Molecular response of *Nicotiana attenuata* to herbivory and solar UVB: laboratory and fieldwork

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Chapter 1

General Introduction

1.1. Evolution of plant defense

The Solar system including Earth began to form around 4.6 billion years ago (bya) (Whipple, 1964), and 750 million years later (3.85 bya), the first life on Earth emerged (Carlin, 1980; Oro et al., 1990). The first living organisms were directly exposed to solar radiation. The lack of a protective ozone layer forced them to develop mechanisms to cope with extreme levels of ultraviolet (UV) radiation. Hence, resistance to UV may be one of the most ancient defense mechanisms on Earth (Hoiczyk and Hansel, 2000).

The appearance of oxygen-producing cyanobacteria 2.8 to 3.46 bya (Peterson et al., 2001; Manetas, 2004) dramatically altered the conditions of terrestrial life. Cyanobacteria were responsible for producing the oxygen-enriched atmosphere on which so much extant life depends. In addition, because the oxygen produced by cyanobacteria creates ozone in the presence of UV radiation, the activity of cyanobacteria led to the formation of the active ozone shield in the stratosphere. Without the production oxygen and ozone layer, life as we know it today would not exist (Sagan, 1957, 1973).

Microbiota were the first life forms to colonize land habitats 2.8 to 3.1 bya (Battistuzzi et al., 2004). 2.7 bya, first eukaryotes have already emerged (Feng et al., 1997; Hedges, 2002). Long after the emergence of the first eukaryotes, the earliest green alga, which is known to be the common ancestor of all land plants, originated 1.5 bya (Lewis and McCourt, 2004; Yoon et al., 2004), preceding the adaptive radiation of land plants 425–490 mya (Sanderson, 2003).

Nicotiana is a genus of herbaceous plants and shrubs in the Solanaceae family. By analyzing the sequences of the small subunit of ribulose-1,5-bisphosphate carboxylases (RuBCs), it was proposed that the Nicotiana clade split with Petunia about 27 million years ago, and with Solanum species about 28 million years ago. These data suggest relatively recent speciation of this plant genus (Ioerger et al., 1990), which comprises some 60–76 naturally occurring species (Subhashini, 1973; Chase et al.,
Most of the *Nicotiana* species occur in the Americas and Australia, except for a single species found in Africa. Two species, *N. tabacum* and *N. rustica*, are important agriculturally (Chase et al., 2003; Knapp et al., 2004). The wild tobacco species *Nicotiana attenuata*, which displays a sophisticated arsenal of responses to various environmental stresses, is a model system for study of plant-herbivore interactions (Kessler and Baldwin, 2002; Kessler, 2006).

Plants have coexisted with insects for approximately 400 million years. (Whalley and Jarzembskowki, 1981; Engel and Grimaldi, 2004). During this time, the interactions between flora and fauna have driven coevolutionary and reciprocal changes, resulting in a complex mosaic of plant defenses and insect counter-defenses. For example, coevolution resulted in spatial, temporal and specific inducible responses to specialist herbivores (Breedlove and Ehrlich, 1968; Voelckel and Baldwin, 2004; Bartel, 2005; Mallory and Vaucheret, 2006; Becerra, 2007; Agrawal et al., 2012; Ali and Agrawal, 2012). However, not all insects are harmful to the plants. When feeding on *Nicotiana attenuata*, *Tupiocoris notatus* mirid bugs induce direct and indirect defenses that make plants more resistant against other, more damaging herbivores (vaccination); however, their feeding does not directly reduce fitness of plants (Halitschke et al., 2011). Moderate herbivory, under certain conditions, can enhance primary plant production; therefore, it can be considered as a mutualistic interaction (Agrawal, 2000; de Mazancourt et al., 2001). Nevertheless, the effects of herbivores on plant fitness are predominantly negative. Plants’ interactions with herbivores have shaped the evolution of specific plant defenses.

In response to plant defenses, herbivores evolved counter-mechanisms that allowed them to cope with or overcome certain plant defense traits. Some insects became specialist herbivores dependent upon specific host plants. Ecological advantages of specialization may stem from optimal exploitation of host plants, including appropriate timing of critical life stages, development of specific resistance to plant defenses, facilitation of mating, avoidance of competition with generalists, and avoidance of natural enemies that cannot survive on the specialist's host (Voelckel and Baldwin, 2004; Reudler et al., 2011; Ali and Agrawal, 2012).

One example of specialization is the tobacco hornworm, *Manduca sexta* (Sphingidae), a typical specialist herbivore of *N. attenuata*. This herbivore has developed mechanisms for selectively sequestering, secreting and/or detoxifying nicotine, a neurotoxic alkaloid found in *Nicotiana* plants (Appel and Martin, 1992;
Wink and Theile, 2002). Although *M. sexta* possesses mechanisms for nicotine detoxification, its performance is still negatively affected by nicotine (Appel and Martin, 1992; Steppuhn et al., 2004). Consequently, *M. sexta* has been widely used to investigate plant-insect interactions involving *N. attenuata*. Such investigations may eventually facilitate control of herbivores on crops. Although RNAi methods to silence gene expression in Lepidopteran insects are still difficult (Terenius et al., 2011), new methods have developed increasingly successful (Kumar et al., 2012).

### 1.2. Chemical ecology and plant defense

Almost all life on earth depends on energy from sunlight. The green leaves responsible for capturing this energy are the primary, and usually also the exclusive, food of more than 50% of known insect species. Yet only particular plant species have nutritional characters that make them suitable for insects (Trager, 1941; Fraenkel and Blewett, 1943; Wolf, 2006). Plant nutrient content often affects both plant-herbivore and higher levels of multitrophic interactions (Thompson et al., 2005). The dependence of insects on plants for subsistence combined with their susceptibility to plant toxins makes plant chemistry of central importance in plant-insect interactions (Schultz, 1988).

Chemical ecology refers to chemically mediated interactions between organisms and their biotic and abiotic environment. This field covers a broad range of chemical interactions such as communication (volatiles, pheromones), mutualistic interactions (pollination, symbiotic, etc.), defenses (against pathogen, herbivore, etc.) and protection (against UV light, drought, etc.) (Hartmann, 2008). Although Gottfried S. Fraenkel was the first entomologist who recognized the essential role of plant secondary metabolites in plant-insect interactions (Fraenkel, 1959), Ernst Stahl is frequently considered the seminal pioneer of chemical ecology (Hartmann, 2008).

A number of theories have been proposed as predictive frameworks to describe the chemistry of plants' defense strategies. About 60 years ago, Lommis proposed the first defense theory, the *Growth Differentiation Balance Theory* (Loomis, 1953; Herms and Mattson, 1992; Stamp, 2004). Later, several other important theories such as the *Biochemical Co-evolutionary Theory* (Ehrlich and Raven, 1964), the *Plant Apparency Theory* (Feeny, 1976; Rhoades and Cates, 1976), the *Optimal Defense Theory* (McKey, 1974), the *Carbon/Nutrient Balance Theory* (Bryant et al., 1983), and the *Resource-Availability Theory* (Coley et al., 1985) have been formulated. Although many studies have supported predictions of these theories (Baldwin and Krebs, 1981; Bazzaz et al.,
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1987; Mole, 1994; Berenbaum, 1995; Cronin and Hay, 1996; Ohnmeiss and Baldwin, 2000; Boege and Dirzo, 2004; Hull-Sanders and Eubanks, 2005; Pavia and Toth, 2008; Van de Waal et al., 2009; Siemens et al., 2010; Diezel et al., 2011; Endara and Coley, 2011; Alba et al., 2012), none of the current defense theories is able to cover all aspects of defense and evolution in nature. This is because of conceptual, ideological and theoretical limitations inherent in the theories. For example, while studies usually concentrate on an individual trait over a short period of time, plants have evolved a complex arsenal of different defense strategies over millions of years (Schultz, 1988; Baldwin et al., 1994; Gershenzon, 1994; Koricheva, 2002; Stamp, 2003; Koricheva et al., 2004; Agrawal, 2011). Nevertheless, advances in the “omic” era (genomic, transcriptomic, proteomic and metabolomic) and comprehensive meta-analysis approaches have recently been developed and applied to questions of evolutionary ecology (Baldwin, 2012; Nordmann et al., 2012).

1.3. *N. attenuata*: an attractive model for plant ecological studies

Plant defense strategies can be generally divided into: (1) Structural defense such as leaf toughness, the epidermis, trichome, thorns (Levin, 1973; Cooper and Owensmith, 1986; Eisner et al., 1998; Lev-Yadun, 2003); (2) Chemical defenses such as alkaloids, phenolics, terpenoids, and fatty acid derivatives; (3) Proteins and enzymes: defensins, digestive enzyme inhibitors, protease inhibitors including trypsin and chymotrypsin, hydrolytic enzymes (chitinases, glucanases, lysozymes) (Bednarek and Osbourn, 2009). Examples of all these strategies can be found in *N. attenuata*, a plant native to the north-western United States. *N. attenuata* germinates from long-lived seed banks following periodic burns. Since this plant may be the only available food after fire, the newly emerged seedlings are usually heavily

![Figure 1. Trichomes on bud (A), leaf (B), and stem (C) of *N. attenuata*](image)
attacked by local herbivore communities (Baldwin and Morse, 1994; Preston and Baldwin, 1999). To optimize fitness, *N. attenuata* plants grow rapidly and use specific inducible defense mechanisms. The deployment of these specific defenses can be divided into five "layers".

The first layer involves the selective perception of environmental stresses, initiation of signal transduction to re-configure the transcriptome and proteome. The second layer involves the activate accumulation of direct defense metabolites such as toxic chemicals, repellents, and digestibility reducers. The third layer of defense involves the so-called indirect defense metabolites, which mediate higher trophic-level defensive interaction to provide information (volatiles) or nutritional encouragement (nectar) and attract entomophages. In addition, *N. attenuata* plants also can “count” the number of attacks and their severity in their activation of a fourth layer of defense, tolerance. A fifth layer of defense involves alteration of flowering time to prevent egg deposition by *M. sexta* moths.

**1.3.1. Signal transduction and activation of defense after perception of herbivore-associated elicitors**

In *N. attenuata*, accumulation of jasmonic acid (JA) orchestrates defense responses in response to wounding or *M. sexta* attack. It was proposed that fatty acid-amino acid conjugates (FACs) in the oral secretions of *Manduca sexta* are recognized by hypothetical receptor(s), before or after being modified on the wounded leaf surfaces, and activate plant defense against herbivores in *N. attenuata* (Halitschke et al., 2001; VanDoorn et al., 2010; Bonaventure, 2012). For example, in response to synthetic FACs treatment, *N. attenuata* plants accumulate mitogen-activated protein kinases (MAPKs) and WRKY transcription factors. More than 500 genes respond to *M. sexta* feeding, resulting in accumulation of JA and its bioactive form JA-Ile, which play critical signaling roles in plant (Halitschke et al., 2001; Hermsmeier et al., 2001; Halitschke and Baldwin, 2003; Paschold et al., 2007; Wu et al., 2007; Skibbe et al., 2008; Oh et al., 2012).

A number of reports suggest that *N. attenuata* is able to distinguish between herbivore damage and more general, but less destructive, mechanical wounding (Halitschke and Baldwin, 2003). In addition, transcriptional responses of *N. attenuata* attacked individually by two common herbivores, *M. sexta* and the piercing/sucking mirid bugs *Tupiocoris notatus*, indicated that *N. attenuata* is able to detect and differentially respond to the presence of herbivore-associated elicitors (HAEs) associated with particular insect species (Voelckel and Baldwin, 2003). The interactions...
between plant and herbivore depend not merely on the herbivore species, but also on the environmental context: accumulation of phytohormones and trypsin protease inhibitors under *M. sexta* or *T. notatus* attack differed under two ultraviolet B (UVB) exposure regimes, which indicated that abiotic stimuli can change the plant's interpretation of the same insect-related stimuli or *vice versa* (Dinh et al., 2012). Following perception of external stimuli and initiation of defense cascades, plants need to tune their transcriptional responses to provide the optimal level of defense induction. Plants may employ, for example, post-transcriptional modification and regulation or degradation of specific inhibitors to short-circuit defense-related pathways; or they may simply shut down expression of particular genes after the successful initiation and establishment of defense production. Such negative regulatory feedback loops may explain the transient gene expression patterns seen in response to environmental stresses (Kim et al., 2011). In this signal transduction process, RNA interference (RNAi) is one key mechanism that orchestrates gene expression patterns in *N. attenuata* (Pandey and Baldwin, 2007, 2008; Pandey et al., 2008a; Pandey et al., 2008b; Bozorov et al., 2012).

Although it is widely accepted that JA signaling is the master pathway in response to herbivory, other signaling molecules activate pathways that act synergistically or antagonistically with JA signaling. In *N. attenuata*, such signaling molecules include salicylic acid (SA), ethylene, brassinosteroids, auxin, and gibberellins. It is likely that the balance between several signaling pathways, together with JA signaling, regulate specific defense responses (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; Onkokesung et al., 2010; Gilardoni et al., 2011; Yang et al., 2011; Heinrich et al., 2012).

In general, the first layer of plant defense is the perception, interpretation, and conversion of external stimuli to specific internal signal responses. Transduction of these internal signals leads to specific changes in gene expression and plant biochemistry. However, it has been reported that even within this first layer of defense (i.e., initiation of signal transduction), a specific type of direct defense can be established. One example is the case of threonine deaminase (NaTD) in *N. attenuata*. The NaTD enzyme is required for the production of Ile in JA-Ile biosynthesis, and it is therefore very quickly induced in the leaves after herbivore attack. However, the activity of TD in ingested food simultaneously reduces the levels and availability of threonine, an essential amino acid, in the midgut of *M. sexta* larvae, thereby playing roles in both signaling and direct defense (Gilardoni et al., 2011).
1.3.2. Direct defense mechanisms

Plant chemicals can be grouped into two major categories: primary and secondary metabolites. Primary metabolites are traditionally defined as substances directly involved in growth, development, or reproduction and include sugars, proteins, amino acids, and nucleic acids (Ramawat et al., 2009). Plants use many secondary metabolites in defense against specialist and generalist herbivores (Hartmann, 2007). The isolation of morphine by Friedrich Wilhelm Sertüner about 200 years ago is generally considered as the beginning of plant secondary product research (Hartmann, 2007). However, the concept of secondary metabolites was first used and/or defined by Kossel (Bourgaud et al., 2001).

Plant secondary compounds are usually classified into four groups: (1) nitrogen-containing compounds that include alkaloids, cyanogenic glycosides, glucosinolates, non-protein amino acids, proteinase inhibitors, and lectins; (2) phenolic compounds (phenylpropanoid phenolics, coumarins, lignans, lignin, polyketide phenolics, quinones, phenylpropanoid-polyketide phenolics, flavonoids, condensed tannins); (3) terpenoids; and (4) fatty acid derivatives (Ramawat et al., 2009).

Nitrogen-containing compounds and, in particular, alkaloids are a well-known group of chemical compounds that contain nitrogen atoms in basic form. Some alkaloids are directly toxic to herbivores; for example, cactus (Lophocereus schotti) produces pilocereine and lophocereine alkaloids which are very toxic to eight species of Drosophila of the Sonoran Desert (Kircher et al., 1967). Alkaloids help plants to reduce herbivory loads and increase visits of pollinators to flowers functioning as partial repellents (Adler et al., 2001). Many alkaloids are involved in allelopathy and strongly inhibit seed germination (Wink, 1983; Levitt et al., 1984; Lovett and Potts, 1987). In general, alkaloids are considered as constitutive defenses.

In N. attenuata, nicotine, a major alkaloid, is one of the most effective constitutive defense metabolites in nature. Nicotine is produced in the roots. When the plant is challenged by herbivores, jasmonic acid induces and catalyzes its transport to leaves, where it is stored in vacuoles (Baldwin, 1999; Ohnmeiss and Baldwin, 2000; Winz and Baldwin, 2001; Steppuhn et al., 2004). Nicotine is released when herbivores damage the leaves. Once consumed and absorbed by the insect, it activates nicotinic acetylcholine receptors in the insects, which impairs the insect's central nervous system, directly suppressing insect performance and reproduction (Breer et al., 1985; Ohnmeiss and Baldwin, 2000; Steppuhn et al., 2004) and resulting in death at high doses (Casida et al., 1999).
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*N. attenuata* and many other plants possess digestive enzyme inhibitors including trypsin, chymotrypsin, and subtilisin protease inhibitors (PIs). PIs work as direct defenses by limiting the digestibility of amino acids in ingested food, thereby negatively affecting herbivore growth and survival (Van Dam et al., 2001; Glawe et al., 2003; Steppuhn and Baldwin, 2007).

Another group of nitrogen-containing compounds are the glucosinolates found in sixteen plant families (and primarily in the family Brassicaceae). There are about 150 identified glucosinolates and breakdown products that are well-known for their fungicidal, bacteriocidal, nematocidal and allelopathic properties. Normally, glucosinolates are accumulated in inactive form; however, they become activated after herbivore attack by mixing with specific myrosinase enzymes in mechanically broken tissues (Fahey et al., 2001; Halkier and Gershenzon, 2006; Wentzell and Kliebenstein, 2008; Clarke, 2010).

**Phenolic compounds:** The primary role of plant phenolics is in defense against abiotic stress. By absorbing biologically damaging wavelengths of light, phenolics function as UV screens that prevent oxidative damage (Landry et al., 1995). In addition, plants use various phenolic compounds as pigments, growth regulators, and anti-herbivore defenses. Their many functions helps explain the ubiquity of phenolic compounds in the plant kingdom (Daniel et al., 1999; Lattanzio et al., 2006). *N. attenuata* accumulates several UV-absorbing phenolic compounds such as rutin, chlorogenic acid, crypto-chlorogenic acid, dicafeoylspermidine and caffeoylputrescine, which have been shown to be simultaneously protective against herbivores and UVB radiation (Kaur et al., 2010; Dinh et al., 2012).

**Terpenoids** are the most structurally diverse group of plant secondary metabolites. They are known to be involved in diverse plant interactions such as (1) defense against herbivores, (2) defense against pathogenic fungi, (3) attraction of entomophages and pollinators, (4) allelopathic effects and (5) interaction with reactive radicals (Langenheim, 1994; Cheng et al., 2007). *N. attenuata's* terpenoids include a group of 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) that have been shown to constitute a very effective defense against insects, including specialist nicotine-adapted herbivore *M. sexta* (Jassbi et al., 2008; Heiling et al., 2010).

**Fatty acid derivatives:** Fatty acids are the biosynthetic precursors of the wax layer that covers aerial surfaces of plants and functions as a waterproof barrier to
prevent desiccation. Waxes therefore provide the first protective layer against environmental stresses (PostBeittenmiller, 1996). Several other fatty acid derivatives, such as jasmonic acid, play essential roles as plant regulators (Weber, 2002; Farmer et al., 2003; Fonseca et al., 2009a; Mosblech et al., 2009). Many fatty acids and their derivatives are well known for their antifungal and bactericidal properties (Kabara et al., 1972; Andersen et al., 1994), attraction of predators (Halitschke et al., 2008; Allmann and Baldwin, 2010), and abilities to deter oviposition by herbivores (De Moraes et al., 2001; Kessler and Baldwin, 2001) and suppress herbivore fecundity (Vancanneyt et al., 2001).

1.3.3. **Indirect defense**

Plants emit many herbivore-induced plant volatiles (HIPVs), with terpenoids acting as the dominant attractants of the carnivorous natural enemies of herbivores (Dicke, 1994; Choh and Takabayashi, 2007; Garms et al., 2008; Mumm et al., 2008). HIPVs can be eavesdropped upon by other plants and used as alarm signals to induce indirect defenses (extrafloral nectar and VOCs) in neighboring unattacked plants (Kost and Heil, 2006). In *N. attenuata*, trans-\(\alpha\)-bergamotene, linalool, and cis-3-hexene-1-ol have been shown to attract predators of herbivores in the field, such as the big-eyed bugs *Geocoris* spp. (Kessler and Baldwin, 2001; Kessler et al., 2004; Schuman et al., 2012), thus helping *N. attenuata* to increase its fitness in nature (Schuman et al., 2012).

1.3.4. **Tolerance**

Defense responses are costly for plants and may decrease plant fitness by diverting resources from growth and reproduction (Harvell, 1990; Baldwin, 1998). As an alternative to active defense, plants have evolved mechanisms to tolerate herbivore damage (Mizumachi et al., 2006; Newingham et al., 2007). Tolerance, which consists of compensatory adjustments in growth, morphology and physiology to mitigate the effects of damage, has been shown to be an efficient response to environmental stresses (Agrawal, 2000). In some extreme cases, being attacked might even prove advantageous because of the overcompensation phenomenon, in which plants damaged by herbivores attain greater fitness than those not attacked at all (Paige and Whitham, 1987; Agrawal, 2000). Under severe herbivore load, *N. attenuata* activates tolerance mechanisms including, for example, rapid changes in sink-source relations, which increases allocation of sugars to the roots and diverts resources to a less vulnerable location (Schwachtje et al., 2006). *N. attenuata* may also reconfigure primary metabolism to reduce the negative
fitness consequences of defense (Schwachtje and Baldwin, 2008), or suppress re-growth while until cessation of attack in order to save resources for later re-growth (Zavala and Baldwin, 2006).

1.3.5. **Pollinator selection**

Because pollination facilitates outcrossing, plants *invest in* pollen and nectar production to attract the services of pollinators (Barrett, 2003; Kessler, 2012). As a reflection of the value of outcrossing, it appears that plants will selectively abort the formation of self-pollinated seeds compared to outcrossed seeds (Levri, 1998). Achievement of optimal fitness in outcrossing species involves a balance between investment in attraction of pollinators and repellence of herbivores. Such a balance becomes especially critical when a single species acts as both pollinator and herbivore, as is the case with *N. attenuata* and *M. sexta*. On one hand, plant fitness is dramatically reduced by feeding of herbivores that consume reproductive (e.g., flowers, buds) and vegetative (e.g., leaves and stems) tissues. On the other hand, fitness may also be compromised by excessive diversion of resources to defense, which decreases the supply of resources available for reproduction (Kessler, 2012). In addition, if defenses are so repulsive that they keep away pollinators, plant fitness will suffer further.

As a solution to this dilemma, *N. attenuata* may opt to change the chemistry of flowers to recruit different, non-herbivorous pollinators (Mothershead and Marquis, 2000; Kessler and Baldwin, 2011). By changing important traits related to oviposition, such as flower numbers, flowering time, floral color, shape and size, pollen number and size, all of which are traditionally considered to be the result of pollinator-mediated selection (Mothershead and Marquis, 2000; Gomez, 2003; Kessler and Baldwin, 2011), plants can continue to recruit pollinators while reducing their attractiveness to the most damaging herbivore species. By shifting from night flowers to morning-open flowers when being attacked by *M. sexta* larvae, *N. attenuata* changes its main pollinator from night-active hawkmoths, such as *M. sexta*, to day-active hummingbirds (Kessler et al., 2010; Kessler and Baldwin, 2011). To further increase reproductive success, the plant varies nicotine concentrations in nectar; nicotine has a partial repellent effect on hummingbirds that encourages visitation of multiple plants, thereby enhancing outcrossing (Kessler et al., 2012).

Although it is convenient to divide plant defenses into discrete layers, with each defense layer having a specific function, plants normally activate multiple and defense
layers in order to respond to environment stimuli. Such multifaceted activation of different defense layers allows for synergistic effects and achievement between layers that results in optimal defense. One example is the synergistic function of nicotine with antidigestive trypsin protease inhibitors (TPIs) in *N. attenuata*. To compensate for the reduced nutritional quality of leaves caused by high levels of antidigestive TPIs, *Spodoptera exigua* consumes more leaf tissue. Such increased consumption would result in increased damage if TPIs were not paired with another defense. However, when TPIs accumulated in combination with nicotine, *S. exigua* larval performance was reduced (Steppuhn and Baldwin, 2007). Another example of synergistic defense in *N. attenuata* is the combination of physically defensive trichomes with chemically defensive O-acyl sugars. When *M. sexta* feeds on sugar-rich trichomes, the insect converts the sugars to branched chain aliphatic acids that attract *Pogonomymex rugosus* predators (Weinhold and Baldwin, 2011).

**Objectives of this thesis** is to explore regulatory mechanisms and interactions mediating plant defense against herbivores, and the effect of solar UVB light on plant defense in *Nicotiana attenuata*. The thesis will be introduced in five chapters as follow:

- ✓ Chapter 1: Introduction.
- ✓ Chapter 2: DICER-like proteins and their role in plant-herbivore interactions in *Nicotiana attenuata*.
- ✓ Chapter 3: A *Nicotiana attenuata* Herbivore Elicitor-Regulated 1 (NaHER1) protein uncovers a central role for abscisic acid in plant defense against herbivores.
- ✓ Chapter 4: UVB radiation and 17-hydroxygeranyllinalool diterpene glycosides provide durable resistance against mirid (*Tupiocoris notatus*) attack in field-grown *Nicotiana attenuata* plants.
- ✓ Chapter 5: Discussion
OVERVIEW OF MANUSCRIPTS

MANUSCRIPT I

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 this manuscript, we 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. *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 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.

Tohir A. Bozorov carried out all experiments, analyzed data and drafted the manuscript; Shree P. Pandey, Sang-Gyu Kim, and Klaus Gase participated in the design and coordination of the study; Maria Heinrich participated in plant-treatment, I participated in plant-treatment experiments and wrote the manuscript. Professor Ian T. Baldwin conceived the study and wrote the manuscript.
MANUSCRIPT II

A *Nicotiana attenuata* Herbivore Elicitor-Regulated 1 (NaHER1) protein uncovers a central role for abscisic acid in plant defense against herbivores.

Son Trương Đinh, Ian T. Baldwin and Ivan Gális

In review at Plant Cell

In this manuscript, we identified a novel FAC-regulated protein in *N. attenuata* (NaHER1) and show that it is an indispensable part of the FAC signal transduction pathway. *N. attenuata* plants silenced in the expression of NaHER1 by RNA interference (irHER1) were unable to amplify their defenses beyond basal, wound-induced levels in response to OS elicitation. *M. sexta* larvae performed two fold better when reared on irHER1 plants. In parallel to impaired jasmonate signaling and metabolism, irHER1 plants were more drought-sensitive and showed reduced levels of abscisic acid (ABA) in the leaves, suggesting that silencing of NaHER1 interfered with ABA metabolism. Because irHER1 plants accumulated significantly more ABA catabolites after exogenous ABA application, and OS-induced defenses in irHER1 plants could be efficiently restored by the application of exogenous ABA, we conclude that NaHER1 acts as a natural suppressor of ABA catabolism after herbivore attack that, in turn, activates full defense profile and resistance against herbivores.

I designed the research, conducted experiments, analyzed data, and wrote the manuscript. Professor Ian T. Baldwin designed experiments and wrote the manuscript. Ivan Gális designed, conducted experiments, and wrote the manuscript.
MANUSCRIPT III

UVB radiation and 17-hydroxygeranyllinalool diterpene glycosides provide durable resistance against mirid (*Tupiocoris notatus*) attack in field-grown *Nicotiana attenuata* plants.

Son Trương Đinh, Ivan Gális, and Ian T. Baldwin

Published in *Plant, Cell and Environment* DOI: 10.1111/j.1365-3040.2012.02598.x

In this manuscript, the responses of *Nicotinana attenuata* on *Tupiocoris notatus* and *Manduca sexta* attack under different solar UVB fluence were evaluated.

Solar UVB increased the accumulation of jasmonic acid, jasmonoyl-L-isoleucine and abscisic acid, all phytohormones that regulate plant defense against biotic and abiotic stress. *N. attenuata* plants experimentally protected from UVB were more infested by mirids in three consecutive field seasons. Among defense metabolites measured, 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) showed strongly altered accumulation patterns and mirid bugs (*Tupiocoris notatus*) preferred to feed on HGL-DTGs-silenced plants when other UVB protecting factors were eliminated by UVB filters. We conclude that UVB exposure not only stimulates UV protective screens but also affects plant defence mechanisms, such as HGL-DTGs accumulation, and modulates ecological interactions of *N. attenuata* with its herbivores in nature.

Son Trương Đinh, Ivan Gális, and Professor Ian T. Baldwin designed the research, conducted experiments, analyzed data, and wrote the manuscript.
Chapter 2

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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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-like 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.


Introduciton

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; Voeckel 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; Voeckel 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. 2008a), 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) small RNAs (Bartel 2004; Sunkar and Zhu 2007). These small RNAs 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 small RNAs: microRNA (miRNA), heterochromatin-associated small-interfering RNAs (hc-siRNA), trans-acting siRNA (tasi-RNA), natural antisense siRNA (nasi-RNA) and secondary siRNAs, so as to reduce transcript accumulations (Papp et al. 2003; Vaucheret 2006). Four DCL proteins with partially 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; Reinhardt et al. 2002). DCL2 is responsible for processing 22–24 nt nasi-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, nasi-RNAs, secondary viral small RNAs and siRNAs from transgenes (Bouche et al. 2006; Rajagopal 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 small RNAs 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 are called the double stranded RNA precursors of small RNAs that are critical to plants’ ability to respond to the abiotic and biotic stresses that orchestrate changes in corresponding transcripts involved in resistance (Philips et al. 2007; Pandey et al. 2008b; Ruiz-Ferrer and Voinnet 2009). When plants are stressed, they alter their small RNA 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 small RNA populations changed dramatically (Pandey et al. 2008b). OS from larvae can mimic herbivore attack in N. attenuata (McCread and Baldwin 1997; Hallischke et al. 2001). Double-stranded precursors of these small RNAs are produced by RNA-directed RNA polymerases (RDRs), which are key enzymes of the RNA 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 DCLs (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 a key role in regulating the timing of embryogenesis in Arabidopsis and in moss (Kraiwesh et al. 2010; Nodine and Bartel 2010). We characterized the three ir-dcl plants’ responses to insect herbivory under greenhouse 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 NaDCLs 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 transcrip
tomic responses.

Results

Identification and characterization of DCLs 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 have 64–66% and 64–74% similarity respectively to other plant DCL orthologs (Supporting Figure S1). DNA gel blot analysis showed that all 4 endogenous DCLs 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 QPCR 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
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, wounded 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 DCLs 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 DCLs, 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 NaDCLs, 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
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 S2A; 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 NaDCLs 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-dcls. Rosette-stage ir-dc2 and ir-dc4 x ir-dc2 plants were significantly smaller compared to WT.

(C) Quantitative data of 32-day-old WT and ir-dcl rosette leaf diameter, leaf length and width. Mean (±SE) of ten replicates per line. Asterisks indicate significant differences (** 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 these genotypes tended to tip over. Further, comparisons of our ir-dcl lines showed clear similarities to Arabidopsis dcl-mutants (Gasciollii et al. 2005; Xie et al. 2005; Adenc 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. attenuate 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; Hallischke 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 (Hallischke 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 NaDCLs 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 NaDCLs (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
Silencing NaDCLs 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 NaDCLs 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 (GE Platform: GPL3527, GE 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.nlm.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 stress-related 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 MAPKKK-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 Camara et al. 2009), was also significantly down-regulated in ir-dcl3 plants (Table 1). On the other hand, jasmonate-resistance 1 like (JAR1) transcripts were 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
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 plants [% (mol mg⁻¹) protein] after 72 h and 120 h of OS elicitation. W+OS.

(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 < 0.001; **P < 0.01; *P < 0.05) 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 DCLs (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 DCLs 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)
components of the MAP signaling cascades act upstream of JA biosynthesis (Heinrich et al. 2011; Wu et al. 2007). Down-regulated MAPK signaling 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 (Coronotina 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 have 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
stages of flowering growth. A similar phenotype has been observed in dac4 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-elicitation (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 activity (Figure 7), demonstrating a phenotype similar to ir-coll plants (Paschold et al. 2007). This suggests that NaDCL3 may be involved in the regulation of the NaCO11 receptor complex or of downstream genes of the NaCO11-mediated defense pathway.

Transcript abundance of all NaDCLs 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 Buhzt 2008; Dunoyer et al. 2010a, 2010b). NaDCL3 and NaDCL4 transcripts appeared to be dimly 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 dicaffeyl 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 (Torey 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 (287 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 TRI-ZOL 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-solaneaous 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 1000 bootstrap replicates (Molecular Evolutionary Genetic Analysis; Tamura et al. 2007).

DICER-like Proteins in Plant-insect Interactions

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 T2 generation. Transgenic seeds were germinated on agar plates containing 35 mg L-1 hygromycin B. Ten seeds of each transgenic T1 line were randomly selected and inbred through the T2 generation to obtain homozygous lines. Southern blot hybridization was performed to determine lines harboring a single insertion using a 32P-labeled fragment of the hptII gene as probe. Homozygous T2 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, ACT1 was used as an endogenous reference. The 2−ΔΔCT 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 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 D2-JA, 40 ng of D6-SA, 40 ng of D6 ABA, and 40 ng of 13C2-JA-ile (as internal standards) were homogenized by reciprocal shaking in GenoGrinder (http://www.speccsp.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-Quadrupole-MS with a ProntoSil column (C18; 5 µm, 50 × 2 mm).

Secondary metabolite analysis

The secondary metabolites analysis was performed 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 deionized water (v/v), pH 4.8). Leaf material was homogenized with GenoGrinder (http://www.speccsp.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 (Jongma 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), pH 7.6) 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 Mata-moros (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/chrRNA and hybridization to an N. attenuata transcriptome specific microarray (GEO GPL3527) 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 log2 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 (NaD clan), JN032014 (NaD clan), JN032015 (NaD clan), JN032016 (NaD clan), FM986780 (NaD clan), FM986781 (NaD clan), FM986782 (NaD clan), FM986783 (NaD clan), NM_09986 (At clan), NM_111200 (At clan), NM_01161190 (At clan), NM_122039 (At clan), XM_002302643 (Pd clan), XM_002315083 (Pd clan), XM_002324148 (Pd clan), XM_002303848 (Pd clan), XM_002515051 (Pd clan), XM_002514764 (Ro clan), XM_002516114 (Ro clan), XM_002523486 (Ro clan).
Chapter 2 – Dicer-like protein

Acknowledgments

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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 protease inhibitors. *Plant Mol. Rep.* DOI: 10.1007/s11105-011-0388-0.


Chapter 2 – Dicer-like protein

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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-dcl2. (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 EcoRI, XbaI or XhoI 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 abscistic 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), (B), (D), JA-ile. (C), SA and (D), ABA phytohormones. (+1) node rosette leaves were wounded and 20 μL of deciliated 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-dc14 silenced plants. (A), JA, (B), JA-ile, (C), SA and (D), ABA phytohormones.

Figure S7. Dicaffeoyl spermidine and caffeoylpdtrescine levels differed after OS elicitation in ir-dcl plants from WT plants. Ir-dc3 reduced dicaffeoyl spermidine and caffeoylptrescine level, whereas ir-dc14 increased dicaffeoyl spermidine and reduced caffeoylpdtrescine 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.

Figure S8. Silencing NaDCL2, NaDCL3 and NaDCL4 did not change chlorogenic acid and rutin levels after OS elicitation. Means (±SE) of six replicates per treatment.

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
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

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

**NaDCL1**
CTGTTTGCAATGAGCTGGATGCAGAGGATATTATCGATGTCAGATGGATTATTTATTA
TTGCTCGGACTGTGAAGAACAAAGGCAGCTCTTGTCTGCTAGAGGGCCAAATAGAAG
TTACAGAGTCTMGATGGGCTCYCTTAARAGCTTCTCAGTAAAGATGATGACCAT
TGATATKGGATGTTGATGGAGCCATCACCACACTCCTTGAGGCCAAGGCA
TATCT

**NaDCL2**
GATGCAACCTAGCCGAGGCACTTCCCCATGAAATGCTGAGAAGCTAGTTAATGTTCG
ATACCTGGGAATCAGCTAGATCAAGAACCTCCATGATCTTTGCTGTTGAGCT
CTAACTCAGGATCTTACATGCTACTGGAGATCCACGATGCTATCAGCGCTTGGAAT
TTCTTGGAGATTCAGTGCTAGATTATGCTGTTACAGCACATCTCATTTCAAATATCC
GGGACTGTCT

**NaDCL3**
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TCACCTGACTGAATCGGAGGCTCCTGATAAAAAGGAAGGTCTTTACAACACTTTCTGCA
TCTCAATCTCGTTGACCACATACCCGACTACGGTTTGATAGAGCTTACTGGGGATGAA
AGAGCTGATAAGA

**NaDCL4**
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TAGAGATGGGACAGCTGATAAGGAAAAACCCAGAAAGAGCATTTTGGCTTTCTGCTCC
CAGCTGCTGCGGTTGTCAGCACAGCAAGCCAGGTCATAGAAGACTCT
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.
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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.
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Table S2 List of primers used for the study.

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Chapter 3

A *Nicotiana attenuata* Herbivore Elicitor-Regulated 1 (NaHER1) Protein Uncovers a Central Role for Abscisic Acid in Plant Defense against Herbivores

Sơn Trưởng Đinh, Ian T. Baldwin and Ivan Gális
ABSTRACT

*Nicotiana attenuata* plants can distinguish the damage caused by herbivore feeding by perceiving herbivore-associated elicitors, such as the fatty acid-amino acid conjugates (FACs) in oral secretions of *Manduca sexta* larvae. However, the transduction of FAC signal into downstream plant defense responses remains largely unknown. We identified a novel FAC-regulated protein in *N. attenuata* (NaHER1) and show that it is an indispensable part of the FAC signaling. *N. attenuata* plants silenced in the expression of NaHER1 by RNA interference (irHER1) were unable to amplify their defenses beyond basal, wound-induced levels in response to OS elicitation. *M. sexta* larvae performed two fold better when reared on irHER1 plants, which released less volatile organic compounds (indirect defense) and had strongly reduced levels of several direct defense metabolites after herbivore attack. In parallel to impaired jasmonate signaling, irHER1 plants were more drought-sensitive and showed reduced levels of abscisic acid (ABA) in the leaves. Because irHER1 plants accumulated significantly more ABA catabolites after exogenous ABA application, and OS-induced defenses in irHER1 plants could be efficiently restored by the application of exogenous ABA, we conclude that NaHER1 acts as a natural suppressor of ABA catabolism after herbivore attack that, in turn, activates full resistance against herbivores.


INTRODUCTION

During the estimated ca. 400 million years of coexistence of plants with herbivorous arthropod insects (Whalley and Jarzembowski, 1981; Sanderson, 2003; Engel and Grimaldi, 2004), many plants have evolved the ability to discriminate simple wounding from herbivore-associated damage (Wu and Baldwin, 2010; Bonaventure, 2012). This discrimination ability allows plants to tailor their defense responses to the attack of specific herbivores and thereby attain higher fitness and survival rates in natural environments in which defense-growth tradeoffs frequently determine plant performance (Reymond et al., 2000; Howe and Jander, 2008). Some plants have been shown to discriminate between the attack of generalist and specialist herbivores, or
insects from different feeding guilds, through the perception of specific herbivore elicitors associated with the particular insect species (Heidel and Baldwin, 2004; Diezel et al., 2009; Rodriguez-Saona et al., 2010; Bidart-Bouzat and Kliebenstein, 2011; Chung and Felton, 2011; Ali and Agrawal, 2012; Kawazu et al., 2012). A number of herbivore-associated elicitors (HAEs) that mediate these specific recognition responses have already been identified: fatty acid-amino acid conjugates (FACs), caeliferins, glucose oxidase, β-glucosidase, inceptin, oligouronides, and lipases (Alborn et al., 1997; Schafer et al., 2011; Bonaventure, 2012; Erb et al., 2012).

The identification of HAEs, and their apparent structural diversity is consistent with the expectations of strong (co)evolutionary interactions between herbivores and their host plants (Ehrlich and Raven, 1964; Voelckel and Baldwin, 2004; Agrawal et al., 2012; Ali and Agrawal, 2012). FACs found in oral secretions (OS) of many Lepidopteran larvae are introduced into wounds during feeding on the plants; they are biosynthesized from fatty acids, such as linolenic- and linoleic acids, of plant origin which are conjugated with glutamine or glutamic acid in the insect gut. Because FACs play an essential role in larval digestion and nitrogen utilization (Yoshinaga et al., 2008) and do not occur in intact plants due to the apparent absence of the FAC-conjugating enzyme, FACs provide plants with an accurate and reliable signal of herbivore attack. FAC perception ability has been demonstrated in both monocots (maize) and dicot plants (eggplant, *N. attenuata*), suggesting an ancient origin or convergent evolution of FAC perception (Halitschke et al., 2001; Truitt et al., 2004; Schmelz et al., 2009). Because FACs in OS are rapidly metabolized on wounded leaf surfaces, it is still not completely clear if FACs function alone, or in conjunction with some of their metabolic products, to activate downstream defenses against herbivores in *N. attenuata* (Halitschke et al., 2001; VanDoorn et al., 2010; Bonaventure, 2012). In contrast to the well-established activity of FACs as insect elicitors, the signal pathways that mediate the transduction of FAC perception into defense responses are less well known (Bonaventure, 2012).

What is clear is that jasmonate (JA) signaling plays a central role in the transduction process. When synthetic FACs are applied to mechanical wounds, *N. attenuata* plants transiently accumulate dramatically larger amounts of JA and its bioactive form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), and consequently, activate both quantitatively and qualitatively adapted (tuned) defense responses compared to standardized mechanical wounding (Halitschke et al., 2001; Halitschke et al., 2003; Giri et al., 2006; von Dahl et al., 2007; Gaquerel et al., 2009). The first committed step in JA biosynthesis is the oxygenation of α-linolenic acid by 13-lipoxygenase (LOX3),
followed by the activity of several other chloroplast- and peroxisome-localized enzymes (Vick and Zimmerman, 1984; Halitschke and Baldwin, 2003). JA is then conjugated with isoleucine to form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), known to be the major endogenous bioactive jasmonate regulating downstream defense responses in plants (Kang et al., 2006; Fonseca et al., 2009b). When any of the many JA-Ile biosynthetic genes are silenced by RNAi or antisense approach, FAC-elicited herbivore resistance in *N. attenuata* is dramatically attenuated (Halitschke and Baldwin, 2003; Kessler et al., 2004; Heiling et al., 2010; Kallenbach et al., 2012). In the presence of JA-Ile, JAZ repressors that physically interact with the positive transcription factors of JA signaling, such as MYC2 proteins, are recruited to the SCF COI1 E3 ubiquitin ligase complex, ubiquitinated and consequently degraded by 26S proteasome. The removal of JAZ repressors releases MYC2, and other related transcription factors, that trigger downstream JA responses (Lorenzo et al., 2004; Chini et al., 2007; Dombrecht et al., 2007; Thines et al., 2007; Kazan and Manners, 2008; Sheard et al., 2010; Fernandez-Calvo et al., 2011; Hiruma et al., 2011; Kazan and Manners, 2012a, b; Oh et al., 2012). In *N. attenuata*, these defense responses include defense proteins such as trypsin proteinase inhibitors (TPIs), and enzymes that are directly involved in the biosynthesis of secondary metabolites that function as direct defense metabolites such as 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), caffeoylputrescine (CP), dicaffeoylspermidine (DCS), and nicotine, and the activation of indirect defense, such as the biosynthesis and release of volatile organic compounds (VOCs) from the leaves, or the production of extrafloral nectar that attracts natural enemies of herbivores (Kessler and Baldwin, 2001; Van Dam et al., 2001; Halitschke and Baldwin, 2003; Steppuhn et al., 2004; Kost and Heil, 2008; Radhika et al., 2008; Skibbe et al., 2008; Heiling et al., 2010; Kaur et al., 2010; Heinrich et al., 2012; Kessler et al., 2012; Schuman et al., 2012).

In Arabidopsis, several plant hormones have been shown to have synergistic or antagonistic effects on JA signaling. For example, synergistic and antagonistic interactions between SA and JA in defense have been reported (Takahashi et al., 2004; Beckers and Spoel, 2006), suggesting that the relative concentration of each hormone, JA and SA, affects the outcome of response (Mur et al., 2006). NPR1 protein regulates the crosstalk between SA and JA (Spoel et al., 2003), and ethylene and methyl jasmonate synergistically induced several members of the pathogenesis-related genes in groups 1 and 5 (Xu et al., 1994). ABA antagonized the JA-ethylene signaling in response to biotic and abiotic stresses (Anderson et al., 2004), however, ABA elicits JA responses via an interaction with MYC2 transcription factors (Dombrecht et al., 2007).
In fact, MYC2 known to be the central transcription factor in JA signaling in Arabidopsis, was first reported as an ABA-regulated gene (Abe et al., 2003). Conversely to effects of ABA on JA signaling, JA can have positive effects on ABA signaling, for example by mediating the expression of the ABA receptor PYL4 in leaves (Lackman et al., 2011). Crosstalk among JA and SA with other signaling pathways, such as ET, auxin, gibberellins, cytokinins (Pieterse et al., 2012), and the highly interconnected signaling networks of JA, SA, ET, nitric oxide (NO), and reactive oxygen species (ROS) in response to pathogen attack remain topics of intensive investigation (Sato et al., 2010).

In *N. attenuata*, synergistic or antagonistic interactions of plant hormones such as salicylic acid (SA), ethylene, brassinosteroids, auxin, and gibberellins with JA signaling in mediating defense and developmental responses are also widely known and most of these have been shown to be elicited by FAC elicitation (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; von Dahl et al., 2007; Onkokesung et al., 2010; Gilardoni et al., 2011; Yang et al., 2011; Heinrich et al., 2012). These interactions suggest that phytohormone crosstalk very likely plays an important role in the organization and execution of optimal defense responses against insects in the real world. Previously, the multifaceted role of ABA suggested that ABA may function to fine-tune many stress responses (Atkinson and Urwin, 2012); however, whether this modulatory activity also includes plant defense responses against herbivores is not established.

In addition, to the elicitation of phytohormone signaling by FACs in *N. attenuata*, many other FAC-elicited responses are known in this plant making it one of the best developed molecular models in plant-insect interactions. For example, FAC elicitation is known to activate mitogen-activated protein kinases (MAPKs), WRKY transcription factors, more than 500 herbivory-responsive genes, and cause changes in resources allocation and accumulation of secondary metabolites such as nitrogen-containing compounds (nicotine, TPIs), phenolic compounds (caffeoylputrescine), terpenoids (17-hydroxygeranyllinalool diterpene glycosides, terpenoid volatiles), and fatty acid derivatives (volatile oxylipins) (Halitschke et al., 2001; Halitschke et al., 2003; Giri et al., 2006; Schwachtje et al., 2006; Paschold et al., 2007; von Dahl et al., 2007; Wu et al., 2007; Gaquerel et al., 2009; Meldau et al., 2009; Kaur et al., 2010; VanDoorn et al., 2010). Although the FAC receptor(s) has not yet been identified, an analysis of FAC-responsive genes by Super SAGE (Gilardoni et al., 2010) recently identified Lectin Receptor Kinase 1 (NaLecRK1) that is rapidly induced by FACs and *M. sexta* OS that affects both SA and JA signaling pathways. A functional analysis of
NaLecRK1 found that FACs modulated and/or integrated several phytohormone signal transduction pathways during herbivore attack (Gilardoni et al., 2010; Gilardoni et al., 2011).

Here, we identify another FAC-regulated protein, NaHER1, in \textit{N. attenuata} plants and functionally locate it downstream of herbivory perception and upstream of JA-regulated defenses and metabolite accumulation. The negative impact of \textit{NaHER1} silencing on free ABA levels and the experimentally demonstrated positive effect of ABA signaling on the accumulation of several well-known anti-herbivore defense metabolites presented in this study highlights for the first time the involvement of ABA in FAC-elicited JA signaling, and the essential role played by ABA in the activation of plant innate defense against herbivores.

\textbf{RESULTS}

\textit{NaHER1} is specifically induced by FACs but not JA

In an extensive search for herbivory-elicited genes and their regulators, we focused on genes strongly responding to WOS treatment (a standardized mechanical wounding treatment immediately treated with insect OS) but less to wounds treated with water (WW). The transcripts corresponding to an \textit{N. attenuata} cDNA fragment Na\textunderscore454\textunderscore06813 (named \textit{NaHER1}) accumulated strongly and specifically 1 h after WOS but remained at significantly lower levels after WW treatment (Fig. 1A). As suggested from the lack of response to WW that is already associated with a significant burst of JA in \textit{N. attenuata}, \textit{NaHER1} induction proved to be independent of JA signaling: \textit{NaHER1} was, similar to WT, strongly induced in \textit{N. attenuata} plants silenced in JA biosynthesis (irLOX3) and perception (irCOI1) (Paschold et al., 2007; Kallenbach et al., 2010) (Fig. 1C). In addition, \textit{NaHER1} was not induced by direct application of MeJA to the leaves in a lanolin paste (Fig. 1B), even when combined with WW. MeJA is rapidly de-esterified to JA in \textit{N. attenuata} leaves (Wu et al., 2008b) and it is therefore routinely used as functional probe of JA signaling. Despite the lack of regulation by JA, \textit{NaHER1} strongly responded to synthetic FAC (18:3-Glu) found in the OS of \textit{M. sexta} larvae (Fig. 1B).

Using a previously published microarray data (Kim et al., 2011), we extracted the \textit{NaHER1} expression profile from a GEO microarray data repository, which revealed that \textit{NaHER1}, apart from its activation in OS elicited leaves, was also strongly but transiently induced in systemic untreated leaves, as well as being moderately elevated in
the roots of *N. attenuata* after OS elicitation of rosette leaves (Supplemental Fig. 1). Notably, the root transcript abundances detected by microarrays were significantly lower compared to the very strong signals found in the leaves (Supplemental Fig. 1). In summary, the intrinsic gene expression patterns of *NaHER1*, inferred from transcript abundances, indicated that it might convey specific herbivory-imprinted signals from the putative FAC receptor to converge them with the more downstream JA signaling pathway in plants attacked by herbivores.

To further explore the function of *NaHER1* gene in defense, we used RNAi-mediated gene silencing to knock-down the expression of *NaHER1* in *N. attenuata* and selected three well-silenced, single-insert containing and independently-transformed lines irHER1-6/4, -8/6, and -9/6 for further analysis (See Materials and Methods and Supplemental Fig. 2 for more details).

**Figure 1. NaHER1 is regulated by FACs independently of JA.** (A) *NaHER1* transcript abundances (± SE, n≥3) in WT leaves wounded and immediately treated with 20 µL of water (WW), or 1:5 water-diluted oral secretions (WOS) from *M. sexta* larvae. Samples were collected at designated time points and analyzed by qPCR. (B) *NaHER1* transcript abundances in WT plants wounded and treated with OS (WOS), or with the most abundant FAC in *M. sexta* oral secretions, N-linolenoyl-L-Glu (18:3-Glu) (FAC) suspended in water (WFAC), and complemented with 150 µg (0.625 µmol) of MeJA in 20 µL of lanolin paste as described; 20 µL of pure lanolin was used as control; samples were collected after 1 h treatment. (C) *NaHER1* transcript abundances in JA biosynthesis (irLOX3) and perception (irCOI1) deficient plants after WOS and WOS+MeJA treatments, samples were collected after 1 h treatment.
irHER1 plants are impaired in indirect defense

In the next experiment, we asked if NaHER1 could directly connect OS perception to OS-specific plant defenses in *N. attenuata*. *N. attenuata* plants emit a blend of volatile organic compounds (VOCs) after herbivore attack that functions as an indirect defense by attracting natural enemies of herbivores (Pare and Tumlinson, 1999; Kost and Heil, 2006; Schuman et al., 2009). VOCs are at the forefront of plant defenses, as some are instantly released from the mechanically disrupted tissues after herbivore attack (Pare and Tumlinson, 1999; Kessler and Baldwin, 2001; Huang et al., 2012; Schuman et al., 2012), frequently being modified by constituents in OS other than FACs (Allmann and Baldwin, 2010), while others are released from the entire plant, usually within one light cycle of the start of the attack (Loughrin et al., 1994). Because WW treatment does not induce volatiles such as the strongly FAC- and OS-elicited α-bergamontene in *N. attenuata* (Wu et al., 2008a), examination of indirect defenses in irHER1 plants presented a unique opportunity to parse OS (FAC)-specific responses by contrasting WW and WOS treatments. In the systemic leaf, i.e. 8-th leaf above the WOS-treated leaf, four groups of VOCs were released at reduced levels from irHER1 plants compared to wild type (WT) plants. This included the green leaf volatiles (GLVs): cis-3-hexenyl-3-methylbutyrate, cis-3-hexenyl-2-methylbutyrate, and cis-3-hexenylbutyrate; monoterpens: α-terpineol, D-limonene, and β-pinene; diterpenes: trans-α-bergamotene; and benzenoid/phenylpropanoid derivatives: methylsalicylate, methylbenzoate and benzylalcohol. Interestingly, the levels of these volatiles in irHER1 plants equaled the amounts released from WW-treated WT plants (Fig. 2), suggesting that the OS signal required to activate the VOC release was blocked in irHER1 plants. The local VOC emissions were similarly affected in irHER1 (Supplemental Fig. 3), consistent with the hypothesis that NaHER1 mediates *N. attenuata* responses to OS and/or FACs.
Figure 2. WOS-induced volatile emissions are compromised in irHER1 plants. Mean (± SE, n≥6) relative emission rates of (A) green leaf volatiles, (B) monoterpenes, (C) diterpenes, and (D) benzenoid/phenylpropanoid derivatives released from the systemic leaves of WT and irHER1 plants treated with WW or WOS. One local rosette leaf in each 44 d-old *N. attenuata* plant was mechanically wounded and treated with 20 µL water (WW) or diluted OS (WOS) 18 h before the start of volatile collections. Volatiles were collected for 3 h from the head space of a systemic leaf at position +8 (sys +8), relative to the leaf undergoing its transition from source to sink (position 0). Different letters show significant differences determined by one-way ANOVA, followed by a Fisher PLSD post hoc test (*P* ≤0.05). SE, standard error.
irHER1 plants are poorly defended against attack from *M. sexta* larvae

Because *NaHER1* is specifically induced by FACs (Fig. 1), and irHER1 plants were compromised in indirect defenses (Fig. 2), we decided to examine direct defense responses in irHER1 plants and performance of *M. sexta* on these plants. We first quantified the levels of defense metabolites HGL-DTGs (Heiling et al., 2010), caffeoylputrescine (CP), dicaffeoylspermidine (DCS) (Kaur et al., 2010) and TPIs (Steppuhn and Baldwin, 2007), as well as the transcript levels of two genes involved in their regulation, *NaMYB8* (Kaur et al., 2010) and *NaMYC2* (cDNA fragment Na_454_00400, Supplemental Fig. 4), which is the homolog of Arabidopsis *MYC2* gene (AB000875) (Abe et al., 2003).

Silencing *NaHER1* in *N. attenuata* reduced the expression of *NaMYB8* and *NaMYC2* (Fig. 3B), which translated into reduced levels of CP, DCS, HGL-DTGs, and TPIs in the plants (Fig. 3C). The reduced levels of defense metabolites resulted in the better performance of *M. sexta* larvae fed on the irHER1 plants, which grew up to two times larger on irHER1 plants compared to WT (Fig. 3A).

**Figure 3. Silencing of *NaHER1* suppressed plant defense and increased performance of *M. sexta* specialist herbivore.** (A) Fresh masses (± SE, n≥10) of *M. sexta* neonates feeding on WT and irHER1 plants recorded at designated time points to estimate the specialist herbivore performance. (B) Relative transcript abundances (± SE, n=5) of *NaMYB8* and *NaMYC2* genes determined by qPCR. (C) Mean (± SE, n=5) accumulations of secondary metabolites caffeoylputrescine (CP) and total HGL-DTGs in WT and irHER1 leaves fed by *M. sexta* neonates for 4 d. (D) Mean (± SE, n=5) TPI activity determined in local WOS-treated leaves 24 h after elicitation. Asterisks represent significant differences between WT and irHER1 lines determined by ANOVA, followed by a Fisher PLSD post hoc test (* P ≤ 0.05; *** P ≤ 0.01). SE, standard error; FM, fresh mass; CHA eq., chlorogenic acid equivalents.
irHER1 plants show symptoms of ABA-deficiency

While studying defense responses of irHER1 plants in the glasshouse, we noticed that irHER1 plants wilted faster than did WT when, accidentally, not watered for a few days. This observation suggested an ABA-related function of NaHER1, and a possible connection between ABA and JA signaling. To examine this hypothesis, we checked transpiration rates in the glasshouse and found that, indeed, both irHER1 lines had significantly higher transpiration rates compared to WT plants (Fig. 4B; ANOVA, Fisher's PLSD test, \( P < 0.05 \)). We then conducted a water competition experiment by pairing initially size-matched WT and irHER1-6/4 in one pot to provide equal conditions for both genotypes, and observed growth parameters of the plants under increasing drought stress. The drought symptoms started to develop after 4 d without watering in both WT and irHER1 plants, however, at this time point the irHER1-6/4 plants almost stopped growing while WT plants continued to elongate. After 12 d without water, WT plants were twice as tall, and flowered while none of the irHER1 plants entered the flowering stage (Fig. 4A). These results suggested that irHER1 plants either had lower ABA levels or were affected in ABA signaling, such as ABA

Figure 4. Silencing of NaHER1 affects transpiration rates and plant growth under drought stress. (A) WT (no arrows) and irHER1-6/4 (white arrows) plants after 12 d without watering. (B) Transpiration rates (± SE, n=4) determined by a LI-6400 Portable Photosynthesis System in the leaves of glasshouse-grown WT and irHER1 plants. (C) Water loss (± SE, n=9) from detached leaves of WT and irHER1 plants determined at designated time points after spray with 0.5% ethanol (control), or with 0.5% ethanol supplied with 300 µM ABA (ABA). Transpiration rates were compared by ANOVA, followed by a Fisher PLSD post hoc test; water loss data in C were analyzed by Mann-Whitney U test (* \( P \leq 0.05 \); ** \( P \leq 0.01 \)).
sensitivity that has been shown to regulate water loss via control of guard cells in stomata (Cutler et al., 2010). To examine this possibility, we measured water loss in detached leaves: while the leaves were not different under control conditions, irHER1 leaves lost more water compared to WT after spraying a 300 µM ABA solution on the leaves (Fig. 4C, Mann-Whitney U test). These data suggested that irHER1 plants are unlikely to be compromised in ABA biosynthesis but, rather, deficient in ABA perception and/or more rapidly catabolize the exogenously applied ABA, resulting in higher water loss from the leaves.

irHER1 plants accumulate less JA and ABA

To assess the possible function of NaHER1, simultaneously in local and systemic signaling, we determined phytohormone and defense metabolite levels in four different parts of the leaves, while only part 2 was treated with WOS (Fig. 5). A similar system was previously used to study the systemic defense responses in N. attenuata leaves (Wu et al., 2007; Stork et al., 2009), providing one local and three systemic samples from each treated leaf. In most zones, the levels of phytohormones JA and ABA determined 30 min after WOS, and secondary metabolites CHA, CP, DCS, HGL-DTGs determined 48 h after WOS, were reduced in the two examined irHER1 lines compared to WT plants (Fig. 5). This effect was particularly clear in the case of ABA; it was not limited to the directly wounded section of the leaf that experienced the most stress and water loss due to open wounds, it affected the entire leaf. The levels of defense metabolites in irHER1 leaf parts (Fig. 5) are consistent with the previous data from the whole-leaf treatments (Fig. 3).

Exogenous application of JA does not restore defense metabolite levels in irHER1

JA complementation by exogenous application of MeJA is an established procedure in our group to complement impaired JA levels in several JA-deficient plant genotypes such as antisense LOX3 (asLOX3), irWRKY3 and irWRKY6; MeJA treatment fully restores JA-mediated defense responses in these plants (Halitschke and Baldwin, 2003; Skibbe et al., 2008). Because reduced levels of HGL-DTGs, CP, DCS and reduced TPI activity could be due to the reduced JA and/or JA-Ile levels in irHER1 plants, we applied MeJA to irHER1 and WT plants to test this hypothesis. To our surprise, the local and systemic levels of CP, DCS and HGL-DTGs in irHER1 were not restored to WT levels when WOS was combined with MeJA treatment (Fig. 6). Only in the case of chlorogenic acid (CHA), a metabolite which is not strongly JA-dependent in its accumulation, the levels while still lower, the difference in CHA content between WT and irHER1 plants became non-significant. Together with the previously demonstrated lack of regulation of NaHER1 by JA (Fig. 1B, C), these results demonstrated that NaHER1 protein regulates JA responses via a signaling pathway that is independent of JA signaling. Since ABA levels were lower in irHER1 leaves, this phytohormone became the first suspect (Fig. 5).
Figure 5. Silencing of NaHER1 affects ABA, JA and defense metabolite levels. Rosette stage *N. attenuata* leaves were treated with WOS in zone 2 and samples were collected after 30 min and 48 h, and analyzed by LC-MS/MS: ABA, JA, JA-Ile, SA (± SE, n=4) and HPLC: chlorogenic acid, caffeoylputrescine, dicaffeoylspermidine, HGL-DTGs (± SE, n=4), respectively. Leaves were divided into four equal parts during sampling and each part was analyzed separately. Asterisks represent significant differences between WT and irHER1 lines of the same sampling zone, determined by one-way ANOVA, followed by a Fisher PLSD post hoc test (P ≤0.05). SE, standard error; FM, fresh mass; CHA eq., chlorogenic acid equivalents. Red dashed lines show treated areas.
Silencing the ABA receptor partially phenocopies irHER1 plants

Since irHER1 accumulated less ABA (Fig. 5), and ABA is known to influence local and systemic responses of plants attacked by root herbivores in other systems (Erb et al., 2011; Erb et al., 2012), we hypothesized that NaHER1 modulates ABA signaling in an FAC-dependent manner. To examine this hypothesis, we silenced the NaPYL4 (cDNA fragment Na_454_17098 (named NaPYL4, Supplemental Fig. 5), an N. attenuata homolog of the Pyrabactin Resistance 1–Like 4 (PYL 4) gene in N. tabacum, which has been shown to function as ABA receptor by Lackman et al., 2011 (Lackman et al., 2011). In the same report, jasmonate signaling was shown to involve PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco plants.

We used virus-induced gene silencing (VIGS) to test if silencing of PYL4 can affect OS-elicited defense signaling in N. attenuata plants. At the same time, we
silenced *NaHER1* in WT plants with an independent VIGS construct for direct comparison, as the interpretation of VIGS results can be influenced by the virus (TRV) presence in the inoculated plants (See Supplemental Fig. 6 for silencing efficiency of *NaHER1* and *NaPYL4*). Although reduction in JA and JA-Ile levels remained below the significance levels in *NaHER1*-VIGS plants, possibly due to a lower efficiency of silencing compared to stable irHER1 lines, both hormone levels were significantly reduced in *NaPYL4*-VIGS plants, along with the JA-elicited defense metabolites, CP and DCS (Fig. 7). These results were consistent with the hypothesis that ABA signaling modulates WOS-triggered defense responses in *N. attenuata*. Interestingly, the levels of HGL-DTGs were only reduced in irHER1-VIGS leaves, prompting a more detailed analysis of these metabolites in the future (also see Discussion).

**Figure 7.** ABA signaling is required for induction of FAC-dependent defenses. Wild type *N. attenuata* plants were silenced by VIGS in the expression of *NaHER1* or *NaPYL4* (ABA receptor). (A) Mean (± SE, n=6) phytohormone contents (ABA, JA, JA-Ile) in mechanically wounded leaves treated with 20 µL diluted OS from *M. sexta* (WOS), collected after 1 h and analyzed by LC-MS/MS. (B) Secondary metabolites (caffeoylputrescine, dicafeoyl spermidine, HGL-DTGs) in the leaves attacked by *M. sexta* neonates (one per leaf) for 4 d and analyzed by HPLC. Different letters show significantly differences between samples determined by one-way ANOVA, followed by Fisher PLSD post hoc test (*P* ≤0.05). SE, standard error; FM, fresh mass; CHA eq., chlorogenic acid equivalents.
Exogenous application of ABA restores defense in irHER1 leaves. Mean (± SE, n=4) secondary metabolite contents in rosette stage WT and irHER1 leaves wounded with a pattern wheel in zone 2 and treated with 5 µL of diluted OS from *M. sexta* immediately after treatment, leaves were sprayed with 0.5% (v/v) ethanol in water (control) or 100 µM ABA diluted in 0.5% ethanol (ABA) to complement ABA deficiency in irHER1 plants. Samples were collected after 48 h and analyzed by HPLC. Asterisks represent significant differences \( P \leq 0.05 \) between WT and irHER1-6/4 plants determined by Student’s \( t \)-test: * \( P \leq 0.05 \), ** \( P \leq 0.01 \), *** \( P \leq 0.001 \); SE, standard errors; FM, fresh mass; CHA eq, chlorogenic acid equivalents.
While treatment with MeJA failed to restore defense metabolite levels in irHER1 plants, we decided to use exogenous ABA treatment to increase the internal ABA pools (Fraser and Whenham, 1989), and see if the restoration of ABA levels would complement the interrupted NaHER1 function. After ABA complementation, the levels of CHA, CP, DCS, HGL-DTGs returned, in both local and systemic tissues to WT levels as almost no statistically significant differences could be observed between WT and irHER1 leaves sprayed with 100 µM ABA (Fig. 8). Considering the role of ABA in NaHER1 function, we also examined the transcript levels of NaHER1 at 0, 1, and 4 h after treatment with 100 µM ABA but the levels remained unchanged compared to control treatment (Supplemental Fig. 7), further confirming the specificity of NaHER1 elicitation by FACs.

Silencing of NaHER1 promotes ABA catabolism in the leaves

Endogenous ABA levels, like those of all phytohormones, are tightly regulated by a dynamic balance of both biosynthesis and catabolism, which is correlated with development, biotic and abiotic stresses (Cutler and Krochko, 1999). In addition, ABA levels are further regulated by hormone transport between roots and leaves (Seo and Koshiha, 2002; Jiang and Hartung, 2008; Umezawa et al., 2010; Seiler et al., 2011). To address a possible impact of NaHER1 silencing on ABA biosynthesis, we quantified transcript levels of NaABA1 (cDNA fragment Na_454_00091, Supplemental Fig. 8) encoding a putative N. attenuata zeaxanthin epoxidase protein in ABA biosynthesis, a putative homolog of Nicotiana plumbaginifolia (X95732), and A. thaliana zeaxanthin epoxidase (AT5G67030) gene (Marin et al., 1996; Nambara and Marion-Poll, 2005; Wang et al., 2011). In addition, we also quantified the transcript levels of NaPDR12 (cDNA fragment Na_454_00067, Supplemental Fig. 9), which is a putative homolog of A. thaliana ATP-binding cassette (ABC) transporter Pleiotropic Drug Resistance Transporter 12 (AtPDR12/ABCG40), known to be involved in ABA transport (Kang et al., 2010). However, the transcript levels of NaABA1 and NaPDR12 were not consistent with any of the observed differences in ABA levels in WT and irHER1 plants (Supplemental Fig. 10), suggesting that an alternative mechanism, such as an accelerated ABA catabolism was involved in NaHER1 function. To gradually stimulate endogenous ABA metabolism, we detached the leaves of irHER1 and WT plants and let them desiccate on a filter paper for up to 3 h. We then analyzed ABA metabolites and found that irHER1 plants accumulated more phaseic acid (PA), dihydrophaseic acid (DPA), and abscisic acid...
Figure 9. *NaHER1* silencing alters ABA metabolism in *N. attenuata*. Mean (± SE, n=3) accumulations of ABA-derived metabolites in detached leaves of WT and irHER1 plants supplied via petiole with 2 mL 0.5% (v/v) ethanol in water supplemented with 0.5% (w/v) mannitol and 0.02% (v/v) OS from *M. sexta* larvae (control), or with control solution supplied with 100 µg ABA (ABA). Samples were collected after 3 h and analyzed by LC-MS/MS for individual ABA metabolites. Different letters show significant differences between samples determined by one-way ANOVA, followed by a Fisher PLSD post hoc test (P ≤0.05). SE, standard errors; 7(9)-OH-ABA, 7(9)-hydroxy-ABA; PA, phaseic acid; neoPA, neophaseic acid; PA-Me, phaseic acid-methyl ester; DPA, dihydrophaseic acid; DPA-Me, dihydrophaseic acid methyl ester; ABA-Me, ABA methyl ester. The levels of ABA metabolites were determined relative to D₆-ABA internal standard.
glucose ester (ABA-GE) (Supplemental Fig. 11), suggesting an accelerated catabolism of ABA in detached irHER1 leaves. To enhance the levels of endogenous ABA and stimulate NaHER1 expression, we stem-fed ABA into the leaves by placing the petioles of detached leaves into 2.0 mL of control solution (0.5% mannitol + 0.02% oral secretions from *M. sexta* + 0.5% ethanol) or the same control solution supplemented with 100 µg ABA for 3 h. In this experiment, ABA was metabolized into four major metabolites, 7-OH-ABA, 9-OH-ABA, neoPA, and PA that significantly increased in WT leaves, and all of them attained concentrations clearly higher in irHER1 leaves compared to those of WT leaves. The levels of DPA, although not significantly changed by exogenous ABA feeding in WT, were also significantly higher in irHER1 leaves (Fig. 9). These results suggested that NaHER1 silencing affected ABA levels by promoting its catabolism. In other words, the function of FAC-regulated NaHER1 gene is to inhibit ABA catabolism that positively contributes to, and converges with, JA signaling to amplify the accumulation of defense metabolites (Fig. 10).

**NaHER1 silencing affects the overall plant fitness**

Because defense responses can be costly for plants (Harvell, 1990; Baldwin, 1998), we speculated that irHER1 plants could have more seed capsules than WT under herbivore pressure in the glasshouse, since the cost of inducible defense would not impact the fitness of plants regularly supplied with sufficient amounts of nutrients (Baldwin, 1998; Zavala et al., 2004). In contrast to our expectations, irHER1 plants showed delayed flowering and produced fewer seed capsules (and flowers at some stages) than WT (Supplemental Fig. 12, Mann-Whitney U test comparisons). As this was true for both control unattacked plants, and plants being continuously attacked by *M. sexta* larvae, we propose that NaHER1, despite its relatively low transcript levels detected in non-induced plants (Fig. 1), can also play a significant role in a plant’s development, possibly due to response to other stresses and/or optimizing a plant’s response to watering regimes. In addition, the costs not incurred by irHER1 plants in avoiding the production of defenses when attacked by herbivores was clearly overshadowed by the negative fitness impact of the loss of NaHER1 function.
DISCUSSION

Plants have evolved sophisticated strategies and extreme plasticity to adapt to – and survive – unpredictable environmental stresses. The evolution of perception mechanisms of biotic and abiotic stresses and the efficient conversion, integration and transduction of external stimuli into downstream signaling networks have allowed plants to mount appropriately tailored and scaled defense responses. During herbivory, a major reconfiguration of plant signaling and metabolism occurs to prevent further mechanical damage and loss of photosynthetic tissues, desiccation from wounds, repair wounds and rebuild damaged cell walls and, in some cases, also to bunker resources to organs distal and inaccessible to feeding herbivores (Schwachtje et al., 2006; Schwachtje and Baldwin, 2008). While jasmonate signaling is known to play a major role in these processes, it is becoming evident that more than one hormone is required to assure properly coordinated growth and defense responses in plants (Genoud and Metraux, 1999; Moubayidin et al., 2009; Robert-Seilaniantz et al., 2011; Choudhary et al., 2012). Here, we show, by analysis of the FAC-mediated signaling and the FAC-regulated protein NaHER1 that a typical drought stress hormone, ABA, interacts with JA signaling to enable \textit{N. attenuata} plants to mount a full defense response against chewing herbivores. These data complement the recently recognized role of ABA in regulating JA-mediated defense responses against necrotrophic pathogens (Adie et al., 2007; Fan et al., 2009).
FAC-mediated signaling in *N. attenuata*

JA signaling is a core mechanism in plant defense against herbivores. While wound responses in most, if not all, plants are associated with a rapid JA burst, occurring just minutes after mechanical damage, different plants display very different defense responses when attacked by herbivores. In some plants, such as lima beans, repeated mechanical damage can closely mimic herbivory-induced spectrum of VOCs emitted from the plants (Mithofer et al., 2005) but other plants perceive herbivore-associated molecular patterns (HAMPs) to mount herbivore-specific defense responses. For example, mechanically wounded *N. attenuata* plants do not produce substantial amounts of TPIs or VOCs, unless OS containing FAC elicitors is introduced into the wounds (Halitschke et al., 2001; Wu et al., 2007; Wu et al., 2008a). Although the accumulation of some other metabolites, such as caffeoylputrescine (CP) and dicaffeoylspermidine (DCS) can be increased by wounding, possibly due to their dual role as antimicrobial and anti-herbivory substances, their amounts greatly increase when FACs are introduced into wounds. Interestingly, core forms of defense metabolites HGL-DTGs undergo a typical conversions into dimalonylated forms, which is triggered by herbivory (Halitschke and Baldwin, 2003; Steppuhn et al., 2004; Kang et al., 2006; Paschold et al., 2007; Steppuhn and Baldwin, 2007; Heiling et al., 2010; Kaur et al., 2010; Woldemariam et al., 2012). Despite a clear role for insect elicitors, such as FACs, in tailoring plant defenses against herbivores, the signal perception mechanisms and transduction of FAC signal remain largely unknown.

ABA/JA crosstalk in herbivory and pathogen resistance

Given the complexity of plant defenses, it is expected that other signaling pathways will strongly interact with FAC-signaling in plant-insect interactions; for example, salicylic acid (SA), ethylene, brassinosteroids and their signaling pathways have already been shown to act both synergistically and antagonistically with JA during herbivore attack in *N. attenuata* (Voelekel et al., 2001; Rayapuram and Baldwin, 2007; Onkokesung et al., 2010; Yang et al., 2011). However, until now, the role of a typical drought-related hormone ABA in defense against herbivores has not been well defined, although its role was anticipated due to herbivory-mediated exposure of cells leading to an excessive water loss. In addition, plant defense against necrotrophs has already been shown to depend on ABA signaling (Adie et al., 2007; Fan et al., 2009).

The combination of JA-deficiency and impaired ABA accumulation in the WOS-treated leaves of irHER1 plants was an unexpected phenotype (Fig. 5), suggesting that NaHER1 influenced both hormone metabolic pathways. Alternatively, NaHER1 could have affected ABA levels, which in turn impaired plants in JA accumulation and response (Fig. 7). Previously, in potato and tomato ABA was required for wound-induced JA accumulation (Pena-Cortes et al., 1995) and ABA signaling was also required for normal JA signal transduction (Pena-Cortes et al., 1993; Pena-Cortes et al., 1995).
1995), indicating a close relationship and coordination of these two hormonal pathways. The T-DNA-insertion causing an overexpression of NINE-CIS-EPOXYCAROTENOID DIOXYGENASE5 (NCED5), ABA biosynthetic enzyme, not only increases ABA levels but also promotes JA biosynthesis (Fan et al., 2009). When we measured JA and JA-Ile levels after WOS treatment supplementation with exogenous ABA, a significant increase in JA and JA-Ile levels compared to WOS alone was observed (Supplemental Fig. 13), supporting a positive role of ABA in JA biosynthesis and/or accumulation in *N. attenuata* plants during herbivory.

While higher levels of ABA compromises Arabidopsis’s resistance to various *Pseudomonas syringae* strains, a biotrophic pathogen, the *cds2-1D* mutants became more resistant to necrotrophic fungus *Alternaria brassicicola*, consistent with the increased JA levels and the role of ABA in resistance against necrotrophic pathogens. In addition, ABA regulated the expression of several defense genes, affected JA biosynthesis, and thus contributed to Arabidopsis defense against another oomycete necrotrophic pathogen, *Pythium irregular* (Adie et al., 2007). While both ABA and JA levels strongly increased upon pathogen infection 6-12 h post infection, the accumulation of JA was strongly compromised in ABA deficient mutant aba2-12, thus establishing the role of ABA in plant defense against necrotrophic pathogen-imposed stress. Whether these results applied to JA-dependent plant defense against herbivores was not yet known, due in large part to the limitations of Arabidopsis plants as plant-insect model (i.e. Arabidopsis does not respond to FACs).

**ABA contribution to defense involves FAC perception and NaHER1 function**

The use of the *N. attenuata* ecological model for plant-insect interactions with its particularly strong natural responses to FACs allowed the identification of several FAC-responsive genes. The expression of *NaHER1* showed only a weak induction by wounding but burst of transcripts after WOS treatment, which coincided with JA and JA-Ile accumulations, but was independent of JA. In contrast, JA and JA-Ile levels were compromised in irHER1 plants, together with the accumulation of several defense metabolites, and plants became more susceptible to attack from the caterpillars of *M. sexta*. In addition, irHER1 plants showed several ABA deficient phenotypes.

In *N. tabacum*, the overexpression of *NtPYL4*, which is a functional ABA receptor, suggested that crosstalk between JA and ABA signaling pathways can affect alkaloid biosynthesis (Lackman et al., 2011). The accumulation of nicotine alkaloids was suppressed in tobacco hairy roots overexpressing *NtPYL4*, suggesting a negative role of ABA in the roots. Interestingly, in the leaves, MeJA induced transient expression of *NtPYL4* with a peak at 30 min. In contrast, in roots, *NtPYL4* expression decreased with a maximum of fivefold reduction after 2 h of MeJA treatment. A differential transcriptional response of ABA receptor in the roots and leaves suggests a possible differential role of ABA in these organs, consistent with the observed positive effect of
ABA on leaf defense metabolites (and JA accumulation) found in this study (Fig 10). While exogenous application of MeJA did not restore WT defenses, exogenous ABA recovered WT defense levels in irHER1 plants, indicating that JA signal transduction indeed requires ABA to induce a full defense response. This was independently confirmed by silencing the ABA receptor \textit{NaPYL4} which reduced the accumulation of JA, JA-Ile and several leaf defense metabolites in these plants (Fig. 7).

A convergence of JA and ABA signaling in Arabidopsis has already been shown at the level of a key transcription factor MYC2 (Abe et al., 2003; Yadav et al., 2005), pointing to the most likely downstream target of JA-ABA crosstalk in defense against herbivores. In \textit{N. attenuata}, \textit{NaMYC2} transcript levels were reduced in irHER1 plants (Fig. 3), together with another transcription factor \textit{NaMYB8} known to control biosynthesis of CP and DCS during herbivore attack (Kaur et al., 2010). Because the Arabidopsis MYC2 was initially identified as an ABA-regulated gene, it is possible that ABA, together with JA contributes to the accumulation of MYC2 protein in the cells. As MYC2 protein levels are titrated by the physical interaction with and inhibition of JAZ repressors, lower amounts of ABA (and MYC2) could lead to a stronger repression of JA signaling, as more JAZ would have to be degraded to release the MYC2 regulators of JA signaling from their inhibition. A crosstalk of ABA signaling with signaling pathways such as SA (Zabala et al., 2009), ethylene (Anderson et al., 2004), cytokinin (Ha et al., 2012), nitric oxide (Hancock et al., 2011) and gibberellins (Seo et al., 2006) provide additional possibilities for an indirect action of ABA on JA as the balance of several phytohormones, rather than one hormone often determines the responses (Mauch-Mani and Mauch, 2005; Stamm and Kumar, 2010; Peleg and Blumwald, 2011). For example, the interaction between ABA and SA determines the interaction of rice and \textit{Magnaporthe grisea}, revealing a strong negative effect of ABA on SA-dependent gene expression in rice (Jiang et al., 2010).

While local and systemic CP and DCS levels were consistently reduced in irHER1 plants, total levels of another typical defense metabolite HGL-DTGs in \textit{N. attenuata} determined by HPLC-ELSD showed much less consistent patterns, sometimes being comparable to WT levels (Fig. 3, 5, 6, & 7). To understand this inconsistency, we conducted additional experiments to analyze the relative amounts of 10 individual HGL-DTGs by highly selective LC-MS/MS analysis in samples showing comparable levels of total HGL-DTG in irHER1 and WT plants. A clear pattern in the accumulation of various forms of HGL-DTGs was observed that provided plausible explanation for variable HGL-DTG patterns detected by HPLC-ELSD method (Supplemental Fig. 14). In \textit{N. attenuata}, four groups of HGL-DTGs (precursor, core, malonylated and dimalonylated) exist; while the first three groups showed increased levels in irHER1 plants, only the dimalonylated compounds (nicotianoside II, nicotianoside V, nicotianoside VII) were reduced. This was highly consistent with the previously reported conversion of precursor, core and malonylated HGL-DTGs to dimalonylated
ones in response to WOS treatment (Heiling et al., 2010). Because total HPLC-ELSD-detected HGL-DTGs represents a sum of all individual forms, the impaired FAC-driven metabolic shift and lack of dimalonylation was likely masked by the accumulation of the upstream metabolites.

**FAC perception regulates ABA homeostasis via NaHER1**

After establishing the role of ABA in FAC and NaHER1-mediated defense against herbivores, we focused on how NaHER1 controls ABA levels during herbivory. ABA metabolism and homeostasis is dynamically controlled by ABA biosynthesis and catabolism (Cutler and Krochko, 1999; Mauch-Mani and Flors, 2009; Dolferus et al., 2011). In addition, the levels of endogenous ABA may fluctuate dramatically in response to variable environmental conditions such as drought (Cutler and Krochko, 1999; Okamoto et al., 2009). The catabolic inactivation of ABA in Arabidopsis occurs via two major pathways, oxidation and conjugation (Oritani and Kiyota, 2003; Nambara and Marion-Poll, 2005). The ABA 8'-hydroxylase gene has been isolated and shown to function as a key enzyme in ABA homeostasis (Krochko et al., 1998; Nambara and Marion-Poll, 2005), together with glucosyltransferases that catalyze the conjugation of abscisic acid-glucose esters (ABA-GE) (Xu et al., 2002; Nambara and Marion-Poll, 2005). In Arabidopsis, the overexpression of glucosyltransferase UGT71B6 caused higher accumulations of ABA-GE, and reduced levels of PA and DPA; however, it only marginally effected free ABA levels (Priest et al., 2006). Similarly, mutation in ABA 8'-hydroxylase in *A. thaliana* only marginally increased ABA levels and decreased PA content, whereas the overexpression of ABA 8'-hydroxylase reduced ABA and increased PA and DPA (Umezawa et al., 2006). The lack of dramatic changes in free ABA levels in plants altered in ABA catabolism suggests a strong feedback control in ABA biosynthesis and metabolism, which is also reflected in the expression levels of key metabolic enzymes (Kushiro et al., 2004; Nambara and Marion-Poll, 2005).

In irHER1, the levels of ABA were transiently reduced at 30 min (Fig. 5) to 1 h (Fig. 7) after WOS treatment, which was not reflected in reduced expression levels of ABA biosynthetic gene *NaABA1* encoding key biosynthetic enzyme zeaxanthine oxidase, or putative ABA transporter *NaPDR12* (Supplemental Fig. 10) in *N. attenuata*. In contrast, several ABA derived catabolites accumulated more in irHER1 plants, suggesting that a more rapid catabolism of ABA could account for the reduction in ABA levels in irHER1. Because this increase was not limited to a single ABA catabolite, it is likely that NaHER1 simultaneously suppresses the activity of several ABA catabolic enzymes to promote ABA accumulation. This positively affects JA accumulation and signaling, and ultimately leads to higher accumulation of defense metabolites and better defense in herbivore attacked *N. attenuata* plants (Fig. 10). Further experiments including the identification of ABA catabolic genes, such as microarray experiments with irHER1 and WT plants will help in the future to fully
understand the function of NaHER1, and possibly allow for the discovery of novel ABA-metabolic genes and/or their regulators in *N. attenuata*.

**NaHER1 in plant development**

ABA metabolism and function are two critical factors involved in normal plant growth and development. Plants impaired in ABA biosynthesis or sensitivity commonly exhibit severe growth retardation, dwarfing and wilting phenotypes (Cheng et al., 2002; Gonzalez-Guzman et al., 2002; Nambara and Marion-Poll, 2005; Arend et al., 2009). In addition, ABA plays a crucial role in the regulation of gas exchange via the control of stomatal aperture and it regulates transpirational water loss and desiccation during drought, therefore affecting total CO$_2$ uptake and photosynthesis (Farquhar and Sharkey, 1982; Pei et al., 1998; Schroeder et al., 2001; Gonzalez-Guzman et al., 2012; Kusumi et al., 2012). Although the *NaHER1* gene was induced in a FAC-dependent manner, *NaHER1* silencing caused other pleiotropic changes in growth and development when plants are grown in glasshouse. Higher transpiration rates, accelerated wilting, delay in flowering time, and reduced seed capsule numbers compared to WT (Fig. 4 & Supplemental Fig. 12) suggested that NaHER1 gene may have -- first -- acquired a role in plant growth and development, which -- only later -- became associated with FAC-signaling and defense responses. Interestingly, the OS fraction isolated from *Pieris brassicae* and *Spodoptera littoralis* larvae suppressed wound-induced water loss in Arabidopsis (Consales et al., 2012). Because *S. littoralis* OS is known to contain FACs (Maffei et al., 2004a), Arabidopsis homologue of HER1 may be involved in this response and, upon putative perception and transduction of FAC signal, promote ABA accumulation and regulation of guard cells to reduce water loss from the OS-treated wounds. Interestingly, such response would have to be disconnected from defense signaling, as Arabidopsis plants did not accumulate more JA in response to treatment with a typical FAC, venticin (Schmelz et al., 2009).

Because JA signaling pathway is known to affect plant development, especially flower, seed maturation, and anther dehiscence in Arabidopsis, tomato and *N. attenuata* (Feys et al., 1994; Li et al., 2004; Paschold et al., 2007), impaired JA signaling pathway could have also directly contributed to irHER1 reproductive phenotypes, such as delayed flowering and reduced number of seed capsules in irHER1 plant (Supplemental Fig. 12).

Overall, a simple model including the regulation of stomata by NaHER1 and ABA is not sufficient to explain irHER1 phenotype, as for example, widely open stomata with higher transpiration rates should allow equal or higher emissions of VOCs. In contrast, VOCs emissions from irHER1 plants were strongly reduced (Fig. 2), providing strong evidence for NaHER1 and ABA function beyond the control of guard cells, revealing a direct contribution of endogenous ABA to JA signaling and plant defense.
MATERIALS AND METHODS

Plant material, growth conditions and plant treatments

The seeds of *Nicotiana attenuata* Torr. ex Watson wild type (WT) and transgenic plants were sterilized and germinated on Gamborg’s B5 medium as described in (Krugel et al., 2002). Previously established *N. attenuata* transgenic lines silenced in JA biosynthesis (irLOX3) and JA-Ile perception (irCOI1) were used (Paschold et al., 2007; Heiling et al., 2010; Kallenbach et al., 2010). The irHER1 *N. attenuata* plants silenced in the expression of novel herbivore elicitor-regulated protein (*NaHER1*) were obtained by transformation with an inverted repeat construct containing a 297 bp long sequence of *NaHER1* as shown in Supplemental Fig. 2A & B. Three homozygous single-insertion lines in T2 generation (irHER1-6/4, irHER1-8/6, irHER1-9/6) were selected after verification by Southern blot hybridization (Supplemental Fig. 2C) and growth on hygromycin-containing media as recommended in (Gase et al., 2011). Plants were maintained under standard glasshouse conditions: 16 h light at 24-28 °C, and 8 h dark at 20-24 °C. To simulate herbivory in WOS treatments, leaf lamina were punctured by rolling a serrated fabric pattern wheel three times on each side of the midvein (W), and the fresh puncture wounds were immediately treated with 20 µL of *M. sexta* oral secretions (OS) diluted 1:5 in distilled water. Otherwise, wounds were treated with water (WW), or leaves remained untreated as controls (Halitschke et al., 2001).

In water competition experiment, plants were kept under standard glasshouse conditions until rosette stage and, then, watering was stopped for 15 d (31 to 46 d after germination). The pictures were taken at regular intervals to document plant wilting, reduced growth and other water-deficiency stress symptoms (Fig. 4A shows plants stressed for 12 d). At 47 d after germination, and 15 d of drought stress, water was re-supplied to all plants and maintained at normal level until termination of experiment.

In methyl jasmonate (MeJA) treatments, 7.5 mg of MeJA was dissolved in one mL of melted lanolin and 20 µL of this lanolin paste, containing equivalent of 150 μg of MeJA, was applied on the leaf. An aliquot of 20 µL pure lanolin was used in control treatments (Baldwin et al., 1996; Halitschke et al., 2000).

In ABA treatments, 100 μM ABA solution, previously shown to activate systemic signaling in tomato (Pena-Cortes et al., 1989), was used to examine the role of ABA in local and systemic defense of *N. attenuata*. An aliquot of approximately 200 µL freshly prepared ABA solution in 0.5% ethanol/distilled water (v/v) was directly sprayed on intact leaves; approximately 200 µL of 0.5% ethanol solution was used in control treatments. Approximately 200 µL of 300 μM ABA or 0.5% ethanol solutions were used to spray isolated leaves of filter paper in water loss experiment.
Phytohormone analyses

Phytohormone extractions were performed as described in (Dinh et al., 2012) and individual phytohormones were analyzed by liquid chromatography - tandem mass spectrometry (LC-MS/MS) on a Varian 1200 Triple-Quadrupole-LC-MS system (Varian, Palo Alto, CA, USA). The mobile phases consisted of solvent A (0.05% (v/v) formic acid in water) and solvent B (methanol). The elution profile was: 0:00-1:30/15% B in A; 1:30-4:30/15 to 98% B in A; 4:30-17:00/98% B in A, 17:00-18:00/98 to 15% B in A; 18:00-20:00/15% B in A, and with a flow, time/flow (min/mL), of: 0:00-1:00/0.4; 1:00-1:30/0.4 to 0.2; 1:30-15:00/0.2; 15:00-15:30/0.2 to 0.4; 15:30-20:00/0.4. Multiple-reaction monitoring (MRM) mode was used to monitor analyte parent ion to product ion transitions as described previously (Wang et al., 2007; Owen et al., 2009; Tureckova et al., 2009); see Supplemental Table 1 for detailed information about the collision-induced dissociation energy (CID), precursor (m/z) and product (m/z). D2-JA, JA13C6-Ile, D6-ABA, and D4-SA were used to quantify JA, JA-Ile, ABA and SA accordingly. The levels of individual ABA metabolites were quantified by using D6-ABA internal standard.

Analysis of secondary metabolites and volatiles

Extraction of secondary metabolites was performed as described previously (Dinh et al., 2012). Leaf extracts were analyzed on an HPLC instrument equipped with PDA detector, essentially as described in (Oh et al., 2012). External calibration curve using an authentic standard with six data points (serial dilutions) was used to quantify chlorogenic acid contents (CHA). Caffeoylputrescine (CP) and dicaffeoylspermidine (DCS) contents were quantified using CHA calibration curves, and expressed as CHA equivalents in all graphs. Peak areas were used to estimate total HGL-DTGs contents in HPLC chromatograms detected by Evaporative Light Scattering Detector (ELSD) after HPLC separation used for secondary metabolites. Volatile organic compounds (VOCs) were collected from the head-space of selected, treated or systemic, leaves and analyzed as described in (Oh et al., 2012) by gas chromatography (GC)-MS.

Analysis of gene expression by quantitative real-time PCR (qPCR)

Total RNA was extracted by TRIzol® reagent (Invitrogen) as recommended by manufacturer and treated with RNase-free DNase-I (Fermentas, St. Leon-Rot, Germany) to remove all DNA contaminations following the manufacturer’s protocol. cDNA synthesis, real-time quantitative PCR (qPCR), and calculation of relative transcript abundances of genes of interest were performed as described in (Pfaffl et al., 2002; Dinh et al., 2012). The primer sequences used for qPCR (SYBR green assays) are listed in Supplemental Fig. 15.
Virus induced gene silencing (VIGS)

The cDNA fragment of *NaHER1* and *NaPYL4* (Supplemental Fig. 2A & 5) were inserted into pTV00 vector to create constructs used for silencing of *NaHER1* and *NaPYL4* expression in *N. attenuata*, respectively. The transformed pTV-PYL4 and pTV-HER1 plasmids were then transferred to *Agrobacterium tumefaciens* for plant transformation. pTV00 plasmid without gene insert was used as an empty vector control and pTV00 plasmid carrying a 206-bp fragment of phytoene desaturase (*pds*) gene from *N. benthamiana* was used as visualization indicator for silencing efficiency (bleaching leaves).

The *Agrobacterium tumefaciens* strains containing pTV plasmids were inoculated in YEB media (10 g L⁻¹ yeast, 10 g L⁻¹ peptone and 5 g L⁻¹ sodium chloride), 50 mg L⁻¹ kanamycin, and shaked at 200 rpm overnight at 28°C until reaching the optical density at 600 nm (OD600) of 0.4 - 0.6. Cells were harvested by centrifugation at 4,000 rpm for 12 min at 20°C and pellets resuspended in 10 mL solution of 5 mM MgCl₂ and 5 mM MES. Before infiltration, each solution containing the *A. tumefaciens* cells carrying the construct of interest was combined with an equal volume of similarly prepared *A. tumefaciens* culture carrying pBINTRA6 plasmid encoding an intron-disrupted RNA1 of tobacco rattle virus (TRV) that is essential for virus multiplication.

Approximately 5 d after transfer from small Teku to larger 1 L pots with soil (ca. 25 days after germination), *N. attenuata* seedlings were pressure-infiltrated on three youngest expanding leaves with a syringe containing *Agrobacterium* solution. After two days in dark and high humidity conditions, the plantlets were grown in the culture room at 20–22°C with a 16/8-h light/dark regime until bleaching in pTV-PDS plants has developed and plants were ready for treatments.

Measurement of TPI activity

TPI activity was measured by a radial diffusion assays as described in (Jongsma et al., 1994). Briefly, plant tissues were ground in liquid nitrogen and 100 mg of fine powder from each sample in microcentrifuge tube was suspended in 300 μL of extraction buffer containing 0.1 M TRIS-HCl, pH 7.6, 5% polyvinylpolypyrrolidone, 0.2 % phenylthiourea, 0.5% diethylithiocarbamate, and 0.05 M Na₂EDTA (Jongsma et al., 1994). After thorough vortexing and homogenization, samples were centrifuged at 12,000 g for 20 min at 4°C and supernatants (100-150 μL) were transferred to a new tube. Cleared supernatants were kept on ice until further analysis. A serial dilution of five bovine serum albumin (BSA) concentrations, ranging from 0.03 - 0.50 mg/mL were used as calibration curves to quantify total protein content in extracts by Bradford method (Bradford, 1976). TPI activities were determined by radial diffusion assays using bovine trypsin protease (Sigma) dissolved in agar (50 μg/25mL agar). A series of soybean trypsin protease inhibitor (Sigma) in the same plate, concentrations ranging
from 0.29 - 4.59 µM, were used to generate external calibration curves for TPI quantifications (Jongsma et al., 1994).

**Statistical analyses**

Data were analyzed with SPSS statistic 17.0 (SPSS Inc., Chicago, Illinois, USA) and INFOSTAT version 2011e (Universidad Nacional de Córdoba, Argentina). Student’s t-test, analysis of variance (ANOVA) followed by *post hoc* Fisher’s protected least significant difference (PLSD) were used. Comparisons of means were calculated at a minimal 0.05 level of significance. Data in water loss experiment (Fig. 4C), and differences in seed capsules and flower numbers (Supplemental Fig. 12) were analyzed by Mann-Whitney U Test.

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**AUTHOR CONTRIBUTIONS**

Son Trường Đinh designed the research, conducted experiments, analyzed data, and wrote the manuscript. Ian T. Baldwin designed experiments and wrote the manuscript. Ivan Gális designed, conducted experiments, and wrote the manuscript.

Supplemental figure 1. *NaHER1* is systemically regulated after WOS treatment. Patterns of local and systemic normalized signal intensity of *NaHER1* transcript abundances (± SE, n=3) after WW and WOS treatments, and in control untreated plants extracted from a previously published microarray data set (Kim et al., 2011). WT leaves were wounded and immediately treated with 20 µL of water (WW), or 1:5 water-diluted oral secretions (WOS) from *M. sexta* larvae, and collected at designated time points.
Supplemental Figure 2. Sequences, silencing construct and transgenic plant information. (A) Full length mRNA sequence of NaHER1; bold underlined sequence was used to create inverted repeat structure (hairpin) and independently silence NaHER1 by VIGS and RNAi. (B) Physical map of pSOL8CBP plant binary transformation vectors used to generate irHER1 plants. The pSOL8CBP vector contains a strong constitutive cauliflower mosaic virus 35S (CaMV) promoter. cDNA fragment shown in (A) was cloned into the two multiple cloning sites in reverse orientation, which was separated by a pyruvate orthophosphate dikinase (pdk i3) intron to form an inverted repeat construct. (C) Southern blot analysis confirming the presence of a single T-DNA insertion in the N. attenuata genome of each transgenic line used in the study. A 10 μg aliquot of genomic DNA prepared from each of three independently transformed lines (irHER1-6/4, -8/6 and -9/6) was digested with XbaI restriction enzyme and separated by gel electrophoresis. Radioactively-labeled DNA fragment of the hygromycin phosphotransferase gene (hptII ) was used as a hybridization probe. (D) Silencing efficiency of NaHER1 in three independent NaHER1-silenced lines determined by qPCR.
Supplemental Figure 3. *NaHER1* silencing reduces emissions of several isoprenoid and phenylpropanoid/benzenoid volatiles from local WOS-treated *N. attenuata* leaves. Mean (± SE; n≥6) releases of volatile compounds from locally-treated leaves of WT and irHER1 plants. A single local leaf in 35 d-old WT or irHER1 plants was mechanically wounded and treated with 20 µL of diluted OS from *M. sexta* (WOS). 18 h after treatment, volatiles were collected from the head space of the same leaf for 7 h and analyzed by GC-MS. Different letters show significant differences between samples determined by one-way ANOVA, followed by a Fisher PLSD *post hoc* test (P ≤0.05). The volatile blends emitted from irHER1 leaves were strongly suppressed in the production of terpenoids (α-pinene, α-terpineol, β-pinene, D-limonene, (E)-α-bergamotene, α-duprezianene), and phenylpropanoid/benzenoids (benzaldehyde, benzyl alcohol). SE, standard error.
Supplemental Figure 4. The sequence of the NaMYC2. The cDNA sequence of bHLH-domain NaMYC2 was obtained after searching cDNA libraries of N. attenuata using authentic A. thaliana (AB000875) and N. benthamiana MYC2 (GQ859153) sequences.
Supplemental Figure 5. cDNA fragment of NaPYL4 gene. The cDNA sequence of a putative *N. attenuata* ABA receptor *NaPYL4* was obtained after searching cDNA libraries of *N. attenuata* using authentic *N. tabacum* *PYL4* (AJ966358) sequence. The bold and underlined sequence was used to design VIGS construct and silence *NaPYL4*. 

>Na_454_17098

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1  CAAAAGAGCC  CCATTAGCGA  CCACTTTTAC  AGTTTACGAC  ATAAAGAATCA  TCAGCTCTCC
61  TTTCATTTCA  GATCAAAATC  TAACAAACCT  TGGCCAAAAA  AAAATGCTCTC  CTAGTTTCTCC
121  AGATTCATCT  GTTTTACTCC  AAAGAATAAG  CTTCAACTCT  ACTTCTGATT  TTGCCCTGTA
181  ACAATCTCAG  CAATTACAAA  GGGGTACTAT  GGGGTACCTT  AGTACGACAC  AAGTCCCAGA
241  TCCGTTGTGC  CGATTCCATA  CCTACCCCGT  GGGGCTCACA  CAGTGGCTCT  CGCCGGTGT
301  CACGGGATT  TCCCGCCCCG  TCTCCACCGT  ATGGTCAGTG  GTCCGGCGGT  TCGACAACCC
361  TCAAGCATAC  AAGCCTTCTG  TCAAGAGCTG  CCACGTCATC  GTAGGGGATG  GTGAGCTCGG
421  CACTCTCGGC  GAGGTTCCGAG  TGATCTCTGGG  CCTTCCAGCTG  CGGTCCAGCA  CGGAAAGACT
481  CGAGATCCTC  GAGGACGAGC  GACATGCTAT  TAGCTTTTGC  GTAGTGCCGTG  GAGACGACCG
541  ACTCGCAGAAAT  TACCGTTCCG  TCACCACACT  CCAACGGGAA  CTTGTCTGGTG  AGGGGAGCCG
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841  CCTGGAACCT  TTACAGATCA  TGTGCTTTAT  ATAAACTCCT  GGTGTCATC  ACTTGTTGAA
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961  TTTTTTGTTC  CTCATGCTCT  TGTATTAAA  TTTATCTTCTG  TATTGTGGA  AATAA
```
Supplemental Figure 6. NaHER1 and NaPYL4 silencing efficiency by VIGS. Relative transcript abundances (± SE, n=4) of NaHER1 and NaPYL4 determined by qPCR. NaHER1 and NaPYL4 were silenced by VIGS method (see Material and Method for more details). Leaves were wounded and immediately treated with 20 µL of 1:5 water-diluted oral secretions (WOS) from M. sexta larvae. Samples were collected after 1 h and analyzed by qPCR. Asterisks represent significant differences determined by ANOVAs, followed by Fisher’s PLSDs post hoc test ($P \leq 0.05$), *** $P \leq 0.001$; SE, standard errors.

Supplemental Figure 7. ABA does not induce NaHER1 expression. Relative transcript abundances (± SE, n=5) of NaHER1 determined by qPCR. The leaves of N. attenuata (one per plant) were treated with 20 µL of 100 µM ABA in lanolin paste. Samples from treated leaves were collected at designated time points and analyzed by qPCR.
Supplemental Figure 8. The sequence of NaABA1 gene. The cDNA sequence of a putative \textit{N. attenuata} NaABA1 was obtained after searching cDNA libraries of \textit{N. attenuata} using authentic \textit{A. thaliana} (AT5G67030) and \textit{Nicotiana plumbaginifolia} (X95732) ABA1 sequence.
**Supplemental Figure 9.** The cdna sequence of a putative *N. attenuata* ABA transporter *NaPDR12* was obtained after searching cdna libraries of *N. attenuata* using authentic *A. thaliana* *PDR12* (NM_101421) sequence.

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Supplemental Figure 10. Silencing of NaHER1 gene does not affect expression of ABA biosynthetic gene NaABA1 or expression of ABA transporter NaPDR12. Rosette stage N. attenuata plants were treated with WOS in zone 2. Samples were collected at designated time points, extracted and analyzed by real-time PCR for NaABA1 and NaPDR12 transcripts.
Supplemental Figure 11. *NaHER1* silencing alters ABA metabolism, JA and JA-Ile accumulation in detached leaf. Mean (± SE, n=4) levels of ABA metabolites, JA, JA-Ile, and SA in detached *N. attenuata* leaves collected at designated time points and analyzed by LC-MS/MS. Metabolite levels were compared by ANOVAs, followed by Fisher's PLSDs post hoc test (* P ≤ 0.05). SE, standard error.
Supplemental Figure 12. Silencing NaHER1 does not affect rosette diameter but delays flowering time and reduces seed capsule numbers in *N. attenuata*. Plants were maintained under continuous attack from *M. sexta* neonates (MS) or left untreated (Cont), and flower and seed capsule numbers (± SE, n=10) were counted at designated time points. Different letters show significant differences between samples determined by Mann-Whitney U test (*P* ≤0.05). Note that the flower numbers at the day 41 and 42 were present with their own y-axis.
Supplemental Figure 13. ABA affects JA accumulation of OS-elicited plants. Leaves of *N. attenuata* (one per plant) were mechanically wounded and immediately treated with 20 µL of diluted OS from *M. sexta* (WOS) or WOS supplemented with 100 mM ABA. Samples were collected after 4 d and analyzed by LC-MS/MS for phytohormone contents (± SE, n=4). Samples were compared by one-way ANOVA, followed by a Fisher PLSD *post hoc* test; different letters show significantly differences between samples (*P* ≤0.05). FM, fresh mass.
Supplemental Figure 14. Accumulation of individual HGL-DTGs in WT and irHER1 plants. Mean (± SE, n=4) accumulation of total and 10 individual HGL-DTGs determined by HPLC and LC-MS/MS, respectively. One leaf in each of the 47-d-old WT and irHER1 plants was treated with 20 µL diluted OS from *M. sexta* (WOS) and local and systemic samples [8th leaf above the local WOS leaf (sys+8 in figure)] were collected after 48 h and analyzed by HPLC and LC-MS/MS. Asterisks represent significant difference between WT and irHER1 plants determined by one-way ANOVA, followed by a Fisher PLSD post hoc test; * P ≤ 0.05, ** P < 0.01, *** P < 0.001. SE, standard error.
Supplemental Figure 15. Specific primer sequences used in qPCR (SYBR) analyses.

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NaPYL4  | 5'-CCATTAGCGACCACTCTTAC-3' | 5'-GGAGCTTTATTTGAGG-3' |
### Supplemental Table 1. Multiple reaction monitoring (MRM) transitions used for LC-MS/MS quantifications of phytohormone levels.

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<td>3 ABA aldehyde</td>
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REFERENCES


Kusumi, K., Hirotsuka, S., Kumamaru, T., and Iba, K. (2012). Increased leaf photosynthesis caused by elevated stomatal conductance in a rice mutant


Chapter 4

UVB radiation and 17-hydroxygeranyllinalool diterpene glycosides provide durable resistance against mirid (*Tupiocoris notatus*) attack in field-grown *Nicotiana attenuata* plants.

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UVB radiation and 17-hydroxygeranyllinalool diterpene glycosides provide durable resistance against mirid (Tupiocoris notatus) attack in field-grown Nicotiana attenuata plants

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ABSTRACT

Depending on geographical location, plants are exposed to variable amounts of UVB radiation and herbivore attack. Because the role(s) of UVB in the priming and/or accumulation of plant defence metabolites against herbivores are not well understood, we used field-grown Nicotiana attenuata plants to explore the effects of UVB on herbivore performance. Consistent with previous reports, UVB-exposed plants accumulated higher levels of ultraviolet (UV)-absorbing compounds (rutin, chlorogenic acid, cryptochlorogenic acid and diacetylphloroglucinol). Furthermore, UVB increased the accumulation of jasmonic acid, jasmonoyl-L-isoleucine and abscisic acid, all phytohormones which regulate plant defence against biotic and abiotic stress. In herbivore bioassays, N. attenuata plants experimentally protected from UVB were more infested by mirids in three consecutive field seasons. Among defence metabolites measured, 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) showed strongly altered accumulation patterns. While constitutive HGL-DTGs levels were higher under UVB, N. attenuata plants exposed to mirid bugs (Tupiocoris notatus) had still more HGL-DTGs under UVB, and mirids preferred to feed on HGL-DTG-silenced plants when other UVB protecting factors were eliminated by UVB filters. We conclude that UVB exposure not only stimulates UV protective screens but also affects plant defence mechanisms, such as HGL-DTGs accumulation, and modulates ecological interactions of N. attenuata with its herbivores in nature.

Key-words: Manduca sexta (tobacco hornworm); N. attenuata; Tupiocoris notatus (mirid bug); 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs); plant–herbivore interactions.

INTRODUCTION

The solar radiation that penetrates the Earth’s atmosphere and impinges on plants spans a wide range of wavelengths that include ultraviolet (UV) light. According to ISO solar irradiance categories [ISO 21348:2007(E)], UV light is subdivided into 10 groups; among these, UVB is classified as having wavelengths between 280 and 315 nm. UVB is a well-known stress factor for plants because it can damage DNA and, as a consequence, affect growth and development (Stapleton 1992; Ballare et al. 1996; Mazzarino et al. 1999; Chimphango et al. 2007). In response to UVB, plants have evolved various defence mechanisms such as nucleotide excision, mismatch and double-strand break repair which can, to some extent, repair damaged DNA (Emanuel & Scheinfeld 2007). In addition, plants produce a large variety of flavonoid derivatives and phenolic compounds which strongly absorb UVB and thereby function as UVB screens that protect cells from damage (Begg, Stolzer-Jehle & Wellmann 1985; Li et al. 1993; Middleton & Teramura 1993; Kootstra 1994; Landry, Chapple & Last 1995; Reuber, Bornman & Weissenbock 1996).

Since herbivores frequently avoid directly being exposed to high UVB irradiation as well as their predators, they normally feed on the lower side of the leaves which make it harder to distinguish between direct and plant-mediated effects of UVB on insect herbivores. However, UVB radiation has been shown to influence survival, behaviour and performance of herbivores and their enemies as well (Kuhlmann & Mueller 2011). The mortality rate of shield beetles Cassida rubiginosa Mueller (Coleoptera, Chrysomelidae) when exposed to UVB radiation was higher and pupal mass lower (Bacher & Luder 2005). Survivorship and egg production of spider mites Tetranychus urticae were strongly reduced when exposed to supplemental UVB (Ohtsuka & Osakabe 2009). Growth and survivorship of Precis coenia Hbn. (Lepidoptera: Nymphalidae), a specialist herbivore of longleaf plantain Plantago lanceolata L., were not affected, while the growth of Trichoplusia ni (Hbn.) (Lepidoptera: Noctuidae), a generalist, was reduced by exposure to UVB (McClymond & Berenbaum 1999). While there are
many direct harmful effects of UVB radiation on insect performance (Ballare et al. 2011), negative effects can also be indirect and mediated by the defence signalling of the host plant.

Apart from direct impact on target organisms, the interactions among UVB radiation, plants, herbivores and pathogens have been examined in several studies. Their results suggest that UVB, while causing environmental stress, can also play important ecological roles in plant–herbivore and –pathogen interactions (Green & Fluhr 1995; Ballare et al. 1996; Rousseaux et al. 1998). In general, the impact of UVB on biotic interactions is highly variable, depending on the species of plants and herbivores. For example, exposure to solar UVB and insect herbivory elicited partially overlapping gene expression and phenolic profiles in Nicotiana attenuata and Nicotiana longiflora plants (Izaguirre et al. 2003, 2007). In addition, UVB affected the tri-trophic interaction between host plants (Brassica oleracea), herbivores (Platella xylostella) and their parasitoids (Cotesia plutellae) (Fogg et al. 2007). In poplar (Populus trichocarpa), exposure to twice-ambient doses of biologically effective UVB showed minimal effects on photosynthesis, growth, leaf area and biomass, but the methanol-extractable foliar phenolics increased and, consequently, the larval performance on UVB-exposed plants decreased (Warren, Bassman & Eigenbrode 2002). Thrips (Calothrips phaseoli) preferred to feed on non-UV-exposed soybean plants while actively avoiding direct UVB exposure (Mazza et al. 1999b). Lindroth et al. (2000) examined the performance of two generalist herbivores, army worms (Spodoptera litura) and cutworms (Graphania mutata), on two populations of white clover (Trifolium repens L.), ‘Huia’ and ‘Tienhoven’, finding a differential response of these generalist herbivores to their host plants depending on UVB exposure. In contrast, Anttila et al. (2010) reported that UVB had neither effects on the autumnal moth nor effects on the accumulation of phenolic compounds or phenoloxidase activities in the mountain birch plants (Anttila et al. 2010). In broccoli (R. oleracea L. cv. ‘batrisy’, Brassicaceae), a high level of UVB exposure decreased biomass and led to high accumulations of flavonoids; however, UVB did not alter the attractiveness of plants to herbivores, such as thrips, whiteflies and aphids (Kuhlmann & Muller 2009). These contrasting results suggest that genotypic factors, both in UVB-exposed plants and insects, can significantly influence the outcome of plant–herbivore interactions (Lindroth et al. 2000).

Jasmonic acid (JA) signalling is the main regulatory pathway that controls herbivore-induced defence responses in plants (Mueller et al. 1993). In N. attenuata, several defence metabolites such as nicotine, trypsin proteinase inhibitors (TPIs), 17-hydroxysterolylcinnamal diterpene glycosides (HGL-DTGs), caffeoylputrescine (CP) and icaffeoylspermidine (DCS) have been shown to strongly affect performance of herbivores in the glasshouse and, more importantly, in the plant’s natural habitat, the Great Basin Desert, UT, USA. The accumulation of these compounds, mediators of direct plant defence, and the emission of volatile organic compounds (VOCs) that are required for indirect defence of N. attenuata are all under the direct control of the JA signalling pathway (Steppuhn et al. 2004; Steppuhn & Baldwin 2007; Heiling et al. 2010; Kaur et al. 2010). Among the various types of JA metabolites, (+)-7-isojasmonoyl-L-isoleucine (JA-Ile) was identified as the major endogenous bioactive jasmonate in plants (Fonseca et al. 2009).

Since it is difficult to distinguish between plant responses to UVB light per se (i.e. UVB-specific pathway) and plant responses to the physical damage caused by UVB radiation (i.e. non-specific signalling pathway) (Jenkins 2009), the mechanisms involved in UVB interference with plant signalling are still largely unknown. Recently, UV RESISTANCE LOCUS 8 (UR8) gene was identified as a specific receptor of UVB in Arabidopsis plants (Rizzini et al. 2011), suggesting that plants actively perceive UVB and regulate downstream signalling pathways that involve phytohormones such as gibberellic acid (GA), auxin, ethylene, JA and salicylic acid (SA) (Kazan & Manners 2011). In a putative UVB signalling pathway, silencing the expression of RNA-directed RNA polymerase 2 impaired growth and reproductive performance of UVB-exposed N. attenuata plants, both in the field and in the glasshouse, suggesting that smRNAs are involved in regulation of UVB-dependent signalling and defences (Pandey & Baldwin 2008). So far, most experiments examining plant–UVB responses have been conducted under glasshouse conditions, using UVB-emitting lamps to generate elevated UVB levels, which may have created unbalanced and/or unrealistic spectral ratios between UVB and photosynthetically active radiation (PAR) influencing the natural behaviour of both plants and insects (Caldwell & Flint 1994; Deckmyn, Martens & Impens 1994; Kakani et al. 2003). Field experiments based on natural interactions occurring under natural fluences of PAR and UVB are therefore ideal experimental conditions to understand the roles of UVB in plant–insect interactions, and mechanisms activated by UVB perception.

Here, we report the results of experiments conducted during three field seasons (2009–2011) in which diminished UVB-exposed (dUVB) plants were significantly more infested by Tupiocoris notatus insects. Natural UVB exposure enhanced the JA burst which accompanies most plant–insect interactions and altered the accumulation of HGL-DTGs. We propose that natural UVB exposure potentiates the accumulation of HGL-DTGs, which, together with the previously reported role of TPs (Glawe et al. 2003), contribute to the defence of N. attenuata against T. notatus in nature.

**MATERIAL AND METHODS**

**Plant material and growth conditions**

Seeds of N. attenuata, originally collected in Utah, were sterilized and germinated on Gamborg’s B5 medium as described in (Kruegel et al. 2002). Previously characterized

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transgenic lines (line name/silenced gene) irPMT/N. attenuata putrescine N-methyltransferase (Steppuhn et al. 2004), irPI/N. attenuata TPI (Steppuhn & Baldwin 2007), irGGPS/N. attenuata geranylgeranylpyrophosphate synthase (Heiling et al. 2010) and irMYB8/ N. attenuata MYB8 transcription factor (Kaur et al. 2010) were used in the field experiments. Empty vector (EV)-transformed plants were used as controls. All plants were maintained in an irrigated field plot at the Lyle Ranch Preserve, near Santa Clara, UT, under Animal and Plant Health Inspection Service (APHIS) release permit 06-242-3r-2a. After germination, the seedlings were kept for 2 weeks in a shade house and subsequently transplanted to the field as previously described in (Diezel, Kessler & Baldwin 2011). The seedlings were placed in two contrasting UVB conditions: strongly attenuated UVB (dUVB) and near-ambient UVB (UVB exposure) with no significant change in photosynthetic active radiation (Supporting Information S1). Six to 10 replicate canopies, half for UVB or dUVB exposure, located in a plowed field plot were used for each experiment. Variable amounts of solar UVB were achieved by placing plants under wooden frames covered on the top and sides with clear polyester film (Melinex® 506, thicknesses of 100 micron or OPTIX Acrylic Sheet, Model: MC-09) that removed ~90% of UVB or transparent film (Husky, Clear Plastic Sheeting, Model: RSHK-403-SOC-U, product of Poly-America LP, Grand Prairie, TX, USA) that allowed transmission of more than 75% of solar UVB (Supporting Information Fig S1). Although levels of PAR and UVB radiation were measured during the experiment, the values differed by the time of the day and cloud cover; therefore, transmittance values which are independent of time and weather effects are presented. UVB fluences of 300–400 μW cm⁻² and the PAR fluences of 1200–1500 μmol m⁻² s⁻¹ are typically measured on sunny days at the field plot. During the experiment, 20–30 cm of unobstructed space from the ground to the surrounding canopies allowed for air circulation and also for natural infestations of insects. Under both types of canopies, humidity, temperature and light intensity (except for UVB) were comparable throughout experiments.

Plant treatments and screening for herbivore damage in the field

Approximately 6-week-old plants were used in all growth seasons in 2009–2011. To simulate herbivore feeding in the field, plants were punctured by rolling a fabric pattern wheel twice on each side of the midvein and immediately applying 20 μL of Manduca sexta oral secretions (OS) diluted 1:5 with distilled water, in a treatment dubbed wounding and applying OS (WOS) elicitation (Halitschke et al. 2001).

In no-choice feeding experiments, two neonates of M. sexta or five adults of T. notatus were kept in clip cages affixed to the 4th stem leaf of N. attenuata plants (counting from the shoot-to-root direction), and allowed to feed continuously for 3 days. M. sexta eggs and larvae were kindly provided by Dr Carol Miles, Department of Biological Sciences, Binghamton University. T. notatus were collected from native populations of N. attenuata in Utah prior to the start of the experiments. After 3 days, insect survivorship and leaf damage were scored, and leaf parts exposed to herbivores were collected and stored at ~20 °C (in a freezer). Samples were transported to the laboratory on dry ice, extracted and subjected to transcript, phytohormone and secondary metabolite measurements. To estimate damage from native herbivores, plants were observed and damage was scored and categorized based on the characteristic feeding behaviour of the different insect species in the field plot. The canopy damage was calculated as the total leaf area damaged; leaf damage was first scored individually on each leaf of the plant and then divided by the number of the leaves on each plant. The average damage of replicate plants (see Figure captions for sample sizes) in each experiment is shown.

Phytohormones analysis

Phytohormone content [JA, JA-ile, abscisic acid (ABA)] was determined as described previously (Wang et al. 2007). In brief, approximately 200 mg leaf tissue was aliquoted in Eppendorf tubes containing three steel balls with 1 mL of ethyl acetate that was spiked with labelled internal standards (40 ng D₆-ABA, 40 ng D₆-SA, 40 ng JA-CD₃-ile and 200 ng D₆-JA) and homogenized in Geno/Grinder 2000 (SPEX CertiPrep, Metuchen, NJ, USA) by vigorously shaking tubes at 1000 strokes per minute for 2 min, and then centrifuged at 8000 g for 10 min at 4 °C. The supernatants were collected and evaporated to near dryness in a vacuum concentrator at 30 °C (Eppendorf, Hamburg, Germany). Dry pellets were re-suspended in 550 μL of 70% methanol and centrifuged at 16 100 g at 4 °C for 20 min. In addition, 450 μL of the supernatants was transferred to clean high-performance liquid chromatography (HPLC) vials and phytohormones were analysed on a Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/ MS) with a Varian 1200 Triple-Quadrupole-LC-MS system (Varian, Palo Alto, CA, USA).

Analysis of secondary metabolites

Samples were aliquoted in Eppendorf tubes containing three steel balls with 1 mL of extraction buffer (50 mM acetate buffer in 40% methanol, pH 4.8) per each 100 mg of tissue and homogenized in Geno/Grinder 2000 by shaking tubes at 1000 strokes per minute for 2 min, and then centrifuged at 8000 g for 20 min at 4 °C. The supernatants were collected and transferred to clean 1.5 mL tubes and re-centrifuged at 16 100 g at 4 °C for 20 min. Furthermore, 500 μL of cleared supernatants were transferred to HPLC vials and analysed on an HPLC as described by Kaur et al. (2010). External calibration curves with six data points were used to quantify nicotine, chlorogenic acid (CHA) and rutin. CP and DCS were quantified using CHA calibration curves and expressed as CHA equivalents in text. Peak
areas were quantified to estimate total HGL-DTGs contents in HPLC chromatograms, detected by evaporative light scattering detector (ELSD) after HPLC separation. For individual HGL-DTGs analysis, extracts were diluted 50 times using a 10x dilution of original extraction buffer in 40% MeOH, and analysed on LC-MS/MS as previously described in Heiling et al. (2010).

Isoleucine determination

Approximately 200 mg leaf tissue was homogenized in a Geno/Grinder 2000 using 2 mL Eppendorf tubes and three steel balls in 1 mL of 40% methanol in 0.1 N HCl solution. Samples were homogenized at 1000 strokes per minute for 2 min, and centrifuged at 16 100 g for 10 min at 4 °C. Clear supernatants were transferred to glass vials for analysis on an LC-MS/MS. The analytical method was a modified protocol described in Jander et al. (2004). Chromatography was performed using an Agilent 1200 HPLC system (Agilent Technologies, Boeblingen, Germany) and Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 μm, Agilent Technologies). As mobile phases, 0.05% formic acid in water (solvent A) and acetonitrile (solvent B) were used in a gradient mode. The elution profile was 0–1 min, 100% A; 1–3 min, 0–100% B in A; 3–4 min 100% B; and 4–7 min 100% A; solvent flow was 0.6 mL min⁻¹. The column temperature was maintained at 25 °C. The HPLC was coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbo spray ion source operated in positive ionization mode. The instrument parameters were optimized by infusing pure standard compounds (amino acid standard mix, Fluka, St. Louis, MO, USA). The ion spray voltage was maintained at 5500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 ps, curtain gas at 35 psi, heating gas at 70 psi and collision gas at 2 psi. Multiple reactions monitoring (MRM) was used to monitor analyte parent ion → product ion as described by Jander et al. (2004). External calibration curve for quantification of amino acids was generated by using commercial isoleucine standard (Fluka). Analyst 1.5 software (Applied Biosystems) was used for data acquisition and data processing.

Analysis of gene expression by quantitative real-time PCR (qPCR)

Total RNA extracted by TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) as recommended by manufacturer was treated with DNase-I (Fermentas, St. Leon-Rot, Germany) to remove DNA contaminations following the manufacturer’s protocol. Aliquots containing 500 ng of total RNA were reverse transcribed using oligo(dT)18 and SuperScript reverse transcriptase II (Invitrogen). Real-time quantitative PCR with SYBR Green I (qPCR Core Kit for SYBR Green I; Eurogentec, Köln, Germany) was performed with MX3005P Multiplex qPCR system (Stratagene, La Jolla, CA, USA). mRNA concentrations were calculated from external calibration curves of fivefold dilution series of cDNAs containing six data points. Relative transcript abundances of genes of interest were calculated after normalizing raw signal values with the N. attenuata ELONGATION FACTOR 1α (NeEF1α) values used as an internal standard as described in Pfaffl et al. (2002). The specific primer sequences used for qPCR are listed in Supporting Information Fig. S2D.

TPI analysis

TPI analysis was performed by radial diffusion assay as previously described (Van Dam et al. 2001).

Kinase activity assay

Four biological replicates in the same treatment were pooled and kinase activity assay was performed by in-gel kinase activity assay as previously described (Wu et al. 2007a).

Statistical analyses

Data were analysed with StatView 5.0 (SAS Institute), SPSS statistic 17.0 (SPSS Inc., Chicago, IL, USA) and INFOSTAT version 2011e (Universidad Nacional de Córdoba, Argentina). Analysis of variance (ANOVA) followed by post hoc Fisher’s protected least significant difference (PLSD) or Tukey test was used; comparison of means was calculated at the 0.05 level of significance. When appropriate, to avoid pseudoreplication, the mean values of plants under a replicate canopy were used in the analysis. Data on canopy damage, survivorship, leaf area damaged (Fig. 5a, Supporting Information Figs S3 & S4) and number of T. notatus infested on plants (Fig. 5c) were analysed by Kruskal–Wallis tests.

RESULTS

UVB exposure promotes phenylpropanoid accumulations

Plants minimize the destructive effects of UVB radiation by enhancing the accumulation of UVB-absorbing screens, most commonly, phenolic compounds. Consistent with previous reports, the levels of several phenolic compounds such as rutin, CHA, crypto-CHA (CHA isomer) and DCS were higher in UVB-exposed N. attenuata leaves, although the same compounds at lower levels were also found in dUVB-exposed plants. Rutin was significantly higher in UVB-exposed plants (Fig. 1; ANOVA, F₁,₂ = 14.873, P = 0.0084) and it remained significantly elevated after simulated herbivory treatment, that is wound (W) leaves and applying OS from M. sexta to the puncture wounds (WOS elicitation; Fig. 1) (24 h: ANOVA, F₁,₂ = 75.977, P = 0.0001; 48 h: ANOVA, F₁,₂ = 12.847, P = 0.0116; 72 h: ANOVA, F₁,₂ = 92.692, P < 0.0001). In contrast, the levels of CHA and crypto-CHA were initially not significantly different (see constitutive levels at 0 h in Fig. 1, CHA: ANOVA, F₁,₂ = 1.882, P = 0.2192; crypto-CHA: ANOVA, F₁,₂ = 0.780, P = 0.4111).

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but they became significantly higher at 24, 48 and 72 h after WOS elicitation compared to dUVB-exposed plants (Fig. 1; CHA: 24 h: \( F_{1, 8} = 9.630, P = 0.0210 \); CHA: 48 h: \( F_{1, 8} = 24.081, P = 0.0027 \); CHA: 72 h: \( F_{1, 8} = 7.926, P = 0.0305 \); Crypto-CHA: 24 h: \( F_{1, 8} = 8.105, P = 0.0293 \); crypto-CHA: 48 h: \( F_{1, 8} = 18.832, P = 0.0049 \); crypto-CHA: 72 h: \( F_{1, 8} = 6.983, P = 0.0384 \)). Similarly, the accumulation of DCS was positively affected by UVB exposure in WOS-elicited leaves 24 h after elicitation (Fig. 1; \( F_{1, 8} = 7.375, P = 0.0348 \)). In summary, while phenolic contents were generally higher in UVB-exposed plants, under simulated herbivory condition, UVB exposure showed additive effects on the accumulation of CHA isomers and phenolamides in *N. attenuata* leaves. The differential amounts of UV screens found in plants confirmed that our field experimental design had effectively provided plants with different levels of UVB fluence from the natural source.

**Defence-related phytohormones in differentially UVB-exposed plants**

In *N. attenuata*, mechanical wounding and herbivore attack resulted in strong transient accumulations of JA, and its bioactive form JA-Ile, and treatment of puncture wounds with OS amplified these wound-induced phytohormone bursts. Because UVB exposure is known to activate some of the JA biosynthetic genes (Izaguirre et al. 2005) and another stress hormone, ABA, has been implicated in systemic defence responses against herbivores (Erb et al. 2011), we examined the levels of these hormones in elicited and control leaves of *N. attenuata* exposed to differential levels of UVB.

UVB did not alter the constitutive levels of JA, JA-Ile and ABA. In contrast, UVB-exposed *N. attenuata* plants accumulated significantly more JA, JA-Ile and ABA 1 h after WOS elicitation (Fig. 2; JA-1 h: \( F_{1, 8} = 7.806, P = 0.0314 \); JA-Ile-1 h: \( F_{1, 8} = 13.705, P = 0.0010 \), Supporting Information Fig. S2A ABA-1 h: \( F_{1, 8} = 7.761, P = 0.0318 \)).

Threonine deaminase (TD), an enzyme involved in the conversion of threonine to α-ketoisobutyrate (a precursor of isoleucine), contributes to defence of plants against herbivores by degrading of the essential amino acid, threonine, in insect guts. Previously, silencing *NaTD* transcripts reduced JA-Ile accumulations and defence in *N. attenuata* (Kang et al. 2006). Under UVB exposure, *NaTD* accumulated more at 1, 3 and 12 h after WOS elicitation (Fig. 2; 1 h:...
Chapter 4 – Solar UVB, N. attenuata, M. sexta, and T. notatus

Figure 2. Natural levels of UVB exposure influence phythohormone, isolectine and associated transcript levels. Mean ±SE, n = 3 accumulation of JA, JA-Ile, isolectine and transcript abundances NaTD after OS elicitation. Single Nicotiana attenuata leaves from individual plants were mechanically wounded and treated with 20 μL oral secretions from Manduca sexta larvae (5x diluted in distilled water). Samples were collected at designated time points, extracted and analysed by LC-MS/MS (JA, JA-Ile, isolectine) and by qPCR for transcript accumulation (NaTD). Values in the near-ambient UVB (control) or diminished UVB (dUVB) treatments at corresponding time points were compared by ANOVA, Fisher’s PLSDs. *P = 0.05. The experiment was conducted in the field during the 2010 season. FM, fresh mass.

**ANOVA, F(1,3) = 8.099, *P = 0.0466; 3 h: ANOVA, F(1,9) = 9.197, *P = 0.0290; 12 h: ANOVA, F(1,9) = 6.520, *P = 0.0433.** Additionally, isolectine levels were higher both constitutively and at 1 h after WOS induction in UVB-exposed plants (Fig. 2: 0 h: ANOVA, F(1,17) = 14.167, *P = 0.0197; 1 h: ANOVA, F(1,17) = 7.943, *P = 0.0372). Overall, UVB showed significant effects on JA, JA-Ile, ABA, isolectine accumulations and strongly up-regulated NaTD transcript accumulations in WOS-elicited plants.

**Solar UVB differentially affects TPI transcripts and protein activity**

Previously, proteinase inhibitor genes in *N. attenuata* and *Solanum nigrum* plants were shown to be transcriptionally regulated, and directly contribute to defence against herbivores in these plants (Van Dam et al. 2001; Hartl et al. 2010). To examine if solar UVB affected the expression of *N. attenuata TRYPsin PROTEINase INHIBITOR 1* (NaTPI), we analysed kinetic accumulation of NaTPI gene transcripts as well as TPI activity after WOS elicitation of plants exposed to different UVB regimes in the field.

UVB-exposed *N. attenuata* plants had higher constitutive levels of TPI activity (Fig. 3: ANOVA, F(1,13) = 11.258, *P = 0.0047). However, when strongly elicited by WOS, TPI activity attained similar levels in both UVB treatments (UVB and dUVB exposure). Interestingly, NaTPI transcripts accumulated less under UVB exposure at early time points after WOS elicitation, namely at 0.5 and 1 h (Fig. 3: 0.5 h: ANOVA, F(1,13) = 7.460, *P = 0.0341; 1 h: ANOVA, F(1,13) = 6.401, *P = 0.0447), and then showed a trend towards higher accumulations at 12 h after WOS (Fig. 3: ANOVA, F(1,13) = 2.183, *P = 0.1900). Taken together, UVB increased constitutive TPIs (despite lower transcript levels); however, it did not show any priming effect on TPI activity after WOS elicitation, suggesting that herbivore elicitors exert a dominant role in the activation of TPI defences.

UVB exposure showed complicated effects on HGL-DTGs accumulations

HGL-DTGs are a diverse group of defence compounds derived from terpenoid metabolism in *N. attenuata* plants. The accumulation of individual HGL-DTGs is a very
dynamic process, and the acylation steps in the production of malonylated HGL-DTGs are known to be JA dependent. In addition, HGL-DTGs levels vary dramatically in different tissues over development (Heiling et al. 2010). Because UVB showed a marginal positive effect on JA and JA-Ile after WOS elicitation, we analysed the levels of HGL-DTGs as a representative of a class of JA-regulated metabolites to examine if these increased hormone levels translated into metabolic changes before and 1, 12, 24 and 48 h after WOS elicitation (Fig. 4).

Constitutive levels of HGL-DTGs were generally higher in UVB-exposed plants, suggesting a JA-independent but UV-dependent mechanism that controls HGL-DTGs levels in field-grown plants. Of 10 analysed HGL-DTGs, only nicotianamide II was found at lower levels in UVB-exposed compared to dUVB-exposed plants (Fig. 4; ANOVA, $F_{1,9} = 14.491, P = 0.0089$). Surprisingly, UVB-exposed plants were strongly depleted in all measured HGL-DTGs just 1 h after WOS elicitation and recovered only 12 h after treatment of the plants. This was not observed in dUVB-exposed plants that showed exactly the opposite trend, that is their HGL-DTGs levels increased as had typically been observed in glasshouse-cultivated *N. attenuata* plants (Fig. 4; Heiling et al. 2010). In the glasshouse, plants are completely deprived of UVB due to strong UV absorption by glass plates.

In order to examine the correlation of HGL-DTG accumulations with the expression of HGL-DTG biosynthetic genes, we analysed transcript levels of 1-deoxy-D-xylulose-5-phosphate synthase (*NaDXS*). The expression of this gene was lower under UVB exposure at 0.5 h after WOS elicitation (Supporting Information Fig. S2B; ANOVA, $F_{1,9} = 8.874, P = 0.0247$); however, UBV slightly (but significantly) increased *NaDXS* transcript levels at 12 h after WOS elicitation (Supporting Information Fig. S2B; ANOVA, $F_{1,9} = 8.782, P = 0.0252$). This expression pattern can partially explain the gradual recovery of depleted HGL-DTGs in WOS-treated and UVB-exposed plants; attenoside, nicotianamide IV, nicotianamide VI and all three dimalonylated HGL-DTGs were similar in all plants 48 h after WOS elicitation (Fig. 4; ANOVA, Fisher's LSD: *p* ≤ 0.05; **p** ≤ 0.01).

Taken together, UVB generally showed a positive effect on the accumulation of most HGL-DTGs at constitutive levels. Under severe stress of simulated herbivory, HGL-DTGs were strongly depleted in plants under normal field levels of UVB fluence but not in plants under dUVB, revealing an unexpected interaction between UVB and diterpene metabolism seen only when plants are grown under field conditions.

**T. notatus are strongly attracted to plants grown under dUVB fluences**

The natural habitat of *N. attenuata* is known for its naturally high fluences of UVB and rich herbivore community that attacks *N. attenuata* plants. We screened for natural herbivore damage on UVB-exposed and dUVB-exposed plants: *T. notatus* (mirids), an abundant herbivore of *N. attenuata*, and other insects such as *M. sexta*, flea beetles, Noctuidae, *Empoasca* sp., tree crickets, aphids and thrips. Herbivore damage was observed and categorized based on the characteristic feeding behaviour of these herbivores using standardized methods. In three consecutive field seasons (2009–2011), *T. notatus* insects showed a strong preference to feed on dUVB-exposed plants. Although the absolute values of canopy damage varied among years, UVB-exposed plants were always less damaged and/or infested by mirids.

In the 2009 field season, mirids heavily attacked dUVB-exposed plants and practically no damage was found on plants exposed to ambient levels of UVB (Fig. 5a; Kruskal–Wallis test, $\chi^2 = 9.466, P = 0.0020$). In the 2010 season with low herbivore occurrence, *T. notatus* were only found on dUVB-exposed plants (Fig. 5c), and in the 2011 field season, dUVB-exposed plants were again significantly more damaged compared to UVB-exposed plants (Fig. 5a; Kruskal–Wallis test, $\chi^2 = 5.707, P = 0.0169$). These results

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Figure 4. Natural levels of UVB exposure strongly affect the dynamics of individual HGL-DTGs after OS elicitation. Mean (±SE; n = 3) of HGL-DTGs accumulations after OS elicitation. Single *Nicotiana attenuata* leaves from individual plants were mechanically wounded and treated with 20 μL oral secretions from *Manduca sexta* larvae (5x diluted in distilled water). Samples were collected at different time points, extracted and analysed on LC-MS/MS. Values in the near-ambient UVB (control) or diminished UVB (dUVB) treatments at the corresponding time points were compared by ANOVA, Fisher’s PLSD. *P* ≤ 0.05, **P** ≤ 0.01, ***P*** ≤ 0.001. (a) precursor; (b) core HGL-DTGs; (c) malonylated HGL-DTGs; (d) dimalonylated HGL-DTGs. The experiment was conducted in the field during the 2010 season. FM, fresh mass.
Figure 5. Lower HGL-DTGs levels in UVB-diminished plants correlate with increased damage from *Tuta absoluta* in the field. Mean (±SE; n = 3) of average *T. absoluta* damage per plant in field seasons 2009 and 2011 (a), accumulation of jasmonates (JA and JA-Ile) and total HGL-DTGs in no-choice experiments (b) and occurrence of feeding and mirid reproduction on transgenic plants silenced in the expression of major groups of defence metabolites in *N. attenuata*, the experimental treatments resulted from a factorial combination of UVB and genotypes (c). In no-choice experiments (2011), plants were exposed to either five adult *T. absoluta* or two Manduca sexta neonates enclosed in a clip cage on a single *N. attenuata* leaf of separate plants. Attacked leaves were collected after 3 d and analysed by LC-MS/MS (JA, JA-Ile) and HPLC-PDA/ELS (total HGL-DTGs). Comparisons of near-ambient UVB (control) or diminished UVB (dUVB) were performed by Kruskal–Wallis test for the canopy damage and number of *T. absoluta* (a and c) and anovas, Fisher’s PLSDs test for JA, JA-Ile, total HGL-DTGs levels (b), *p* < 0.05; **p** < 0.01, nd, not detectable; FM, fresh mass.

suggest that either the UVB radiation itself and/or one or more of the UVB-induced compounds could be responsible for the mirid phenotype. Damage from other herbivores such as flea beetles, Noctuidae or tree crickets was less severe, averaging less than 1% of total canopy (Supporting Information Fig S3; Kruskal–Wallis test, *P* > 0.05). Although the *Empoasca* sp. damage was higher on UVB-exposed plants in the 2009 season (Supporting Information Fig S3; Kruskal–Wallis test, *F* = 9.466, *P* = 0.002), this was not observed in the following two years. *Empoasca* spp. is not a typical herbivore of *N. attenuata* and the 2009 year infestation may have resulted from first planting and subsequently mowing a large alfalfa field adjacent to the field plot as part of another experiment (Kallenbach et al. 2012). Alfalfa is a preferred host plant of *Empoasca* leafhoppers.

Herbivore-attacked plants produce various defensive metabolites in order to deter invaders or suppress their performance. As a result, herbivores choose weak and/or less-defended plants (or their parts) for feeding, which likely explains their frequent movement among leaves or plants. In order to distinguish if UVB exposure makes *N. attenuata* less attractive or more defended against mirids and other insects, we performed no-choice feeding assays with *T. absoluta* and *M. sexta* larvae that were enclosed in small plastic clip cages affixed to the leaves for 3 days. No differences in damage inflicted by the two herbivore species or their survivorship were observed between plants exposed to the different UVB fluences (Supporting Information Fig S4 Kruskal–Wallis test, *P* > 0.05). These results suggest that natural UVB exposure may have changed the attractiveness of plants to mirids due to the accumulation and/or emission of secondary metabolites in attacked leaves.

To gain more information about the possible mechanisms of UVB action, we analysed phytohormone and secondary metabolite levels in the tissues that had been enclosed in the clip cages used in the bioassays. Consistent with earlier results, UVB exposure increased JA and JA-Ile accumulations when the plants were attacked by *M. sexta* larvae – a situation equivalent to WOS elicitation (Fig 5b; JA: *ANOVA, F* = 6.652, *P* = 0.0128; JA-Ile: *ANOVA, F* = 6.622, *P* = 0.0212). However, this elevation of jasmonates did not translate into higher accumulations of defence compound such as nicotine (which showed the opposite trend), CP, DCS, CH4, total HGL-DTGs as determined by HPLC, or 10 individual HGL-DTGs as determined by LC-MS/MS. All these comparable levels after 3 days of feeding by *M. sexta* larvae (Fig 5b, Supporting Information Figs S5 & S6: *ANOVA, Fisher’s PLSD, P* > 0.05).

JA-Ile levels were significantly higher in UVB-exposed plants attacked by *T. absoluta* (Fig 5b; *ANOVA, F* = 7.952, *P* = 0.0182) compared to dUVB-exposed plants. The increased levels of JA-Ile were associated with increased levels of total HGL-DTGs (Fig 5b; *ANOVA, F* = 9.776, *P* = 0.0096), due to increases in three dimonolylated HGL-DTGs as determined by LC-MS/MS analysis (Supporting Information Fig S6 *ANOVA, Fisher’s PLSD, P* < 0.05). It should be noted that the observed changes in phytohormone levels differed extremely in their magnitude, with large increases in jasmonates after the leaf chewing of *M. sexta* larvae, compared to the small changes in the leaves exposed to the cell piercing–sucking mirids. These observations suggest that UVB may contribute differentially to interactions of plants with herbivores from different feeding guilds, such as the specialist chewing herbivore *M. sexta* with its associated strong hormonal responses in attacked plants and the highly attenuated responses elicited by the piercing–sucking *T. absoluta*, perhaps as a result of its ‘stealthily’ feeding behaviour.
HGL-DTGs alter attractiveness of *N. attenuata* to *T. notatus*

From the no-choice feeding assays, a positive association between UVB exposure and HGL-DTGs accumulation was observed in plants attacked by *T. notatus*, which suggested that HGL-DTGs could be one of the important determinants in host choice of *T. notatus*. Because CHA, crypto-CHA, CP and TPI were also affected by UVB (Figs 1 & 3), a factorial combination experiment including differential UVB fluences (UVB exposure and dUVB exposure) and genotypes (lack of individual defence metabolites) was conducted to determine the possible role of UVB fluence and those defence metabolites on mirids performance in the field. Equal numbers of EV (positive controls with their natural levels of defensive metabolites), irMYB8 (plants depleted in CP and DCS, and reduced levels of CHA and crypto-CHA (Kaur et al. 2010), irPI (plants deficient in TPIs (Steppuhn & Baldwin 2007), irPMT (plants compromised in nicotine production and accumulation) (Steppuhn et al. 2004) and irGGPPS (plants with strongly reduced HGL-DTGs levels) (Heiling et al. 2010) were planted in a randomized spatial design under near-ambient UVB and dUVB conditions in the 2010 field season. This season was associated with relatively low abundances of herbivores and no *T. notatus* was observed on field-grown plants with ambient levels of UVB exposure. This was consistent with the data from the 2009 field season when mirids were found only to strongly attack dUVB plants. Lower than 2% damage was found on UVB-exposed plants in 2011, suggesting that under favourable conditions, mirids can colonize *N. attenuata* under natural conditions in the field.

The data of our factorial experiment with plants silenced in the expression of different secondary metabolites suggested that UVB-associated protection of plants against mirids is not mediated by a single class of compounds as none of the transformants were actually attacked by mirids under near-ambient UVB. However, of 57 mirids observed on plants grown under dUVB canopies, irMYB8 and irPI plants harboured on average 3.7 *T. notatus* individuals, while irGGPPS had on average 13.5 mirids per plant. No *T. notatus* was observed feeding on irPMT or EV control plants. By conducting statistical analysis of the data, we found a statistically significant difference among the five different genotypes (Kruskal–Wallis test, \( \chi^2 = 10.329, \quad P = 0.035 \)) with a mean rank of 5.50 for EV and irPMT, 10.00 for irMYB8, 10.38 for irPI and 16.50 for irGGPPS. There was no significant difference between EV, irPMT, irMYB8 and irPI in the number of feeding *T. notatus* (Kruskal–Wallis test, \( \chi^2 = 5.850, \quad P = 0.119 \)), which suggests that the reduction or deficiency in protective metabolites production and accumulation, such as nicotine, CP, DCS, CHA, crypto-CHA, and TPI activity, even under low UVB levels did not significantly affect the performance of *T. notatus*. In contrast, irGGPPS plants were preferentially attacked compared to the other genotypes. Because HGL-DTGs were strongly influenced by variable UVB levels, associated with minute induced levels of JA, and mirid feeding induced more HGL-DTGs under UVB (Fig. 5b), it is reasonable to conclude that HGL-DTGs contribute to the protection of *N. attenuata* plants against *T. notatus* as one of the UVB-mediated mechanisms in the field.

irGGPPS plants accumulate more phenolic sunscreens

Phenolic compounds are well-known for their UV-absorbing sunscreen functions and plants are well known to accumulate more phenolic compounds in response to UVB exposure. Rutin, CHA and crypto-CHA (CHA isomer) accumulated more in UVB-exposed *N. attenuata* leaves (Fig. 4). Interestingly, irGGPPS plants, strongly reduced in HGL-DTGs accumulation, accumulated constitutively more CHA, crypto-CHA and rutin compared to EV and the levels accumulated increased when plants were exposed to UVB (Fig. 6, see Supporting Information Table S1 for full ANOVA and Tukey post hoc test comparisons). These

![Graph showing UVB, Gen, CHA, Crypto-CHA, and Rutin levels with ANOVA and Tukey post hoc test comparisons](image_url)
results are consistent with the hypothesis that both HGL-DTGs and phenolic compounds may contribute to UV protection as discussed in the following sections.

**DISCUSSION**

Virtually all life depends on the sunlight-fuelled photosynthetic process in green plants. Plants expose themselves to and actively seek out light, which exposes them naturally to UVB as a ubiquitous and unavoidable stress when grown under real-world conditions. Although UVB represents only a small fraction of the sun's electromagnetic radiation, it strongly affects all living organisms due to its negative effects on DNA (Caldwell et al. 2007). Since terrestrial plants cannot escape UVB exposure, they have evolved defence mechanisms to protect themselves. Here, we show that UVB-exposed *N. attenuata* plants not only accumulate higher amounts of UV-protecting screens but also respond differentially to the community of insect herbivores that attack them in their natural habitat.

**Crosstalk of biotic stress and UVB signalling**

In several previous reports, jasmonate signalling has been shown to intersect with both UVB- and herbivory-related responses. For example, the octadecanoid signalling pathway was required to mediate UVB-induced responses that largely overlapped with the wound-induced responses of tomato plants (Conconi et al. 1996). Solar UVB radiation and *M. sexta* herbivory induced partially overlapping transcriptional profiles in *N. longiflora* plants (Izaguirre et al. 2003). Although solar UVB and insect herbivory induced partially overlapping phenolic profiles in *N. attenuata* and *N. longiflora* (Izaguirre et al. 2007), UVB did not directly affect the jasmonate levels in the plants (Demkura et al. 2010). In another report with broccoli, plants responded independently and specifically to UVB and herbivores (Kuhlmann & Muller 2009). From these examples, it seems that in some plant–UVB–herbivore interactions, crosstalk between UVB and defence signalling occurs. Consistent with this hypothesis, we found that UVB exposure altered phytohormone levels, gene expression and metabolic profiles of *N. attenuata* in natural habitat, some of which counteracted *T. notatus* infestations in UVB-exposed *N. attenuata* plants. Possible mechanisms involved in these interactions are discussed in the next section.

**Effects of UVB on jasmonate metabolism**

Previously, the attractiveness of *Arabidopsis* to diamondback moths (*P. xylostella*) was altered, apparently in a JA-dependent manner, in response to UVB (Caputo, Rutitzky & Ballare 2006). UVB exposure directly increased JA and ethylene levels, which together with reactive oxygen species mediated the UVB-induced stress and gene expression responses in *Arabidopsis thaliana* (Mackerness et al. 1999). Consistent with these reports, solar UVB exposure in plants growing in their native habitats increased the OS-elicited JA and JA-IIe bursts (Fig. 2), as well as hormone levels elicited by attack from *M. sexta* or *T. notatus* herbivores (Fig. 3b). In contrast, jasmonate levels were not affected by UVB exposure in *N. attenuata* in another experiment conducted under simulated UVB in the glasshouse (Demkura et al. 2010), which may reflect differences in experimental conditions. Previously, it was proposed that the unbalanced UVB and PAR levels of the glasshouse environment could result in aberrant results (Caldwell & Flint 1994; Caldwell, Flint & Searles 1994; Deckmyn et al. 1994; Kakani et al. 2003). From our experience and other literature reports (Demkura et al. 2010), when tobacco plants are exposed to UVB in the glasshouse or growth chamber, visible morphological changes such as leaf rolling, reduced growth and leaf surface glazing are frequently observed in UVB-exposed plants; however, we have never seen such morphological responses in field-grown plants even though UVB fluences of 300–400 µW cm⁻² and the PAR fluences of 1200–1500 µmol m⁻² s⁻¹ are typically measured on sunny days at our field plot. This suggests that a balanced light spectrum, in particular, naturally high PAR levels, may be essential to obtain consistent results between different UVB light-source experiments.

**Effects of UVB on defence metabolites**

Similarly to previous experiments with *N. longiflora* (Izaguirre et al. 2003), solar UVB suppressed *NaTPI* expression in *N. attenuata* at early time points after WOS elicitation (0.5 and 1 h; Fig. 3). However, after 12 h, UVB exposure appeared to increased *NaTPI* levels by approximately twofold (non-significant difference; *P = 0.1900*) compared to UVB-exposed plants (Fig. 3). This result is consistent with the earlier work when *NaTPI* expression in UVB-exposed plants significantly increased 24 h after simulated herbivory treatment. Because the gene expression of *NaTPI* in the other work was examined only at 24 h post-elicitation (Demkura et al. 2010), the importance of lower levels of *NaTPI* transcripts at early time points observed here remains unclear. Indeed, the lower transcript abundances of *NaTPI* in UVB-exposed plants after WOS elicitation did not result in lower TPI activities, which remained comparable in both types of UVB-exposed plants treated with WOS (Fig. 3). In contrast, constitutive levels of TPI activity were higher in UVB-exposed plants, despite lower levels of transcripts in these plants (Fig. 3). Therefore, post-transcriptional modifications seem to be crucial for the activation of TPI defences, which disconnected transcript levels from the observed protein activities (McManus et al. 1994). As discussed further, we propose that UVB priming of defence plays important role when plants interact with insects that do not cause significant damage on plants, and otherwise feed stealthily on the plants in the field.

Previously, it was shown that the rapid OS-elicited JA burst in *N. attenuata* requires the activation of two MAP kinases, salicylate-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) (Kallenbach et al. 2010), suggesting that protein phosphorylation is important.
for JA biosynthesis and downstream JA signalling. Because SIPK and WIPK are known to regulate many other stress responses in tobacco (Wu et al. 2007a; Meldau, Wu & Baldwin 2009), we hypothesized that UVB could target these enzymes to promote plant defence under field conditions. In Lycopersicon peruvianum, three MAP kinases (LeMPK1, LeMPK2 and LeMPK3) were induced by UVB exposure; while LeMPK3 is highly homologous to WIPK in tobacco, LeMPK1 and LeMPK2 are highly similar (approximately 95%) to SIPK (Holley et al. 2003). In maize, a phosphate dikinase was regulated post-translationally by phosphorylation after UVB exposure (Casati et al. 2005), and, in human, UVB induced and activated p38 MAP kinase (Chouinard et al. 2002). In our experiment, the NaSIPK and NaWIPK kinase activity showed a slight trend towards higher activity after 3, 12 and 24 h after WOS elicitation (Supporting Information Fig. S2C); however, the significance of this trend remains to be examined in detail. Alternatively, other changes in the sensitivity of stress signalling pathways could provide another mechanism for UVB action such as enhancing the sensitivity to JA as previously described in tobacco plants (Demkura et al. 2010).

**UVB-regulated metabolites provide enhanced resistance to mirids**

Because JA signalling orchestrates defences against herbivores and necro- trophic pathogens in N. attenuata plants, silencing of JA biosynthetic genes such as NaLOX3 results in highly vulnerable plants when exposed to natural herbivores (Kessler et al. 2004). Silencing of the NaCOIII gene, an essential component in JA perception, made N. attenuata irCOIII plants vulnerable to attack from T. notatus, flea beetles and leafhoppers in the field (Paschohl, Halitschke & Baldwin 2007). Similarly, silencing of genes that impair JA levels and/or signalling, such as N. attenuata transcription factors WRKY3 and WRKY6, resulted in less-defended plants which were highly susceptible to grasshoppers and T. notatus (Skibbe et al. 2008). Interestingly, the UVB-mediated suppression of thrips performance on N. attenuata was abolished in plants defective in JA accumulation (as-lox; Demkura et al. 2010). While these results demonstrate that JA is essential for plant resistance to herbivores, it does not explain which parts of JA-dependent metabolism are responsible for protection against herbivores from different feeding guilds. Previous differential display-reverse transcriptase PCR experiments revealed that T. notatus attack induced a different set of genes compared to those that respond to attack of M. sexta caterpillars (Voelkel & Baldwin 2003). How four major groups of defence metabolites and their interaction with UVB could contribute to plant defence against herbivores is discussed further.

**HGL-DTGs**

The plants lacking JA (or JA signalling) are typically more vulnerable to T. notatus infestations. Because JA controls, apart from other defences, the accumulation of HGL-DTGs, it suggests that the presence of HGL-DTGs may be an important factor contributing to T. notatus performance on N. attenuata in natural habitats. Indeed, plants silenced in the expression of geranylgeranyl diphosphate synthase plants (irGGPPS) with highly reduced HGL-DTGs levels were more damaged by T. notatus, grasshoppers and flea beetles in the field (Helling et al. 2010). By counting T. notatus feeding on irGGPPS plants, we directly confirmed the higher attractiveness of these plants to mirids when grown under UVB-absorbing canopies (Fig. 5c). Although UVB-exposed plants were significantly less attacked over three consecutive field seasons, and had constitutively higher HGL-DTGs levels (Fig. 4), the effect was not solely due to HGL-DTGs because HGL-DTG-silenced irGGPPS plants were still resistant to mirids under UVB. It is also likely that the effect of UVB does not involve the priming of JA signalling for resistance against mirid bugs. Mirid feeding did not induce a significant JA burst; however, they were strongly affected by HGL-DTGs levels in a UVB-dependent manner. Because M. sexta feeding induced a strong JA burst associated with accumulation of large amounts of HGL-DTGs in UVB-independent manner, we propose that UVB could be actually important for natural defence against herbivores associated with low levels of JA signalling.

*T. notatus* clearly preferred to feed on plants with low levels of HGL-DTGs (irGGPPS). However, lower HGL-DTGs cannot explain why *T. notatus* did not colonize irGGPPS plants under UVB fluence. In addition to the possibility that UVB radiation could directly influence *T. notatus* host choice, other unexamined UVB-induced compounds could deter *T. notatus*. UVB exposure is known to induce plant volatiles emissions (Eichholz et al. 2011), some of which function as indirect defences against herbivores (Pare & Tumlinson 1999; Kessler & Baldwin 2001; Schuman, Barthel & Baldwin 2012) and deterr oviposition (McCallum et al. 2011). Bleeker et al. (2009) reported that some volatile terpenoid compounds such as *p*-cymene, *α*-terpinene, *α*-phellandrene, ringiberene and curcumene have repellent effects on whiteflies (Bleeker et al. 2009). HGL-DTGs are derived from terpenoid metabolism; it is possible that the volatile terpenoid compounds in N. attenuata were also induced by UVB fluence and repelled *T. notatus*.

Not all HGL-DTG-impaired *N. attenuata* plants have shown compromised resistance against *T. notatus*. The ectopic overexpression of A. thaliana jasmonic acid O-methyltransferase (AtJM) gene in N. attenuata lowered HGL-DTG levels, as well as other defences, but these 35S-jmt-1 plants showed similar mirid damage as EV (Stitz, Baldwin & Gaugler 2011). Because 35S-jmt-1 plants convert most of their JA to methyl jasmonate (MeJA), accumulating 82 times more MeJA 1 h after WOS elicitation compared to EV (Stitz et al. 2011), it is possible that these unusually high levels of volatile MeJA could play a repellent role against *T. notatus*. It is interesting to note that the dominant sagebrush of the Great Basin desert, Artemisia tridentata, which produces substantial amounts of MeJA in its volatile oils (Preston, Laue & Baldwin 2001) is also never
attacked by mirids (I.T. Baldwin, unpublished results). Further experiments on volatile measurement and choice test need to be conducted to falsify the hypothesis that UVB-induced volatiles may contribute to the host choice of *T. notatus*.

**Phenylpropanoid pathway**

In artificial diet experiments, rutin increased feeding by Japanese beetle (*Popillia japonica* Newman) and CHA mildly stimulated beetle feeding at concentrations lower than 31.6 mM; CHA became a mild deterrent at high concentrations (~100 mM) (Fulcher et al. 1998). Overexpression of the phenylalanine ammonia-lyase (PAL) enzyme in tobacco resulted in 10 times higher levels of CHA and twice the rutin levels compared to sense-suppressed plants, however; *M. sexta* caterpillars, previously reared on artificial diet or tobacco leaves, showed no feeding preference for either leaves, over or underexpressing PAL, suggesting that CHA and rutin do not influence feeding, either positively or negatively by *M. sexta* larvae (Eichenscer, Bi & Felton 1998). In our field experiments, the UVB-exposed plants contained constitutively higher levels of rutin and CHA that may have contributed to the low infestation rates of these plants by mirid bugs. Previously, using irMYB8 plants, Kaur et al. (2010) showed that silencing of two major tobacco phenolamides derived from the phenylpropanoid pathway, CP and DCS, increased the performance of *M. sexta* larvae on the irMYB8 transgenic plants. In our field experiment, although UVB-protected irMYB8 attracted more *T. notatus* than EV, this difference was not statistically different (Fig. 5c).

**TD**

A potentially interesting phenotype was found when we analysed the levels of *NaTD* expression under different UVB regimes. While TD catalyses the conversion of threonine to α-ketobutyrate in the biosynthetic pathway of isoleucine (required for JA-Ile), enzymes such as arginase and TD are known to metabolize essential amino acids in insect digestive tracts, and, as a consequence, suppress herbivore performance (Chen et al. 2005). In tomato, gene duplication resulted in two TD enzymes, one of them being inducible by herbivore feeding and involved in direct defence (Gonzales-Vigil et al. 2011). *NaTD* (and consequently leaf isoleucine levels) increased more in UVB-exposed plants after WOS elicitation than dUVB exposure (Fig. 2). However, as this increase occurred only in response to the WOS treatment, we assume that TD may be targeting chewing herbivores whose attack is associated with strong, feeding-elicited JA bursts in *N. attenuata* plants (Stork et al. 2009). As *T. notatus* attack did not elicit a detectable JA burst (Fig. 5b), it is unlikely that TD was directly involved in the UVB-mediated resistance against these insects.

**TPIs**

While TPIs are essential for the protection of *N. attenuata* and other plants against chewing insects, which is consistent with the strong inducible character of this gene, the role of TPIs in defence against sucking insects is less known. A previous comparison between two ecotypes of *N. attenuata*, Utah (a TP producing ecotype) and Arizona (lacking TP activity due to the nonsense-mediated mRNA decay (NMD) of the gene’s transcripts) (Wu et al. 2007b), suggested that TPIs could be one of the important factors that determines colonization of *N. attenuata* by *T. notatus* (Glawe et al. 2003). In our experiment, although irPl plants attracted more mirids in the dUVB environment compared to EV, this difference was not significant (Fig. 5c).

**Could HGL-DTGs function as novel antioxidant/protectant in *N. attenuata***?

While UVB exposure elicited the expected accumulation of phenoic sunscreens, the complicated pattern of HGL-DTG accumulation observed under field conditions provides a novel insight into UVB effects on plant metabolism. However, the severe depletion of HGL-DTGs in response to simulated herbivory in UVB-exposed plants still waits for an explanation. The analysis *NaDXS* expression, an enzyme which supplies isoprene for terpenoid biosynthesis (Lichtenthaler 1999), did not correlate with the observed decline in HGL-DTG levels. This suggests that mechanism(s) other than biosynthesis, such as degradation and/or flux of HGL-DTGs into another interconnected pathway, are responsible for the UVB-dependent decline in HGL-DTG contents.

Previously, UVB has been shown to modulate the interplay between terpenoids and flavonoids in peppermint plants. Field-grown peppermint could adapt better to increased UVB irradiation compared to plants cultivated in the growth chamber. In addition, UVB-exposed peppermint plants accumulated more terpenoid compounds such as pulegone, linalool, (E)-b-caryophyllene and gammaene (Dolzenko et al. 2010). In rice, UV irradiation induced expression *DAX* up to ninelfold compared to non-irradiated controls (Kim, Kim & Chang 2005), suggesting that terpenoids may, directly or indirectly, contribute to UV resistance in plants.

Mechanical wounding and application of insect oral secretions (WOS) or insect chewing activity are sources of oxidative stresses, leading to the activation of antioxidant mechanisms. In our system, perhaps, the UVB triggered an antioxidant response that utilizes HGL-DTGs, which would not occur in glasshouse-grown or dUVB-exposed plants in the field. The results from experimental treatments involving factorial combinations of UVB and different genotypes are consistent with this scenario. They showed that plants silenced in total HGL-DTGs production (irGGPPS plants) constitutively accumulated more CHA, crypto-CHA and rutin, three well-known natural UV screens in plants, and the accumulation of these metabolites was amplified when the irGGPPS plants were exposed to UVB (Fig. 6, see Supporting Information Table S1 for full *anova* and Tukey post hoc test comparisons). These results are consistent with the hypothesis that HGL-DTGs or and HGL-DTG-metabolized compounds participate in UVB resistance;
consequently, the lack of HGL-DTGs in irGGPPS plants may elicit a compensatory response upon exposure to UVB that results in amplified levels of CHA, crypto-CHA and rutin, sunscreens that may accumulate in response to the perception of UVB-induced damage.

CONCLUSIONS

Our results suggest that solar UVB is one of the essential factors that determine the attractiveness of N. attenuata to T. notatus by affecting accumulation of HGL-DTGs. UVB signalling may fine-tune defence pathways at post-transcriptional and post-translational levels, honing defence responses under combined natural stress conditions. In addition, we propose that UVB may play an important role in regulation of plant-insect interactions that do not involve strong JA bursts.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

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UVB and defence against herbivores

Figure S1. (A) UVB absorbance of UVB opaque and UVB transparent foils used for the canopies under which plants were grown (see D). The absorbance spectra of plastic foils were determined by a Tecan Infinite M200 Reader (Tecan Group). Transmittance of UVB (B) and photosynthetic active radiation (C) under near-ambient UVB (control) or diminished UVB (dUVB) condition. UVB fluence was measured inside and outside the canopies with a Digital Ultraviolet Radiometer, Model 6.2 UVB (Solartech Inc.), photosynthetic active radiation (PAR) was measured by a Li-190SA quantum sensor connects directly to a Li-COR Li-250A light meter (http://www.licor.com). The percentage of transmittance was calculated by dividing value measured under the canopy by the values measured outside the canopy and expressed as a percentage. (D) Design of one of 10 replicate canopies under which plants were grown. The height of the canopy was raised as plants grew and foil was added to the sides to ensure that stray radiation did not impinge on plants as the sun moved through the sky. PAR values under the different canopies did not differ and foils were changed 2–3 week as they solarized.

Figure S2. Mean (±SE, n ≥ 3) levels of ABA (A), relative abundance of NaDXS transcripts (B) and NaSIPK and NaWIPK activity in OS elicited N. attenuata leaves (C). Single leaves were mechanically wounded and the resulting puncture wounds were treated with 20 μL of oral secretions from M. sexta larvæ (5x diluted in distilled water). Samples were collected 0, 0.5, 1, 3, 12 and 24 h after OS elicitation, extracted and analysed by LC-MS/MS (ABA) and qPCR (NaDXS). Protein kinase activity was determined by in-gel kinase activity assay (NaSIPK and NaWIPK). Comparisons of near-ambient UVB (control and assigned as ‘+’ in C) or diminished UVB (dUVB, assigned as ‘−’ in C) were determined for individual time points by ANOVAs, Fisher’s PLSDs: *P ≤ 0.05. SE, standard error; FM, fresh mass. (D) Specific primer sequences used for qPCR in the SYBR Green assays.

Figure S3. Herbivore damage in season 2009 and 2011. Mean (±SE, n ≥ 11) levels of herbivore damage grown under near-ambient UVB (control) or diminished UVB (dUVB) treatments. Types of herbivore damage were identified and categorized based on the characteristic feeding damage of the different herbivore taxa. Comparisons between near-ambient UVB (control) or diminished UVB (dUVB) treatments were performed by Kruskal-Wallis test: **P = 0.01. SE, standard error.

Figure S4. Leaf damage and survivorship of T. notatus and M. sexta under different UVB fluences. Mean (±SE, n ≥ 7) survivorships of two different native herbivore species from different feeding guilds feeding on EV plants exposed to near-ambient UVB (control) or diminished UVB (dUVB) treatments in the 2011 field season. Plants were attacked by either 5 adult T. notatus or two M. sexta neonates enclosed in a clip cage on a single N. attenuata leaf of separate plants. Observations were made after 3 d. Comparisons between treatments were performed Kruskal-Wallis test; SE: standard error.

Figure S5. Accumulation of nicotine, CHA, CP and DCS in no-choice experiment under different UVB fluences. Mean (±SE, n ≥ 7) accumulation of nicotine, caffeoylputrescine (CP), chlorogenic acid (CHA) and dichalcosylpyridine (DCS) in leaves of plants exposed to near-ambient UVB (control) or diminished UVB (dUVB) and attacked by two different native herbivore species from different feeding guilds. Plants were attacked by either 5 adult T. notatus or two M. sexta neonates enclosed in a clip cage on a single EV N. attenuata leaf of separate plants. Attacked leaves were collected after 3 d and analysed by HPLC-PDA. Comparisons between near-ambient UVB (control) or diminished UVB (dUVB) treatments were made for each insect by ANOVA test, Fisher’s PLSDs: *P = 0.05. The experiment was conducted in the field during the 2011 season. SE, standard error; CHA eq. chlorogenic acid equivalents.

Figure S6. Accumulation of individual HGL-DTGs after 3 d feeding by different insects. Either five adult T. notatus or two M. sexta neonates were kept in a clip cage on a single leaf of EV N. attenuata. Attacked leaves were collected after 3 d, extracted and analysed by LC-MS/MS. Comparisons near-ambient UVB (control) or diminished UVB (dUVB) treatments were made for each insect by ANOVA test, Fisher’s PLSDs: *P = 0.05, **P = 0.01, ***P = 0.001, (A) precursor; (B) core HGL-DTGs; (C) malonylated HGL-DTGs; (D) dimalonylated HGL-DTGs. The experiment was conducted in the field during the 2011 season. SE, standard error; FM, fresh mass.

Table S1. Full ANOVA and Tukey post hoc test comparisons for chlorogenic acid, crypto-chlorogenic acid and rutin.

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Supplemental Figure 1. A: UVB absorbance of UVB opaque and UVB transparent foils used for the canopies under which plants were grown (see D). The absorbance spectra of plastic foils were determined by a Tecan Infinite M200 Reader (Tecan Group). Transmittance of UVB (B) and photosynthetic active radiation (C) under near ambient UVB (control) or diminished UVB (dUVB) condition. UVB fluence was measured inside and outside the canopies with a Digital Ultraviolet Radiometer, Model 6.2 UVB (Solartech Inc.), photosynthetic active radiation (PAR) was measured by a Li-190SA quantum sensor connects directly to a Li-COR Li-250A light meter (http://www.licor.com). The percentage of transmittance was calculated by dividing value measured under the canopy by the values measured outside the canopy and expressed as a percentage. D: Design of one of 10 replicate canopies under which plants were grown. The height of the canopy was raised as plants grew and foil was added to the sides to ensure that stray radiation did not impinge on plants as the sun moved through the sky. PAR values under the different canopies did not differ and foils were changed 2-3/week as they solarized.
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D. The specific primer sequences used for qPCR (SYBR) are as followed.

*NaTD* forward primer: 5’-TAAGGCATTTGTGGGGAGGC-3’
*NaTD* reverse primer: 5’-TCTCCCTGTTTGCAATTGGA-3’
*NaEF1a* forward primer: 5’-CCACACTCCCATGGGTCA-3’
*NaEF1a* reverse primer: 5’-CGCATGGCTCCCTCACAGAAAAC-3’
*NaDXS* forward primer: 5’-ATTGATGACAGACAAAGCTGTTT-3’
*NaDXS* reverse primer: 5’-TATCTCAGTAGAGCCACTCTC-3’

**Supplemental Figure 2.** Mean (± SE, n=3) levels of ABA (A), relative abundance of *NaDXS* transcripts (B) and NaSIPK and NaWIPK activity in OS elicited *N. attenuata* leaves (C). Single leaves were mechanically wounded and the resulting puncture wounds were treated with 20 μL of oral secretions from *M. sexta* larvae (5X diluted in distilled water). Samples were collected 0, 0.5, 1, 3, 12 and 24h after OS elicitation, extracted and analyzed by LC-MS/MS (ABA) and qPCR (*NaDXS*). Protein kinase activity was determined by in-gel kinase activity assay (NaSIPK and NaWIPK). Comparisons of near ambient UVB (control and assigned as “+” in C) or diminished UVB (dUVB, assigned as “-” in C) were determined for individual time points by ANOVAs, Fisher’s LSDs: * P ≤ 0.05. SE, standard error; FM, fresh mass. D. Specific primer sequences used for qPCR in the SYBR Green assays.
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Mean (± SE, n≥11) levels of herbivore damage grown under near ambient UVB (control) or diminished UVB (dUVB) treatments. Types of herbivore damage were identified and categorized based on the characteristic feeding damage of the different herbivore taxa. Comparisons between near ambient UVB (control) or diminished UVB (dUVB) treatments were performed by Kruskal-Wallis Test: ** $P \leq 0.01$. SE, standard error.
Supplemental Figure 4. Leaf damage and survivorship of *T. notatus* and *M. sexta* under different UVB fluences.
Mean (± SE. n≥7) survivorships of two different native herbivore species from different feeding guilds feeding on EV plants exposed to near ambient UVB (control) or diminished UVB (dUVB) treatments in the 2011 field season. Plants were attacked by either 5 adult *T. notatus* or two *M. sexta* neonates enclosed in a clip-cage on a single *N. attenuata* leaf of separate plants. Observations were made after 3 d. Comparisons between treatments were performed Kruskal-Wallis Test; SE: standard error.
Supplemental Figure 5. Accumulation of nicotine, CHA, CP and DCS in no-choice experiment under different UVB fluences.

Mean (± SE, n=7) accumulation of nicotine, caffeoylputrescine (CP), chlorogenic acid (CHA) and dicafeoylsermidine (DCS) in leaves of plants exposed to near ambient UVB (control) or diminished UVB (dUVB) and attacked by two different native herbivore species from different feeding guilds. Plants were attacked by either 5 adult T. notatus or two M. sexta neonates enclosed in a clip-cage on a single EV N. attenuata leaf of separate plants. Attacked leaves were collected after 3 d and analyzed by HPLC-PDA. Comparisons between near ambient UVB (control) or diminished UVB (dUVB) treatments were made for each insect by ANOVA test, Fisher’s PLSDs: * P ≤ 0.05. SE, standard error. CHA eq, chlorogenic acid equivalents. The experiment was conducted in the field during the 2011 season.
Supplement Figure 6. Accumulation of individual HGL-DTGs after 3 d feeding by different insects.
Either five adult *T. notatus* or two *M. sexta* neonates were kept in a clip-cage on a single leaf of EV *N. attenuata*. Attacked leaves were collected after 3 d, extracted and analyzed by LC-MS/MS. Comparisons near ambient UVB (control) or diminished UVB (dUVB) treatments were made for each insect by ANOVA test, Fisher’s PLSDs: * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. A: precursor; B: core HGL-DTGs; C: malonylated HGL-DTGs; D: dimalonylated HGL-DTGs. SE: standard error; FM: fresh mass. The experiment was conducted in the field during the 2011 season.
Chapter 5

Discussion

This work describes novel regulatory mechanisms and interactions mediating plant defense against herbivores, and the effect of solar UVB light on plant defense. Specifically, I describe the function of three dicers in plant development, the regulation of their transcripts during herbivory and their contribution to mediating some defense metabolites such as nicotine and trypsin proteinase inhibitors, possibly via mediating multiple signaling pathways and regulators such as ethylene, JA signaling pathways, mitogen activated protein kinases (MAPKs), and MYB transcription factors. In addition, for the first time, the involvement of ABA signaling and the interaction of ABA-JA signaling were decoded and revealed the crucial function of ABA signaling in regulating herbivory-induced defense metabolites such as HGL-DTGs, caffeoylputrescine, dicaffeoylspermidine, trypsin proteinase inhibitors, and trans-α-bergamotene in local and systemic tissue. Moreover, the positive contribution of solar UVB to defense against piercing-sucking herbivores such as T. notatus, but likely not against the chewing herbivore M. sexta associated with strong JA burst, was discovered. In addition, a new sunscreen function of HGL-DTGs under solar UVB exposure was proposed. Finally, the differences in kinetic accumulation of individual HGL-DTGs under two different solar UVB fluences suggested that a combination of laboratory and field experiments are necessary and best strategy to explore plant physiology in the real ecological context.
5.1. Function of DICER-like proteins in defense regulation

RNA interference (RNAi) is the mechanism to degrade specific mRNA molecules in most higher organisms (Baulcombe, 1996; Wassenegger and Pelissier, 1998; Sharp, 1999). After the findings that (1) a double-stranded RNA is much more effective at producing interference (gene silencing) than single stranded RNA in Caenorhabditis elegans (Fire et al., 1998), and (2) dicer can specifically cleave double-stranded RNAs (dsRNAs) to produce short interfering dsRNAs (Bernstein et al., 2001), RNAi has become a valuable research tool to control gene expression in order to investigate gene function in many areas of plant biology, including ecological research (Gase et al., 2011).

In addition to the function of removing invading nucleic acids, such as transgenes and viruses, it is now clear that RNAi is also involved in defense regulation (Brodersen and Voinnet, 2006). In order to investigate functions of dicer-like proteins (DCL) in N. attenuata in the context of plant-herbivore interactions and development, we isolated four DCLs (namely, NaDCL1, 2, 3, 4) and found that they occur as single-copy genes in the N. attenuata genome. Expression of DCLs is ubiquitous but dependent on tissue, developmental stages, and on environmental stresses (Liu et al., 2009). Here we found that DCL expression in N. attenuata was significantly different among tissues (flowers, source leaf, cauline leaf and stem) and showed a diurnal rhythm, but simulated M. sexta elicitation had only a marginal effect on DCL transcript levels. Although there had been several attempts to silence all DCLs individually, only NaDCL2, 3, 4 could be successfully silenced by the inverted repeat constructs, and a stable ir-dcl1 plant was never successfully generated. It is likely that NaDCL1 functions, similar to its homolog in Arabidopsis and moss, as regulator of embryonic development and maturation (Khraiwesh et al., 2010; Nodine and Bartel, 2010), and this essential function interferes with generation of transgenic plants.

Initially, there was a logical concern that using the RNAi machinery to silence dicers may prove difficult or impossible because of trying to silence genes which themselves play a role in gene silencing. Nevertheless, silencing efficiency of NaDCL2 and NaDCL3 was 85-90%, whereas silencing efficiency for NaDCL4 was 60%. Notably, RNAi strategies were also used to silence DCL1 and AGO1 genes in Arabidopsis allotetraploid plants, but only a 23% reduction in DCL1 levels was reported, that, however, already caused morphological changes (Lackey et al., 2010). Moreover, ir-dcl2, 3, 4 showed numerous phenotypes in plant development,
accumulation of phytohormones, secondary metabolites, and *M. sexta* performance that demonstrates RNAi silencing was efficient to knockdown these gene in ir-*dcl* plants.

Because short dsRNAs influence a wide variety of biological processes (Hannon, 2002), dicer and dicer-like (DCL) proteins mutants exhibited many effects on the development of *C. elegans* (Grishok et al., 2001; Knight and Bass, 2001), *Drosophila melanogaster* (Hutvagner et al., 2001; Wang et al., 2006), Arabidopsis (Liu et al., 2009). In *N. attenuata*, we also observed morphological changes in rosette leaf diameter, leaf length and width, and alterations in root development. Changes in morphology were also found in Arabidopsis *dcl* mutants (Gaschiolli et al., 2005; Xie et al., 2005; Adenot et al., 2006; Marin et al., 2010).

To investigate the function of NaDCLs in defense, we quantified phytohormones (JA, JA-Ile, ABA, and SA), defense metabolites such as nicotine, TPIs, and performed bioassays using *M. sexta* specialist herbivores. Because defense metabolites such as nicotine were reduced in both ir-*dcl3* and ir-*dcl4* plants, and TPIs were reduced in ir-*dcl3*, but these metabolites were at the same levels in ir-*dcl2* as in WT plants, *M. sexta* caterpillars performed better on ir-*dcl3* and ir-*dcl4* but not on ir-*dcl2*. It is known that dicers can work in cooperation; however, they can also show specific functions to regulate transcript abundances (Lee et al., 2004; Gaschiolli et al., 2005; Xie et al., 2005). Interestingly, the reductions in nicotine in ir-*dcl3* and ir-*dcl4* were lost after crossing these plants with ir-*dcl2*. In addition, changes in morphology such as root biomass could be recovered to WT levels when ir-*dcl4* plants were crossed with ir-*dcl2*. These observations strongly suggest a connection/crosstalk between DCLs during defense and development in *N. attenuata*. Analysis with a microarray of the *N. attenuata* transcriptome showed that NaDCLs engage in regulation of genes involved in ethylene and JA signaling pathways, mitogen activated protein kinases (MAPKs), and MYB transcription factors. The overall picture of regulation suggested that the interactions among various molecular players determine, in a complex manner, transcriptional rearrangements during herbivory. These results support previous studies showing that the RNAi machinery contributes to gene regulation during plant-herbivore interactions and development in *N. attenuata* (Pandey and Baldwin, 2007, 2008; Pandey et al., 2008a; Pandey et al., 2008b). In conclusion, our study provides new insights to the regulatory mechanism of DCLs, not limited to plant-herbivore interactions but also in the regulation of plant development.
5.2. **Herbivore-associated elicitor (HAE)-inducible defense signaling**

After long coexistence and coevolution of plants and insects (Whalley and Jarzemkowski, 1981; Engel and Grimaldi, 2004), many plant species evolved specific inducible responses to resist or tolerate herbivores to balance the cost of defense and fitness (Breedlove and Ehrlich, 1968; Voelckel and Baldwin, 2004; Bartel, 2005; Mallory and Vaucheret, 2006; Becerra, 2007; Agrawal et al., 2012; Ali and Agrawal, 2012).

Constitutive defenses are present already before an herbivore attacks. In contrast, inducible defenses are activated only after the plant detects insect attack (Chen, 2008), and thus require specific herbivore-tailored perception systems. While FLS2 in *Arabidopsis thaliana*, and *Solanum lycopersicum*, and toll-like receptor 5 (TLR5) in vertebrates, are well-known receptors of pathogen-associated molecular patterns (PAMPs) (Gomez-Gomez and Boller, 2000; Ausubel, 2005; Mueller et al., 2012; Yoon et al., 2012), none of the receptors for HAEs have been identified in plants. It was predicted that HAEs could be detected by specific plant receptors localized on the plasma membranes, however isolation of such proteins has not been successful (Truitt et al., 2004; Felton and Tumlinson, 2008; Wu and Baldwin, 2010; Bonaventure, 2012; Erb et al., 2012).

Defense theories address the diverse and ubiquitous ways that plants face their enemies, especially why some species are so well defended, while, others are not (Stamp, 2003). The ultimate value of defense is to increase fitness under stress, however, because defense is costly, defense-growth tradeoffs frequently shape plant performance (Reymond et al., 2000; Howe and Jander, 2008). Therefore, plants evoke mechanisms to detect herbivore-associated elicitors (HAEs) and use them as cues to trigger defense (Harvell, 1990). It is obvious that reliability and efficiency of inducible defense will then rely on the selection and perception of a trusted cue(s). The fatty acid-amino acid conjugates (FACs) in *Spodoptera exigua* (Alborn et al., 1997), *Heliothis virescens* and *Helicoverpa zea* (Mori et al., 2001), and in *M. sexta* oral secretion (Halitschke et al., 2001) are an example of such cues. After the finding of FACs as major HAEs in *M. sexta* and their demonstrated recognition by *N. attenuata* (Halitschke et al., 2001), many studies devoted to this signaling pathway have been conducted with special focus in understanding ecological functions of FAC perception. Because FACs regulate majority of inducible defense layers introduced in the Introduction Chapter, *N. attenuata –M. sexta* system became one of the best developed molecular models to
study plant-insect interactions (Halitschke et al., 2001; Halitschke et al., 2003; Giri et al., 2006; Schwachtje et al., 2006; Paschold et al., 2007; von Dahl et al., 2007; Wu et al., 2007; Gaquerel et al., 2009; Meldau et al., 2009; Kaur et al., 2010; VanDoorn et al., 2010). Although one putative receptor for HAEs has been identified in Zea mays (Truitt et al., 2004), and the effects of FACs on membrane potentials, intracellular calcium in Lima bean (Phaseolus lunatus) (Maffei et al., 2004b), as well as their effects on transmembrane ion fluxes is known (Maischak et al., 2007), the actual FAC receptor(s) has not yet been identified. An analysis of FAC-responsive genes by Serial Analysis of Gene Expression (Super SAGE) successfully identified large number of genes induced after FACs application in wounded leaves (Gilardoni et al., 2010). One of them, the Lectin Receptor Kinase 1 (NaLecRK1) was further characterized and shown to mediate defense by affecting both SA and JA signaling pathways (Gilardoni et al., 2011).

Inducible defense signaling requires certain crucial properties such as: (i) specificity, (ii) fast amplification, and (iii) memory (Harvell, 1990). The Nicotiana attenuata Herbivore Elicitor-Regulated 1 (NaHER1) protein was discovered to be one of the indispensable components in FAC-inducible defense that protects N. attenuata from M. sexta herbivory. NaHER1 expression is induced by FACs in oral secretion (OS), independent of JA-signaling. The gene is quickly and transiently induced (peak at 1 h after OS elicitation) in both local and systemic tissues, which indicates a function for this protein in early signal transduction related to the response to M. sexta. Indeed, silencing NaHER1 by RNAi led to reduction in the content of several well-known FAC-induced defense metabolites such as TPIs, HGL-DTGs, CP, and trans-α-bergamotene in local and systemic tissues (Van Dam et al., 2001; Glawe et al., 2003; Wu et al., 2007; Schuman et al., 2009; Heiling et al., 2010; Kaur et al., 2010). Finally, silencing of NaHER1 strongly affected the performance of M. sexta larvae. To my knowledge, the expression pattern of NaHER1 is the unique in terms of specificity to HAEs and independence of JA signaling shown in my thesis. Previous analysis of FAC-responsive genes by Super SAGE successfully identified genes induced after FAC application, and fifteen of these patterns were confirmed by qPCR. As some of these genes were also induced by wounding treatment (Gilardoni et al., 2010), the number of FAC-induced genes discovered by Super SAGE could be overestimated because, for example, the FACs-induced JA but not FACs themselves could induce these genes. Indeed, the NaLecRK1, found by Super SAGE, was partially regulated by JA signaling since this
gene transcript level was higher in irLOX3 and irCOI1 compared to WT (Gilardoni et al., 2011).

It was proposed that inducible defenses could have originally evolved from uninducible or differently induced, related genes because of clear benefits associated with inducible defenses (Harvell, 1990). Without any attack, irHER1 plants showed delayed flowering and produced less seed capsules, which was further amplified under *M. sexta* attack conditions. It makes NaHER1 a possible example of the “original” and “acquired” functions that developed during co-evolution of plants and herbivores. Perhaps, this gene originally regulated only traits related to plant development, and consequently fitness, but over time, while keeping its original function, it engaged in inducible defense mechanisms that further optimized fitness during herbivory.

Despite FACs betraying insects to induce strong plant defenses, the fact that FACs have been found in high concentration in guts of *Spodoptera exigua* (Alborn et al., 1997), *Heliocthis virescens* and *Helicoverpa zea* (Mori et al., 2001), *M. sexta* (Halitschke et al., 2001; Roda et al., 2004) suggests the particular importance of FACs in those insects (Kuhns et al., 2012). Indeed, FACs were proposed to play essential role in larval digestion and nitrogen utilization (Yoshinaga et al., 2008), and recently, an aminoacylase-like protein (L-ACY-1) in *Heliocthis virescens* was shown to hydrolyze FACs into fatty and amino acids (Kuhns et al., 2012). In the future, functional analysis of this protein in *Heliocthis virescens* will likely further clarify the importance of FACs in insect development.

### 5.3. Relationship of phytohormones in plant development and defense after perception of HAEs

After being attacked by *M. sexta* herbivore, *N. attenuata* responds by transient accumulation of several phytohormones including jasmonates, ABA, SA, and ethylene. All these phytohormones are known to positively affect accumulation of defense metabolites and/or consequently increase plant fitness. For example, JA (Paschold et al., 2007), SA (Rayapuram and Baldwin, 2007), ethylene (Voelckel et al., 2001), and ABA signaling pathways described this thesis clearly demonstrate the importance of these phytohormones in plant physiology.

In *N. attenuata*, synergistic or antagonistic interactions among plant hormones such as salicylic acid (SA), ethylene, brassinosteroids, auxin, gibberellins, and JA.
signaling have been widely demonstrated in defense and developmental responses. Importantly, most of these responses have been shown to be regulated by FAC elicitation (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; von Dahl et al., 2007; Onkokesung et al., 2010; Gilardoni et al., 2011; Yang et al., 2011; Heinrich et al., 2012). These interactions suggest that phytohormone crosstalk or the balance between hormonal signaling pathways likely play an important role in the organization and execution of optimal defense responses (Erb et al., 2012). However, in one rather puzzling example, that the responses of *N. attenuata* and *N. longiflora* to UVB were similar to those elicited by simulated *M. sexta* herbivory and only approximately 20% (48 of 241 genes) showed differential expression. Notably, 241 genes were specifically induced by *M. sexta* attack (Izaguirre et al., 2003) and it is now clear that solar UVB does not strongly affect defense metabolites targeted against *M. sexta* (Chapter 4) (Dinh et al., 2012). These observations lead to several questions: (1) Why is there a similarity in response of *N. attenuata* to solar UVB and *M. sexta*? (2) Why are several phytohormones simultaneously changed after herbivore attack? (3) Why is there so much overlap in the signaling systems regulating different responses?

The fundamental function of phytohormones in plants is regulating development that ultimately affects plant reproduction; for example, JA signaling pathway in Arabidopsis, *Lycopersicon esculentum* and *N. attenuata* (Feys et al., 1994; Li et al., 2004; Paschold et al., 2007), SA (Vicente and Plasencia, 2011), ABA (Cheng et al., 2002; Gonzalez-Guzman et al., 2002; Nambara and Marion-Poll, 2005; Arend et al., 2009), auxin (Vanneste and Friml, 2009), cytokinin (Werner et al., 2001), ethylene (Burg, 1973; Ruzicka et al., 2007), and gibberellins (Richards et al., 2001). Crosstalk between phytohormones in regulating plant development has been widely discussed (Rock and Sun, 2005; Spoel and Dong, 2008; Moubayidin et al., 2009; Hoffmann et al., 2011; Choudhary et al., 2012). Perhaps changes after being attacked, especially in terms of phytohormone-mediated reconfiguration of transcriptomes, are the reflections of the dual function of phytohormones in regulation of (i) plant development is to bring the plant along a path from a single seed to multiple seeds, likely, is the priority and (ii) defense is to prevent the loss of reproductive capacity due to stress. Perhaps, it could explain why transcriptional responses are similar after exposure to solar UVB and *M. sexta* attack, and also the phenomenon that any attack could induce multiple phytohormone signaling responses. Interestingly, phytohormones which regulate defense responses also regulate
development was already stated in the \textit{Growth differentiation balance} theory (Loomis, 1953; Herms and Mattson, 1992; Stamp, 2004)

Functional analysis showed that NaHER1 suppressed ABA metabolism to increase the levels of ABA and defense capacity of JA-mediated defense signaling by increasing the potency of, or stabilizing JA signaling during herbivore attack. Silencing \textit{NaHER1} in \textit{N. attenuata} caused significant reductions in levels of two phytohormones (ABA, JA); trypsin proteinase inhibitors and other defense metabolites (17-hydroxygeranyllinalool diterpene glycosides, caffeoylputrescine, dicafeoylspermidine; and volatile organic compounds) in herbivory-elicited plants. Likely as a consequence, \textit{M. sexta} performed better on \textit{NaHER1}-silenced plants. While most of JA-mediated direct defense metabolites were recovered by exogenously applying ABA to the leaves, none of them recovered to WT levels when applying only MeJA, which revealed the essentiality of ABA in JA-mediated defense. In addition, NaHER1 is likely to positively affect plant growth and development via regulation of stomatal aperture or though JA-mediated responses. Silencing the ABA receptor (\textit{NaPLY4}) in an independent experiment further supported our conclusion that ABA is indeed an important signaling component that positively contributes to regulation of plant defense during herbivore attack.

Because the cost of defense in WT plants would be greater than in irHER1, we predicted that irHER1 plants would have more seed capsules than WT under herbivore pressure in the glasshouse. However, WT plants always had more seed capsules than irHER1. WT control (control: without any attack) had more seeds than irHER1 control plants, indicating an important function of HER1-mediated ABA levels and/or signaling in plant development. Additionally, seed capsule number in WT control > WT \textit{M. sexta} attack > irHER1 control > irHER1 \textit{M. sexta} attack (Chapter 3, Supplemental Figure 12), indicating the function of ABA in optimizing plant fitness during defense. These observations further emphasize the dual function of ABA in plant development and defense, in agreement with the finding that defenses are costly, however, essential for fitness (Baldwin, 1998; Zavala and Baldwin, 2004). Moreover, it may explain why ABA-mediated defense metabolites are inducible.

In Arabidopsis, several plant hormones such as JA, SA, ABA, ethylene, auxin, gibberellins, cytokinins, nitric oxide (NO), and reactive oxygen species (ROS) have been
shown to have synergistic or antagonistic effects on each other to regulate defense against environmental stresses (Xu et al., 1994; Spoel et al., 2003; Anderson et al., 2004; Takahashi et al., 2004; Beckers and Spoel, 2006; Mur et al., 2006; Sato et al., 2010; Lackman et al., 2011; Pieterse et al., 2012). In \textit{N. attenuata}, crosstalk between hormones has also been documented (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; von Dahl et al., 2007; Onkokesung et al., 2010; Gilardoni et al., 2011; Yang et al., 2011; Heinrich et al., 2012). Because hormone crosstalk acts antagonistically or synergistically, huge flexibility in regulation of responses to large variety of environmental stresses exits. Hence, no signaling which is dedicated only to defense has been found.

\begin{figure}[h]
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\caption{Proposed mechanism of relationship of phytohormones with plant development and defense}
\end{figure}

After perception of stress stimuli, several phytohormones initially respond and crosstalk between them lead to transcriptional responses that affect plant development and defense. Different color lines represent individual phytohormones. Direction of arrows indicates that different phytohormones affect different angles, antagonism, synergism for optimizing plant reproduction and defense.
5.4. A lesson from field work

Inspired by Ernst Stahl’s motto “mein Laboratorium ist die Natur” (“my laboratory is nature”), leadership of Prof. Ian T. Baldwin, and support of Prof. Ivan Gális during my thesis, I am now addicted to the philosophy of doing ecological experiments in the real world. Only in the real natural environment, where all possible interactions in the realistic and complex ecological networks happen, can the real behavior of living organisms be discovered. It was my pleasure and great luck for me to adopt *N. attenuata* as my major experimental model, and to have the opportunity to experience and perform field work in the Great Basin in Utah, the native habitat of *N. attenuata*. There, one long rain can make vast local areas to bloom with varieties of flowers and insects after years of hibernation. In addition, the unpredicted shifts in local seasons in the field not only challenge experimental procedures but also affect the presence of insect communities. That, I think, makes the beauty of the real world where one can experience un-expected and unpredicted events brought on us by the Earth and this was the driving forces for *N. attenuata* to evolve inducible defense mechanisms and to adapt to such dramatic changes and severe environmental stresses.

Green plants and algae are the only organisms to capture the energy from sunlight to build up all necessary life ingredients such as carbohydrates, fats, proteins and other organic compounds. As a consequence, plants actively seek out light, which exposes them naturally to solar radiation composed of wide range of wavelengths that include potentially harmful ultraviolet (UV) light (Stapleton, 1992; Ballare et al., 1996; Caldwell et al., 2007; Ballare et al., 2011). In addition, plants are eaten by herbivores that become prey of carnivores, and so on, creating a multitude of food webs of huge complexity. In nature, these interactions resulted in extremely stable and durable trophic structures (Strong, 1992; Bohan et al., 2007; Rzanny and Voigt, 2012), often disturbed by human activity and destruction of natural environment. Because it was impossible not to be harmed by many biotic and abiotic environmental stresses that forced plants and subsequently insects to evolve mechanisms of defense and counter-defense, respectively (Ehrlich and Raven, 1964; Breedlove and Ehrlich, 1968; Voelckel and Baldwin, 2004). The multiple interactions between abiotic stresses – biotic stresses – and plants have attracted enormous attention because of the potential applications in agriculture with significant economic effects (Aberle and Malzahn, 2007; Wackers et al., 2007; Meyer et al., 2012). Moreover, unavoidable abiotic stresses such as UVB could increase in the future because of the current progress in depletion of stratospheric
ozone that is likely to threaten agriculture and natural ecosystems in the future (Caldwell et al., 1980; Bachelet et al., 1991; Caldwell and Flint, 1994).

Although it is known that UVB affects herbivore response in *N. attenuata* (Izaguirre et al., 2007; Demkura et al., 2010), *N. longiflora* (Izaguirre et al., 2003; Izaguirre et al., 2007), Arabidopsis (Mackerness et al., 1999; Caputo et al., 2006), tomato (Conconi et al., 1996), there has been no report specifically addressing the effects of UVB on *Tupiocoris notatus* the nature. Moreover, there has been no study focusing on the interactions of *N. attenuata* with its specialist *M. sexta* and *T. notatus* under natural solar UVB fluences. By comparing the effects of solar UVB on JA accumulation and HGL-DTGs, it allowed us to uncover the positive contribution of solar UVB on defense against piercing-sucking feeding behavior insect such as *T. notatus* but most likely not effective against specialist chewing herbivores like *M. sexta*. This results, together with previous finding that *T. notatus* and *M. sexta* attack induced a different set of genes (Voelckel and Baldwin, 2003), provide now better explanation of this phenomenon, at least, for the accumulation of HGL-DTGs in *N. attenuata* that showed great diversity under natural field stress.

It is clear that HGL-DTGs are one of the most efficient defense metabolites in *N. attenuata*, the amount of HGL-DTGs in *N. attenuata* is proportionally equivalent to starch content (mg/g fresh mass) in the aboveground plant tissues, and it continuously increases over time that fully supports for the Optimal Defense Theory (McKey, 1974; Heiling et al., 2010). More ever, HGL-DTGs increase even without any attack, it rather suggests multiple and overlying functions of these diverse natural compounds. Since *N. attenuata* is a fire-chasing annual that occupies and grows in the post-fire nitrogen-rich soils (Baldwin and Morse, 1994), thus being exposed to strong non-shaded sunlight intensities in Utah, which can support high levels of photosynthesis and thus that carbon should not be a limiting nutrient for the plant. In that situation, the high accumulation level of secondary metabolites such as HGL-DTGs could be likely explained, and in combination with the increasing accumulation at reproductive stage, makes these compounds to fit the Growth Differentiation Balance Theory also (Stamp, 2004) (Loomis, 1953; Herms and Mattson, 1992).

In nature, herbivorous insect determinedly seek food and completely depend on their sole suppliers, plants (Caldwell et al., 2007). Some insects such as *Drosophila*, and butterfly *Papilio* are able to perceive light in UVB range (Feiler et al., 1988; Arikawa et al., 1999) and UVB reflected by some flowers can be seen by these insects (Kevan and
Baker, 1983). Honey bees are able to use the Sun as a guide to navigate in nature (Gould et al., 1978; Menzel et al., 2005). Interestingly, *Caliothrips phaseoli*, a phytophagous insect from the same feeding guilds as *T. notatus*, can detect and respond to solar UVB radiation (Ballare et al., 2002; Mazza et al., 2010). The level of UVB exposure strongly affected the dynamics of individual HGL-DTGs after OS elicitation. In addition, *N. attenuata* plants impaired in the accumulation of HGL-DTGs (irGGPPS) constitutively accumulated more phenolic sunscreens (CHA, crypto-CHA and rutin) and this accumulation pattern was even amplified when the irGGPPS plants were exposed to solar UVB. It suggests that the function of HGL-DTGs may relate to a yet unknown sunscreen function under solar UVB exposure. Notably, phenolic compounds are not the only chemical groups able to absorb photons from the sunlight, for example, chlorophyll is the well-known complex compound that can absorb energy from the sunlight but it is not, structurally, a phenolic compound (Leupold et al., 2002). It is notable that the diterpene skeleton of HGL-DTGs comes from the same substrate molecule as the side chain for chlorophyll, and the skeletons for light-absorbing antenna pigments involved in photoprotection (zeaxanthin and related compounds, carotenoids) (Kajiwara et al., 1997; Albrecht et al., 1999; Giuliano et al., 2000). Perhaps, HGL-DTGs exert synergistic effects with some groups of phenolic compounds, for example in reflection, absorption, or scattering of the sunlight fraction to, for example, make *N. attenuata* invisible/less apparent/less attractive to certain insect eyes. Finally, while the kinetic accumulation of individual HGL-DTGs under diminished UVB was comparable to greenhouse experiments, the strikingly different accumulation pattern under natural solar UVB fluence revealed the natural regulation of these compounds which made me to truly appreciate the Ernst Stahl’s slogan “mein Laboratorium ist die Natur”. 

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Summary

Plants have coexisted with insects for approximately 400 million years. During this time, the interactions between flora and fauna have driven coevolutionary and reciprocal changes, resulting in a complex mosaic of plant defenses and insect counter-defenses. In addition, abiotic stress such as UVB radiation dramatically affects plant performance; therefore, mechanisms to recognize environmental stresses, mounting of properly scaled responses and fitness optimization are three essential features for plant survival in nature.

This work describes novel regulatory mechanisms and interactions mediating plant defense against herbivores, and the effect of solar UVB light on plant defense in Nicotiana attenuata. Specifically, I describe the function of three dicer like proteins (NaDCL2, NaDCL3, NaDCL4) in plant development, the regulation of their transcripts during herbivory and their contribution to mediating some defense metabolites such as nicotine and trypsin proteinase inhibitors, possibly via mediating multiple signaling pathways and regulators such as ethylene, JA signaling pathways, mitogen activated protein kinases (MAPKs), and MYB transcription factors. In addition, for the first time, the involvement of ABA signaling and the interaction of ABA-JA signaling were decoded and revealed the crucial function of ABA signaling in regulating herbivory-induced defense metabolites such as HGL-DTGs, caffeoylputrescine, dicaffeoylspermidine, trypsin proteinase inhibitors, and trans-α-bergamotene in local and systemic tissue. Moreover, the positive contribution of solar UVB to defense against piercing-sucking herbivores such as T. notatus, but likely not against the chewing herbivore M. sexta associated with strong JA burst, was discovered. In addition, a new sunscreen function of HGL-DTGs under solar UVB exposure was proposed. Finally, the differences in kinetic accumulation of individual HGL-DTGs under two different solar UVB fluences suggested that a combination of laboratory and field experiments are necessary and best strategy to explore plant physiology in the real ecological context.
Zusammenfassung

Seit 400 Millionen Jahren leben Pflanzen und Insekten in Koexistenz. Während dieser Zeit führten die Interaktion zwischen Flora und Fauna zu koevolutionären, gegenseitigen Anpassungen, die sich in einem komplexen Mosaik von Pflanzenverteidigungen und Gegenmechanismen der Insekten widerspiegeln. Zusätzlich beeinflussen abiotische Stressfaktoren wie UVB-Strahlung die Pflanzen; darum sind Mechanismen, welche die Stressfaktoren der Umwelt wahrnehmen, ein passendes Maß an Gegenreaktionen einleiten und die Fitness optimieren, drei essentielle Faktoren für das Überleben der Pflanzen in der Natur.

Tóm tắt

Sau khoảng 400 triệu năm cuộc sinh sống trên trái đất, sự tương tác giữa thực vật và động vật ảnh hưởng vật lý do chính dẫn tới sự đồng tiến hóa về tình trạng lẫn nhau giữa chúng. Bên cạnh đó, các nhân tố tự nhiên như tia tử ngoại loại B (UVB) cũng có tác động không nhỏ tới sự sinh trưởng, phát triển của động thực vật. Do vậy, các cơ chế giúp nhân biết các nhân tố tác động ngoại cảnh nhằm phân ứng một cách đặc hiệu với từng nhân tố từ độ tối ướt hòa khả năng duy trì nơi giống động vật quan trọng sống còn của thực vật trong tự nhiên.

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**Eigenständigkeitserklärung**


*Jena, den 28. Januar 2013*

Son Truong Dinh

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**Erklärung über laufende und frühere Promotionsverfahren**


*Jena, den 28. Januar 2013*

Son Truong Dinh
1. **PERSONAL INFORMATION**

Surname:    Dinh  
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2. **EDUCATIONAL BACKGROUND**


3. **LANGUAGE PROFICIENCY**

- Vietnamese: Mother tongue.

- English: good.
4. WORK EXPERIENCE

- Institute of Agrobiology - Hanoi Agricultural University from 1999 to 2008 as trainer and researcher.

- Extension and technique transferring activities: participating extension programs on plant tissue culture techniques, production of virus-free potato seed, etc. for technicians in Hanoi, Ninh Binh, Thanh Hoa, Hai Duong, Phu Yen, Bac Ninh.

5. RESEARCH INTERESTS

- Ecology, molecular ecology.

- Plant cell, tissue, organ culture, molecular biology and application of them for micropropagation, crop production and genetic manipulation of plants in order to improve the disease resistance, quality improvement.

6. PUBLICATION

Scientific Articles


Book chapter


Talk Presentations

Poster Presentations

- **Dinh T.S.** UVB radiation provides durable DTG-mediated resistance to mirid (*Tupiocoris notatus*) attack in field-grown *Nicotiana attenuata* plants. 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2012.


7. **AWARDS**

- Four years scholarship (2008-2012) from Vietnam Ministry of Agricultural and Rural Development and Deutscher Akademischer Austauschdienst (DAAD) for PhD study in Germany.


- Establishing protocol for micro-propagation of pineapple and got the Award "Advanced science and technologies" in 2004 by Ministry of Agricultural and Rural Development (co-author).

*Jena, 28th January 2013*

Son Truong Dinh
List of Publications

Scientific Articles

Book chapter

Talk Presentations

Poster Presentations
- Dinh T.S. UVB radiation provides durable DTG-mediated resistance to mirid (Tupiocoris notatus) attack in field-grown Nicotiana attenuata plants. 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2012.
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Jena, ngày 28 tháng 01 năm 2013

Dinh Trương Sơn

Thank you! Dankeschön! Xin cảm ơn!