protein 2 (PLP2) plays an essential role in cell death execution and differentially affects biosynthesis of oxylipins and resistance to pathogens. *Mol. Plant Microbe. Interact.* **22,** 469–481.

Lackey E, Ng DW, Chen ZJ (2010) RNAi-mediated down-regulation of DCL1 and AGO1 induces developmental changes in resynthesized *Arabidopsis* allotetraploids. *New Phytol.* **186,** 207–215.

Liu Q, Feng Y, Zhu Z (2009a) Dicer-like (DCL) proteins in plants. *Funct. Integr. Genomics* **9,** 277–286.

Liu Y, Gao Q, Wu B, Ai T, Guo X (2009b) NgRDR1, an RNA-dependent RNA polymerase isolated from *Nicotiana glutinosa*, was involved in biotic and abiotic stresses. *Plant Physiol. Biochem.* **47,** 359–368.

Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A (2010) miR390, *Arabidopsis* TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *Plant Cell* **22,** 1104–1117.

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.

Meldau S, Wu J, Baldwin IT (2009) Silencing two herbivory-activated MAP kinases, SIPK and WIPK, does not increase *Nicotiana attenuata*'s susceptibility to herbivores in the glasshouse and in nature. *New Phytol.* **181,** 161–173.

Moreno JE, Tao Y, Chory J, Ballare CL (2009) Ecological modulation of plant defense via phytochrome control of jasmonate sensitivity. *Proc. Natl. Acad. Sci. USA* **106,** 4935–4940.

Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* **312,** 436–439.

Nodine MD, Bartel DP (2010) MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis. *Genes Dev.* **24,** 2678–2692.

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, Gaquerel E, Gase K, Baldwin IT (2008a) RNA-directed RNA polymerase3 from *Nicotiana attenuata* is required for competitive growth in natural environments. *Plant Physiol.* **147,** 1212–1224.

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

Papp I, Mette MF, Aufsatz W, Daxinger L, Schauer SE, Ray A, van der Winden J, Matzke M, Matzke AJ (2003) Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol.* **132,** 1382–1390.

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. *Plant J.* **31,** 1–12.

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *Plant J.* **51,** 79–91.

Phillips JR, Dalmay T, Bartels D (2007) The role of small RNAs in abiotic stress. *FEBS Lett.* **581,** 3592–3597.

Qu F, Ye X, Morris TJ (2008) *Arabidopsis* DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc. Natl. Acad. Sci. USA* **105,** 14732–14737.

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev.* **20,** 3407–3425.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. *Genes Dev.* **16,** 1616–1626.

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. *Annu. Rev. Plant Biol.* **60,** 485–510.

Schwachtje J, Baldwin IT (2008) Why does herbivore attack reconfigure primary metabolism? *Plant Physiol.* **146,** 845–851.

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. *Plant Cell* **16,** 2117–2127.

Steppuhn A, Baldwin IT (2007) Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. *Ecol. Lett.* **10,** 499–511.

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. *PLoS Biol.* **2,** E217.

Sunkar R, Zhu J (2007) Micro RNAs and short-interfering RNAs in plants. *J. Integr. Plant Biol.* **49,** 817–826.

Ulm R, Ichimura K, Mizoguchi T, Peck SC, Zhu T, Wang X, Shinozaki K, Paszkowski J (2002) Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1. *EMBO J.* **21,** 6483–6493.

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev.* **20,** 759–771.

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

Wang L, Allmann S, Wu J, Baldwin IT (2008) Comparisons of LIPOXYGENASE3- and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. *Plant Physiol.* **146,** 904–915.

Wang L, Halitschke R, Kang JH, Berg A, Harnisch F, Baldwin IT (2007) Independently silencing two JAR family members impairs levels of trypsin proteinase inhibitors but not nicotine. *Planta* **226,** 159–167.

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 Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **102,** 12984–12989.

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2,** E104.

Yamakawa H, Katou S, Seo S, Mitsuhara I, Kamada H, Ohashi Y (2004) Plant MAPK phosphatase interacts with calmodulins. *J. Biol. Chem.* **279,** 928–936.

Zavala JA, Giri AP, Jongsma MA, Baldwin IT (2008) Digestive duet: Midgut digestive proteinases of *Manduca sexta* ingesting *Nicotiana attenuata* with manipulated trypsin proteinase inhibitor expression. *PLoS One* **3,** e2008.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Protein alignment of NaDCLs with other plant DCL proteins. Regions in black shading indicate similarities of NaDCLs to other plant DCL homologs. (A) NaDCL1, (B) NaDCL2, (C) NaDCL3 and (D) NaDCL4.

Figure S2. Physical map of RNAi plant transformation vectors for *NaDCL2*, *3* and *4* and gene silencing for two independently transformed ir-*dcl*s. (A) Physical map of pSOL8 and pRESC5 RNAi vectors for *NaDCL2*, *3* and *4*. (B) Southern blot analysis confirmed single T-DNA insertions of RNAi vectors. For Southern blotting, 10 μg of genomic DNA of three independently transformed ir-*dcl2*, ir-*dcl3* and ir-*dcl4* lines was digested with *Eco*RI, *Xba*I or *Xho*I restriction enzymes as indicated. A fragment of the *hptII* (hygromycinphosphotransferase) gene was used as probe. (C) Silencing efficiency of *NaDCL2, 3* and *4* in ir-*dcl* crossed plants. Asterisks indicate significant differences ($^{***}P < 0.001$) in Fisher's PLSD test following an ANOVA.

Figure S3. Partial sequences of the three *N. attenuata* DCLs, used for RNAi vector constructs.

Figure S4. Salicylic acid (SA) and abscisic acid (ABA) levels were not significantly changed in *NaDCL*-silenced lines compared to WT after OS elicitation. Rosette leaves (+1) were wounded with a fabric pattern wheel and applied 20 μL of OS. Means (±*SE*) of six replicates per treatment.

Figure S5. Phytohormone levels (JA, JA-Ile, SA and ABA) do not differ from WT level after wounding in *ir-dcl3* silenced plants. (A), JA, (B), JA-Ile. (C), SA and (D), ABA phytohormones. (+1) node rosette leaves were wounded and 20 μL of deonized water was applied. Leaf material was harvested at the different time points (n = 6) after wounding as indicated. Means (±*SE*) represent phytohormone levels measured at the different times after treatment.

Figure S6. Phytohormone levels (JA, JA-Ile, SA and ABA) do not differ from WT levels after wounding in ir-*dcl4* silenced plants. (A), JA. (B), JA-Ile. (C), SA and (D), ABA phytohormones.

Figure S7. Dicaffeoyl spermidine and caffeoylputrescine levels differed after OS elicitation in ir-*dcl* plants from WT plants. Ir-*dcl3* reduced dicaffeoyl spermidine and caffeoylputrescine level, whereas ir-*dcl4* increased dicaffeoyl spermidine and reduced caffeoylputrescine levels after OS and W+JA-Ile treatments. Mean (±*SE*) of six replicates per treatment. Asterisks indicate significant differences between lines and WT ($^{*}P < 0.05$; $^{***}P < 0.001$) in Fisher's PLSD test following an ANOVA.

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Figure S9. Gene ontology annotation of significantly altered transcripts from analyzed microarray data. (A) Clustering of genes in GO biological processes. (B) Clustering of genes in GO molecular function. A total of 861 differentially expressed genes were annotated and clustered according to their GO terms. Categorization is presented as a histogram showing GO category for both ir-*dcl3* and ir-*dcl4* genes including their

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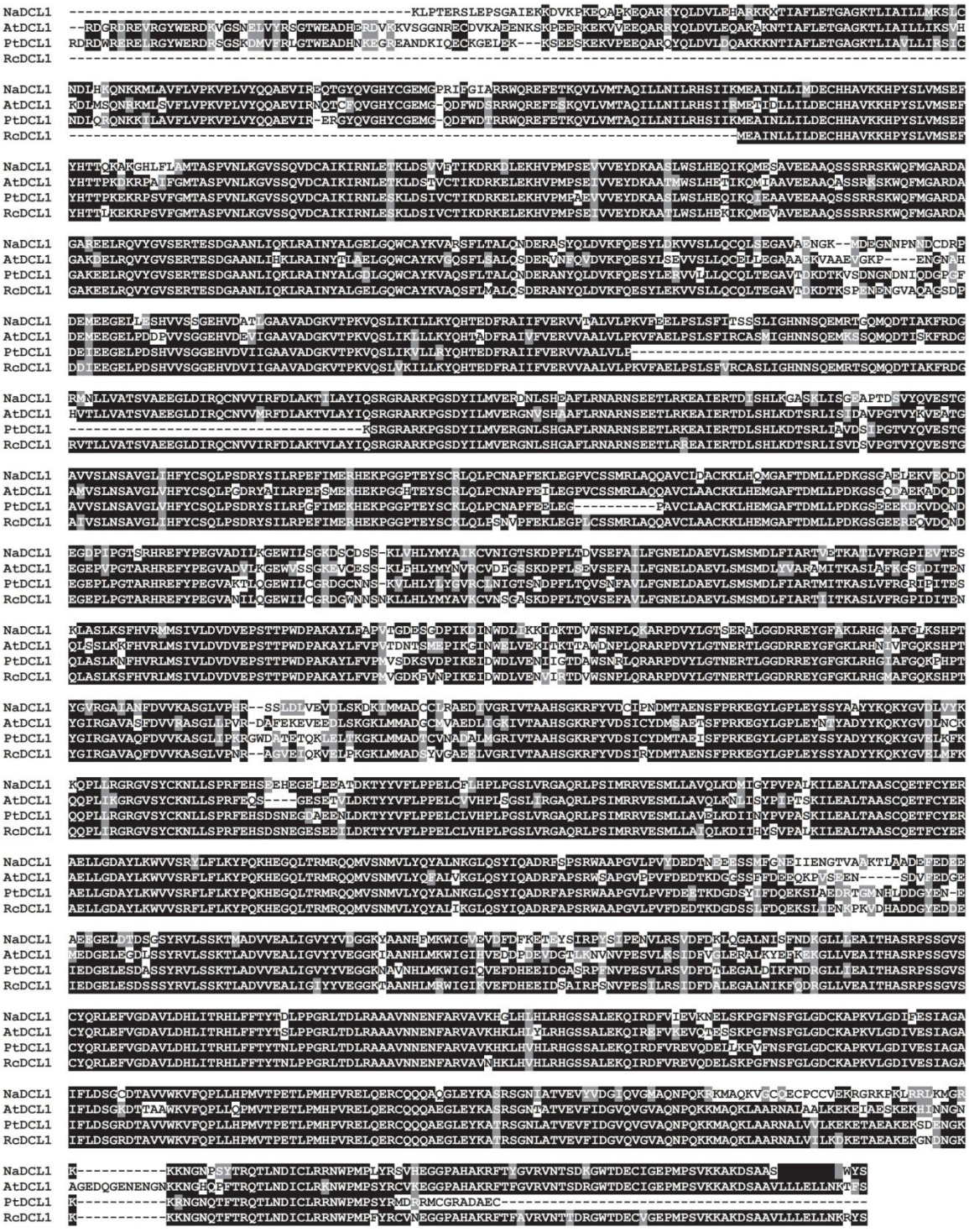
co-regulation. Transcript abundance ratios with fold changes $\geq$ 2 for up-regulated probes and $\leq$ 0.5 down-regulated probes (*t* test, *P* value $<$ 0.05).

Table S1. Microarray data analysis of significantly altered genes in ir-*dcl3* and ir-*dcl4* lines compared to WT.

Table S2. List of primers used for the study

A



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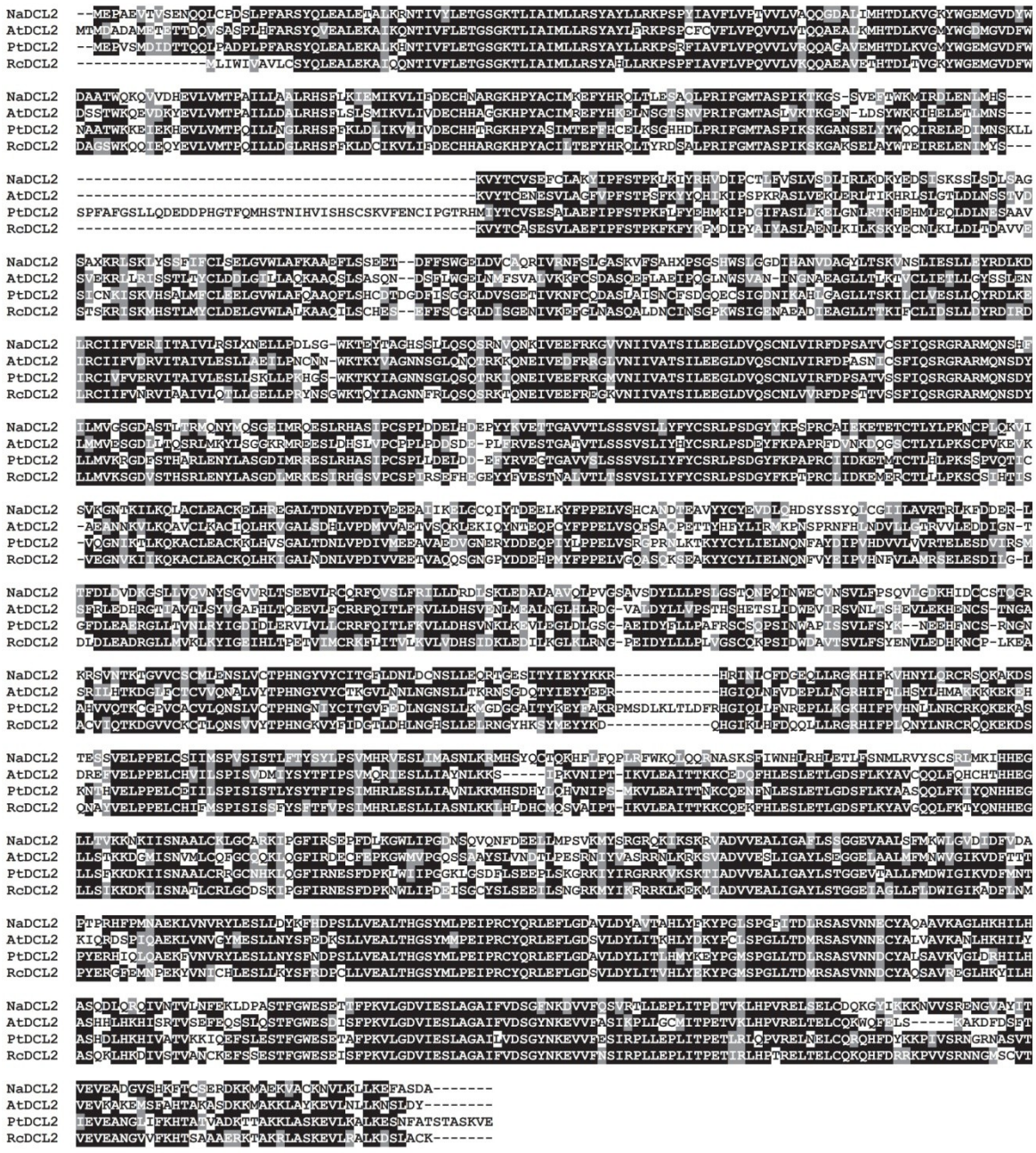

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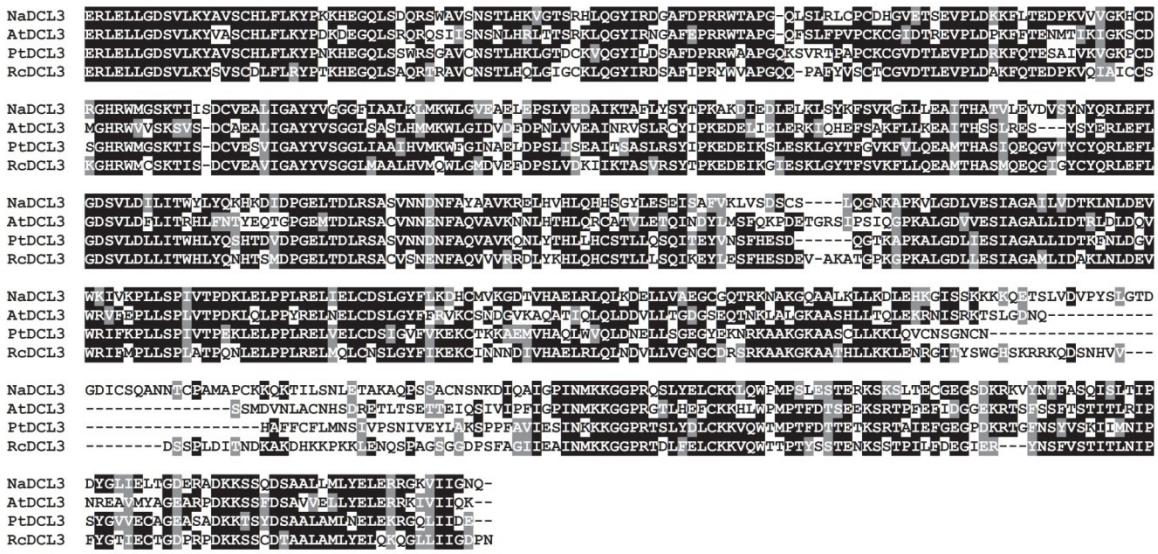

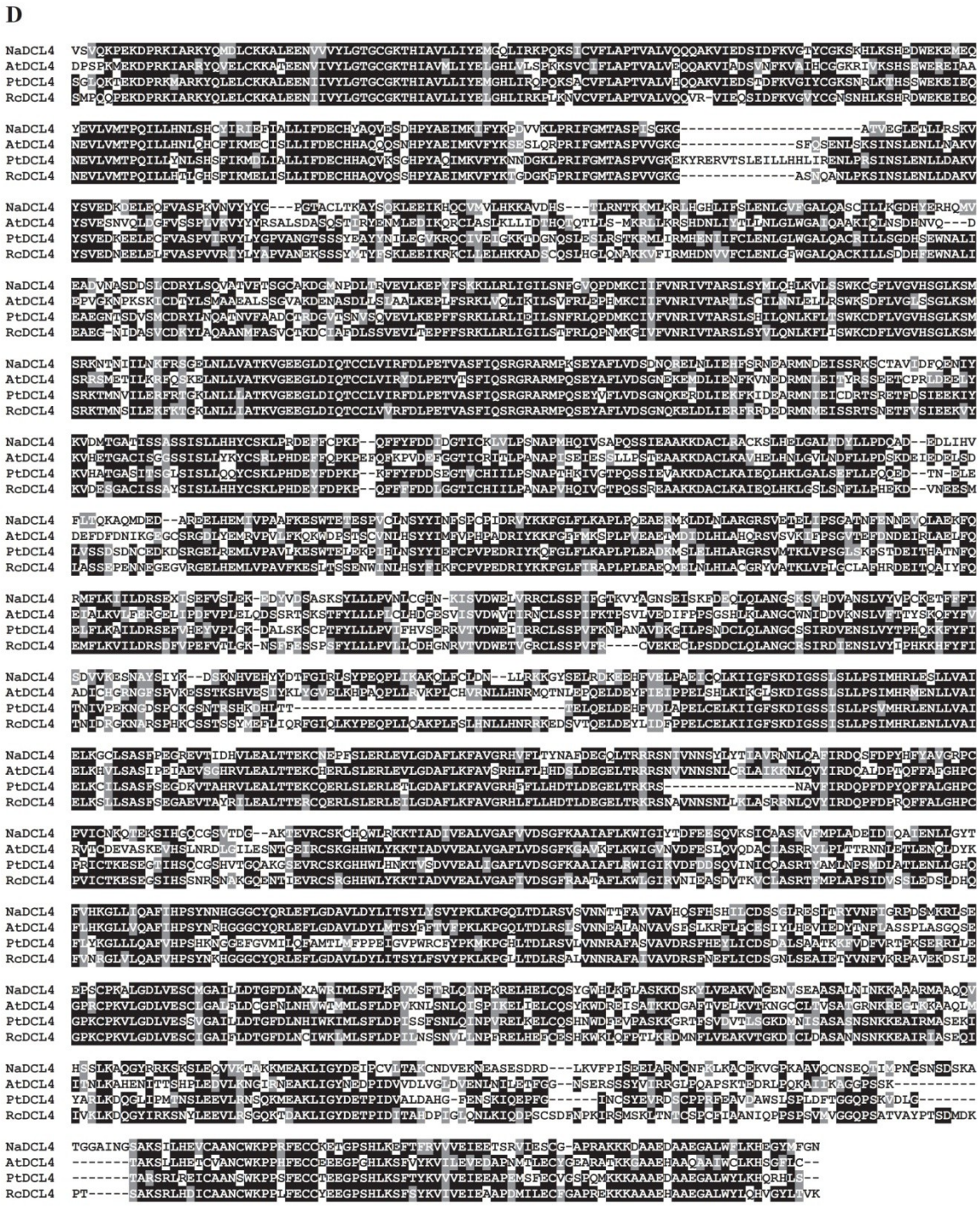

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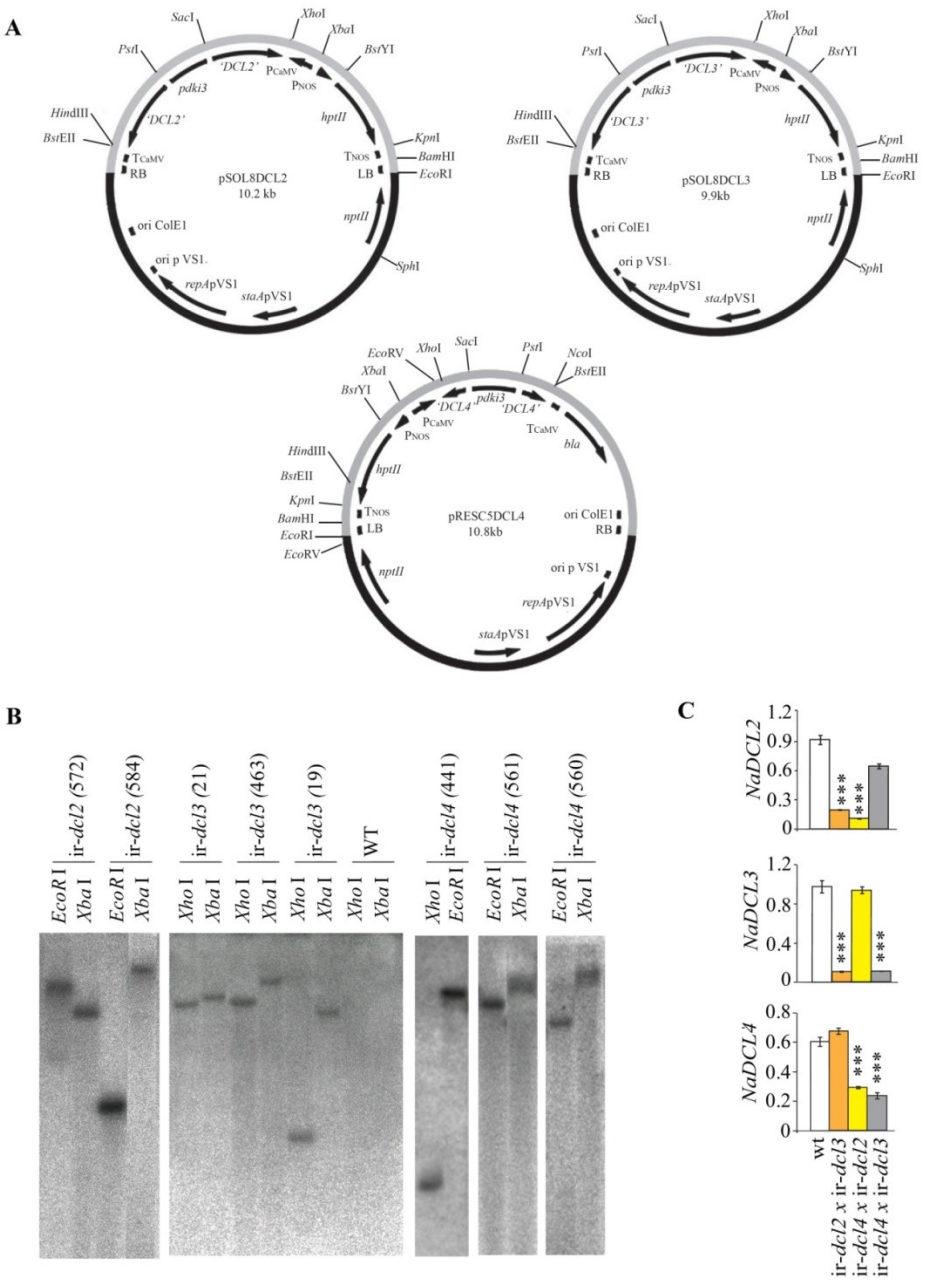

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NaDCL1
CTGTTTGGCAATGAGCTGGATGCAGAGGTATTATCGATGTCGATGGATTTATTTA
TTGCTCGGACTGTAGAAACAAAGGCGACTCTTGTCYTCAGAGGGCCAATAGAAG
TTACAGAGTCTMAGTTGGCGTCYCTTAARAGCTTTCATGTAAGAATGATGAGCAT
TGTATKGGATGTTGATGTTGAGCCATCCACCACTCCTTGGGACCCTGCAAAGGCA
TATCT

NaDCL2
GATGCACCTACGCCGAGGCACTTCCCCATGAATGCTGAGAAGCTAGTTAATGTTCG
ATACTTGGAATCACTGCTAGATTACAAGTTCCATGATCCTTCTCTGCTTGTTGAAGCT
CTAACTCACGGATCTTACATGCTACCTGAGATTCCACGATGCTATCAGCGCTTGGAAT
TTCTTGGAGATGCAGTGCTAGATTATGCTGTTACAGCACATCT CTATTTCAAATATCC
GGGACTGTCT

NaDCL3
TGCAAGAAACTGCAGTGGCCTATGCCTAGTTTGGAGTCAACAGAGCGTAAATCCAAG
TCACTGACTGAATGCGGTGAAGGCTCTGATAAAAGGAAGGTTTACAACACTTTTGCA
TCTCAAATCTCGTTGACCATACCCGACTACGGTTTGATAGAGCTTACTGGGGATGAA
AGAGCTGATAAGA

NaDCL4
GTTGTTGTATATCTGGGAACGGGCTGTGGGAAAACTCATATAGCCGTCTTGCTTATC
TAGAGATGGGACAGCTGATAAGGAAACCCCAGAAGAGCATTTGCGTGTTTCTTGCTCC
CACTGTGGCGTTGGTGCAACAGCAAGCCAAGGTCATAGAAGACTCT

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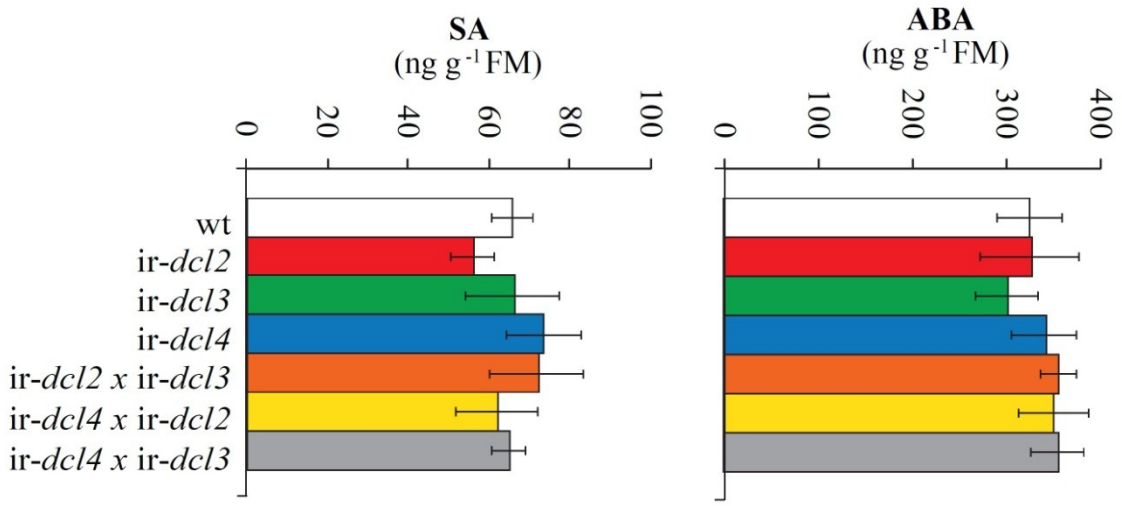

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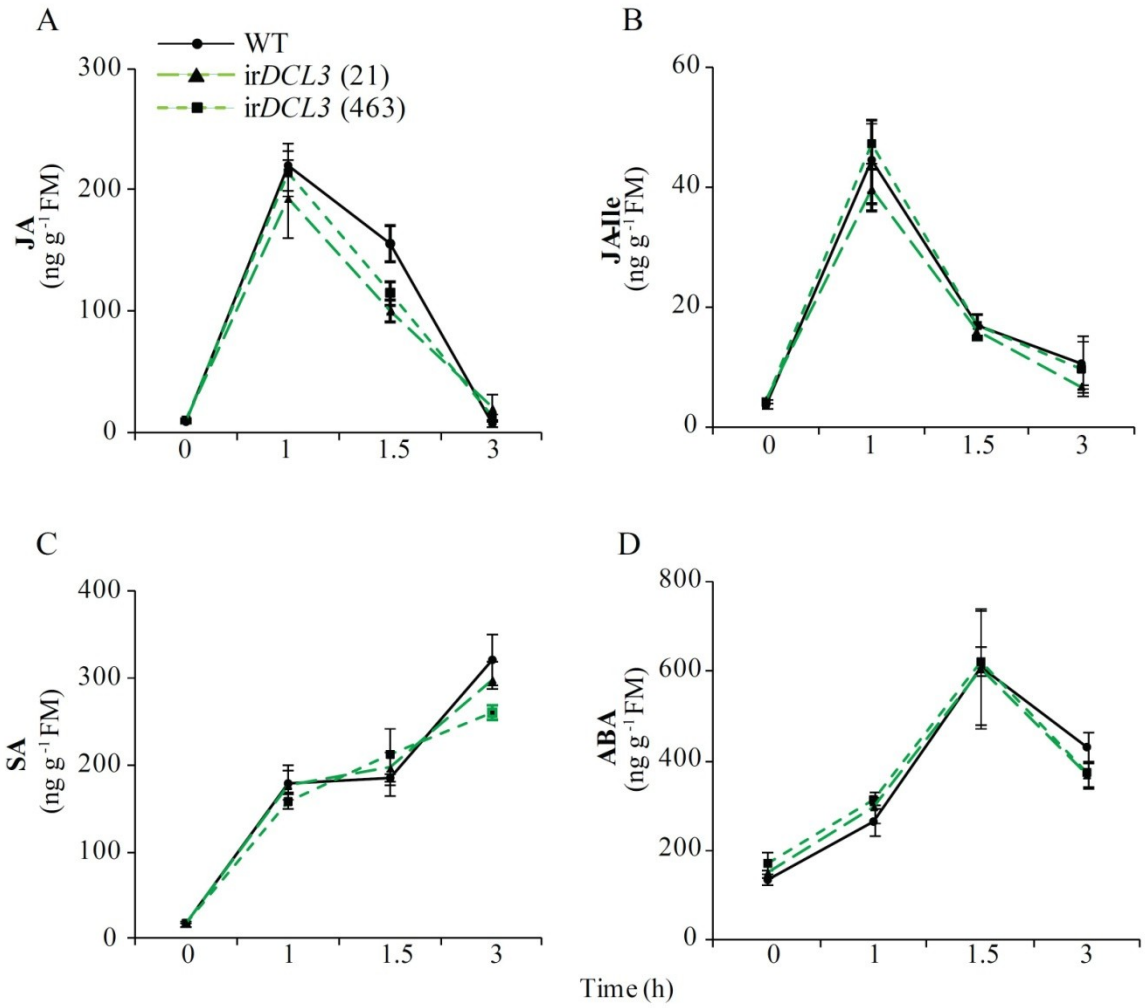

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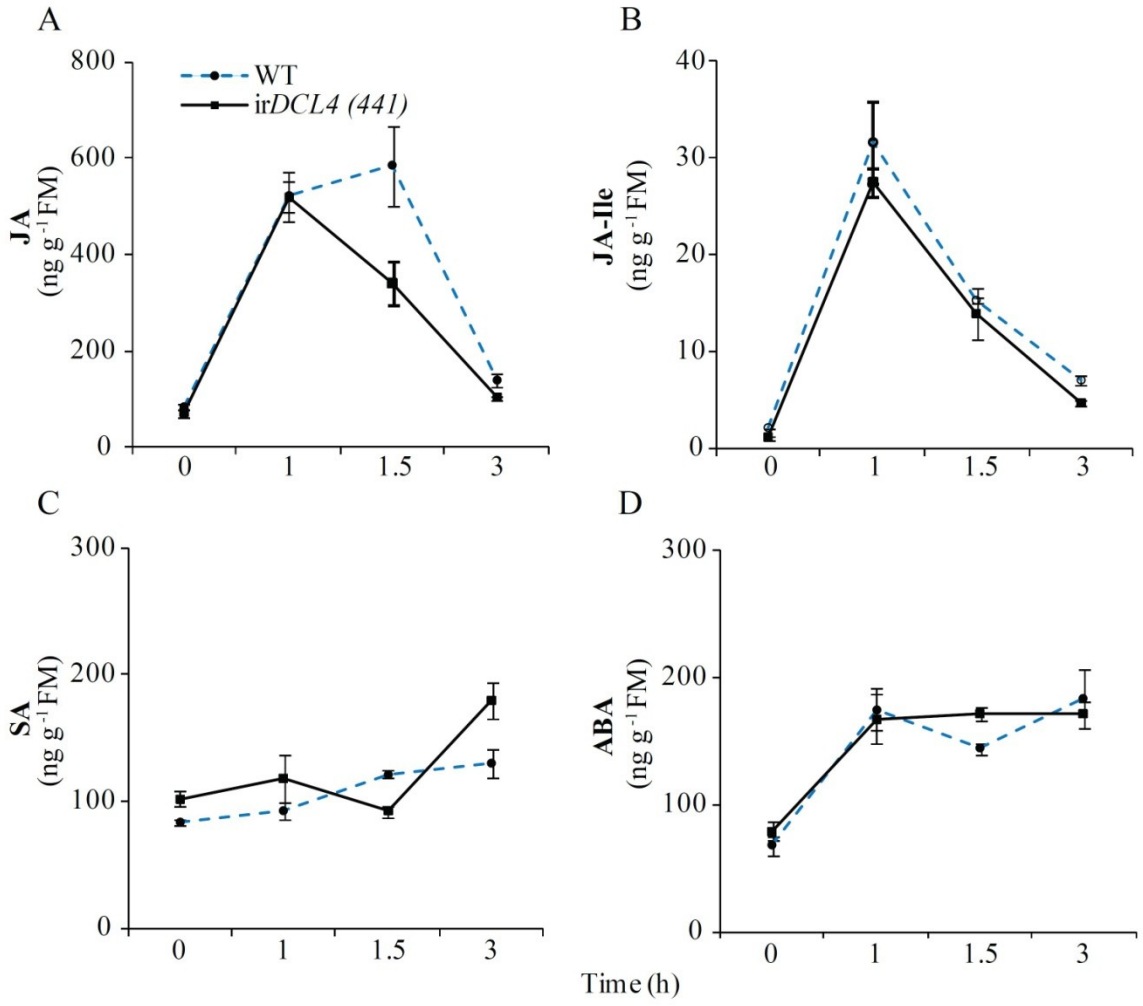

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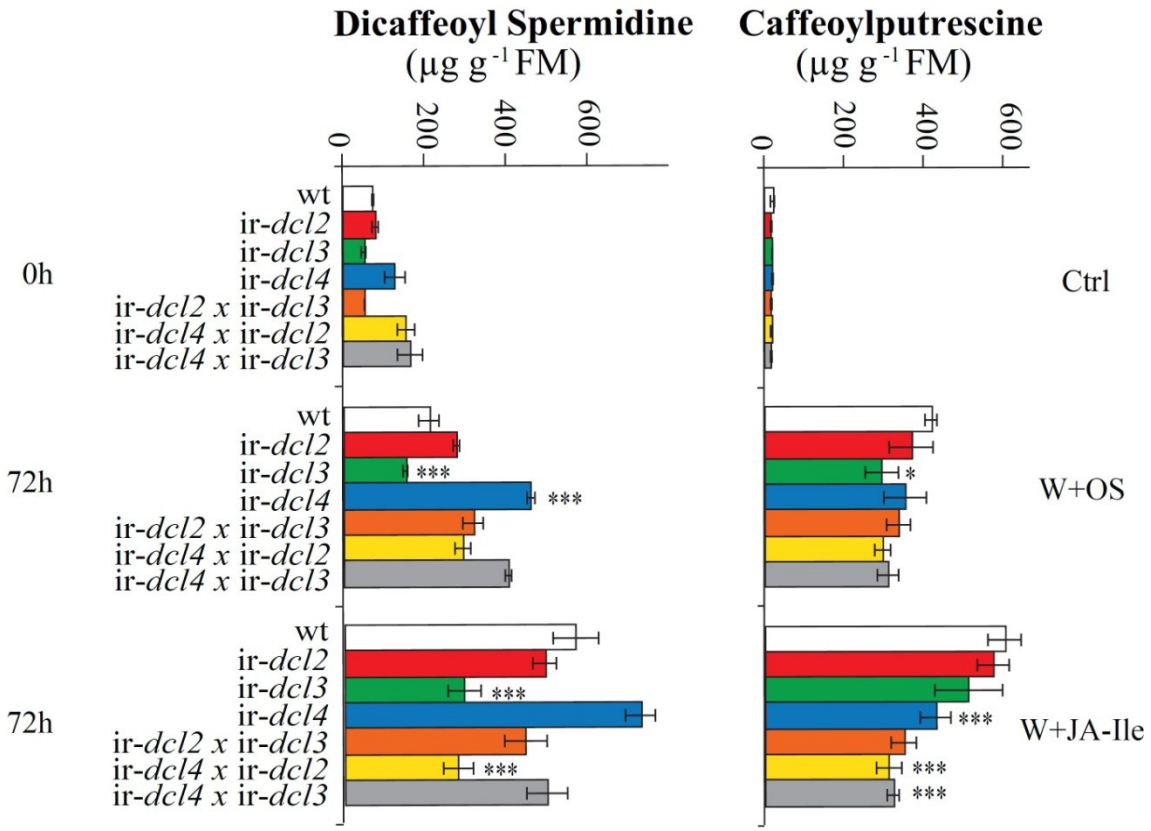

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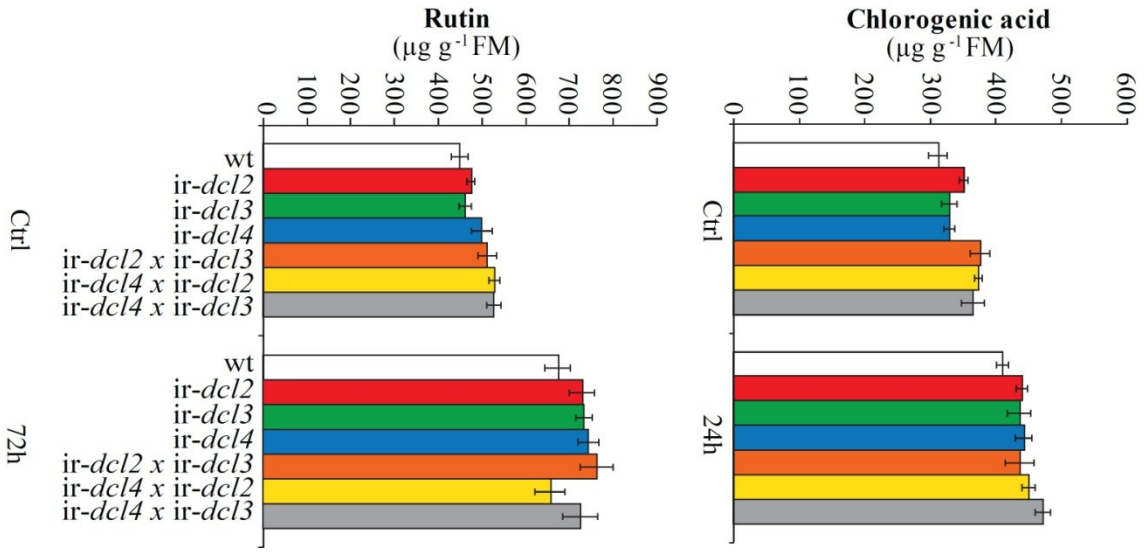

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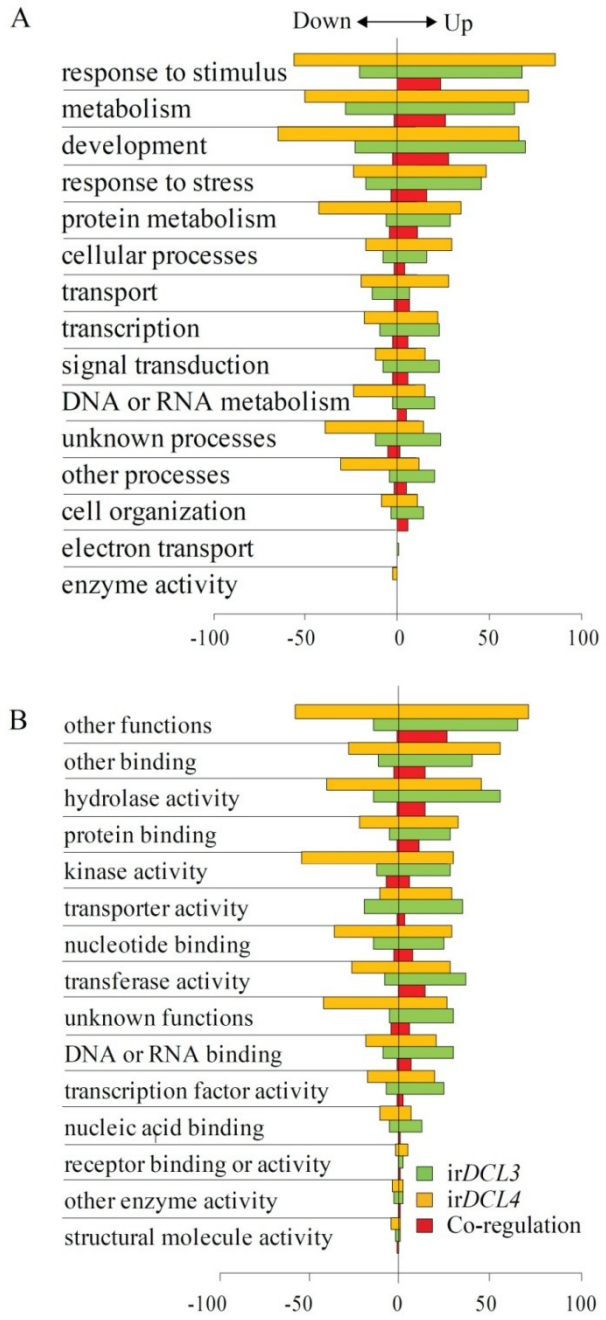

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Table S2 List of primers used for the study.

Name	Primer
NaDCL1-RAC5'	ACTGAATCTGTTGGTGCCTCC
NaDCL1-RAC3'	AGAGCAGAGCTTCTTGGAGATGCC
NaDCL2-RAC5'	TCACCAGGAATGAGCCATCC
NaDCL2-RAC3'	CGTTCGGATGGGAGTCCGAAACTAC
NaDCL3-RAC5'	AAGTGATTGGCGTGGTCCACCC
NaDCL3-RAC3'	CTTACATTCTTGCACTAACGCGTAG
NaDCL4-RAC5'	CAAGGACCTCATACTGTTCC
NaDCL4-RAC3'	CAAGGACCTCATACTGTTCC
NaDCL1-For	GATGAGACTTCAATCCAAATGCC
NaDCL1-Rev	TCTTGGTGGAGATCGAAGGG
NaDCL2-For	TCTTGGTGGAGATCGAAGGG
NaDCL2-Rev	TCTTGGTGGAGATCGAAGGG
NaDCL3-For	CAAGCGAATAATACATGTCCTGCT
NaDCL3-Rev	TGGCCGTCTCTAAATTCGAAAG
NaDCL4-For	CAGAAGCTTGAAGAGATAAAACACCA
NaDCL4-Rev	TTGTGTTCCTGAGAGTGCTATGATC
NaTPI-For	TCAGGAGATAGTAAATATGGCTGTTCA
NaTPI-Rev	ATCTGCATGTTCCACATTGCTTA
Actin-For	CCACACTTCCCACATTGCTGTCA
Actin-Rev	CGCATGTCCCTCACAGCAAAAC

Chapter 5

Identification and profiling of miRNAs during herbivory reveals jasmonate-dependent and -independent patterns of accumulation in *Nicotiana attenuata*

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Submitted to BMC Plant biology.

Identification and profiling of miRNAs during herbivory reveals jasmonate-dependent and -independent patterns of accumulation in *Nicotiana attenuata*

Abstract

Background

Plant microRNAs (miRNAs) play key roles in the transcriptional responses to environmental stresses. However, the role of miRNAs in responses to insect herbivory has not been thoroughly explored. To identify herbivory-responsive miRNAs, we first identified conserved miRNAs in the ecological model plant *Nicotiana attenuata* whose interactions with herbivores have been well-characterized in both laboratory and field studies.

Results

We identified 59 miRNAs from 36 families, and two endogenous *trans*-acting small interfering RNAs (tasiRNA) targeted by miRNAs. We characterized the response of the precursor and mature miRNAs to simulated attack from the specialist herbivore *Manduca sexta* by quantitative PCR analysis and used ir-*aoc* RNAi transformants, deficient in jasmonate biosynthesis, to identify jasmonate-dependent and -independent miRNA regulation. Expression analysis revealed that groups of miRNAs and tasiRNAs were specifically regulated by either mechanical wounding or wounding plus oral secretions from *M. sexta* larvae, and these small RNAs were accumulated in jasmonate-dependent or -independent manners. Moreover, cDNA microarray analysis indicated that the expression patterns of the corresponding target genes were correlated with the accumulation of miRNAs and tasiRNAs.

Conclusions

We suggest that miRNAs and tasiRNAs play an important role in *N. attenuata*'s responses to herbivore attack, a hypothesis that will be tested in future work.

Keywords: miRNA, tasiRNA, jasmonate, anti-herbivore defense, *Manduca sexta, Nicotiana attenuata*

Background

A group of non-coding small RNAs (smRNAs) plays an important role in transcript regulation by binding to their target sequences, resulting in transcriptional degradation, transcriptional or translational inhibition of the targets [1-5]. The smRNAs are classified into two major classes: microRNAs (miRNAs), and small interfering RNAs (siRNAs). Primary transcripts of miRNAs are processed into precursors of miRNAs that form secondary stem-and-loop structures, which are processed by the ribonuclease DICER-like 1 (DCL1) into miRNA/miRNA* duplexes, which are subsequently incorporated into the RNA induced silencing complex (RISC) [5-7]. The siRNAs are further classified into *trans* acting siRNAs (tasiRNAs), chromatin-associated *cis* acting siRNAs, and natural antisense siRNAs, based on their biogenesis [8-10]. Biogenesis of tasiRNAs is regulated by miRNAs, which direct cleavage of primary tasiRNA (*TAS*) transcripts encoding tasiRNAs, resulting in second-strand RNA synthesis by RNA-dependent RNA polymerases (RdRs). The double-stranded RNAs are diced by DCL4 to generate tasiRNAs in *Arabidopsis thaliana* [9-11].

Plant miRNAs and siRNAs are involved in several developmental processes [12]: embryogenesis [13], organ polarity [14], leaf formation [15], root development [11, 16], phytohormone signaling [17, 18], and flowering time [19]. Plant defense signaling is also regulated by miRNAs in response to different abiotic stresses [20, 21] including heat, cold, drought [22, 23], and UV-B radiation [24]. For example, *A. thaliana* miR399 (Ath-miR399), induced during phosphate starvation, targets the ubiquitin-conjugating E2 enzyme involved in phosphate uptake from the soil [25]. Under drought stress, Ath-miR159 regulates *MYB33* and *MYB101* transcription factors, which activate abscisic acid responses during seed germination [26]. Ath-miR398 regulates Cu/Zn-superoxide dismutase genes, which detoxify superoxide radicals [27]. A recent study reported that several miRNAs are induced upon mechanical wounding in tobacco leaves and roots [28].

Plant miRNAs are also involved in biotic interactions. Ath-miR393 is induced by flagellin-derived PAMP peptide 22, and targets the F-box protein and transport

inhibitor response 1, which plays a key role in antibacterial responses [29]. Ath-miR160, Ath-miR167, and Ath-miR825 are induced in response to infection by *Pst* DC3000 hrcC [20], and *A. thaliana* and *Nicotiana tabacum* plants infected by TYMVp69 virus accumulate high levels of miR156, miR160, and miR164 [30, 31]. Plant miRNAs are also involved in beneficial interactions with bacteria: miR482, miR1512, and miR1515 play a role during rhizobial infection in *Glycine max* nodulation with *Bradyrhizobium japonicum* [32]. However, little is known about the role of plant miRNAs in the response to insect herbivores.

The wild tobacco *Nicotiana attenuata* and its herbivore community have become an ecological model system for the study of plant-herbivore interactions. During attack by insect herbivores, *N. attenuata* rapidly induces jasmonate-mediated defense responses, which reconfigure primary and secondary metabolism [33, 34]. Jasmonates comprise jasmonic acid (JA), its derivatives and conjugates; the jasmonates and in particular, the active hormone jasmonoyl-isoleucine (JA-Ile) regulate most defenses against chewing herbivores [35]. Fatty acid amino acid conjugates (FACs) in oral secretion (OS) from larvae of the specialist herbivore, *Manduca sexta*, trigger jasmonate-mediated direct and indirect defenses in *N. attenuata*, such as nicotine accumulation, proteinase inhibitor production, diterpene glycoside biosynthesis, and emission of green leaf volatiles [36-38]. Transgenic plants impaired in jasmonate biosynthesis or signaling show increased susceptibility to herbivory in both glasshouse and field studies [35, 39-41].

OS-elicitation dramatically changes the smRNA population in *N. attenuata* [42], and two major components of the smRNA pathway, RdRs and DCL proteins, function in biotic and abiotic stress responses [42-45]. Silencing of *N. attenuata RdR1*, *DCL3*, and *DCL4* results in impaired defense responses against *M. sexta* herbivory [42, 44, 45]. Silencing either *NaRdR1* or *NaDCL4* impairs jasmonic acid (JA) accumulation, and co-silencing *NaDCL3* and *NaDCL4* reduces JA levels, indicating that RdR1/DCL4-mediated smRNAs are critical regulators of responses to insect herbivory.

To deepen our understanding of the roles that smRNAs play in plant-insect interactions, we identified primary miRNA (*MIR*) transcripts and *TAS* transcripts encoding tasiRNAs in a transcriptome database of *N. attenuata* [46], and computationally analyzed secondary stem-and-loop structures of *MIR* transcripts. To understand the role of jasmonates in regulating miRNAs, we examined miRNA accumulation in jasmonate-deficient *allene oxide cyclase* (*AOC*) RNAi lines. The AOC protein provides a precursor for JA biosynthesis [47]. Expression analysis of miRNAs and tasiRNAs with their putative target genes provides evidence for a key role of plant smRNAs in the response to herbivory.

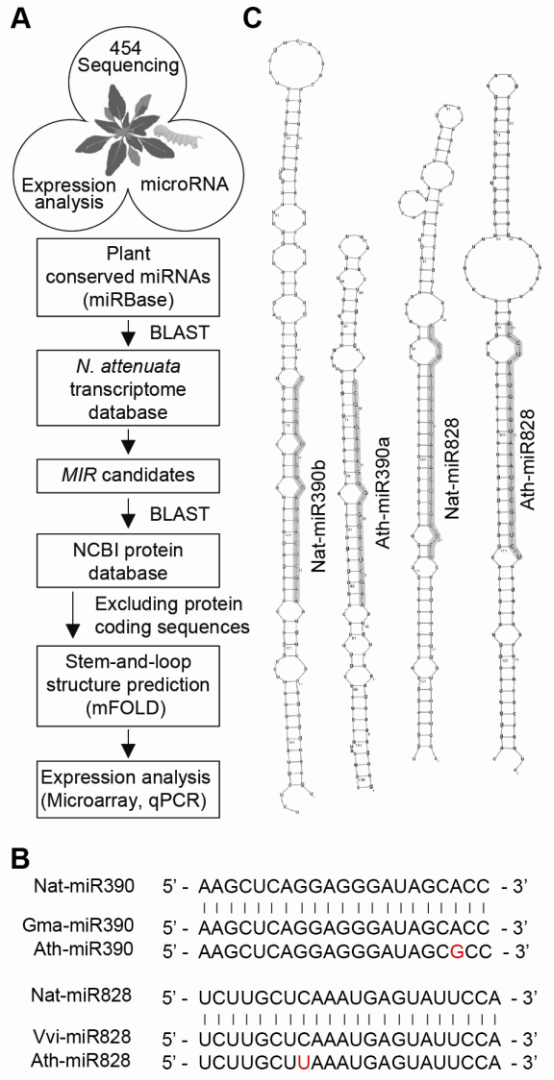

Figure 1. Identification and prediction of miRNAs in *N. attenuata.*

(A) A workflow depicting miRNA identification in *N. attenuata*. *MIR*, primary miRNA transcript. (B) Examples of conserved *N. attenuata* miRNAs with orthologs in plant species. Ath, *Arabidopsis thaliana*; Gma, *Glycine max*; Nat, *Nicotiana attenuata*; Vvi, *Vitis vinifera*. (C) Stem-and-loop structures of Nat-miRNA390 and Nat-miR828 precursors. Hairpin structures are compared to *Arabidopsis* miRNA orthologs based on their miRNAs sequence similarity. The miRNAs are highlighted in structures.

Results and discussion

Identification of conserved miRNAs and their precursors in *N. attenuata*

To identify conserved miRNAs in *N. attenuata*, we used a 454-transcriptome database of *N. attenuata* to conduct a BLAST search against conserved plant miRNAs in the miRBase

Table 1. Identification and prediction of miRNAs in *N. attenuata*.

Nat-MIR family members	MFE	miRNA	Length (nt)	GC content (%)	Hit in miRBase	E-value
Nat-miR156a	-55.1	ugacagaagagagugagcaca	21	47.6	bna-miR156	0.001
Nat-miR156b	-76.5	ugacagaagagagugagcaca	21	47.6	bna-miR156	0.001
Nat-miR157	-43.9	uugacagaagauagagagcac	21	42.9	ath-miR157	0.001
Nat-miR159a	-62.3	uuuggauugaagggagcucua	21	42.9	ath-miR159	0.001
Nat-miR159b	-90.6	uuuggauugaagggagcucua	21	42.9	ath-miR159	0.001
Nat-miR159c	-104.3	uuuggauugaagggagcucua	21	42.9	ath-miR159	0.001
Nat-miR160a	-61.6	ugccuggcucccuguaugcca	21	61.9	ath-miR160	0.001
Nat-miR160b	-63.3	ugccuggcucccuguaugcca	21	61.9	ath-miR160	0.001
Nat-miR162	-43.9	uggaggcagcgguucaucgauc	22	54.5	csi-miR162	0.0001
Nat-miR164	-74.2	uggagaagcagggcacgugca	21	61.9	ath-miR164	0.001
Nat-miR166a	-59.8	ucggaccaggcuucauucccc	21	61.9	ath-miR166	0.001
Nat-miR166b	-51	ucggaccaggcuucauucccc	21	61.9	ath-miR166	0.001
Nat-miR166c	-71.8	ucggaccaggcuucauucccc	21	61.9	ath-miR166	0.001
Nat-miR167a	-42	ugaagcugccagcaugaucua	21	47.6	ath-miR167	0.001
Nat-miR167b	-47	ugaagcugccagcaugaucua	21	47.6	ath-miR167	0.001
Nat-miR168	-78.3	cccgccuugcaucaacugaau	21	52.4	aly-miR168	0.001
Nat-miR169a	-86.1	cagccaaggaugacuugccga	21	57.1	ath-miR169	0.001
Nat-miR169b	-70.5	uagccaaggaugacuugccugc	22	50.0	bna-miR169	0.001
Nat-miR171a	-70.1	ugauugagccgcgucaauauc	21	47.1	vvi-miR171	0.001
Nat-miR171b	-61.4	ugauugagccgcgucaauauc	21	47.1	vvi-miR171	0.001
Nat-miR171c	-44.9	ugauugagccgcgucaauauc	21	47.1	vvi-miR171	0.001
Nat-miR171d	-60.4	ugauugagccgcgucaauauc	21	47.1	vvi-miR171	0.001
Nat-miR172a	-53.4	ugagaaucuugaugaugcugcau	23	39.1	vvi-miR172	0.001
Nat-miR172b	-49.5	ugagaaucuugaugaugcugcau	23	39.1	vvi-miR172	0.001
Nat-miR172c	-61.7	ugagaaucuugaugaugcugcau	23	39.1	vvi-miR172	0.001
Nat-miR172d	-35.2	ugagaaucuugaugaugcugcau	23	39.1	vvi-miR172	0.001
Nat-miR319a	-97.5	uuggacugaagggagcucccu	21	57.1	ath-miR319	0.001
Nat-miR319b	-89.7	uuggacugaagggagcucccu	21	57.1	ath-miR319	0.001
Nat-miR319c	-95.4	uuggacugaagggagcucccu	21	57.1	ath-miR319	0.001
Nat-miR390a	-67.6	aagcucaggagggauagcacc	21	57.1	gma-miR390	0.001
Nat-miR390b	-58.1	aagcucaggagggauagcacc	21	57.1	gma-miR390	0.001
Nat-miR393a	-41.7	uccaaagggaucgcauugaucc	21	45.5	ath-miR393	0.0004
Nat-miR393b	-54.4	uccaaagggaucgcauugaucc	21	45.5	ath-miR393	0.0004
Nat-miR394a	-41.7	uuggcauucuguccaccuccau	22	50.0	vvi-miR394	0.0004
Nat-miR394b	-82.3	uuggcauucuguccaccuccau	22	50.0	vvi-miR394	0.0004
Nat-miR396	-56.4	uuccacagcuuucuugaacug	22	42.9	ath-miR396	0.001
Nat-miR397	-49.9	ucauugagugcagcguugaug	22	47.6	ath-miR397	0.001
Nat-miR398	-69.1	uguguucucaggucaccccuu	21	52.4	ath-miR398	0.001
Nat-miR399	-	ugccaaagaagauuugccccgu	21	52.4	ptc-miR399	0.001
Nat-miR403	-38.2	uuagauucacgcacaaacucg	21	42.9	ath-miR403	0.001
Nat-miR408	-47.6	augcacugccucuucccuggc	21	61.9	ath-miR408	0.001
Nat-miR413	-	cuaguuucucuuguucugcuu	21	38.1	ath-miR413	0.015
Nat-miR414	-	uccucuucaucaucaucuuc	21	40.0	ath-miR414	0.074
Nat-miR477	-54.8	acucucccucaagggcuucug	21	57.1	aqc-miR477	0.001
Nat-miR478	-33.3	ugacaugucuuauauuuuuag	20	23.8	ptc-miR478	0.005
Nat-miR482	-53.1	uuuccaauuccacccauuccua	21	40.9	sly-miR482	0.0004

Nat-miR828	-46.7	ucuugcucaaaugaguauucca	21	36.4	vvi-miR828	0.0004
Nat-miR845a	-	ugcucugauaccaaauugaug	22	38.1	ath-miR845	0.003
Nat-miR845b	-	uggcucugauaccaauugau	22	40.0	vvi-miR845	0.004
Nat-miR1128	-85.4	uacuacucccuccguuucaa	20	45.0	ssp-miR1128	0.081
Nat-miR1133	-88.1	cauauacucccuccgucccugaaa	21	50.0	tae-miR1133	0.017
Nat-miR1446	-48.7	uucugaacucucucccucaa	20	45.0	ptc-miR1446	0.003
Nat-miR1863a	-	gcucugauaccauguuaacu	24	40.0	osa-miR1863b	0.008
Nat-miR1863b	-	gacucugauaccauguuaaaaua g	20	28.0	osa-miR1863	0.02
Nat-miR1919	-87.1	aggcgagtcatctgtgacagg	21	57.1	sly-miR1919	0.029
Nat-miR2911	-67.3	ggccgggggacggacuggga	20	80.0	peu-miR2911	0.014
Nat-miR5281a	-51.9	cauauaaauugaaacggagggag	23	39.1	mtr-miR5281b	0.13
Nat-miR5281b	-69.9	cauauaaauugaaacggagggag	23	39.1	mtr-miR5281b	0.13
Nat-miR5281c	-34.2	cauauaaauugaaacggagggag	23	39.1	mtr-miR5281b	0.13

(www.mirbase.org) (Figures 1A and 1B). This search identified 59 potential miRNAs distributed in 36 families (Table 1). We used the BLASTX algorithm against the NCBI protein database to check that the putative primary transcripts of miRNAs were non-coding. Web-based mFOLD software (http://mfold.rna.albany.edu/) was used to predict secondary stem-and-loop structures. Of the identified miRNA-precursors, 52 had stem-and-loop structures (Figure 1C and Additional file 1), which were created with minimum free energies (MFE) ranging from ΔG = -97.5 kcal mol^{-1} to -33.3 kcal mol^{-1} (Table 1) with an average MFE of -62.1 kcal mol^{-1}. This average MFE is comparable to that found in *A. thaliana* (-59.5 kcal mol^{-1}), higher than in the red alga *Porphyra yezoensis* (-41.7 kcal mol^{-1}) and lower than in the monocots rice (-71.0 kcal mol^{-1}) and wheat (-72.4 kcal mol^{-1}) [22, 48]. Only seven predicted miRNA-precursors transcripts did not form stem-and-loop structures or were not stable (Table 1). We identified several *N. attenuata* (Nat) miRNA families (Nat-miR403, Nat-miR478, Nat-miR482, Nat-miR1128, Nat-miR1133, Nat-miR1446, Nat-miR1863, Nat-miR2911, and Nat-miR5281) which were not reported in *N. tabaccum* [28, 49]. Among these, Nat-miR478, Nat-miR482, Nat-miR1128, Nat-miR1133, Nat-miR1446, Nat-miR1863, and Nat-miR5281are absent in *A. thaliana* but are close homologues to those in other plant species (Table 1).

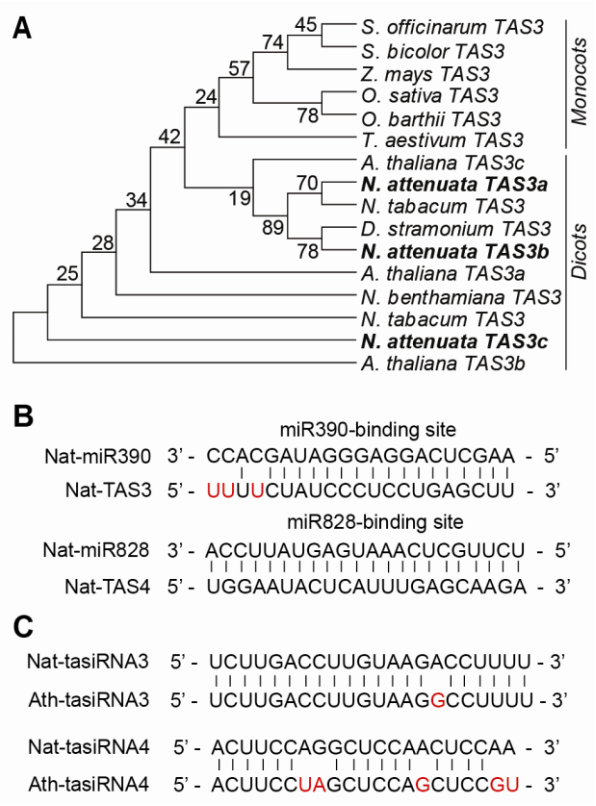

Figure 2. Identification of miRNA-regulated tasiRNAs in *N. attenuata*. (A) Phylogenetic analysis of three *TAS3* transcripts in *N. attenuata*. Nucleotide sequences of three *TAS3* transcripts were aligned with *TAS3* orthologs of monocotyledonous and dicotyledonous plants. Distance values were calculated using the neighbor-joining method with 1000 bootstrap replicates. (B) Binding site of Nat-miR390 and Nat-miR828 in *TAS3* and *TAS4* transcript, respectively. (C) Conserved tasiRNAs in *N. attenuata* and *A. thaliana*.

Next, we designed probes to detect *N. attenuata* miRNAs on RNA blots (Additional file 2). We performed northern blot hybridization using 40 µg of total RNA extracted from rosette leaves to detect selected miRNAs. Accumulation of miRNAs varied (Additional file 3). Accumulation of Nat-miR159, Nat-miR171, Nat-miR172, and Nat-miR319 was high compared to Nat-miR157, Nat-miR393, Nat-miR396, and Nat-miR828 in leaves from rosette-stage plants. For further analyses of mature and precursor miRNA abundance, we used real-time quantitative PCR (qPCR) with specific primer sets (Additional files 4 and 5).

Identification of conserved tasiRNAs in *N. attenuata*

Four families of endogenous tasiRNAs (*TAS1*, *TAS2*, *TAS3*, and *TAS4*) identified in *A. thaliana* are regulated by miRNAs [9, 50]. We found three *TAS3* transcripts and one *TAS4* transcript in *N. attenuata* (Figure 2), and constructed a phylogenetic tree with their homologs from different plant species to examine the evolutionary relationships of *TAS3* expressed in dicotyledonous and monocotyledonous plant species [50, 51]. Not surprisingly, *NaTAS3* members were grouped amongst members of the dicotyledonous plant species (Figure 2A).

Because *TAS3* and *TAS4* transcripts contain the binding sites of miR390 and miR828, respectively [9, 11, 52], we blasted Nat-miRNA390 and Nat-miRNA828 against the *TAS* transcripts (Figure 2B), and compared the *N. attenuata* tasiRNAs with those in *A. thaliana* (Figure 2C). Sequence analysis indicated that binding sites of miRNA and tasiRNA sequences are highly conserved in *N. attenuata* and *A. thaliana*.

Wound- and OS-inducible miRNAs

Next, we examined the abundance of miRNAs in leaves of rosette-stage WT plants changed after wounding and the application of diluted *M. sexta* OS (W+OS) or water to wounds (W+W, as a control for W+OS) versus no treatment (control) (Figure 3). W+OS treatment faithfully mimics the majority of responses elicited by *M. sexta* feeding [36, 38], but has the distinct advantage of allowing the time of elicitation to be precisely controlled (as opposed to the sporadic nature of *M. sexta* larval feeding behavior) and hence greatly increases the reproducibility of transcriptional analyses.

The abundance of mature Nat-miR159, Nat-miR160, Nat-miR167, Nat-miR396, Nat-miR403, Nat-miR408, and Nat-miR828 increased after both W+W and W+OS treatments, abundances of which are not differed (Figure 3A). We categorized these miRNAs wound-inducible miRNAs. Twelve conserved miRNA families (Nat-miR156, Nat-miR164, Nat-miR166, Nat-miR168, Nat-miR171, Nat-miR172, Nat-miR319, Nat-miR390, Nat-miR393, Nat-miR394, Nat-miR398, and Nat-miR1446) were significantly increased in W+OS treated leaves compared to control and W+W treated leaves (Figure 3B) and these were classified as OS-inducible miRNAs.

Ath-miR164 is known to negatively regulate ORE1/NAC2 transcription factors which are involved in age-dependent senescence [53] and mutations in ORE1/NAC2 delays chlorophyll loss in old leaves of *A. thaliana* [53]. We found a NAC transcription factor containing a Nat-miR164 binding site in *N. attenuata* (Figure 6). The NAC transcripts were significantly reduced in W+W and W+OS

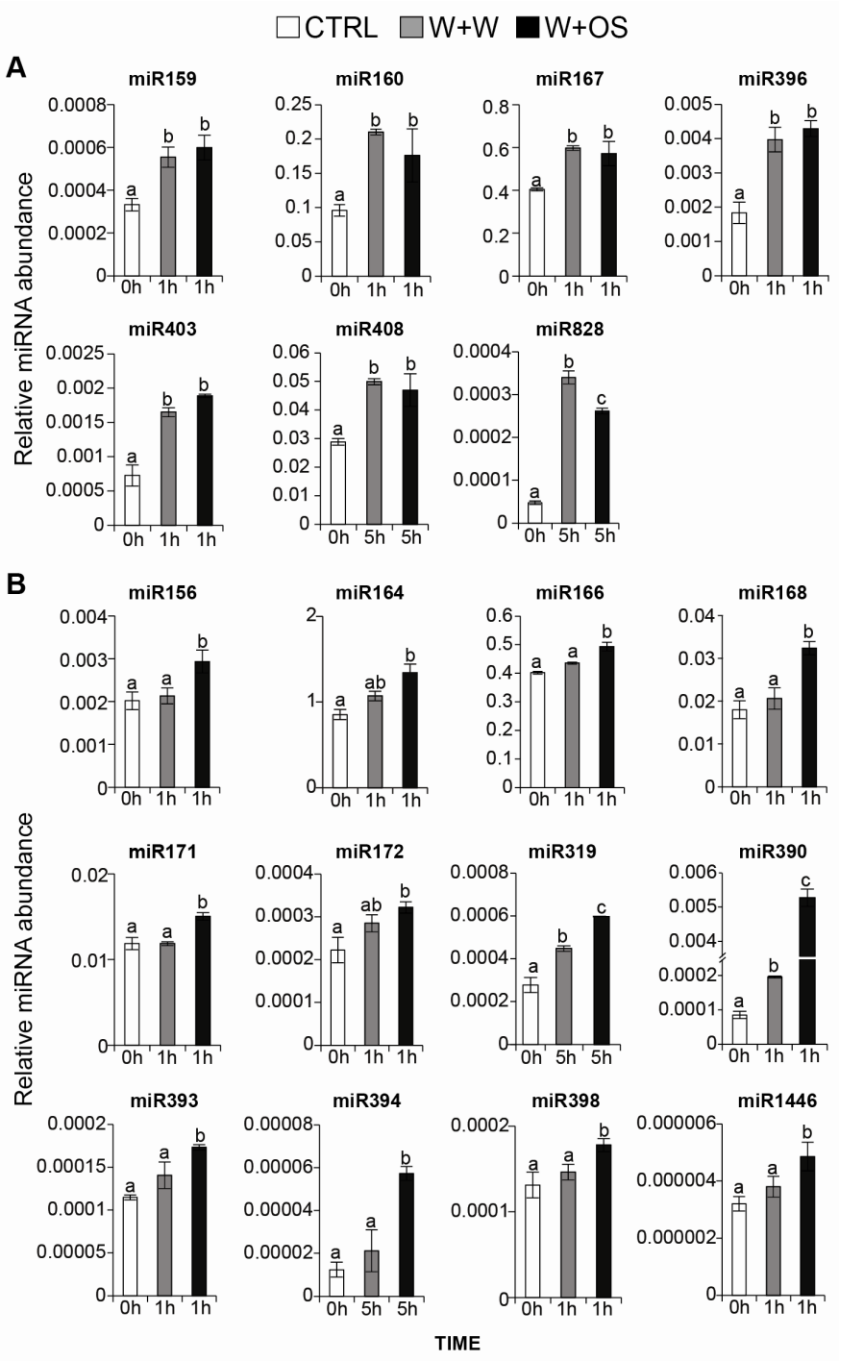

Figure 3. Wound- and OS-inducible miRNAs.
(A) Wound-inducible miRNAs. (B) OS-inducible miRNAs. Effects of W+W and W+OS on relative transcript abundance of *N. attenuata* miRNAs. For each sample, one leaf on the rosette of a 32-day-old WT plant was left untreated (control, CTRL) or treated with wounding plus water (W+W) or wounding plus oral secretions of the larvae of the specialist *M. sexta* (W+OS) and harvested 1 h or 5 h post treatment. Shown are mean (± SE) levels of three replicates. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD tests following an ANOVA.

elicited leaves (Figure 6), which correlated with elevated Nat-miR164 levels after OS-elicitation (Figure 3B). This result may explain how *M. sexta* attack delays senescence in *N. attenuata* [54].

The most significant change in miRNA transcripts after W+OS treatment was for Nat-miR390, which showed a 75-fold increase compared to control leaves and a 30-fold increase compared to W+W treated leaves (Figure 3B). Ath-miR390 cleaves *TAS* transcripts, resulting in production of tasiRNA3 mediated by the RdR6/DCL4 complex [11, 52]. TasiRNA3 controls the transcription of auxin response factors

(ARFs) 2, 3, and 4, which regulate leaf morphology and lateral root growth in *A. thaliana* [11, 16, 52]. Overexpression of *TAS3* leads to an increased number of lateral roots, and the knock-out mutant shows impaired lateral root growth [11]. Abundance of mature Nat-tasiRNA3 in W+OS treated leaves was increased (Figure 5A) and Nat-tasiRNA3 was regulated in a JA-independent manner (Figure 5B). The targets of tasiRNA3, transcripts homologous to *A. thaliana ARF2*, *3* and *4* were significantly reduced after W+OS elicitation (Figure 6), suggesting that Nat-miR390 could affect the architecture of roots and thereby regulate the production of nicotine, which is synthesized in the roots, or promote tolerance of herbivory by increasing mineral uptake or sugar storage in roots. Our previous study shows that silencing *NaDCL4*, which in turn reduces the accumulation of tasiRNA3, dramatically impairs root growth and nicotine accumulation in *N. attenuata* [45].

JA-dependent miRNAs

The key role of jasmonates in mediating responses to herbivory is well established and it was not surprising to find that the levels of jasmonates dramatically increased in W+OS treated leaves compared to W+W treatment (Additional file 6) [37]. Silencing of *NaAOC* reduces JA accumulation in W+OS treated leaves by 90-100% (Additional file 6) and is known to silence the production of JA-mediated defenses [47]. OS-elicitation of this genotype allowed us to understand which miRNAs are regulated by jasmonates.

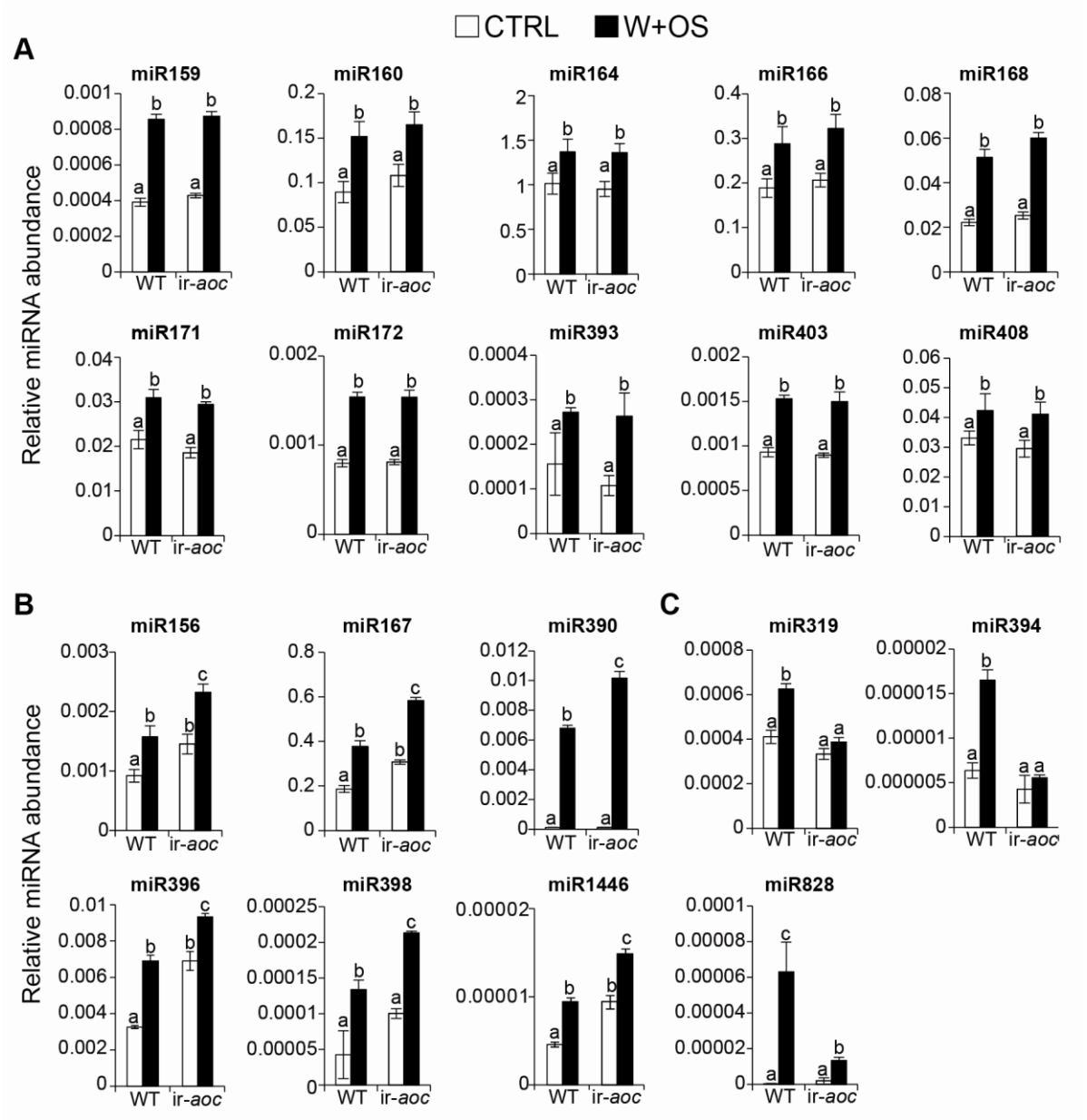

Figure 4. JA-independent and dependent miRNAs accumulation in response to OS-elicitation.

(A) W+OS inducible and JA-independent miRNAs. (B) W+OS inducible miRNAs accumulated more in ir-*aoc* plant. (C) W+OS inducible and JA-dependent miRNAs. Shown are mean (± SE) levels of miRNAs in control and W+OS elicited leaves in wild-type (WT) and ir-*aoc* plants. For each sample, one leaf on the rosette of a 32-day-old WT and ir-*aoc* plant was left untreated (control, CTRL) or treated with wounding plus oral secretions of the larvae of the specialist herbivore *M. sexta* (W+OS) and harvested 1 h or 5 h post elicitation. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD tests following an ANOVA.

Abundance of 10 miRNAs (Nat-miR159, Nat-miR160, Nat-miR164, Nat-miR166, Nat-miR168, Nat-miR171, Nat-miR172, Nat-miR393, Nat-miR403, and Nat-miR408) increased after W+W and W+OS treatments, but did not differ between WT and ir-*aoc* plants (Figure 4A). We considered these miRNAs as JA-independent miRNAs. The abundance of Nat-miR156, Nat-miR167, Nat-miR390, Nat-miR396, Nat-miR398, and Nat-miR1446 were significantly higher in both control and W+OS elicited leaves of ir-*aoc* than of WT plants (Figure 4B), indicating that jasmonates or JA-signaling negatively influences levels of these miRNAs, regardless of treatment. Nat-miR390 abundance was higher in ir-*aoc* only after W+OS treatment (Figure 4B). While abundance of Nat-miR319 and Nat-miR394 were induced by W+OS treatment in WT (Figure 3B), they were not induced by W+OS treatment in ir-*aoc* plants (Figure 4C). We considered these as OS-inducible JA-dependent miRNAs. Wound-inducible Nat-miR828 was induced in both W+W and W+OS treatments in WT (Figure 3A), but their levels were not dramatically induced in elicited JA-deficient ir-*aoc* plant compared to WT (Figure 4C).

One main target of miR319 in *A. thaliana* is TCP (TEOSINTEBRANCHED/CYCLOIDEA/PCF) transcription factors, which positively regulates jasmonate biosynthesis [18]. The TCP4 protein can bind a promoter of *LIPOXYGENASE2* (*LOX2*) in *A. thaliana* [18]. We do not yet know the orthologs of *A. thaliana* TCPs in *N. attenuata*, but the timing of Nat-miR319 induction was similar with that of W+OS elicited JA accumulation and the induction of *NaLOX3*, which is the functional homolog of *A. thaliana LOX2* [40, 55]. W+OS treatment amplifies the wound-induced JA accumulation (Additional file 6) [36, 38] and does the same for Nat-miR319 (Figure 3B). This suggests that Nat-miR319 could play a role in the fine-tuning regulation of jasmonate biosynthesis [18] during herbivory.

Nat-miR828 and Nat-tasiRNA4 were increased by W+W and W+OS treatments in WT (Figure 3A), but not in ir-*aoc* plants (Figures 4C and 5). Levels of Nat-miR828 dramatically decreased in ir-*aoc* plants compared to WT and its primary transcript as well (Figure 4C and Additional file 7). One target of miR828 is

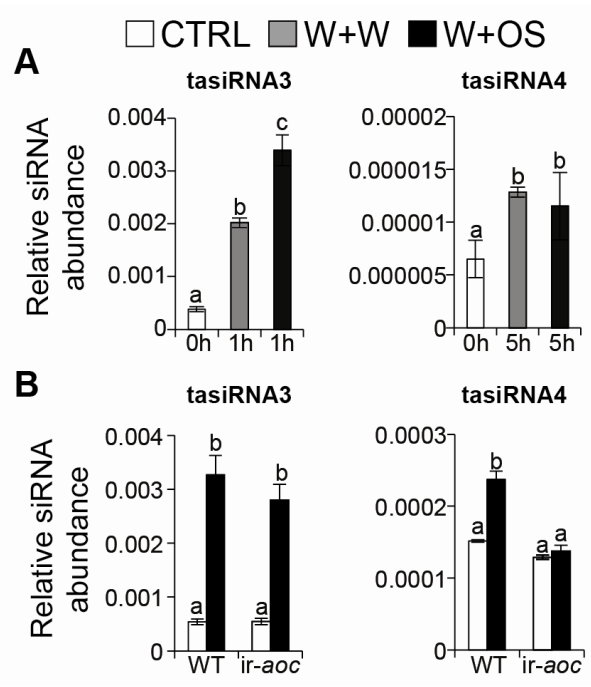

Figure 5. Accumulation of tasiRNAs in response to OS-elicitation.
(A) Abundance of tasiRNA3, which is a target of the OS-inducible Nat-miR390, significantly increased in OS-elicited plant compared to control (CTRL) and W+W treatment. Abundance of tasiRNA4, which is regulated by the wound-inducible Nat-miR828, was significantly increased by wounding. (B) Accumulation of JA-independent tasiRNA3 and JA-dependent tasiRNA4 after OS-elicitation. For each sample, one leaf on the rosette of a 32-day-old plant was treated with wounding plus oral secretions of the larvae of the specialist herbivore *M. sexta* (W+OS) and harvested 1 h or 5 h post treatment. Untreated plants served as control. Shown are mean (± SE) levels of three replicates. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA.

a *TAS4* transcript, which encodes tasiRNA4 processed by RdR6/DCL4 proteins [9, 56]. TasiRNA4 targets several MYB transcription factors which regulate phenylpropanoid biosynthesis [9, 56]. Several phenylpropanoid defense metabolites increase in *N. attenuata* during herbivory and silencing of *DCL4* alters the accumulation of dicaffeoyl spermidine and caffeoylputrescine in W+OS-elicited leaves [45]. A jasmonate-inducible MYB transcription factor, NaMYB8, is involved in plant defense against herbivory and phenylpropanoid biosynthesis [57, 58]. Although we were not able to find a MYB transcription factor containing a Nat-tasiRNA4 binding site in our current cDNA library, sequence conservation of miR828 and tasiRNA4 in *A. thaliana* and *N. attenuata* will guide the identification of targets which regulate secondary metabolite production.

Comparison between primary and mature miRNA transcript accumulation

We checked the accumulation of *MIR* transcripts using previously reported microarray data [46] (Additional file 8) and confirmed these responses by qPCR (Additional file 7). Accumulation of *MIR156*, *MIR159*, *MIR164*, *MIR166*, *MIR167*,

MIR168, *MIR172*, *MIR393*, *MIR396*, *MIR398*, *MIR403*, and *MIR1446* was not correlated to the abundance of their mature miRNAs. Changes in transcript levels of *MIR160*, *MIR171*, *MIR319*, *MIR390*, *MIR408*, and *MIR828* reflected the transcript accumulation of their mature miRNAs. Generally, a weak relationship between *MIR* and mature miRNA accumulation has been reported [59]. Our data also suggests that the processing of *MIR* transcripts is more important than the transcription of *MIR* genes in herbivory-induced miRNA regulation.

Identification of miRNA targets and their transcript accumulation

It is well-established that miRNAs and tasiRNAs bind to their target mRNAs by perfect or imperfect complementarity [5]. Such complementarity permits the identification of miRNA targets in databases. We blasted miRNA sequences against an in-house transriptomic database using BLASTN algorithms with default parameters allowing 1-4 mistmatches. We identified 111 potential targets, inluding targets of tasiRNAs (Additional file 9).

Putative Nat-miRNA targets included transcription factors in the ARF, NAC (NAM, ATAF1/2 and CUC2), HD-ZIP III, AP2-like, MYB, and GRAS domain proteins (Additional file 9). *ARF6* and *ARF8* were putative targets of wound-inducible Nat-miR167, and their transcripts decreased concurrently with the accumulation of Nat-miR167 (Figure 6). Potential targets of OS-inducible Nat-miR171, GRAS domain transcription factors, were down-regulated in OS-elicited plants (Figure 6). We found that transcripts of several OS-inducible Nat-miR156 targets annotated as *SQUAMOSA* promoter binding proteins (SBPs) were significantly down-regulated after W+OS treatment. Abundance of Nat-miR172 increased significantly in W+OS treated leaves (Figure 3B) and consistently, transcripts of its putative targets, AP2-like proteins, were significantly down-regulated in W+W and W+OS treated leaves (Figure 6). We validated the microarray data by qPCR analysis (Additional file 10), and the result showed similar expression patterns (Additional file 11).

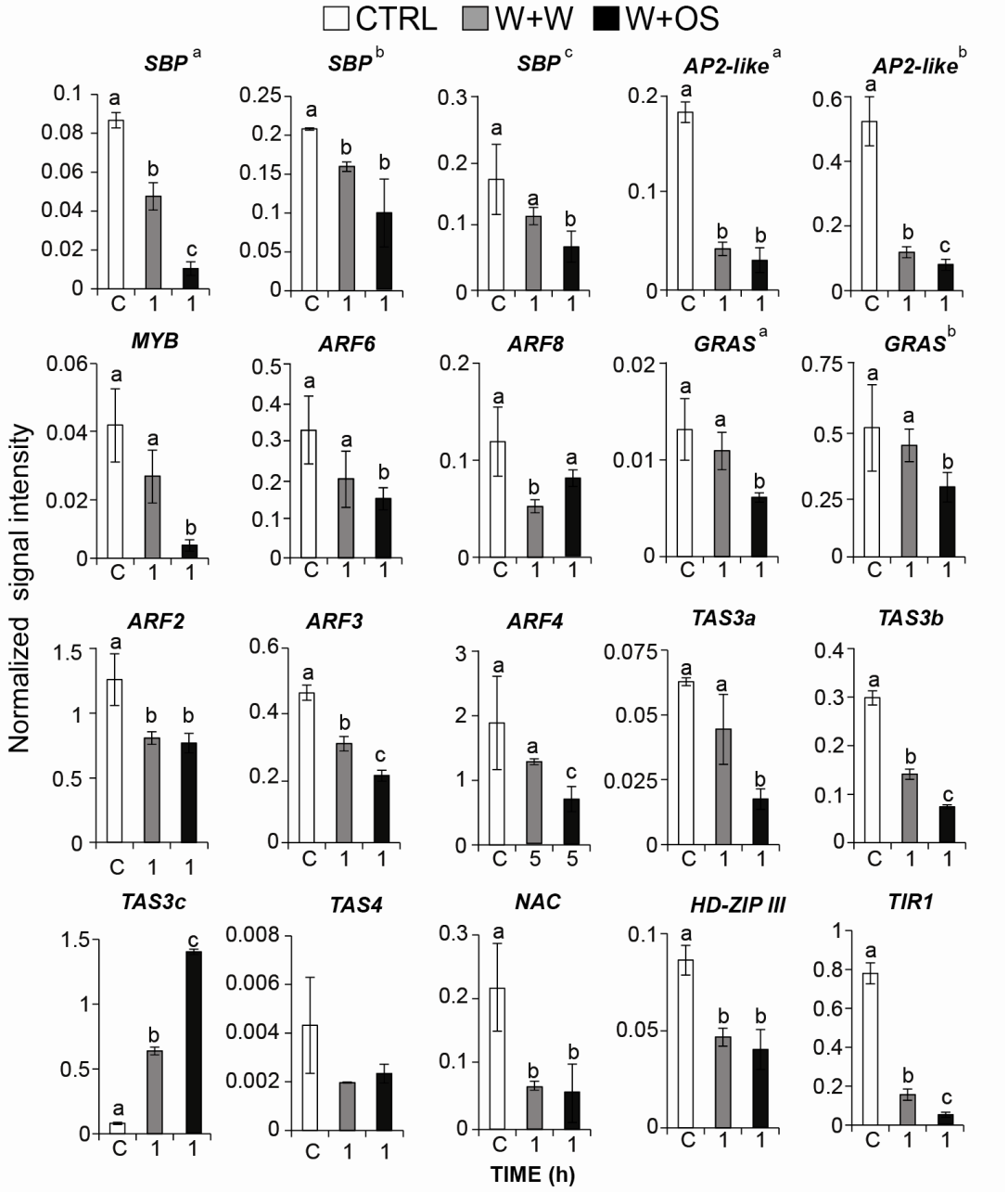

Figure 6. Target genes expression of miRNAs and tasiRNAs.
Mean (± SE) levels of normalized transcript abundance of miRNAs and tasiRNAs target genes after W+W and W+OS treatments. Untreated plants served as control (CTRL, C). Signal intensities in microarray data was normalized using the 75th percentile value and log2-transformation. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA. For identification of the target genes, see Additional file 9.

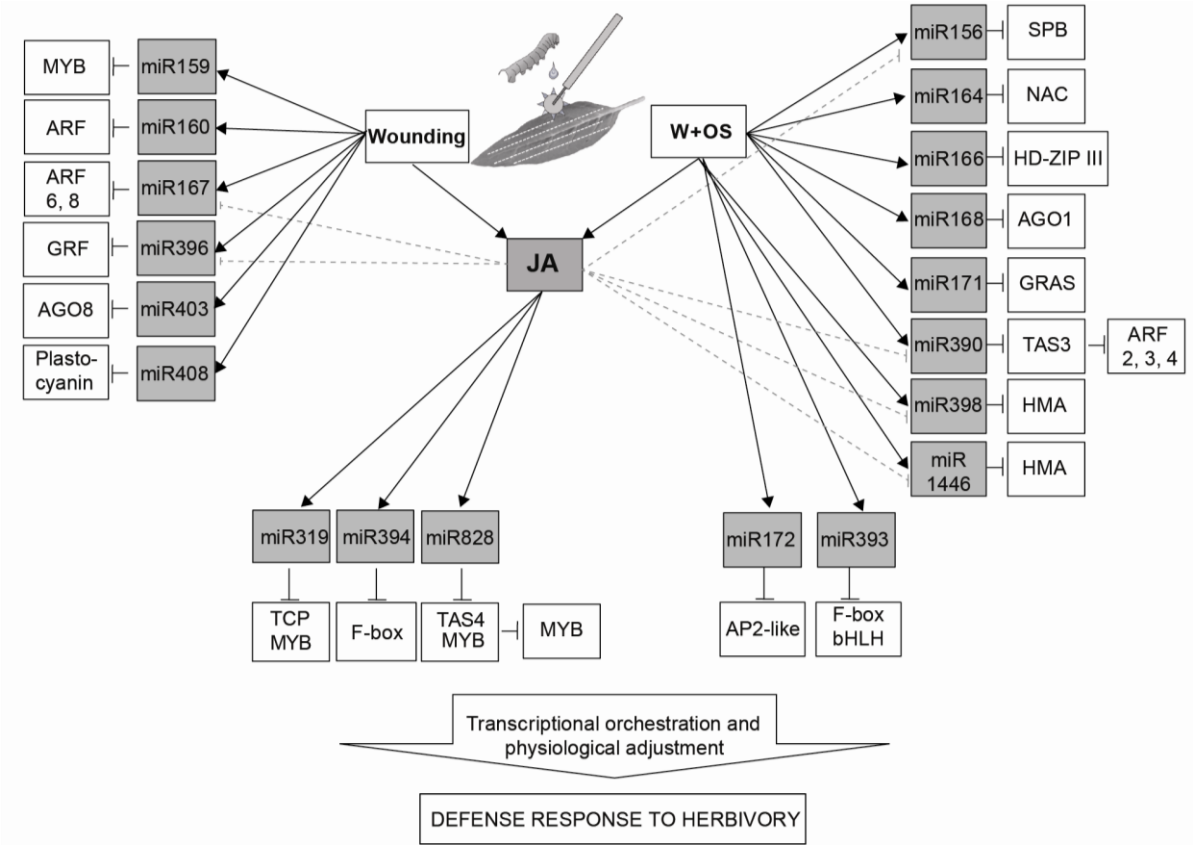

Figure 7. Summary of herbivory-responsive miRNAs and tasiRNAs in *N. attenuata*.

Conclusion

In order to build a database of plant miRNAs functionally involved in plant-insect interactions, we investigated the accumulations of miRNAs and their targets in *N. attenuata* after OS-elicitation. We classified W+OS-induced miRNAs and tasiRNAs into four groups: JA-dependent or –independent wound-inducible and OS-inducible miRNAs or tasiRNAs (Figure 7). Herbivore-attacked *N. attenuata* plants induce defense metabolites and tune their physiology to tolerate insect attack. This study shows that W+OS elicitation, a rigorous means of mimicking herbivore attack, rapidly changed the expression of miRNAs involved in flowering time, root morphology, senescence, hormone regulation, and metabolite synthesis.

In future work, we will experimentally characterize the function of these W+OS regulated JA-dependent and JA-independent miRNAs and their targets.

Methods

Plant material and growth conditions

WT and JA-deficient ir-*aoc* RNAi lines of the 30th inbred generation of the *N. attenuata* (Torrey ex. Watson) (originally collected in Southwestern Utah, USA) were used for the experiments. Seeds were germinated on MS basal medium supplemented with vitamin B5 (GB5, Duchefa, http://www.duchefa.com). For treatment, the leaf at the +1 node of the rosette (youngest fully-expanded leaf) was wounded with a fabric pattern wheel, and either 20 uL MilliQ water (W+W) or 20 uL *M. sexta* OS (diluted 5 x with sterile water) (W+OS) were applied. Leaves at the same position from untreated plants served as controls. Plants were grown in a glasshouse under 16/8 h (long day) (26-28^{o}C) supplemental light from Master Suns-U PIA Agro 400-W sodium lights.

Identification of miRNAs and precursors, and prediction of miRNA targets

To identify conserved miRNA and siRNA, we blasted all conserved plant miRNAs present in miRBase (www.mirbase.org) against *N. attenuata*'s in house 454-transriptome database using default search parameters allowing 1 or 2 mismatches (Figure 1A). Hits of 20 to 24 nt sequences against non-coding transcripts with up to four nucleotide mismatches were selected as candidates to check for predicted miRNA secondary structure. The public web-based mFOLD server (http://mfold.rna.albany.edu/) was used to predict secondary stem-and-loop structures using default parameters, folding temperature (37^{0}C) and ionic conditions (1MNaCl) with minimum free energy (MFE) formations (Table 1). Predicted miRNA-precursors were additionally depicted using RNAshapes (http://bibiserv.techfak.uni-bielefeld.de/rapidshapes) [60] to create stem-and-loop structures (Additional file 1). For *TAS3* identification we used the following *TAS3* orthologs: *Saccharum officinarum* (EU327139), *Sorghum bicolor* (EU327137), *Zea*

mays (EU327127, EU293143), *Oryza barthii* (GQ420228), *Triticum aestivum* (EU327134), *A. thaliana TAS3a* (AT3G17185), *TAS3b* (AT5G49615), *TAS3c* (At5g57735), *Nicotiana tabacum* (FJ804751, FJ804743), *Nicotiana benthamiana* (FJ804742), *Datura stramonium* (FJ804744). A neighbor-joining tree was built for *TAS3* transcripts using MEGA4 with group evaluation and 1000 bootstrap replicates [61].

RNA extraction and RNA blot hybridization

RNA blot hybridization was performed as described by Molnar et al. (2007) [62]. Extracted total RNA was treated with DNase I (Fermentas; http://www.fermentas.com) according to the manufacturer's protocol. Enzymes were removed by phenol-chloroform extraction and total RNA was re-isolated by ethanol precipitation. A denaturing 15% polyacrylamid gel containing 7M Urea was prepared using the BIORAD Mini-Protean 3 Cell system (http://www.bio-rad.com). Forty µg of total RNA were denatured with an equal volume of 2x gel-loading dye (Fermentas, http:/www.fermentas.de) at 65^{o}C for 5 minutes. Denatured RNA samples were loaded into the prerun 15% denaturing polyacrylamid gel at 50 V. A ZR small-RNA^{UM} Ladder (Zymo Research, http://www.zymoresearch.com) was also loaded. The gel was run at 150 V until the bromphenol blue in the loading dye reached the bottom of the gel. Next, the portion of the gel containing small RNA (between the bromphenol blue and xylene cyanol) was cut out and blotted onto a nylon membrane (Nytran Supercharge; http://www.whatman.com) by capillary wet transfer overnight. Transferred RNA was crosslinked to the membrane with UV light at 1200 µJ (UV Stratalinker 2400, Stratagene; http://www.stratagene.com). The membrane was pre-hybridized in 5-10 mL of ULTRAhyb-Oligo Hybridization Buffer (http://www.ambion.com) at 40^{o}C for at least 30 min. DNA oligo probes (Additional file 2) were labeled using ^{32}P with U4 polynucleotide kinase (Fermentas; http://www.fermentas.com). The reaction mix was incubated at 37^{o}C for 1 h. Unincorporated nucleotides were separated using a Microspin G-25 column according to the manufacturer's instructions (GE Healthcare,

http://www.gelifesciences.com). The labeled probe was mixed with the hybridization solution and hybridized to the membrane at 40°C overnight. The membrane was washed with a washing solution (2xSSC, 0.1% SDS) at 40°C for 10 minutes, wrapped with plastic saran wrap, and exposed to phospho-imaging plates.

Quantitative real-time PCR (qPCR) and cDNA microarray

One µg of total RNA were reverse-transcribed using SuperScript® II reverse transcriptase following the manufacturer's protocol (Invitrogen; http://www.invitrogen.com). Twenty µg of cDNA was used to perform quantitative real-time PCR with SYBR Green using gene-specific primers (Additional files 4 and 10) designed for *MIR* and target genes. Elongation factor (*NaEF*) was used as reference house-keeping gene for analysis.

For reverse transcription of miRNA and tasiRNAs into cDNA, we used the miScript Reverse Transcription Kit (Qiagen; http://www.qiagen.com). 10 µg of total RNA were used for qPCR (Additional file 5) with the miScript SYBR Green PCR Kit (Qiagen; http://www.qiagen.com) to quantify miRNAs and tasiRNAs. All qPCR data were analyzed using the 2ΔΔCt calculation method [63].

We used a cDNA microarray NCBI GEO database (Platform GPL13527, accession number GSE30287) [46]. For data analyses, raw data was normalized to the 75th percentile and log2-transformed. Comparisons with greater than a 2-fold change were tested by Fisher's PLSD test following an ANOVA.

Phytohormone analysis

JA and JA-Ile were co-extracted from leaf tissue as previously described [64]. One hundred to 150 mg of lamina tissue from control and treated plants were used for phytohormone extraction with 1 ml of ethyl acetate spiked with 200 ng of D_2-JA, and 40 ng of $^{13}C_6$-JA-Ile as internal standards. Fifteen µL of the supernatant were analyzed on a Varian 1200 L Triple-Quadrapol-MS with a ProntoSIL column (C18; 5 µm, 50 × 2 mm).

Statistical Analysis

Data were calculated with the StatView Software using the one-way analysis of variance ANOVA (means were compared by the lowest standard deviation (LSD)) algorithm (SAS Institute Inc., Cary, NC, USA).

Authors' contributions

TAB performed the experimental work and analyzed the data. TAB, ITB and SGK participated in the design of the study. ITB and SGK conceived of the study and edited the manuscript. TAB drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, Qi Y: **DNA methylation mediated by a microRNA pathway**. *Mol Cell* 2010, **38**(3):465-475.
2. Ronemus M, Martienssen R: **RNA interference: methylation mystery**. *Nature* 2005, **433**(7025):472-473.
3. Chellappan P, Xia J, Zhou X, Gao S, Zhang X, Coutino G, Vazquez F, Zhang W, Jin H: **siRNAs from miRNA sites mediate DNA methylation of target genes**. *Nucleic Acids Res* 2010, **38**(20):6883-6894.
4. Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W: **Transcriptional control of gene expression by microRNAs**. *Cell* 2010, **140**(1):111-122.
5. Bartel DP: **MicroRNAs: genomics, biogenesis, mechanism, and function**. *Cell* 2004, **116**(2):281-297.
6. Henderson IR, Zhang X, Lu C, Johnson L, Meyers BC, Green PJ, Jacobsen SE: **Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning**. *Nat Genet* 2006, **38**(6):721-725.
7. Kurihara Y, Watanabe Y: ***Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions**. *Proc Natl Acad Sci U S A* 2004, **101**(34):12753-12758.
8. Ruiz-Ferrer V, Voinnet O: **Roles of plant small RNAs in biotic stress responses**. *Annu Rev Plant Biol* 2009, **60**:485-510.
9. Rajagopalan R, Vaucheret H, Trejo J, Bartel DP: **A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana***. *Genes Dev* 2006, **20**(24):3407-3425.
10. Xie Z, Allen E, Wilken A, Carrington JC: **DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana***. *Proc Natl Acad Sci U S A* 2005, **102**(36):12984-12989.
11. Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A: **miR390, *Arabidopsis* TAS3 tasiRNAs, and their**

AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *Plant Cell* 2010, **22**(4):1104-1117.

12. Chen XM: **Small RNAs and their roles in plant development**. *Annu Rev Cell Dev Bi* 2009, **25**:21-44.
13. Willmann MR, Mehalick AJ, Packer RL, Jenik PD: **MicroRNAs regulate the timing of embryo maturation in *Arabidopsis***. *Plant Physiol* 2011, **155**(4):1871-1884.
14. Kidner CA, Martienssen RA: **Spatially restricted microRNA directs leaf polarity through ARGONAUTE1**. *Nature* 2004, **428**(6978):81-84.
15. Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D: **Control of leaf morphogenesis by microRNAs**. *Nature* 2003, **425**(6955):257-263.
16. Yoon EK, Yang JH, Lim J, Kim SH, Kim SK, Lee WS: **Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in *Arabidopsis* lateral root development**. *Nucleic Acids Res* 2010, **38**(4):1382-1391.
17. Liu Q, Chen YQ: **Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response**. *Biochem Biophys Res Commun* 2009, **384**(1):1-5.
18. Schommer C, Palatnik JF, Aggarwal P, Chetelat A, Cubas P, Farmer EE, Nath U, Weigel D: **Control of jasmonate biosynthesis and senescence by miR319 targets**. *PLoS Biol* 2008, **6**(9):e230.
19. Aukerman MJ, Sakai H: **Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes**. *Plant Cell* 2003, **15**(11):2730-2741.
20. Khraiwesh B, Zhu JK, Zhu J: **Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants**. *Biochim Biophys Acta* 2012, **1819**(2):137-148.

21. Kulcheski FR, de Oliveira LF, Molina LG, Almerao MP, Rodrigues FA, Marcolino J, Barbosa JF, Stolf-Moreira R, Nepomuceno AL, Marcelino-Guimaraes FC *et al*: **Identification of novel soybean microRNAs involved in abiotic and biotic stresses**. *BMC Genomics* 2011, **12**:307.
22. Xin M, Wang Y, Yao Y, Xie C, Peng H, Ni Z, Sun Q: **Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum L.*)**. *BMC Plant Biol* 2010, **10**:123.
23. Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L: **Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa***. *J Exp Bot* 2010, **61**(15):4157-4168.
24. Zhou X, Wang G, Zhang W: **UV-B responsive microRNA genes in *Arabidopsis thaliana***. *Mol Syst Biol* 2007, **3**:103.
25. Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su CL: **Regulation of phosphate homeostasis by MicroRNA in *Arabidopsis***. *Plant Cell* 2006, **18**(2):412-421.
26. Reyes JL, Chua NH: **ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination**. *Plant J* 2007, **49**(4):592-606.
27. Jones-Rhoades MW, Bartel DP: **Computational identification of plant microRNAs and their targets, including a stress-induced miRNA**. *Mol Cell* 2004, **14**(6):787-799.
28. Tang S, Wang Y, Li Z, Gui Y, Xiao B, Xie J, Zhu QH, Fan L: **Identification of wounding and topping responsive small RNAs in tobacco (*Nicotiana tabacum*)**. *BMC Plant Biol* 2012, **12**(1):28.
29. Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhang X, Fromuth N, Coutino G, Coffey M, Jin H: **Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks**. *Plant Mol Biol* 2011, **75**(1-2):93-105.

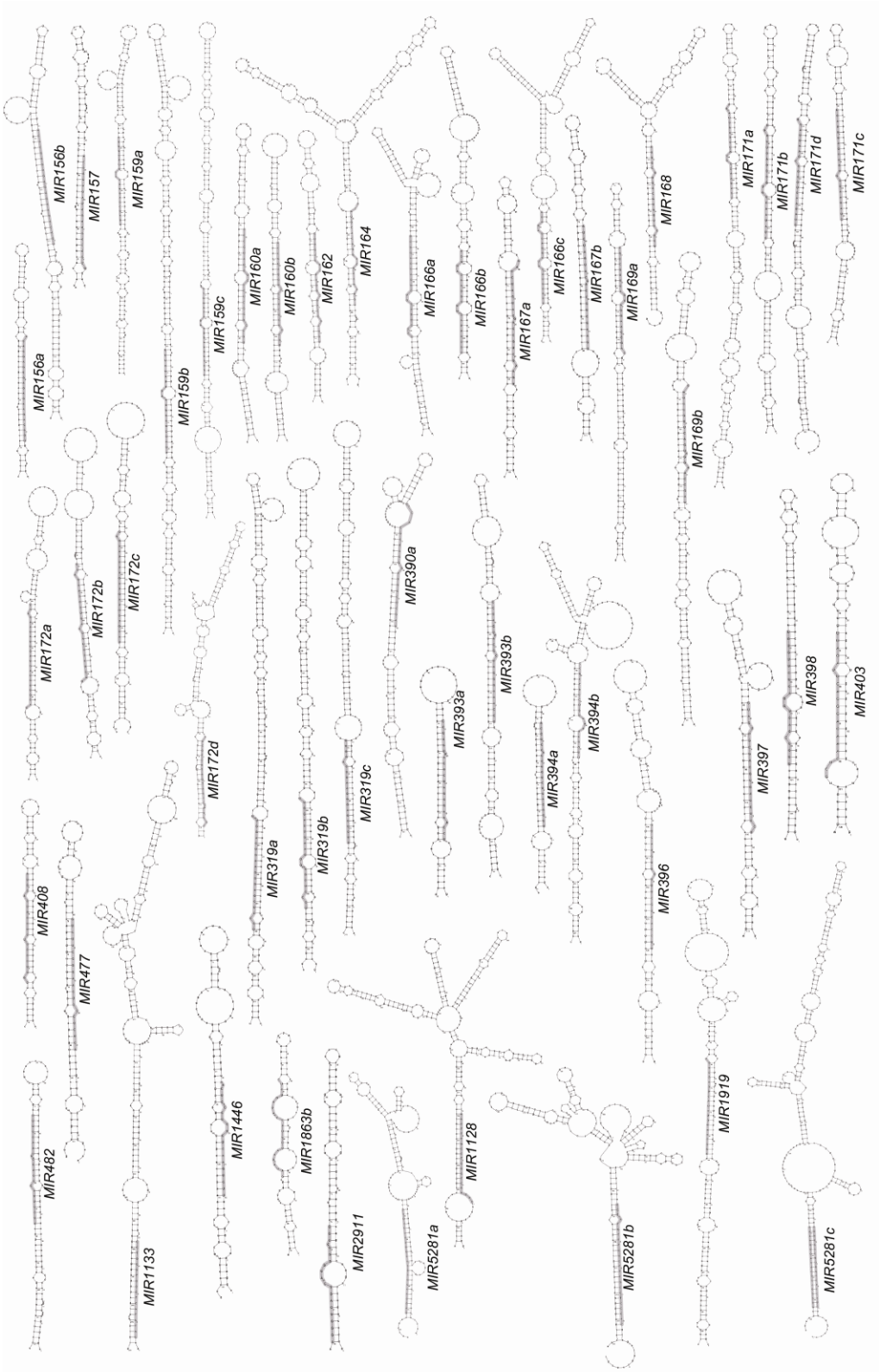
30. Navarro L, Jay F, Nomura K, He SY, Voinnet O: **Suppression of the microRNA pathway by bacterial effector proteins**. *Science* 2008, **321**(5891):964-967.
31. Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC: **P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function**. *Dev Cell* 2003, **4**(2):205-217.
32. Li H, Deng Y, Wu T, Subramanian S, Yu O: **Misexpression of miR482, miR1512, and miR1515 increases soybean nodulation**. *Plant Physiol* 2010, **153**(4):1759-1770.
33. Schwachtje J, Baldwin IT: **Why does herbivore attack reconfigure primary metabolism?** *Plant Physiol* 2008, **146**(3):845-851.
34. Kessler A, Baldwin IT: **Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata***. *Plant J* 2004, **38**(4):639-649.
35. Kang JH, Wang L, Giri A, Baldwin IT: **Silencing threonine deaminase and *JAR4* in *Nicotiana attenuata* impairs jasmonic acid-isoleucine-mediated defenses against *Manduca sexta***. *Plant Cell* 2006, **18**(11):3303-3320.
36. Halitschke R, Schittko U, Pohnert G, Boland W, Baldwin IT: **Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses**. *Plant Physiol* 2001, **125**(2):711-717.
37. Bonaventure G, VanDoorn A, Baldwin IT: **Herbivore-associated elicitors: FAC signaling and metabolism**. *Trends Plant Sci* 2011, **16**(6):294-299.
38. McCloud ES, Baldwin IT: **Herbivory and caterpillar regurgitants amplify the wound-induced increases in jasmonic acid but not nicotine in *Nicotiana sylvestris***. *Planta* 1997, **203**(4):430-435.

39. Halitschke R, Ziegler J, Keinanen M, Baldwin IT: **Silencing of hydroperoxide lyase and allene oxide synthase reveals substrate and defense signaling crosstalk in *Nicotiana attenuata***. *Plant J* 2004, **40**(1):35-46.
40. Kallenbach M, Alagna F, Baldwin IT, Bonaventure G: ***Nicotiana attenuata* SIPK, WIPK, NPR1, and fatty acid-amino acid conjugates participate in the induction of jasmonic acid biosynthesis by affecting early enzymatic steps in the pathway**. *Plant Physiol* 2010, **152**(1):96-106.
41. Paschold A, Halitschke R, Baldwin IT: **Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses**. *Plant J* 2007, **51**(1):79-91.
42. Pandey SP, Shahi P, Gase K, Baldwin IT: **Herbivory-induced changes in the small-RNA transcriptome and phytohormone signaling in *Nicotiana attenuata***. *Proc Natl Acad Sci U S A* 2008, **105**(12):4559-4564.
43. Liu Y, Gao Q, Wu B, Ai T, Guo X: **NgRDR1, an RNA-dependent RNA polymerase isolated from *Nicotiana glutinosa*, was involved in biotic and abiotic stresses**. *Plant Physiol Biochem* 2009, **47**(5):359-368.
44. Pandey SP, Baldwin IT: **RNA-directed RNA polymerase 1 (RdR1) mediates the resistance of *Nicotiana attenuata* to herbivore attack in nature**. *Plant J* 2007, **50**(1):40-53.
45. Bozorov TA, Prakash Pandey S, Dinh ST, Kim SG, Heinrich M, Gase K, Baldwin IT: **DICER-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata***. *J Integr Plant Biol* 2012, **54**(3):189-206.
46. Kim SG, Yon F, Gaquerel E, Gulati J, Baldwin IT: **Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco, *Nicotiana attenuata***. *PLoS One* 2011, **6**(10):e26214.
47. Kallenbach M, Bonaventure G, Gilardoni PA, Wissgott A, Baldwin IT: **Empoasca leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations**. *Proc Natl Acad Sci U S A* 2012, 109(24): 1548-1557.

48. Liang C, Zhang X, Zou J, Xu D, Su F, Ye N: **Identification of miRNA from *Porphyra yezoensis* by high-throughput sequencing and bioinformatics analysis**. *PLoS One* 2010, **5**(5):e10698.
49. Frazier TP, Xie F, Freistaedter A, Burklew CE, Zhang B: **Identification and characterization of microRNAs and their target genes in tobacco (*Nicotiana tabacum*)**. *Planta* 2010, **232**(6):1289-1308.
50. Allen E, Xie Z, Gustafson AM, Carrington JC: **microRNA-directed phasing during trans-acting siRNA biogenesis in plants**. *Cell* 2005, **121**(2):207-221.
51. Shen D, Wang S, Chen H, Zhu QH, Helliwell C, Fan LJ: **Molecular phylogeny of miR390-guided trans-acting siRNA genes (*TAS3*) in the grass family**. *Plant Syst Evol* 2009, **283**(1-2):125-132.
52. Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouche N, Gasciolli V, Vaucheret H: **DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7**. *Curr Biol* 2006, **16**(9):927-932.
53. Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG: **Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in *Arabidopsis***. *Science* 2009, **323**(5917):1053-1057.
54. Schwachtje J, Minchin PE, Jahnke S, van Dongen JT, Schittko U, Baldwin IT: **SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots**. *Proc Natl Acad Sci U S A* 2006, **103**(34):12935-12940.
55. Halitschke R, Baldwin IT: **Antisense *LOX* expression increases herbivore performance by decreasing defense responses and inhibiting growth-related transcriptional reorganization in *Nicotiana attenuata***. *Plant J* 2003, **36**(6):794-807.
56. Luo QJ, Mittal A, Jia F, Rock CD: **An autoregulatory feedback loop involving PAP1 and TAS4 in response to sugars in *Arabidopsis***. *Plant Mol Biol* 2011, doi 10.1007/s11103-011-9778-9.
57. Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis I: **R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are**

essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiol* 2010, **152**(3):1731-1747.

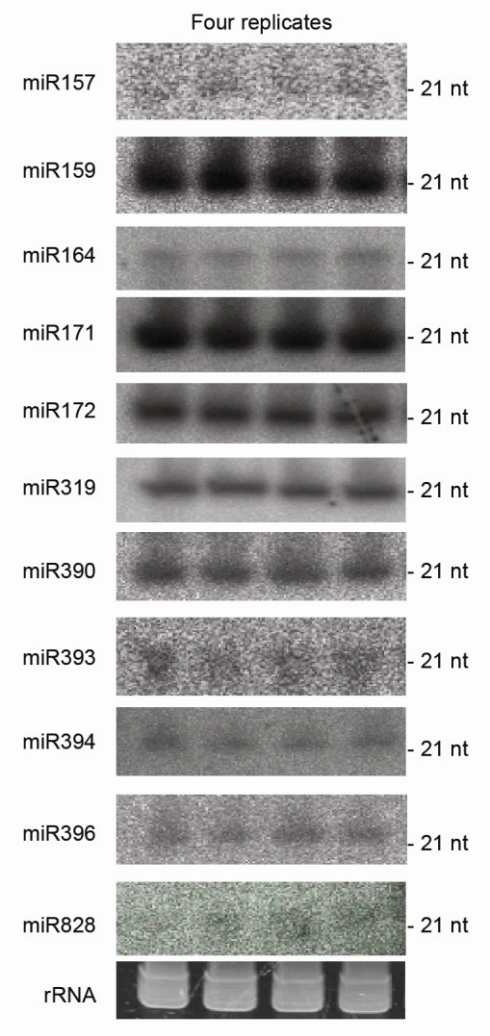
58. Onkokesung N, Gaquerel E, Kotkar H, Kaur H, Baldwin IT, Galis I: **MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-coenzyme A:polyamine transferases in *Nicotiana attenuata***. *Plant Physiol* 2012, **158**(1):389-407.
59. Schwab R, Voinnet O: **miRNA processing turned upside down**. *EMBO J* 2009, **28**(23):3633-3634.
60. Steffen P, Voss B, Rehmsmeier M, Reeder J, Giegerich R: **RNAshapes: an integrated RNA analysis package based on abstract shapes**. *Bioinformatics* 2006, **22**(4):500-503.
61. Tamura K, Dudley J, Nei M, Kumar S: **MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0**. *Mol Biol Evol* 2007, **24**(8):1596-1599.
62. Molnar A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC: **miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii***. *Nature* 2007, **447**(7148):1126-1129.
63. Bubner B, Gase K, Baldwin IT: **Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR**. *BMC biotechnology* 2004, **4**:14.
64. Wang L, Halitschke R, Kang JH, Berg A, Harnisch F, Baldwin IT: **Independently silencing two *JAR* family members impairs levels of trypsin proteinase inhibitors but not nicotine**. *Planta* 2007, **226**(1):159-167.



Additional file 1. Stem-and-loop structures of identified miRNAs in *N. attenuata*.

Additional file 2. List of Small RNA probes used for RNA blot hybridization.

Primer	Sequence
RM157-22	GTTGCTCTCTATCTTCTGTCAA
RM159-21	TAGAGCTCCCTTCAATCCAAA
RM164-21	TGCACGTGCCCTGCTTCTCCA
RM171-21	GATATTGGCACGGCTCAATCA
RM172-23	ATGCAGCATCATCAAGATTCTCA
RM319-21	AGGAGCTCCCTTCAGTCCAA
RM390-21	GGTGCTATCCCTCCTGAGCTT
RM393-22	GGATCAATGCGATCCCTTTGGA
RM394-22	ATGGAGGTGGACAGAATGCCAA
RM396-21	CTTCCCACAGCTTTATTGAAC
RM828-22	TGGAATACTCATTTGAGCAAGA



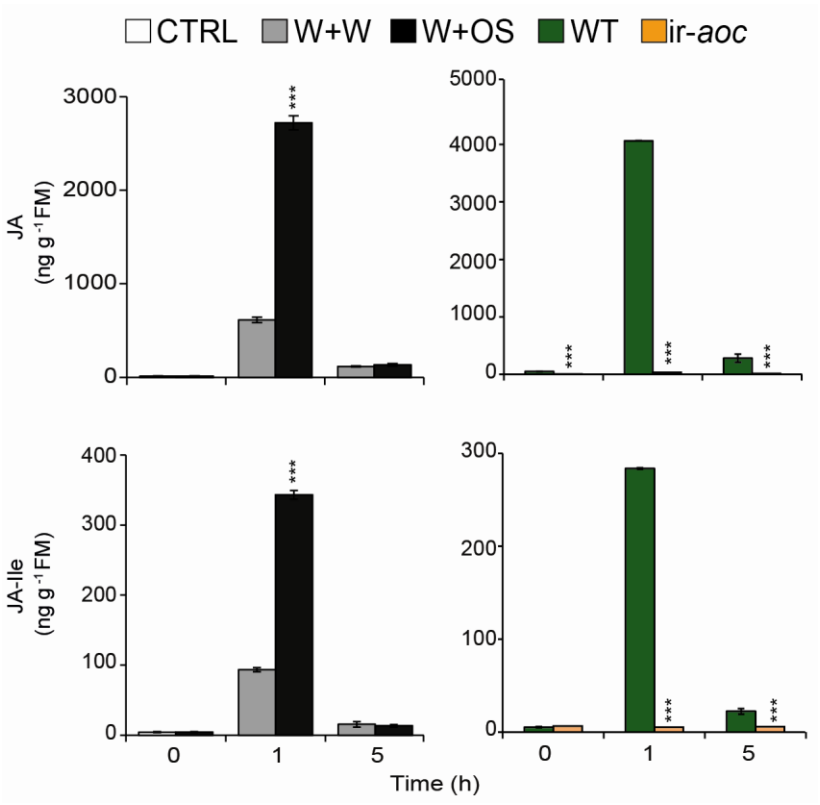
Additional file 3. Accumulation of several miRNAs in rosette leaves of *N. attenuata*. RNA blot hybridization performed to examine the accumulation of miRNAs in rosette leaves of *N. attenuata*. Ethidium bromide staining of rRNA is shown as a loading control.

Additional file 4. List of primers used for qPCR analysis of primary miRNAs.

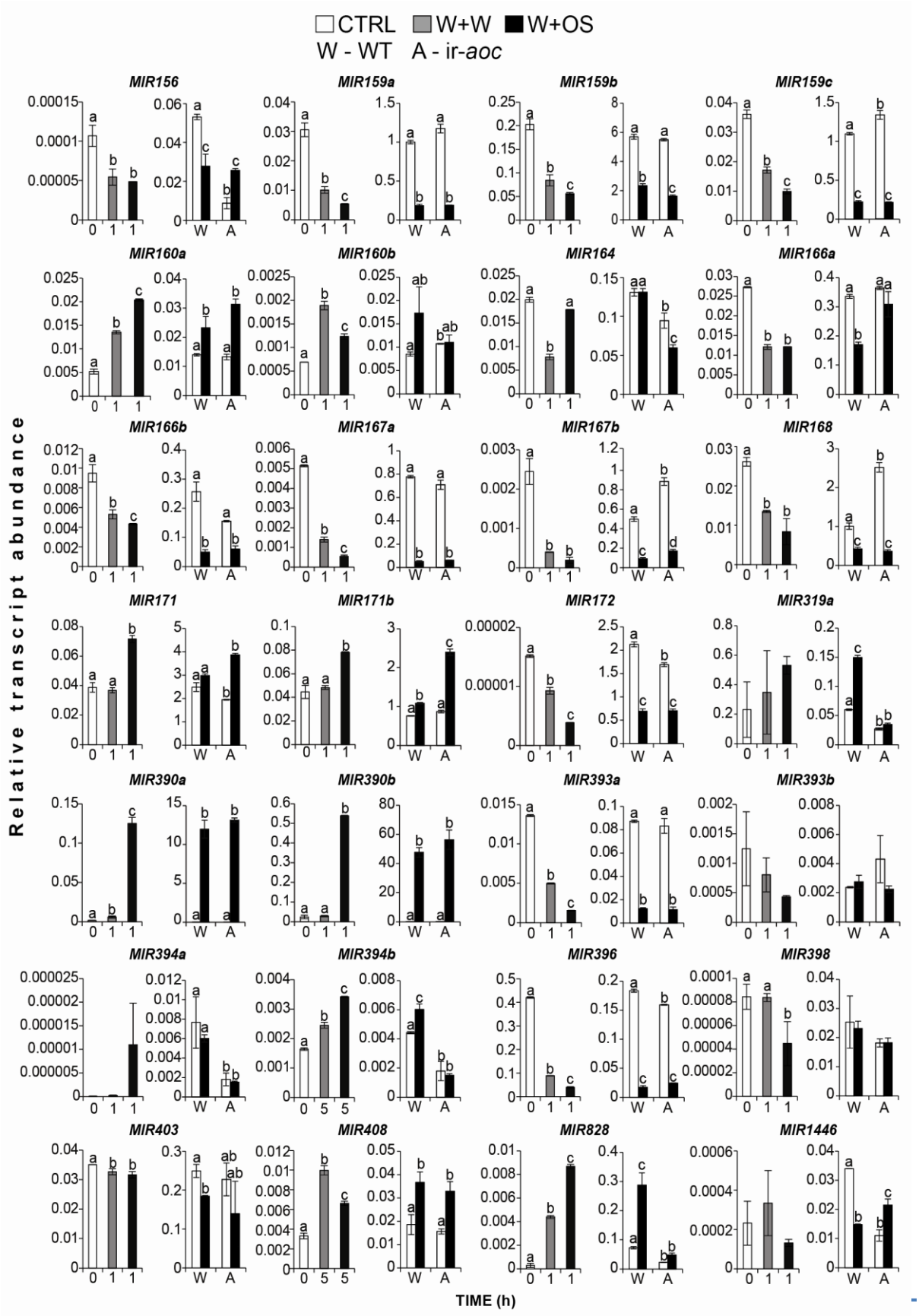
Primer	sequence	Primer	sequence
PCR156A-PF-24	acagaagagagtgagcacacatgg	*PCR319C-PF-24*	agtagcggcaagagctgtcatgt
PCR156A-PR-24	agtgtgacagatagagagcgagca	*PCR319C-PR-24*	agtagttgctctcccgtcatgcaa
PCR159A-PF-26	gagtatctaactggtttgtggcttgc	*PCR390A-PF-24*	gcggttatgtggaaattgcatggg
PCR159A-PR-25	gaagagagatgaatgtagagctccc	*PCR390A-PR-24*	aaactcaggatggatagcgcaacg
PCR159B-PF-26	acctatggatccctcagccctatcta	*PCR390B-PF-24*	aaagctcaggagggatagcaccat
PCR159B-PR-25	gaagagagatgaatgtagagctccc	*PCR390B-PR-24*	aggccatgaaactcaggatggaga
PCR159C-PF-26	gagtatctaactggtttgtggcttgc	*PCR393A-PF-24*	gtggagtattccaaagggatcgca
PCR159C-PR-25	gaagagagatgaatgtagagctccc	*PCR393A-PR-28*	ggagaaatccgaagagatcgcatg atcc
PCR160A-PF-24	atacgtatatgcctggctccctgt	*PCR393B-PF-23*	tggtcttcccagcaactgaagga
PCR160A-PR-24	tatgcttggctcctcatacgccat	*PCR393B-PR-24*	agctccaaaggatgagtccaaagg
PCR160B-PF-24	tgagaaacttggatgtgcctggct	*PCR394A-PF-24*	ttggcattctgtccacctccattg
PCR160B-PR-24	aatgtggtatgcttggctccttgc	*PCR394A-PR-26*	tttgcagagctagtttggcactctgg
PCR164-PF-24	agcagggcacgtgcattactaact	*PCR394B-PF-24*	tctttggcattctgtccacctcct
PCR164-PR-29	gggaagacaggcacatgaagaact aactc	*PCR394B-PR-24*	tggcagtatgcccacctccataat
PCR166A-PF-24	gaggagaatgttgtctggttcgag	*PCR396-PF-25*	ccacagctttcttgaactgcatctg
PCR166A-PR-24	ggaatgaagcctggtccgaaatca	*PCR396-PR-24*	cccacagctttattgaaccgcaac
PCR166B-PF-24	gggaatgttgtttggctcgaggat	*PCR398-PF-24*	cagaggagtgaacatgggaacac a
PCR166B-PR-26	aatgaagcctggtccgacgataccaa	*PCR398-PR-24*	ggtgacctgagaacacaagtgcat
PCR166C-PF-24	ggaatgttgtctggctcgaggaat	*PCR403-PF-24*	cgtttgtgcgtgattctgacaacc
PCR166C-PR-21	aatgaagcctggtccgacgac	*PCR403-PR-24*	gtgcgtgaatctaacaaacacaac c
PCR167A-PF-24	aggtgatgctgccacatgatctga	*PCR408-PF-25*	agaggatagacagggacgaggta ga
PCR167A-PF-24	accactagtagttgaagctgcc	*PCR408-PR-27*	agccagggaagaggcagtgcata gaat
PCR167B-PF-24	acaccactatcagttgaagctgcc	*PCR828-PF-24*	cctttcttgtgaaggcctcttgct
PCR167B-PR-24	accaactaacgggtgaagctacca	*PCR828-PR-26*	cttcatgcccaaatgagtatctcaag
PCR168-PF-24	actgaatcggagactgcggtgaat	*PCR1446-PF-24*	tctctccctcaatggctgctcata
PCR168-PR-24	actgttaccacacagcacagccta	*PCR1446-PR-24*	ctgaactcaatctctcatggctgt
PCR171A-PF-24	ggtgcggttcaatgagaaagcagt	*PCRTAS3A-F-24*	gtgctcgaagtcatggttcgtcta
PCR171A-PR-24	ttgctacacgtgatattggcacgg	*PCRTAS3A-R-24*	agctcaggaaaggataacaccgc a
PCR171B-PF-24	ggtgcggttcaatgagaaagcagt	*PCRTAS3B-F-24*	accttgcaagtccgaggtcttctt
PCR171B-PR-24	tagtgacacgtgatattggcacgg	*PCRTAS3B-R-25*	gctcagaagggatagaaatgagac g
PCR172A-PF-26	gcagctgcagcatcatcaagattcac	*PCRTAS3C-F-24*	acctagccgagtcttcttgacctt
PCR172A-PR-23	cagcatcatcaagattctcacg	*PCRTAS3C-R-24*	aggagaacatgagttgagcggga a
PCR319A-PF-24	agggatttggattagctgccgact	*PCRTAS4-PF-25*	ggagtattgagaaacagaagggcc a
PCR319A-PR-24	acctcccgcatcattcacacattc	*PCRTAS4-PR-26*	gctacatcatcctcgtatcttcatcg
PCR319B-PF-24	acacatgggcggtgataaggttca		

Additional file 5. SmRNA-specific forward primers used for miScript qPCR.

Primer	Sequence
FM156-21	TGACAGAAGAGAGTGAGCACA
FM159-21	TTTGGATTGAAGGGAGCTCTA
FM160-21	TGCCTGGCTCCCTGTATGCCA
FM164-21	TGGAGAAGCAGGGCACGTGCA
FM166-21	TCGGACCAGGCTTCATTCCCC
FM167-21	TGAAGCTGCCAGCATGATCTA
FM168-21	CCCGCCTTGCATCAACTGAAT
FM171-21	TGATTGAGCCGTGCCAATATC
FM172-23	TGAGAATCTTGATGATGCTGCAT
FM319-21	TTGGACTGAAGGGAGCTCCCT
FM390-21	AAGCTCAGGAGGGATAGCACC
FM393-22	TCCAAAGGGATCGCATTGATCC
FM394-22	TTGGCATTCTGTCCACCTCCAT
FM396-21	GTTCAATAAAGCTGTGGGAAG
FM398-21	TGTGTTCTCAGGTCACCCCTT
FM403-21	TTAGATTCACGCACAAACTCG
FM408-21	ATGCACTGCCTCTTCCCTGGC
FM828-22	TCTTGCTCAAATGAGTATTCCA
FM1446-20	TTCTGAACTCTCTCCCTCAA
FMTAS3	TTCTTGACCTTGTAAGACCTTTT MTTGACCTTGTAAGACCCC
FMTAS4-22	AACCTCAACCTCGGACCTTCAT

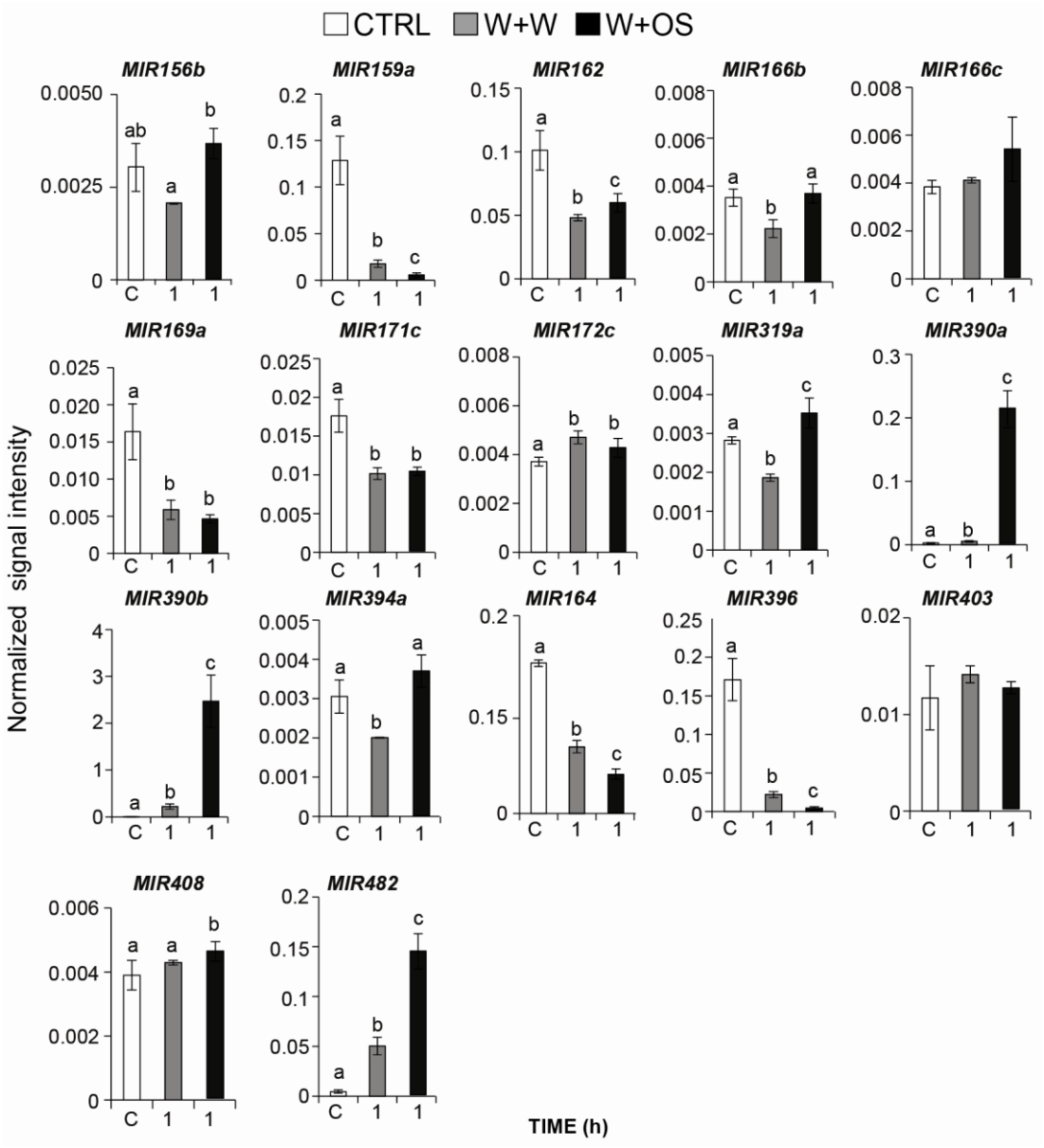


Additional file 6. JA and JA-Ile levels were impaired in ir-*aoc* plants after elicitation. Accumulation of Jasmonic acid (JA) and jasmonoyl isoleucine (JA-Ile) increased in W+W and W+OS treated leaves and these accumulations were dramatically altered in ir-*aoc* plants. For each sample, one leaf on the rosette of a 32-day-old plant was treated and harvested 1 h or 5 h post treatment. Untreated plants served as control. Asterisks indicate significant differences (***, $P < 0.001$) in Fisher's PLSD tests following an ANOVA.


CTRL W+W W+OS
W - WT A - ir-aoc
MIR156
MIR159a
MIR159b
MIR159c
MIR160a
MIR160b
MIR164
MIR166a
MIR166b
MIR167a
MIR167b
MIR168
MIR171
MIR171b
MIR172
MIR319a
MIR390a
MIR390b
MIR393a
MIR393b
MIR394a
MIR394b
MIR396
MIR398
MIR403
MIR408
MIR828
MIR1446
Relative transcript abundance
TIME (h)

Additional file 7. qPCR data showing accumulation of primary miRNA transcripts in W+W and W+OS treated leaves in wild type and ir-*aoc.*

For each sample, one leaf on the rosette of a 32-day-old plants was treated with wounding plus water (W+W) or wounding plus OS (W+OS) and harvested 1 h or 5 h post treatment. Untreated plants served as control (CTRL). Shown are mean (± SE) levels of three replicates. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA.



Additional file 8. Microarray data showing accumulation of primary miRNAs in W+W and W+OS treated leaves of *N. attenuata*.
Mean (± SE) levels of normalized transcript abundance of miRNAs and tasiRNAs target genes after W+W and W+OS treatments. Untreated plants served as control (CTRL). Signal intensities in microarray data was normalized using the 75th percentile value and log2-transformation. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA.

Additional file 9. List of putative miRNA targets containing miRNA binding site.

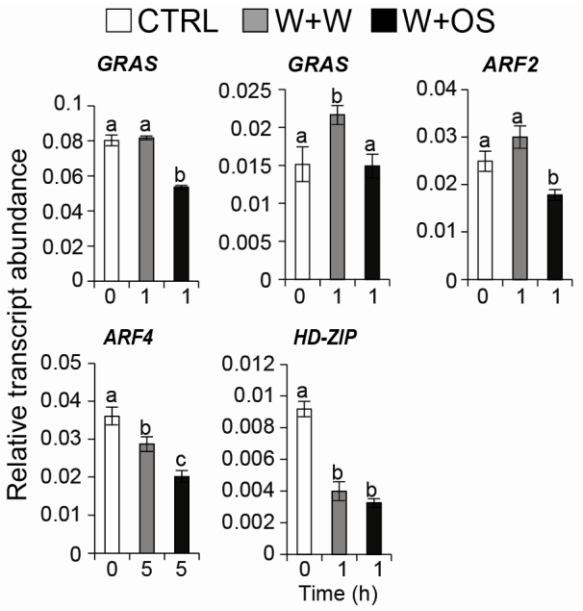
Nat-miRNA and tasiRNA	Putative targets. ID number in 454-cDNA library	BLAST result	Protein
Nat-miR156	Na_454_14995	XP_002271312	Squamosa promoter-binding-like protein 12-like
Nat-miR156	Na_454_32681	n/a	
Nat-miR156	Na_454_32681	n/a	
Nat-miR156	Na_454_40776	n/a	
Nat-miR156	Na_454_11145	XP_002273534	Squamosa promoter-binding-like protein 6
Nat-miR156	Na_454_16374	XP_002270226	Squamosa promoter-binding-like protein 5
Nat-miR156	Na_454_11778 (SBPb)	XP_002275728	Squamosa promoter-binding-like protein 3
Nat-miR156	Na_454_12952	XP_002444771	Squamosa promoter-binding-like protein 17
Nat-miR156	Na_454_29611	XP_002265203	Squamosa promoter-binding-like protein 6-like
Nat-miR156/157	Na_454_16237 (SBPc)	XP_002271312	Squamosa promoter-binding-like protein 12-like
Nat-miR156/157	Na_454_17671 (SBPa)	ACL51015	Squamosa promoter-binding protein
Nat-miR157	Na_454_25255	n/a	
Nat-miR157	Na_454_14252	XP_002280052	Squamosa promoter-binding protein 1
Nat-miR159	Na_454_05653	n/a	
Nat-miR159	Na_454_23594	CAN68216	Hypothetical protein
Nat-miR159	Na_454_29216	n/a	
Nat-miR159/319	Na_454_32439	XP_002298147	GAMYB-like2
Nat-miR159/319	Na_454_11279 (MYB)	XP_002313492	GAMYB
Nat-miR160	Na_454_42902	NP_001234237	Auxin response factor 17
Nat-miR160	Na_454_09731	XP_002273590	Auxin response factor 18
Nat-miR160	Na_454_08900	XP_002519531	Auxin response factor 18
Nat-miR162	Na_454_22807	XP_002303937	Hypothetical protein
Nat-miR164	Na_454_07162	AAT40107	UDP-glucuronic acid decarboxylase 1
Nat-miR164	Na_454_24855	n/a	
Nat-miR164	Na_454_29588	AAQ75123	Salicylic acid-induced protein 19
Nat-miR164	Na_454_38519	AFB82644	NAC transcription factor
Nat-miR164	Na_454_10250	NP_001048872	NAC domain-containing protein 61
Nat-miR164	Na_454_06449	XP_002528436	UDP-glucuronic acid decarboxylase 1
Nat-miR164	Na_454_01423	XP_002528437	UDP-glucuronic acid decarboxylase 1
Nat-miR166	Na_454_10320 (HD-ZIP III)	XP_002338824	Class III HD-Zip protein HDZ32A
Nat-miR166	Na_454_01017	NP_001042315	Homeobox-leucine zipper protein HOX29
Nat-miR167	Na_454_05475	XP_002300855	Auxin response factor 8-1
Nat-miR167	Na_454_39350	XP_002307573	Auxin response factor 8
Nat-miR167	Na_454_00863 (ARF6)	XP_002279808	Auxin response factor 6

Nat-miR167	Na_454_01157	XP_002279808	Auxin response factor 6
Nat-miR167	Na_454_08712 (ARF8)	XP_002266678	Auxin response factor 8
Nat-miR168	Na_454_08455	AAC31213	Ethylene receptor homolog
Nat-miR169	Na_454_25841	XP_002521352	Nuclear transcription factor Y subunit A-3, putative
Nat-miR169	Na_454_14069	CAM32010	Nuclear transcription factor Y subunit A-5, putative
Nat-miR171	Na_454_03756 (GRASa)	XP_002266783	Scarecrow-like protein 6
Nat-miR171	Na_454_17764 (GRASb)	XP_002329873	GRAS family transcription factor
Nat-miR172	Na_454_27816	ACY30435	Apetala 2-like protein
Nat-miR172	Na_454_29286	ACY30435	Apetala 2-like protein
Nat-miR172	Na_454_30374	NP_001233891	Apetala 2-like protein
Nat-miR172	Na_454_40525	ACY30435	Apetala 2-like protein
Nat-miR172	Na_454_27216 (AP-like a)	NP_001233886	AP2 transcription factor SlAP2c
Nat-miR172	Na_454_25470	XP_002322849	Floral homeotic protein APETALA 2
Nat-miR172	Na_454_06076 (AP-like b)	XP_002283045	Floral homeotic protein APETALA 2
Nat-miR390	Na_454_29794	FJ804743	TAS3-like
Nat-miR390	Na_454_41575	FJ804744	TAS3-like
Nat-miR390	Na_454_07467	FJ804751	TAS3-like
Nat-miR393	Na_454_03710	XP_002274892	Auxin signaling F-BOX 2
Nat-miR393	Na_454_10365	NP_001054666	Transport inhibitor response 1
Nat-miR394	Na_454_06761	XP_002514903	F-box only protein 6
Nat-miR396	Na_454_30538	AAT39964	Putative gag polyprotein
Nat-miR396	Na_454_39305	n/a	
Nat-miR396	Na_454_24031	XP_002309001	Putative calcium-transporting ATPase 13
Nat-miR396	Na_454_09390	XP_002264528	Galacturonokinase
Nat-miR397	Na_454_00207	XP_002322961	Laccase 4
Nat-miR397	Na_454_00220	XP_002322961	Laccase 4
Nat-miR397	Na_454_04194	XP_002284473	Laccase 2
Nat-miR397	Na_454_00782	XP_002270890	Laccase 17
Nat-miR397	Na_454_10174	XP_002276415	Laccase 7
Nat-miR397	Na_454_02461	XP_002276415	Laccase 7
Nat-miR397	Na_454_05011	XP_002276415	Laccase 7
Nat-miR397	Na_454_01174	XP_002273875	Laccase 5
Nat-miR397	Na_454_16697	XP_002449706	Laccase 2
Nat-miR397	Na_454_18235	XP_002512915	Laccase 11
Nat-miR397	Na_454_03266	XP_002531562	Laccase 4
Nat-miR397	Na_454_33395	XP_002988572	Laccase-like multicopper oxidase 61
Nat-miR397	Na_454_40396	XP_002988572	Hypothetical protein
Nat-miR399	Na_454_00149	XP_002267490	Probable ubiquitin-conjugating enzyme E2 24

Nat-miR403	Na_454_25058	n/a	
Nat-miR403	Na_454_06067	XP_002332150	Argonaute 8
Nat-miR408	Na_454_01388	XP_002280050	Probable copper-transporting ATPase SynA
Nat-miR408	Na_454_16396	XP_002272263	Uclacyanin-2
Nat-miR413	Na_454_19755	n/a	
Nat-miR413	Na_454_26978	CAC95024	Mitochondrial and plastid RNA polymerase
Nat-miR413	Na_454_12677	n/a	
Nat-miR413	Na_454_09634	XP_002265804	Tubby-like F-box protein 5-like
Nat-miR413	Na_454_21073	n/a	
Nat-miR413	Na_454_01186	n/a	
Nat-miR414	Na_454_05002	n/a	
Nat-miR482	Na_454_24200	n/a	
Nat-miR828	Na_454_29570	BAB02134	MYB family transcription factor-like
Nat-miR828	Na_454_16118	TAS4	
Nat-miR845a	Na_454_18106	n/a	
Nat-miR845a	Na_454_01587	CAB75469	Copia-type reverse transcriptase-like protein
Nat-miR845b	Na_454_23859	n/a	
Nat-miR1128	Na_454_25147	n/a	
Nat-miR1128	Na_454_34675	n/a	
Nat-miR1128	Na_454_38840	n/a	
Nat-miR1133	Na_454_22258	n/a	
Nat-miR1133	Na_454_42945	n/a	
Nat-miR1133	Na_454_04982	XP_002305044	Peroxisomal (S)-2-hydroxy-acid oxidase
Nat-miR1446	Na_454_00571	XP_002282335	Hypothetical protein
Nat-miR1446	Na_454_06733	XP_002285129	PRA1 family protein H
Nat-miR1863	Na_454_31457	n/a	
Nat-miR1863	Na_454_43165	n/a	
Nat-miR1863	Na_454_02660	XP_002282174	Uncharacterized mitochondrial protein
Nat-miR1863	Na_454_09857	XP_002270225	Polypeptide with a gag-like domain
Nat-miR1919	Na_454_19883	XP_002511778	Arsenical pump-driving ATPase
Nat-miR5281	Na_454_20896	NP_190270	Serine protease inhibitor/potato inhibitor I domain-containing protein
Nat-miR5281	Na_454_27481	n/a	
Nat-miR5281	Na_454_32219	n/a	
Nat-miR5281	Na_454_37131	n/a	
Nat-miR5281	Na_454_39764	n/a	
Nat-miR5281	Na_454_23395	AAM63021	GDSL-motif lipase/hydrolase-like protein
Nat-miR5281	Na_454_03064	BAH78126	Hypothetical protein
tasiRNA3	Na_454_00409	ABC69711	Auxin response factor 2
tasiRNA3	Na_454_02948	NP_001234316	Auxin response factor 3
tasiRNA3	Na_454_00881	NP_001233771	Auxin response factor 4

Additional file 10. Primer sequences of miRNA targets.

Primer	Sequence
PCRARF2-F-24	TAACTGCAGACCCTTCACAAGCCA
PCRARF2-R-22	CCACTGATGGTGGCCATAACAT
PCRARF4-F-26	TGGACTTTGAGGAGTCGGTAAGATCC
PCRARF4-R-24	TCCATAGGGAGGTGATATCAGACCTAA
PCRHDZIP2-F-24	ACCATTTGACATCTCAGCATCCGC
PCRHDZIP2-R-24	ACTCAACAGCAGTTCCAGTAGCCT
PCRGRAS-F-24	GGACAAATGCGGCAGCAAGTCAAT
PCRGRAS-R-24	CTGTTGTTGATGACGAGGCACCAT
PCRGRAS2-F-24	CCTCGTCATCAACAGCAGGAACAA
PCRGRAS2-R-26	GCGTTTGAGAATTGTCCGGCCAGTAA



Additional file 11. Transcript abundance of miRNA target genes.
Abundance of miRNA targets after W+W and W+OS treatments. For each sample, one leaf on the rosette of a 32-day-old WT plant was left untreated (control) or treated with wounding plus water (W+W) or wounding plus OS (W+OS) and harvested 1 h or 5 h post treatment. Shown are mean (± SE) levels of three replicates per line. Letters indicate significant differences ($P < 0.05$) in Fisher's PLSD tests following an ANOVA.

Chapter 6

General Discussion

Tohir A. Bozorov

GENERAL DISCUSSION

To understand the ecological functions of plant genes, we require systems to manipulate plant gene expression in wild plants in their native ecologies. We used the ecological model plant *Nicotiana attenuata* (wild coyote tobacco) which is a suitable system to study plant-plant, plant-herbivore and plant-pathogen interactions. Large transcriptional reconfigurations are involved in mediating these interactions. Reverse genetics is a powerful tool to understand the function of individual genes involved in such interactions. Tools based on T-DNA insertions are widely used in plants. In this dissertation, I discuss the most efficient protocol to produce genetically modified plants utilizable in ecological research. To take advantage of this tool, transgenic plants that fulfill the requirements for the structure and stable inheritance of the transgene need to be created and selected. The screening of transgenic plants is a costly and time-consuming procedure and must be optimized. In **manuscript I**, we developed efficient screening strategy of transformants for ecological studies.

Screening transgenes is an integral part of in obtaining appropriate stably-transformed transgenes. The following requirements must be considered: Transformation should not change the plant ploidy level. In contrast to *Arapidopsis*, for other plant species like tobacco, tomato, potato and cotton, the flower size prohibits stable transform procedures via floral dip transformation. In this case, stable transformation via somatic embryos using tissue cultures is required. To initiate callus requires the addition of exo-phytohormones to the basal medium: cytokinin and auxin. Long callus maintenance and misbalance of endo- and exo-phytohormones can change ploidy level in regenerated plants depending on plant species and method (Elkonin et al. 1993). Ten to twenty percent of transformants were found to be autotetraploids in *N. attenuata*. Twenty-four to eighty percent of diploid tomato was found to be tetraploid, depending on the cultivar (Ellul et al. 2003). Polyploidy occurrence can be visually determined in the regenerated plant

in early stage (T_0) by increased leaf thickness, larger leaf lamina, curly lamina apices and wider and longer flowers. The most efficient and conclusive method of determining ploidy levels is flow cytometry.

Lines must contain completely inserted T-DNA without vector backbone sequences and all inserted elements must be functional. Around 10 to 20% of lines transformed using *Agrobacterium* carry insertions with vector backbone sequences or incomplete insertions lacking functional elements, rendering the T-DNA non-functional. Incompletely inserted IR cassettes expressed as sense or antisense fragments without termination sites easily can be segregated without posttranscriptional modifications. I developed DNA diagnostics for detection of T-DNA insertion completeness which permits excluding lines with incomplete T-DNA insertions in early T_0 stages and avoiding putting effort into screening them.

Transformants must be homozygous and harbor a single T-DNA copy in a homozygous state. We have found that in most cases a single copy is sufficient for the expected phenotypic effect. T-DNA insertion should not change the phenotype by interrupting gene regulatory elements or other intron/exonic elements. To avoid this, plants must be transformed in several independent events with the same T-DNA and analyzed. To understand the actual function of gene of interest, it is recommended to use at least two lines among the screened independently transformed lines that must phenotypically correspond to each other. If any of them shows a different phenotype to the others, it should be excluded. Lines must produce or silence enough transcript copies of the transgene or target to demonstrate a strong phenotype.

Moreover, it is also important to design RNAi constructs which are specific to single targets. To avoid off-target effects, it is recommended to choose fragments for RNAi outside of regions which are conserved with other genes; even 21 conserved nucleotides can cause off-target effects. In addition, the determination of silencing efficiency requires primers designed outside of the RNAi fragment, but these primers sometimes give artificially low estimates of silencing efficiency. It is important to design the qPCR amplicon in the 5'- or 3'-prime regions

(Rajeswaran et al. 2012; Rajeswaran and Pooggin 2012). Since RDR6 synthesizes second RNA strands in the 3' to 5' direction, and these are substrates for DCL2 and DCL4. If primers are designed outside of the RNAi fragment in this 3' region, then measured silencing efficiency may not reflect actual silencing efficiency, because primers may amplify the RDR6-generated second strands which are not gene transcripts, but instead part of the RNA silencing machinery. This also depends on the location of the RNAi fragment.

RNA interference or posttranscriptional gene silencing (Fire et al. 1998) phenomena were breakthrough discoveries in animal and plant biology. At present, RNA interference has been found in plants from primitive mosses to higher trees (Blevins et al. 2006; Khraiwesh et al. 2010). RNAi has become an efficient approach in functional genomics, genetics and molecular biological studies. It is the well-established mode of action of smRNAs in silencing pathways that allows using the mechanism for control of gene expression. However, performance of RNAi pathways also varies in response to environmental stresses (Phillips et al. 2007; Ruiz-Ferrer and Voinnet 2009; Kulcheski et al. 2011). Different members of the RNAi pathway were identified and their expression patterns were characterized under stresses in maize, rice, *Arapidopsis* and tobacco (Gasciolli et al. 2005; Schmitz et al. 2007; Liu et al. 2009a; Liu et al. 2009b; Qian et al. 2011; Yang et al. 2011). Previous studies have shown that the expression of RDRs is diurnally regulated, but changed in wounded leaves and dramatically altered during *M. sexta* herbivory in *N. attenuata* (Pandey and Baldwin 2007, 2008; Pandey et al. 2008a). This suggests that RNAi plays a key role in regulating stress-related genes.

DICER-like proteins (DCL) play a pivotal role in the production of smRNAs (Kurihara and Watanabe 2004; Dunoyer et al. 2005; Gasciolli et al. 2005; Liu et al. 2009a). In **Manuscript II**, I demonstrate that all three DCLs are involved in anti-herbivory responses in *N. attenuata*. Microarray-based gene expression analysis showed that DCLs regulate transcriptional changes from the early stage of herbivore attack through secondary metabolite biosynthesis. They interact with each other and act together to regulate transcriptional rearrangements during

herbivory in a complex manner. We stably silenced four *DCL* genes using inverted repeat (IR) RNAi (ir-*dcl*). Several attempts to generate stable ir-*dcl1* lines were not successful because of the function of DCL1-processed miRNAs that regulate embryo development and maturation in *Arapidopsis* and moss (Khraiwesh et al. 2010; Nodine and Bartel 2010).

Notably, silencing efficiency of *DCL2* and *DCL3* was 85-90% whereas silencing efficiency for *DCL4* was 60%. A similar silencing efficiency of both *DCL2* and *DCL3* was found in crosses. Silencing of *DCL4* in crosses either with *DCL2* or *DCL3* also did not change and was comparable to ir-*dcl4* (Figure 3). Clearly, during

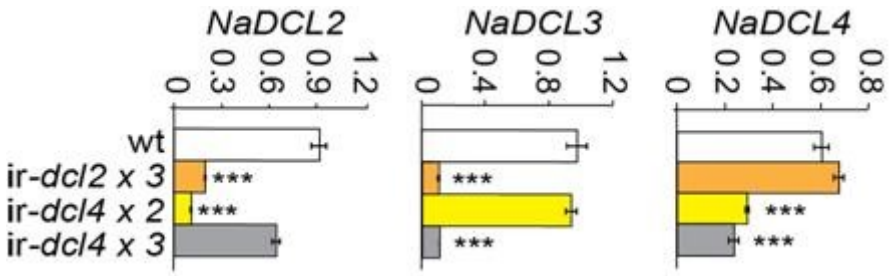


Figure 3. Silencing efficiencies of stable transformed *DCL*-plants. Asterisks indicate significant differences ($P < 0.05$) in Fisher's PLSD test following an ANOVA.

silencing of *DCL2* and *DCL3*, DCL4 is processing *DCL2*- or *DCL3*-siRNAs from respective IRs. In case of *DCL4*-silencing, level of DCL4 protein gradually decreased by *DCL4*-mediated-siRNAs. In the further steps of silencing machinery, *DCL4* transcripts are arrested by DCL2-processed *DCL4*-siRNA. It was reported that DCL2-processed 22 nt length siRNA is involved in transitive silencing of transgenes in *Arabidopsis* which is used for double strand RNA (dsRNA) synthesis by RDR6 (Mlotshwa et al. 2008). This suggests that during *DCL4* silencing, level of dsRNAs is increased which required phased excision by DCL4. Each DCLs has a distinct pattern of dsRNA excision. In *Arapidopsis*, DCL4 is required to phased excision of long dsRNA to produce secondary 21 nt siRNAs which incorporate into the AGO7-RISC complex to trigger posttranscriptional gene silencing (Herr 2005;

Ghildiyal and Zamore 2009). Probably we detected these dsRNA during our estimation of silencing efficiency of DCL4 silencing by qPCR which demonstrated "reduced" silencing efficiency in ir-*dcl4*. To be sure, it would be necessary to determine the protein level of DCL4 in ir-*dcl4* plants.

As I introduced in Chapter I, the phytohormone JA and its conjugate JA-Ile are key regulators of defense responses to herbivory, and accumulate to high levels in the early stage of herbivory attack. JA induction requires activation of MAPK cascades, which activate transcription factors, resulting in the induction of secondary defense metabolites (Seo et al. 2007; Wu et al. 2007; Skibbe et al. 2008). Several MAP kinases were down-regulated in ir-*dcl3* and ir-*dcl4* plants. This suggests that DCL-dependent smRNAs play a role in the regulation of MAPK signaling cascades. This also can be seen in the reduction of JA in ir-*dcl4* plants. In addition, several JA-related transcripts were differently regulated in ir-*dcl* plants. However, JA was not changed in ir-*dcl3* plants, but a JA-Ile conjugate highly accumulated which might be an inactive form of JA-Ile. Decreased JA was observed in the cross between ir-*dcl2* and ir-*dcl3* plants, suggesting that they regulate JA downstream of the regulation of DCL4.

All three DCLs are potentially involved in nicotine biosynthesis or its movement. DCL3 and DCL4 positively regulated nicotine levels whereas DCL2 had a negative effect on nicotine levels. Interestingly, levels of JA-induced nicotine were not only affected by reduce JA levels in ir-*dcl4*, but could also be reduced because of an impaired root system, where nicotine is biosynthesized. Probably, DCL-processed smRNAs regulate the root system and thus increases nicotine levels by enhanced root branching during herbivore stress (see below). DCL2 functionally antagonizes other DCLs (Bouche et al. 2006), and this also can be seen in the case of regulation of nicotine and other metabolites. Silencing *DCL2* together with *DCL4* or *DCL3* restores root development and shows normal WT nicotine accumulation. This suggests that DCL-dependent smRNAs control nicotine and other defenses. In the case of trypsin proteinase inhibitor (TPI) production, DCL3 has a specific role in its regulation. DCL3-dependent smRNAs

are known to methylate DNA. Perhaps one of these smRNAs is involved in regulating the negative regulators of TPI genes, or controls genes involved in JA signaling. In summary, RNAi pathways play a central role in regulation of the response to herbivory, and DCL2/3/4 interact in a complex manner to regulate genes involved in anti-herbivore defenses. These interactions significantly complicate the already challenging task of understanding smRNA function in the regulation of biotic interactions. In future, using DCL-mutant plants, we can identify and characterize DCL-specific smRNAs which are involved in plant and insect interactions.

Small RNAs are key regulators, controlling abundance of transcripts at a transcriptional and posttranscriptional level. However, smRNAs such as miRNA and endogenous siRNAs are differently regulated during abiotic and biotic stress (Zhao et al. 2007; Zhou et al. 2007; Jia et al. 2009; Zhou et al. 2010; Golldack et al. 2011; Ruiz-Ruiz et al. 2011; Zhang et al. 2011; Tang et al. 2012). In **manuscript III**, I discuss the role of miRNAs and endogenous siRNAs in regulation of anti-herbivore responses. I computationally identified 59 miRNAs belonging to 36 miRNA families in *N. attenuata*. Precursors of 52 miRNAs had secondary stem and loop structures with an average minimum free energy (MFE) of ΔG = -62.1 kcal mol^{-1}. This demonstrated that folding energy of Na-pre-miRNAs was close to that known from other dicotyledonous plant species. The MFE for *Arapidopsis* is -59.5 kcal mol^{-1} , for red alga – 47.7 kcal mol^{-1} , and for wheat 72.4 kcal mol^{-1} (Liang et al. 2010; Xin et al. 2010).

Expression analysis of miRNAs and precursors demonstrated different patterns in leaves elicited with oral secretion (OS) from *Manduca sexta* (W+OS treatment). I found that several miRNAs were significantly altered in OS-elicited leaves. As a control we used wounding and water-treated leaves (W+W treatment). However, I also found W+W-inducible miRNAs compared to untreated leaves. Among the OS-inducible miRNAs (those which differed between W+W-elicited and W+OS-elicited leaves) were miR156, miR164, miR166, miR168, miR171, miR172, miR319, miR390, miR393, miR394, miR398, miR1446 and one endogenous

tasiRNA. To elucidate whether the induction of OS-inducible miRNAs was JA dependent, we used JA-deficient lines silenced in the JA biosynthetic gene *allene oxide cyclase* (*AOC*) (Kallenbach et al. 2012). Results revealed that JA negatively and positively regulates miRNA levels. miR319, miR394 and miR828 were found to be JA-dependent. Interestingly, miR390 highly accumulates (around 80 fold) after OS-elicitation. The target of miR390 is non-protein coding TAS3 transcripts which encode tasiRNAs processed by RDR6/DCL4. TasiRNAs target several auxin response factors (ARF2, 3, 4). Expression of TAS3, tasiRNAs, and ARFs were correlated with each other, i.e., smRNA increased and targets decreased during OS elicitation. It has been shown that the *Arapidopsis* TAS3a mutant has an impaired lateral root system. A similar phenotype also was observed in *dcl4* and *rdr6* mutants in *Arapidopsis* (Marin et al. 2010). We observed a similar phenotype in ir-*dcl4* plants of our model system. This indicates that ir-*dcl4* plant does not produce tasiRNAs or they can be substituted by other DCLs which produce non-funtional tasiRNAs. As **Manuscript II** indicates, less nicotine in ir-*dcl4* may be due to a lack of tasiRNAs. In future we will overexpress primary transcripts of MIR390 and *TAS3* to understand their function and ecological relevance. MiR828 which is JA-dependent also targets non-protein coding *TAS4* transcripts encoding tasiRNAs, and MYB transcription factors. In *Arapidopsis*, tasiRNA4 is predicted to target several MYB transcription factors which are involved in phenylpropanoid biosynthesis (Rajagopalan et al. 2006; Luo et al. 2011). Unfortunately I could not find tasiRNA4 target in our current cDNA database. However, the phenylpropanoid-polyamine conjugate dicaffeoyl spermidine was altered in ir-*dcl4* plants. Further work requires the functional characterization of miR828 to understand its role during herbivory.

Summary

Plants have evolved sophisticated and complex strategies to increase plasticity to adapt to abiotic and biotic stress conditions. This requires rapid rearrangements of epigenome and transcriptome which subsequently trigger secondary metabolite production. *Nicotiana attenuata* is a native plant which is highly plastic and competitive and regularly attacked by number of generalist and specialist insect herbivores. *N. attenuata* recognizes herbivores by the content of herbivore oral secretions. Elicitors in oral secretion can rapid induce jasmonic acid response and large-scale transcriptional and metabolic changes. This is required to maximize plant fitness in the face of herbivory. Small RNAs are also regulators of plant defense responses.

To deepen our understanding of the role of smRNAs, we characterized four *Dicer-like* (*DCL*) genes, a key processor of smRNAs in the ecological model plant system *N. attenuata*. Time-course expression analysis of *DCL* transcripts with wounding and OS-elicitation compared to control treatments demonstrated different transcriptional responses. *DCL*s transcripts are diurnally regulated: *DCL1* is highly accumulates during the day-time and *DCL2, 3* and *4* highly accumulated in dark period of the day. In addition we found that DCLs differently express in different tissues. We used reverse genetics approach to describe them in term of ecological relevance. *DCL*s independently regulate or co-regulate jasmonates and defense responses to *M. sexta* herbivory. Herbivores grew larger on ir-*dcl3* and ir-*dcl4* plants. However, silencing DCL3 reduce nicotine and protease inhibitor (TPI) whereas ir-*dcl4* has lower nicotine and altered phenylpropanoid-polyamine conjugates. I found that DCL3 positively regulate nicotine biosynthesis through impaired JA-signaling and DCL4 through impaired root system. In contrast DCL2 has antagonistic effect on DCL4 or DCL3 in term of nicotine production. We found

that among the DCLs DCL3 regulate TPI level. In addition I showed independent transcriptional regulation of DCLs by using microarray tool. Annotation of significantly regulated transcripts indicated that DCLs regulate mainly diverse biological processes such as metabolic responses to stimulus and stresses, development, protein metabolism.

Moreover, to understand the role of microRNAs and endogenous tasiRNAs, I identified 59 miRNAs belonging to 36 MIR families and two miRNA-regulated TAS transcripts, and predicted their stem and loop structures and their targets. The average minimum free energy for stem and loop folding was comparable to *Arapidopsis*. We found *TAS* members using BLAST search by their miRNA-binding site. Results revealed that tasiRNAs are conserved with one in *Arabidopsis*. Quantitative PCR analysis showed that smRNAs had different and specific transcriptional responses to wounding and oral secretions treatment. Moreover, I also found that some oral secretions-induced miRNAs were specifically regulated in a jasmonate-dependent manner. Microarray expression analysis showed miRNA-target abundance which was correlated to miRNA abundance after treatments. Additional qPCR analysis confirmed similar expression pattern of target genes.

Transformed plants are a breakthrough tool to study functions of genes of interest. I contributed to developing an efficient screening procedure for ecological research. I developed and optimized DNA diagnostics of T-DNA inserts in transformed plants and screening strategy.

In conclusion, the function of different DCLs is specific for particular stresses and they also regulate stress responses by interacting with each other. DCL-processed specific small RNAs promote various responses by orchestrating transcriptome. These adjustments optimize physiology allowing the plant to adapt to distinct environmental stresses. Moreover, our optimized screening strategy of transformants allows for greater efficiency to characterize gene of interest in plant.

Zusammenfassung

Pflanzen haben komplexe Strategien entwickelt um variabel auf biotischen und abiotischen Stress zu reagieren. Dafür ist es notwendig innerhalb kurzer Zeit das Epigenom und Transkriptom anzupassen, was wiederum Veränderungen in der Produktion von Sekundärmetaboliten nach sich zieht. *N. attenuata* ist eine natürlich vorkommende Pflanze mit hoher Anpassungs- und Konkurrenzfähigkeit, die regelmäßig Attacken von generalistischen als auch spezialisierten pflanzenfressenden Insekten ausgesetzt ist. *N.attenuata* erkennt die Herbivoren anhand spezieller Bestandteile ihrer oralen Sekrete (OS). Elicitoren aus den OS induzieren sehr schnell eine Jasmonsäure Antwort, sowie umfangreiche transkriptionelle und metabolische Veränderungen. Dies ist notwendig um auch unter Einfluss von Herbivoren die Fitness zu maximieren. Auch kleine RNAs (smRNAs) gehören zu den Regulatoren der Verteidigungsantworten von Pflanzen.

Um unser Wissen über smRNAs zu vertiefen haben wir vier *Dicer-like* (DCL) Gene, welche Hauptkomponenten des smRNA Systems darstellen, untersucht. Die Experimente wurden mit *N. attenuata*, einer Modelpflanze für ökologische Forschung, durchgeführt. Die Durchführung von Expressionsanalysen der DCL Gene nach Verwundung und Behandlung mit OS, ergab Unterschiede im zeitlichen Verlauf der transkriptionellen Änderungen im Vergleich zu den Kontrollen. Die DCL Transkripte werden diurnal reguliert: DCL1 akkumuliert sehr stark tagsüber, DCL2, 3 und 4 hingegen reichern sich vor allem während der Dunkel-Phase an. Zusätzlich fanden wir heraus, dass die DCLs in verschiedenen Pflanzenteilen unterschiedlich expremiert werden. Um eine Charakterisierung der DCLs in Hinblick auf ihre ökologische Relevanz durchzuführen nutzten wir die Methoden der Reversen Genetik. DCLs regulieren gemeinsam oder unabhängig die Jasmonate, sowie der Verteidigungs Antwort auf M. sexta Fraß. Die Herbivoren zeigten ein erhöhtes Wachstum auf den ir-dcl3 und ir-dcl4 Pflanzen. Das stilllegen

von DCL3 sorgte für eine Reduktion der Nikotin und Trypsin Protease Inhibitor (TPI) Level, während die ir-dcl4 Pflanzen niedrigere Nikotin Werte als auch Veränderungen in den Phenylpropanoid-Polyamin Konjugaten aufweisen. Ich konnte herausfinden, dass DCL3 den JA-Signalweg beeinträchtigt, was einen positiven Einfluss auf die Nikotin Biosynthese hat, während DCL4 dies durch eine Störung im Wurzelsystem verursacht. DCL2 hingegen hat, im Hinblick auf die Nikotin Biosynthese, einen negativen Einfluss auf DCL3 oder DCL4. Wir fanden heraus, dass von DCLs DCL3 die TPI Level reguliert werden. Zusätzlich konnte ich mit Hilfe von Microarray Daten zeigen, dass die DCLs transkriptionell unterschiedlich reguliert sind. Die Annotierung signifikant regulierter Transkripte deutet daraufhin das DCLs unterschiedliche biologische Prozesse wie die stoffwechsel Antwort auf Reize und Stress, Entwicklungsprozesse, als auch den Proteinmetabolismus regulieren. Um außerdem die Rolle der microRNAs und endogenen tasiRNAs zu verstehen, habe ich 59 miRNAs die zu 36 verschiedenen MIR Familien gehören, sowie zwei miRNA regulierte TAS Transkripte identifiziert und ihre Stamm und Schlaufen Struktur und als auch ihre Ziele berechnet. Die minimale freie Energie für die Stamm und Schlaufen Faltung war vergleichbar mit den Daten aus Arabidopsis. Wir fanden TAS Mitglieder mithilfe der BLAST Suche anhand ihrer miRNA Bindestelle. Die Daten ergaben, dass die gefundenen tasiRNAs konserviert zu einer aus Arabidopsis sind. Quantitative PCR (qPCR) Analysen zeigten, dass die smRNAs spezifische transkriptionelle Antworten auf Verwundung und OS Behandlung aufweisen. Zusätzlich fand ich heraus, dass die spezifische OS induzierte Regulation einiger miRNAs JA abhängig ist. Microarray Analysen ergaben, dass nach einer Behandlung die Häufigkeit der Transkripte der Zielgene mit der Menge der entsprechenden miRNA korreliert. Zusätzliche qPCR Analysen bestätigten die Ähnlichkeiten mit den Expressionsmuster der Zielgene.

Die Herstellung transformierter Pflanzen stellt für die Erforschung der Funktion spezifischer Gene einen Durchbruch dar. Ich habe dazu beigetragen effiziente Auswahlverfahren für die ökologische Forschung zu etablieren. Ich habe die diagnostischen Verfahren der Analyse von T-DNA Insertionen in

transformierten Pflanzen, sowie die zugehörigen Strategien für die Auswahlmethode entwickelt und optimiert.

Zusammenfassend kann man sagen, dass die Funktion verschiedener DCLs stressspezifisch ist und Stressantworten durch die Interaktion der DCLs untereinander reguliert werden. DCL prozessierte spezifische smRNAs unterstützen verschiedene Reaktionen durch die Manipulation des Transkriptoms. Diese Anpassungen verbessern die Physiologie der Pflanze und ermöglichen ihr eine bessere Anpassung an verschiedene Umweltbedingungen. Außerdem führt unsere optimierte Auswahlstrategie für transformierte Pflanzen dazu, dass Gene mit erhöhter Effizienz charakterisiert werden können.

References

Alborn HT, 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.

Allmann S, Baldwin IT (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. *Science* **329**, 1075-1078.

Baev V, Naydenov M, Apostolova E, Ivanova D, Doncheva S, Minkov I, Yahubyan G (2010) Identification of RNA-dependent DNA-methylation regulated promoters in *Arabidopsis*. *Plant Physiol Biochem* **48**, 393-400.

Baldwin IT (1988) The Alkaloidal Responses of Wild Tobacco to Real and Simulated Herbivory. *Oecologia* **77**, 378-381.

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proc Natl Acad Sci U S A* **95**, 8113-8118.

Baldwin IT (1999) Functional interactions in the use of direct and indirect defences in native *Nicotiana* plants. *Novartis Foundation symposium* **223**, 74-87; discussion 87-94, 160-165.

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

Baldwin IT, Morse L (1994) Up in Smoke .2. Germination of *Nicotiana attenuata* in response to smoke-derived cues and nutrients in burned and unburned soils. *Journal of Chemical Ecology* **20**, 2373-2391.

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297.

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. *Proc Natl Acad Sci U S A* **92**, 8675-8679.

Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W (2006) Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells. *Cold Spring Harb Symp Quant Biol* **71**, 513-521.

Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A, Park HS, Vazquez F, Robertson D, Meins F, Hohn T, Pooggin MM (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Research* **34**, 6233-6246.

Bouche N, Lauressergues D, Gasciolli V, Vaucheret H (2006) An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J* **25**, 3347-3356.

Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, Hollunder J, Meins F, Jr., Kovalchuk I (2010) Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of Dicer-like proteins. *PLoS One* **5**, e9514.

Boyko A, Kovalchuk I (2010) Transgenerational response to stress in *Arabidopsis* thaliana. *Plant Signal Behav* **5**, 995-998.

Bozorov TA, Prakash Pandey S, Dinh ST, Kim SG, Heinrich M, Gase K, Baldwin IT (2012) DICER-like Proteins and Their Role in Plant-herbivore Interactions in *Nicotiana attenuata*. *J Integr Plant Biol* **54**, 189-206.

Bricchi I, Leitner M, Foti M, Mithofer A, Boland W, Maffei ME (2010) Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: early signaling and volatile emission in Lima bean (Phaseolus lunatus L.). *Planta* **232**, 719-729.

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

Chellappan P, Xia J, Zhou X, Gao S, Zhang X, Coutino G, Vazquez F, Zhang W, Jin H (2010) siRNAs from miRNA sites mediate DNA methylation of target genes. *Nucleic Acids Res* **38**, 6883-6894.

Chen D, Meng Y, Yuan C, Bai L, Huang D, Lv S, Wu P, Chen LL, Chen M (2011) Plant siRNAs from introns mediate DNA methylation of host genes. *RNA* **17**, 1012-1024.

Chen X (2005) MicroRNA biogenesis and function in plants. *FEBS Lett* **579**, 5923-5931.

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature* **448**, 666-U664.

Chinnusamy V, Zhu JK (2009) Epigenetic regulation of stress responses in plants. *Curr Opin Plant Biol* **12**, 133-139.

Choi CS, Sano H (2007) Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. *Mol Genet Genomics* **277**, 589-600.

De Vos M, Van Oosten VR, Van Poecke RM, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M, Pieterse CM (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol Plant Microbe Interact* **18**, 923-937.

Devoto A, Turner JG (2005) Jasmonate-regulated *Arabidopsis* stress signalling network. *Physiol Plantarum* **123**, 161-172.

Dicke M (2009) Behavioural and community ecology of plants that cry for help. *Plant Cell and Environment* **32**, 654-665.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiol* **150**, 1576-1586.

Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat Genet* **37**, 1356-1360.

Elkonin LA, Gudova TN, Ishin AG, Tyrnov US (1993) Diploidization in haploid tissue-cultures of sorghum. *Plant Breeding* **110**, 201-206.

Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA, Moreno V (2003) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent [corrected]. *Theor Appl Genet* **106**, 231-238.

Endres MW, Gregory BD, Gao Z, Foreman AW, Mlotshwa S, Ge X, Pruss GJ, Ecker JR, Bowman LH, Vance V (2010) Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. *PLoS Pathog* **6**, e1000729.

Fernandes GW (1994). "Plant mechanical defenses against insect herbivory". *Revista Brasileira de Entomologia* **38** (2): 421–433.

Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811.

Galis I, Gaquerel E, Pandey SP, Baldwin IT (2009) Molecular mechanisms underlying plant memory in JA-mediated defence responses. *Plant Cell Environ* **32**, 617-627.

Gaquerel E, Weinhold A, Baldwin IT (2009) Molecular Interactions between the Specialist Herbivore *Manduca sexta* (Lepidoptera, Sphigidae) and Its Natural Host *Nicotiana attenuata*. VIII. An Unbiased GCxGC-ToFMS Analysis of the Plant's Elicited Volatile Emissions. *Plant Physiology* **149**, 1408-1423.

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* **15**, 1494-1500.

Ghildiyal M, Zamore PD (2009) Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10**, 94-108.

Gilardoni PA, Schuck S, Jungling R, Rotter B, Baldwin IT, Bonaventure G (2010) SuperSAGE analysis of the *Nicotiana attenuata* transcriptome after fatty acid-amino acid elicitation (FAC): identification of early mediators of insect responses. *BMC Plant Biol* **10**, 66.

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 proteome. *Plant Physiology* **142**, 1621-1641.

Golldack D, Luking I, Yang O (2011) Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant Cell Rep* **30**, 1383-1391.

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

Halitschke R, 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.

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

Heil M (2008) Indirect defence via tritrophic interactions. *New Phytol* **178**, 41-61.

Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, Jassbi AR, Baldwin IT (2010) Jasmonate and ppHsystemin regulate key Malonylation steps in the biosynthesis of 17-Hydroxygeranyllinalool Diterpene Glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *Plant Cell* **22**, 273-292.

Hermsmeier D, Schittko U, Baldwin IT (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.

Herr AJ (2005) Pathways through the small RNA world of plants. *Febs Letters* **579**, 5879-5888.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. *Annu Rev Plant Biol* **59**, 41-66.

Hui DQ, Iqbal J, Lehmann K, Gase K, Saluz HP, Baldwin IT (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.

Jia X, Ren L, Chen QJ, Li R, Tang G (2009) UV-B-responsive microRNAs in Populus tremula. *J Plant Physiol* **166**, 2046-2057.

Johannes F, Porcher E, Teixeira FK, Saliba-Colombani V, Simon M, Agier N, Bulski A, Albuisson J, Heredia F, Audigier P, Bouchez D, Dillmann C, Guerche P, Hospital F, Colot V (2009) Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS Genet* **5**, e1000530.

Kallenbach M, Bonaventure G, Gilardoni PA, Wissgott A, Baldwin IT (2012) Empoasca leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. *Proc Natl Acad Sci U S A*.

Kandoth PK, Ranf S, Pancholi SS, Jayanty S, Walla MD, Miller W, Howe GA, Lincoln DE, Stratmann JW (2007) Tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects. *Proc Natl Acad Sci U S A* **104**, 12205-12210.

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.

Karan R, Deleon T, Biradar H, Subudhi PK (2012) Salt stress induced variation in DNA methylation pattern and its influence on gene expression in contrasting rice genotypes. *PLoS One* **7**, e40203.

Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis I (2010) R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. *Plant Physiol* **152**, 1731-1747.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: The emerging molecular analysis. *Annual Review of Plant Biology* **53**, 299-328.

Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W (2010) Transcriptional control of gene expression by microRNAs. *Cell* **140**, 111-122.

Khraiwesh B, Zhu JK, Zhu J (2012) Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochim Biophys Acta* **1819**, 137-148.

Kim SG, Yon F, Gaquerel E, Gulati J, Baldwin IT (2011) Tissue specific diurnal rhythms of metabolites and their regulation during herbivore attack in a native tobacco, *Nicotiana attenuata*. *PLoS One* **6**, e26214.

Kooter JM, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. *Trends Plant Sci* **4**, 340-347.

Kulcheski FR, de Oliveira LF, Molina LG, Almerao MP, Rodrigues FA, Marcolino J, Barbosa JF, Stolf-Moreira R, Nepomuceno AL, Marcelino-Guimaraes FC, Abdelnoor RV, Nascimento LC, Carazzolle MF, Pereira GA, Margis R (2011) Identification of novel soybean microRNAs involved in abiotic and biotic stresses. *BMC Genomics* **12**, 307.

Kurihara Y, Watanabe Y (2004) *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci U S A* **101**, 12753-12758.

Li Y, Luo J, Zhou H, Liao JY, Ma LM, Chen YQ, Qu LH (2008) Stress-induced tRNA-derived RNAs: a novel class of small RNAs in the primitive eukaryote Giardia lamblia. *Nucleic Acids Res* **36**, 6048-6055.

Liang C, Zhang X, Zou J, Xu D, Su F, Ye N (2010) Identification of miRNA from *Porphyra yezoensis* by high-throughput sequencing and bioinformatics analysis. *PLoS One* **5**, e10698.

Liu Q, Feng Y, Zhu Z (2009a) Dicer-like (DCL) proteins in plants. *Funct Integr Genomics* **9**, 277-286.

Liu Y, Gao Q, Wu B, Ai T, Guo X (2009b) NgRDR1, an RNA-dependent RNA polymerase isolated from *Nicotiana glutinosa*, was involved in biotic and abiotic stresses. *Plant Physiol Biochem* **47**, 359-368.

Luo QJ, Mittal A, Jia F, Rock CD (2011) An autoregulatory feedback loop involving PAP1 and TAS4 in response to sugars in *Arabidopsis*. *Plant Mol Biol*.

Mallory AC, Vaucheret H (2006) Functions of microRNAs and related small RNAs in plants. *Nat Genet* **38 Suppl**, S31-36.

Marin E, Jouannet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A (2010) miR390, *Arabidopsis* TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *Plant Cell* **22**, 1104-1117.

Mattiacci L, Dicke M, Posthumus MA (1995) beta-Glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc Natl Acad Sci U S A* **92**, 2036-2040.

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.

Miles PW (1999) Aphid saliva. *Biol Rev* **74**, 41-85.

Mlotshwa S, Pruss GJ, Peragine A, Endres MW, Li J, Chen X, Poethig RS, Bowman LH, Vance V (2008) DICER-LIKE2 plays a primary role in transitive silencing of transgenes in *Arabidopsis*. *PLoS One* **3**, e1755.

Nodine MD, Bartel DP (2010) MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis. *Genes Dev* **24**, 2678-2692.

Onkokesung N, Galis I, von Dahl CC, Matsuoka K, Saluz HP, Baldwin IT (2010) Jasmonic acid and ethylene modulate local responses to wounding and simulated herbivory in *Nicotiana attenuata* leaves. *Plant Physiol* **153**, 785-798.

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

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

Pandey SP, Gaquerel E, Gase K, Baldwin IT (2008a) RNA-directed RNA polymerase3 from *Nicotiana attenuata* is required for competitive growth in natural environments. *Plant Physiol* **147**, 1212-1224.

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

Paschold A, Halitschke R, Baldwin IT (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore- induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *Plant J* **51**, 79-91.

Phillips JR, Dalmay T, Bartels D (2007) The role of small RNAs in abiotic stress. *FEBS Lett* **581**, 3592-3597.

Pontes O, Costa-Nunes P, Vithayathil P, Pikaard CS (2009) RNA polymerase V functions in *Arabidopsis* interphase heterochromatin organization independently of the 24-nt siRNA-directed DNA methylation pathway. *Mol Plant* **2**, 700-710.

Preston LC, Baldwin IT (1999) Positive and negative signals regulate germination in the post- fire annual, *Nicotiana attenuata*. *Biotechnology letters* **80**, 481-494.

Qian Y, Cheng Y, Cheng X, Jiang H, Zhu S, Cheng B (2011) Identification and characterization of Dicer-Like, Argonaute And Rna-Dependent RNA Polymerase gene families in maize. *Plant Cell Rep* **30**, 1347-1363.

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev* **20**, 3407-3425.

Rajeswaran R, Aregger M, Zvereva AS, Borah BK, Gubaeva EG, Pooggin MM (2012) Sequencing of RDR6-dependent double-stranded RNAs reveals novel features of plant siRNA biogenesis. *Nucleic Acids Res*.

Rajeswaran R, Pooggin MM (2012) RDR6-mediated synthesis of complementary RNA is terminated by miRNA stably bound to template RNA. *Nucleic Acids Research* **40**, 594-599.

Rasmann S, De Vos M, Casteel CL, Tian D, Halitschke R, Sun JY, Agrawal AA, Felton GW, Jander G (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant Physiol* **158**, 854-863.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. *Genes Dev* **16**, 1616-1626.

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. *Annu Rev Plant Biol* **60**, 485-510.

Ruiz-Ruiz S, Navarro B, Gisel A, Pena L, Navarro L, Moreno P, Di Serio F, Flores R (2011) Citrus tristeza virus infection induces the accumulation of viral small RNAs (21-24-nt) mapping preferentially at the 3'-terminal region of the genomic RNA and affects the host small RNA profile. *Plant Mol Biol* **75**, 607-619.

Saedler R, Baldwin IT (2004) Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *J Exp Bot* **55**, 151-157.

Schittko U, Hermsmeier D, Baldwin IT (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. II. Accumulation of plant mRNAs in response to insect-derived cues. *Plant Physiology* **125**, 701-710.

Schmelz EA, LeClere S, Carroll MJ, Alborn HT, Teal PE (2007) Cowpea chloroplastic ATP synthase is the source of multiple plant defense elicitors during insect herbivory. *Plant Physiol* **144**, 793-805.

Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT (2005) Specificity in ecological interactions: attack from the same lepidopteran herbivore results in

species-specific transcriptional responses in two solanaceous host plants. *Plant Physiol* **138**, 1763-1773.

Schmitz RJ, Hong L, Fitzpatrick KE, Amasino RM (2007) DICER-LIKE 1 and DICER-LIKE 3 redundantly act to promote flowering via repression of FLOWERING LOCUS C in *Arabidopsis thaliana*. *Genetics* **176**, 1359-1362.

Schwachtje J, Baldwin IT (2004) Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual, *Nicotiana attenuata*. *Seed Sci Res* **14**, 51-60.

Schwachtje J, Baldwin IT (2008) Why does herbivore attack reconfigure primary metabolism? *Plant Physiol* **146**, 845-851.

Selvi BR, Mohankrishna DV, Ostwal YB, Kundu TK (2010) Small molecule modulators of histone acetylation and methylation: a disease perspective. *Biochim Biophys Acta* **1799**, 810-828.

Seo S, Katou S, Seto H, Gomi K, Ohashi Y (2007) The mitogen-activated protein kinases WIPK and SIPK regulate the levels of jasmonic and salicylic acids in wounded tobacco plants. *Plant J* **49**, 899-909.

Shi Q, Li C, Zhang F (2006) Nicotine synthesis in *Nicotiana tabacum* L. induced by mechanical wounding is regulated by auxin. *J Exp Bot* **57**, 2899-2907.

Skibbe M, Qu N, Galis I, Baldwin IT (2008) Induced plant defenses in the natural environment: *Nicotiana attenuata* WRKY3 and WRKY6 coordinate responses to herbivory. *Plant Cell* **20**, 1984-2000.

Smith CM, Boyko EV (2007) The molecular bases of plant resistance and defense responses to aphid feeding: current status. *Entomol Exp Appl* **122**, 1-16.

Sunkar R (2010) MicroRNAs with macro-effects on plant stress responses. *Semin Cell Dev Biol* **21**, 805-811.

Tang S, Wang Y, Li Z, Gui Y, Xiao B, Xie J, Zhu QH, Fan L (2012) Identification of wounding and topping responsive small RNAs in tobacco (*Nicotiana tabacum*). *BMC Plant Biol* **12**, 28.

van Wolfswinkel JC, Ketting RF (2010) The role of small non-coding RNAs in genome stability and chromatin organization. *J Cell Sci* **123**, 1825-1839.

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* **20**, 759-771.

Verhoeven KJ, Jansen JJ, van Dijk PJ, Biere A (2010) Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New Phytol* **185**, 1108-1118.

Verhoeven KJ, van Gurp TP (2012) Transgenerational effects of stress exposure on offspring phenotypes in apomictic dandelion. *PLoS One* **7**, e38605.

von Dahl CC, Winz RA, Halitschke R, Kuhnemann F, Gase K, Baldwin IT (2007) Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *Plant J* **51**, 293-307.

Winz RA, Baldwin IT (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, 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.

Wu J, Hettenhausen C, Schuman MC, Baldwin IT (2008) A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. *Plant Physiol* **146**, 927-939.

Xin M, Wang Y, Yao Y, Xie C, Peng H, Ni Z, Sun Q (2010) Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum L.*). *BMC Plant Biol* **10**, 123.

Yamakawa H, Katou S, Seo S, Mitsuhara I, Kamada H, Ohashi Y (2004) Plant MAPK phosphatase interacts with calmodulins. *J Biol Chem* **279**, 928-936.

Yan Y, Zhang Y, Yang K, Sun Z, Fu Y, Chen X, Fang R (2011) Small RNAs from MITE-derived stem-loop precursors regulate abscisic acid signaling and abiotic stress responses in rice. *Plant J* **65**, 820-828.

Yang H, Wang M, Gao Z, Zhu C, Guo X (2011) Isolation of a novel RNA-dependent RNA polymerase 6 from *Nicotiana glutinosa*, NgRDR6, and analysis of its response to biotic and abiotic stresses. *Mol Biol Rep* **38**, 929-937.

Yoshinaga N, Alborn HT, Nakanishi T, Suckling DM, Nishida R, Tumlinson JH, Mori N (2010) Fatty acid-amino acid conjugates diversification in lepidopteran caterpillars. *J Chem Ecol* **36**, 319-325.

Zhang S, Zhou J, Han S, Yang W, Li W, Wei H, Li X, Qi L (2010) Four abiotic stress-induced miRNA families differentially regulated in the embryogenic and non-embryogenic callus tissues of Larix leptolepis. *Biochem Biophys Res Commun* **398**, 355-360.

Zhang W, Gao S, Zhou X, Chellappan P, Chen Z, Zhang X, Fromuth N, Coutino G, Coffey M, Jin H (2011) Bacteria-responsive microRNAs regulate plant innate immunity by modulating plant hormone networks. *Plant Mol Biol* **75**, 93-105.

Zhao B, Liang R, Ge L, Li W, Xiao H, Lin H, Ruan K, Jin Y (2007) Identification of drought-induced microRNAs in rice. *Biochem Biophys Res Commun* **354**, 585-590.

Zhou L, Liu Y, Liu Z, Kong D, Duan M, Luo L (2010) Genome-wide identification and analysis of drought-responsive microRNAs in *Oryza sativa*. *J Exp Bot* **61**, 4157-4168.

Zhou X, Wang G, Zhang W (2007) UV-B responsive microRNA genes in *Arabidopsis thaliana*. *Mol Syst Biol* **3**, 103.

Ziebell H, Carr JP (2009) Effects of dicer-like endoribonucleases 2 and 4 on infection of *Arabidopsis thaliana* by cucumber mosaic virus and a mutant virus lacking the 2b counter-defence protein gene. *J Gen Virol* **90**, 2288-2292.

Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. *Nat Genet* **39**, 61-69.

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PULICATIONS:

- **Bozorov T.A.**, Baldwin I.T. and S.G. Kim. Identification and profiling of miRNAs during herbivory reveals jasmonate-dependent and -independent patterns of accumulation in *Nicotiana attenuata. BMC Plant Biology*, 22 (1), 209.
- **Bozorov T.A.**, Pandey S.P., Dinh S.T., Kim S.G., Heinrich M., Gase K. and I.T. Baldwin. Dicer-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata*. *Journal of Integrative Plant Biology* 2012, (54:3), 189–206.
- Gase K., Weinhold A., **Bozorov T.**, Schuck S. and I.T. Baldwin. Efficient screening of transgenic plant lines for ecological research. *Molecular Ecology Resources* 2011 (11), 890–902.
- Abdurakhmonov I.Y., Devor E.J., Buriev Z.T., Huang L., Makamov A., Shermatov S.E., **Bozorov T.**, Kushanov F.N., Mavlonov G.T. and A. Abdukarimov. Annotation of small RNA and microRNA mature sequences from developing ovules of *G. hirsutum L*. *BMC Plant Biology* 2008, 8:93.
- **Bozorov T.A.**, Abdurahmonov I.Yu., Buriev Z.T. and A.A. Abdukarimov. Creating of gene knockout constructs. *Dokladii Akademii Nauk of Uzbek Academy of Science*. 2009 (1), 63-65.
- Abdurakhmonov I.Yu,, **Bozorov T.A.**, Buriev Z.T., Shermatov Sh.E. and A. Abdukarimov. RNA interference of cotton HY5 gene in model plant *Arabidopsis*, *Uzbek Biology journal*, 2008 (4), 18-21.

- Buriev Z.T., **Bozorov T.A.**, Abdurahmonov I.Yu., Yakubov M.D. and A.A. Abdukarimov. Somtaic embryogenesis and regeneration of cotton (*Gossypium hirsutum*. L) Coker 312. *Dokladii Akademii Nauk of Uzbek Academy of Science*. 2008 (2), 80-83.
- **Bozorov T.A.**, Abdurahmonov I.Yu., Buriev Z.T. and A.A. Abdukarimov. Silencing of *Arabidopsis* phytochrome genes expression by small interfering RNAs. *Uzbek Biology journal*, 2008 (4), 57-60.
- **T.A. Bozorov**. Isolation of tobamoviruses from *Physalis alkekengi* and elaboration of storing in collection (2004). Collected articles, National University of Uzbekistan. University Press.

PATENTS:

- Abdurahmanov, I.Yu., Buriev, Z.T., **Bozorov, T.A.** and Abdukarimov, A. (2011): Small interfering RNAs (siRNA) for silencing of gene expression in plant cell. Uzbekistan Patent (Patent IAP 04383).
- Abdurahmanov, I.Yu., Buriev, Z.T., **Bozorov, T.A.** and Abdukarimov, A. (2011): Small interfering RNAs (siRNA) for silencing of gene expression in plant cell. Uzbekistan Patent (Patent IAP 04360).

ORAL PRESENTATION:

- **Bozorov T.** Silencing DICER-LIKE 4 (DCL4) impairs defense responses to herbivory in *Nicotiana attenuata*. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2010.
- **Bozorov T.** Silencing DICER-LIKE 4 (DCL4) impairs defense responses to herbivory in *Nicotiana attenuata*. Spring School, „RNAi & the World of Small RNA molecules“, Wageningen, The Netherlands, Apr 2010.
- **Bozorov T.,** Abdurakhmonov I.Yu., Buriev Z.T., Abdukarimov A.A. Silencing of cotton phytochrome genes. “Prospects in Physical-chemical biology and biotechnology”. June 15-16, 2007, Andijan, Uzbekistan.

- **Bozorov T**, Kadirova Z., Kadirova Zu., Vakhabov A.. Isolation of tobamoviruses from Physalis alkekengi and elaboration of storage in collection (2003). Workshop Conference. Bukhara.Uzbekistan.

POSTER PRESENTATION:

- **Bozorov T.A,** Kim S.G. and I.T. Baldwin. Do small RNAs orchestrate defense responses to insect herbivory? ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2011.
- **Bozorov T.A.** Do dicers function independently to regulate defenses during herbivore attack? 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2011.
- **Bozorov T.A**., Pandey S.P., Kim S.G., Baldwin I.T. Do Dicers function independently to regulate defenses during herbivore attack? 6th Genetic Workshop, Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Berlin, DE, Nov 2010.
- **Bozorov T.A**., Pandy S.P., Gase K., Kim S.G. and I.T. Baldwin. Small RNA Roles in *N. attenuata*'s Ecological Interaction. SAB 2010. MPI-CE, Jena, Germany.
- Kumar P., Poosakkannu A., Pandit S., **Bozorov T.,** Kröber W., Kessler D., Schoetner M. and I.T. Baldwin. Plant Mediated RNA Interference for Insect Gene Silencing. SAB 2010. MPI-CE, Jena, Germany.
- **Bozorov T.A**., Pandey S.P., Gase K and I.T. Baldwin. Role of DCL genes in herbivore attack. 8th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Mar 2009.
- **Bozorov T.,** Abdurakhmonov I.Yu., Buriev Z.T., Abdukarimov A.A. Creating RNAi constructs. Actual problem in plant molecular biology, 2008, Tashkent, Uzbekistan.
- **Bozorov T.A.,** Kadirova Z.A., Storage conditions for tobamoviruses from Tomato (2003). Abstracts. 2nd International conference of young scientists. Ukraine.

- Kadirova Z.A., and **Bozorov T.A.** Preparation of antiserum to tomato TMV using immunomodulin as aduvant (2003). Abstracts. XLI International Conference of Young Scientists. Novosibirsk. Russia.

  Kadirova Z.A., and **Bozorov T.A.** Identification of tobamoviruses from leaves of Physalis alkekengi and developing methods of storage and reactivation. (2003). Abstracts. The 7th Workshop-conference of young scientists. Pushino, Russia.

Selbständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Jena, 28.11.2012