Identification of novel regulatory mechanisms involved in plant-herbivore interactions in *Nicotiana attenuata*

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Chapter 1: Introduction

The autotrophic life style of plants makes them the major producers of food on which the majority of heterotrophs depend. Consequently, plants assume a critical position in the food web and affect the life cycles of a great number of heterotrophs. Insect herbivores, the most diverse group of living organisms with diverse feeding adaptations, thrive feeding on plants. Some of these herbivores feed on one or a few related plant species (and are named as specialists) while others feed on a wider groups of plants (named as generalists). The diversity in herbivore species and their feeding specializations signify the magnitude of herbivore-associated stress on plants. In addition to herbivore attack, plants are faced with biotic challenges from the microbe world and intra- or interspecific competitions or abiotic stress including drought, salinity, water-logging, ultraviolet radiation and the like (Ali and Agrawal 2012, Bruce and Pickett 2007, Reymond *et al.* 2004). These stressful conditions considerably undermine plant fitness as is often demonstrated by the huge loss of agricultural produce (Agrawal 2011, Marquis 1984). Hence, to survive and maintain higher overall fitness, plants have to resist or respond to these stressful conditions. I would like to focus on herbivory for the pursuing discussions.

Over the millions of years of evolutionary arms race against herbivores (Labandeira *et al.* 1994), plants have evolved various mechanisms to defend themselves. One of these mechanisms is using preemptive structural fortress (e.g. thorns, trichomes, latex) that ward off herbivores and reduce or avoid attack (Federal 1988, Hanley *et al.* 2007). If herbivore attack is unavoidable, plants produce potent defense compounds that affect the attacking herbivores directly, recruit other organisms to remove the threat (as indirect response) or tolerate. These defense responses are generally grouped into two categories: some are constitutively present while others are

induced only after attack. These mechanisms are discussed in great detail elsewhere (Agrawal 1998, Baldwin 2010, Federal 1988, Halitschke *et al.* 2000, Karban 2011, Kessler and Baldwin 2001, Kessler and Baldwin 2002, Pare and Tumlinson 1999, Stowe *et al.* 2000, Wittstock and Gershenzon 2002).

The effectiveness of plant defense responses is likely context-dependent. In general, a defense strategy that slows down the growth of the attacking herbivore by using direct defense compounds while recruiting predators or parasitoids of the attackers could be reasonably effective in all contexts. In situations where herbivore attack is spatially and temporally predictable, constitutive defenses might be more effective, while plants could be better off protected by inducible defense responses when the attack is unpredictable. The optimality of the type of defense may also depend on plant growth mode (slow growing, long-lived plants versus fast growing, short-lived plants) or availability and type of resources (resource rich versus poor; carbon- versus nitrogen-rich) (Coley *et al.* 1985, Ito and Sakai 2009, Karban 2011, Kempel *et al.* 2011, Kessler and Baldwin 2002, Wittstock and Gershenzon 2002). On the other hand, from the patterns of accumulation of defense compounds in different parts of plants, it could be argued that the extent of plant defense investment is correlated to the relative value that the respective tissue adds to the overall fitness of plants. Accordingly, young leaves or reproductive tissues are predicted to be more defended than old leaves (Mckey 1974, Meldau *et al.* 2012a). Hence, it is fair to conclude that the context in which the attack is made (plant growth conditions and strategies, importance of the tissue, type of herbivore and their feeding adaptations etc.) determines the type of defense deployed by plants and shapes their ecological interactions. Whatever the circumstances may be and whichever is the hypothesis forwarded to explain the

scenario, plants have to defend themselves in a manner that maximizes their fitness. This very fact compels the evolution of mechanisms of regulating plant defense responses.

In direct response to herbivore attack, plants produce potent toxic, anti-nutritive or anti-digestive compounds: alkaloids, phenolamides, terpenoids, cyanogenic glycosides, glucosinolates, proteinase inhibitors and the like (Bennett and Wallsgrove 1994, Hopkins *et al.* 2009, Konno 2011, Mithofer and Boland 2012, Steppuhn *et al.* 2004, Stout *et al.* 1998). These compounds are demonstrated to be effective against herbivores. In some instances, the efficacy of the compounds increased multifold when they were presented to herbivores in combination, a phenomenon known as synergism (Hummelbrunner and Isman 2001, Wittstock and Gershenzon 2002). When discussing the raison d'être of secondary plant compounds in his review, Fraenkel argued that secondary plant substances shape the specificity of plant-insect interactions by playing roles as feeding inducers or deterrents or serving as protective evolutionary adaptations.

Therefore, as adaptive responses, the qualitative and quantitative similarities/differences in the accumulations of secondary metabolites among different families of plants and the respective feeding adaptations of herbivores on these plants reflect the co-evolutionary adaptive history of plants and insects (Ehrlich and Raven 1964, Fraenkel 1959). Put simply, in response to changes in the defense chemical milieu of plants, insects developed effective mechanisms of coping with the defense metabolites (physical sabotage, detoxification or even sequestration of the defense compounds and using them as nutrition or defense); and this adaptation drove the co-evolutionary arms race between plants and their herbivores. Logically, prior to the invention of novel defense compounds, the regulatory mechanisms controlling the biosynthetic pathways have to evolve through, for example, gene duplication and neo-functionalization (Agrawal 2011,

Ehrlich and Raven 1964, Johnson 2011). The current chemical fingerprint of plants, hence, is a signpost for the co-evolutionary trail plants and herbivores shared.

Despite the implicit protective benefits that these defense metabolites confer plants, their production, storage or translocation is costly because scarce resources, that could otherwise be used for important physiological processes like growth and reproduction, are channeled into the production of these metabolites (Brown 1988, Coley*, et al.* 1985, Heil and Baldwin 2002, Kempel*, et al.* 2011, Siemens *et al.* 2010, Zavala *et al.* 2004). Due to the limitations in available resources and the severe intra- and interspecific competitions for these resources, plants have be able to grow fast to outcompete competitors while getting adapted to the stressful environmental conditions. These require optimal investment of resources to growth and defense processes (Berenbaum 1995, Fox 1981, Heil and Baldwin 2002, Herms and Mattson 1992, Ito and Sakai 2009).

In *N. attenuata*, cost-benefit analyses of producing herbivory-induced nicotine or trypsin protease inhibitors were performed and demonstrated the fitness advantages of producing these metabolites in the presence of herbivore attack. The fitness consequences of producing defense metabolites were also described for different plant-herbivore models (Baldwin 1998, Baldwin 2001, Lou and Baldwin 2004, Meldau *et al.* 2012b, Redman *et al.* 2001, Strauss *et al.* 2002, van Dam and Baldwin 1998, Zavala*, et al.* 2004).

To respond to herbivore attack without too much cost, plants have developed mechanisms of regulating the type, amount and duration of defense responses. Many members of the plant transcription factor families (e.g. MYB, bHLH, Homeobox domain, NAC, WRKY, ERF) mediate complex physiological processes in plants including defense against pathogens or

herbivores (De Geyter *et al.* 2012, Endt *et al.* 2002, Onkokesung *et al.* 2012, Singh *et al.* 2002, Todd *et al.* 2010). In *N. attenuata*, silencing two herbivore-inducible WRKY TFs (WRKY 3 and WRKY 6) decreased the accumulation of JA/JA-dependent direct and indirect defenses and affected the performance of the specialist herbivore (*M. sexta*) validating the regulatory roles of the transcription factors in plant defense (Skibbe *et al.* 2008). In another experiment, the regulatory role of a member of the MYB family of TFs (NaMYB8) in the biosynthesis of herbivore-induced phenolamides was described (Kaur *et al.* 2010, Onkokesung*, et al.* 2012). In *N. tabacum* and *N. benthamiana*, nicotine biosynthesis was regulated by transcription factors of the bHLH family (De Boer *et al.* 2011, Shoji and Hashimoto 2011, Shoji *et al.* 2010, Todd*, et al.* 2010, Zhang *et al.* 2012). In *A. thaliana*, three recently identified MYC transcription factors, MYC2, MYC3 and MYC4, were implicated with regulation of various aspects of jasmonate-dependent plant defense responses against pathogens/ herbivores (Cheng *et al.* 2011, Fernandez-Calvo *et al.* 2011, Niu *et al.* 2011). More recently, two jasmonate responsive MYC TFs (MaMYC2a/b) were identified in banana fruit that regulated JA-dependent chilling tolerance (Zhao *et al.* 2013). These are just a few examples that exhibit the important roles that transcription factors, in general, and MYC2 TFs specifically, play in the regulation of development and defense responses in plants.

MYC TFs are key mediators of the jasmonate signaling and response cascade (Cheng*, et al.* 2011, De Geyter*, et al.* 2012, Dombrecht *et al.* 2007, Fernandez-Calvo*, et al.* 2011, Kazan and Manners 2012), which affect many physiological processes in plants: plant defense against herbivores, root growth and flower development among others (Avanci *et al.* 2010, Bari and Jones 2009, Durbak *et al.* 2012, Hause *et al.* 2009, Wasternack and Kombrink 2010).

The jasmonate signaling pathway is induced immediately after herbivore attack and involves the biosynthesis of a huge pool of jasmonic acid (JA). The biosynthesis of herbivore-induced JA is briefly summarized as follows: attack by herbivores results in the release of 18:3 α-linolenic acid (α-LeA) from membranes of chloroplasts by the GLA1 family of lipases. In the chloroplasts, enzymatic processing of α-LeA by lipoxygenase 3 (LOX3), allene oxide synthase (AOS) and allene oxide cyclasse (AOC) enzymes produces oxophytodienic acid (OPDA) that is transported to peroxisomes and converted to JA after three β-oxidation steps (Gfeller *et al.* 2010, Kombrink 2012, Paschold *et al.* 2008, Turner *et al.* 2002, Wasternack 2007).

The herbivore-induced production of JA is a transient process and the highest accumulation is attained few minutes after the initial attack (hence the term JA burst). Relatively, a very small fraction of the JA burst is converted to the bioactive jasmonate, (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile), by an enzyme (s) known as jasmonate resistant (JAR) (Fonseca *et al.* 2009). The receptor complex (SCF^{COI1}) perceives JA-Ile and ubiquitinates the repressors of the jasmonate response (known as jasmonate ZIM domain proteins, JAZ) (Chico *et al.* 2008, Chini *et al.* 2007, Pauwels and Goossens 2011, Thines *et al.* 2007). Presumably, ubiquitination tags the JAZ proteins for degradation by the 26 S-proteasome and releases the MYC2 TFs from repression (Chini *et al.* 2009, Fonseca*, et al.* 2009, Kombrink 2012, Sheard *et al.* 2010, Thines*, et al.* 2007). MYC2 TFs, then, mediate expression of defense genes or transcription factors (Dombrecht*, et al.* 2007, Memelink 2009, Shoji and Hashimoto 2011, Shoji*, et al.* 2010, Todd*, et al.* 2010).

Strikingly, about 2 h after the initial herbivore attack, the level of the herbivore-induced JA-/JA-Ile-burst wanes to the constitutive level (Stork *et al.* 2009). It is suggested that inactivation of JA is performed by conjugating it to amino acids (other than isoleucine), glucose,

inositol or by 12-hydroxylation. Apparently, these processes offer effective mechanisms of attenuation of the JA/JA-Ile burst because none of these compounds are active in mediating the jasmonate response (Miersch *et al.* 2008, Wasternack 2007). Recently, two cytochrome p450 enzymes, CYP94B3 and CYP94C1, were identified in *A. thaliana* and annotated to function in attenuation of the JA-Ile burst through hydroxylation and carboxylation respectively (Heitz *et al.* 2012, Kitaoka *et al.* 2011, Koo *et al.* 2011). The incomplete attenuation of the JA and JA-Ile burst in plants with silenced expression of these enzymes suggest the existence of other complementary inactivation mechanisms in plants.

The jasmonate signaling pathway is a highly conserved signal transduction mechanism found in plants. Its conservation among plant species depicts the long-term association that plants had with herbivores over the course of their evolutionary history. In spite of the overall conservation of jasmonate signaling and response among plant species (Boter *et al.* 2004, Hause*, et al.* 2009, Katsir *et al.* 2008), variations exist. In *A. thaliana*, silencing coronatine insensitive 1 (AtCOI1) impaired all jasmonate responses and resulted in female sterility (Xie *et al.* 1998). In *Lycopersicum esculentum*, silencing COI1 affected ovule and trichome development (Li *et al.* 2004). Interestingly, in *N. attenuata*, plants which were silenced in the COI1 expression (irCOI1 plants) were impaired in JA responses, and were male sterile because they had defects in anther dehiscence (Paschold *et al.* 2007). A variation of JA signaling and response was also observed in *Solanum nigrum* in which extended JA burst and partially redundant SnLOX3, SnJAR4 and SnCOI1 functions were reported (VanDoorn *et al.* 2011). These observations signify the context-dependency of jasmonate responses (Pauwels *et al.* 2009) and underlie the importance of studying regulation of the jasmonate responses across species to completely understand how plants regulate their defense responses against herbivores and how defense responses evolved.

For this work, we raised the following research questions:

- What regulatory mechanisms control the duration, type and extent of defense responses in *N. attenuata*?
- What are the ecological significances of these regulatory mechanisms?
- Are there novel mechanisms of attenuating the herbivore-induced JA-Ile burst in *N. attenuata*?
- How would these mechanisms of attenuation affect the jasmonate signaling cascade in *N. attenuata*?
- How would these mechanisms of attenuation affect direct and indirect plant defense responses in *N. attenuata*?
- How do the attenuation mechanisms affect the interaction of this species with its herbivore community?
- What are the roles of MYC2 in regulation of defense responses in *N. attenuata*?
- What is the level of conservation of the downstream jasmonate signaling in plants of different families?
- How would the fitness and ecology of plants be affected if processes of attenuation of the JA-Ile burst are disarmed?
- Are there other JA/JA-Ile-derived compounds that plants use to switch off JA/JA-Ile burst?

To address these questions, we used the model system that is recently developed by our group to explore the ecological interactions of *N. attenuata* plants with its specialist (e.g. *Manduca sexta;* Sphingidae) and generalist (e.g. *Spodoptera littoralis;* Noctuidae) herbivores (Figure 1). *N. attenuata* is a wild tobacco species native to the Great Basin Desert in Utah, USA.

Seeds of *N. attenuata* lying dormant in seed banks for hundreds of years germinate after "smelling" cues in the smoke of wild fire and flourish rapidly on the temporarily nitrogen-rich environment the fire creates (Baldwin and Morse 1994, Baldwin *et al.* 1994). However, as native plants of the region, these plants are exposed to the biotic and abiotic challenges that the great basin desert incurs on them. From the outset, the seedlings have to deal with drought, intense UV-B radiation, intra-/inter-specific competition and herbivory among others. Adult *M. sexta* moths are nectarivorous and lay their eggs on the underside leaves of their host plants, so that neonates are hatched on their food plants and start feeding right after hatching. Caterpillars of *M. sexta* are specialist herbivores of plants in Solanaceae family and feed on the areal plant parts. *N. attenuata* plants recognize attack by larvae of *M. sexta* when the wounds caused by herbivore feeding are in contact with fatty acid amino acid conjugates coming from the regurgitant of the larvae and respond against it by igniting the jasmonate signaling cascade (Bonaventure 2012, Bonaventure *et al.* 2011, Halitschke *et al.* 2001).

The defense responses of this species to biotic and abiotic stresses, in general, and the involvement of the jasmonate signaling in mediating direct or indirect defense responses, specifically, are being explored in our group using combinations of molecular, analytic and ecological approaches. The chemical defense in *N. attenuata* consists of potent toxic, anti-nutritive or anti-digestion compounds like nicotine, phenolamides, diterpene glycosides and proteinase inhibitors which are deployed against herbivores upon attack. Attacked *N. attenuata* plants also release volatile organic compounds to attract parasitoids, pathogens or egg hunters of the attacking herbivores (Allmann and Baldwin 2010, Kessler and Baldwin 2004, Kessler *et al.* 2004, Kessler *et al.* 2008). In this work, we identified and characterized important regulatory mechanisms of the jasmonate signaling and response and shown their ecological relevance.

In the first manuscript (Manuscript I), a new mechanism of attenuation of the JA-Ile burst by a multifunctional hydrolase is described. Manuscript II reviewed known transcription factor-based regulatory mechanisms of plant inducible defenses, while manuscript III reported the identification and characterization of one of the bHLH transcription factors, MYC2, in *N. attenuata*.

A

B

Figure 1. The model system used in this work dealt with **(A)** *Nicotiana attenuata* that grows in the field site in Utah, USA, and **(B)** one of the specialist herbivores, *Manduca sexta*. Photos were taken by Danny Kessler.

Chapter 2: Manuscript overview

Manuscript I

Jasmonoyl-l-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-l-isoleucine levels and attenuates plant defenses against herbivores.

Melkamu G. Woldemariam, Nawaporn Onkokesung, Ian T. Baldwin and Ivan Galis

Published: The Plant Journal (2012), 72: 758–767

In this manuscript, we identified a novel hydrolase, jasmonoyl-L-isoleucine hydrolase 1 (JIH1), that is involved in hydrolysis of the bioactive jasmonate (JA-Ile) and attenuates herbivore-induced JA-Ile burst. JIH1 is a homologue of the *Arabidopsis thalina* indole acetic acid alanine resistant 3 (IAR3) which functions in hydrolysis of amino acid conjugates of indole acetic acid and releases active IAA. We showed that JIH1 has enzymatic activity against some amino acid conjugates of JA and IAA *in vitro*, though its highest activities were against JA-Ile and IAA-Ala. *Nicotina attenuata* plants that were silenced in the expression of JIH1 (irJIH1 plants) accumulated higher levels of JA-Ile, and consequently, more defense compounds. In the field, irJIH1 attracted significantly more *Geochoris pallens*, predators of *M. sexta* egg, than wild type plants. By hydrolyzing the bioactive jasmonate, JA-Ile, JIH1 attenuates herbivore-induced JA-Ile burst and contributes to regulation of plant defense responses.

The study was conceived of by Dr. Ivan Galis and Professor Ian T. Baldwin, who participated at all stages. I designed and executed the experiments described in the manuscript, analyzed the data and wrote the manuscript. Nawaporn identified the gene and performed the initial screening. The manuscript was refined in consultation with the authors of the paper.

Manuscript II

Transcriptional regulation of plant inducible defenses against herbivores: a mini-review

Woldemariam Melkamu G. Woldemariam, Ian T. Baldwin & Ivan Galis

Published: Journal of Plant Interactions (2011), 6:2-3, 113-119

In this manuscript, we reviewed transcriptional regulation of plant defense responses taking MYC2 as a central point of discussion. We reviewed early plant responses to herbivore attack, perception of the initial signaling and transduction into downstream processes taking the jasmonate signaling as a focal point. We, then, reviewed the known roles that MYC2 transcription factors play in regulation of plant defense responses and suggested a working model for the possible regulatory function of MYC2 transcription factors.

The outline for the review was inspired by Dr. Ivan Galis and Professor Ian T. Baldwin. I wrote the first draft, which was developed further after consultation with the authors.

Manuscript III

NaMYC2 transcription factor regulates plant defense responses in *Nicotiana attenuata.*

Melkamu G. Woldemariam, Son Truong Dinh, YoungJoo Oh, Ivan Galis and Ian T. Baldwin

Accepted for publication: BMC Plant Biology

In this manuscript, we identified a MYC2 transcription factor in *N. attenuata* and characterized its role in regulation of plant defense against herbivores. We used a reverse genetic approach and silenced the expression of MYC2 using Virus Induced Gene Silencing. After verifying the efficiency of silencing, we used transcriptomic, metabolomic and ecological methods to determine its regulatory function. We found that MYC2 is involved in regulation of nicotine and phenolamide biosynthesis in addition to its role in transcriptional regulation of a number of genes.

The research idea was inspired by Dr. Ivan Galis and Professor Ian T. Baldwin, who participated at all processes. I designed the experiments, collected and analyzed the data. Son Truong Dinh and Youngjoo Oh participated in the experimental processes. I wrote the manuscript, which was refined in consultation with the authors.

Chapter 3:

Jasmonoyl-l-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-l-isoleucine levels and attenuates plant defenses against herbivores

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Jasmonoyl-L-isoleucine hydrolase 1 (JIH1) regulates jasmonoyl-L-isoleucine levels and attenuates plant defenses against herbivores

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NaJIH1 Gene Bank accession number: JQ660367.

SUMMARY

For most plant hormones, biological activity is suppressed by reversible conjugation to sugars, amino acids and other small molecules. In contrast, the conjugation of jasmonic acid (JA) to isoleucine (Ile) is known to enhance the activity of JA. Whereas hydroxylation and carboxylation of JA-Ile permanently inactivates JA-Ile-mediated signaling in plants, the alternative deactivation pathway of JA-Ile by its direct hydrolysis to JA remains unstudied. We show that *Nicotiana attenuata* jasmonoyl-L-isoleucine hydrolase 1 (JIH1), a close homologue of previously characterized *indoleacetic acid alanine resistant 3* (*IAR3*) gene in Arabidopsis, hydrolyzes both JA-Ile and IAA-Ala *in vitro*. When the herbivory-inducible *NaJIH1* gene was silenced by RNA interference, JA-Ile levels increased dramatically after simulated herbivory in irJIH1, compared with wild-type (WT) plants. When specialist (*Manduca sexta*) or generalist (*Spodoptera littoralis*) herbivores fed on irJIH1 plants they gained significantly less mass compared with those feeding on wild-type (WT) plants. The poor larval performance was strongly correlated with the higher accumulation of several JA-Ile-dependent direct defense metabolites in irJIH1 plants. In the field, irJIH1 plants attracted substantially more *Geocoris* predators to the experimentally attached *M. sexta* eggs on their leaves, compared with empty vector plants, which correlated with higher herbivory-elicited emissions of volatiles known to function as indirect defenses. We conclude that NaJIH1 encodes a new homeostatic step in JA metabolism that, together with JA and JA-Ile-hydroxylation and carboxylation of JA-Ile, rapidly attenuates the JA-Ile burst, allowing plants to tailor the expression of direct and indirect defenses against herbivore attack in nature.

Keywords: indoleacetic acid alanine resistant 3, jasmonoyl-L-isoleucine hydrolase 1, JA-Ile, IAA-Ala, jasmonate signaling, *Nicotiana attenuata*, *Manduca sexta*.

INTRODUCTION

Plants are often attacked by herbivores with a wide range of feeding modes and preferences. In response, plants evolved diverse defense mechanisms, such as constitutive mechanical barriers, thorns and trichomes (Federal, 1988), replete with chemical defenses to fend off attackers. When these barricades are breached, plants produce inducible defense metabolites that directly affect the survival of the attackers or slow their growth and/or reproduction. In addition, plants release blends of volatile organic compounds to summon predators or parasitoids of the attackers (Kessler and Baldwin, 2002; Howe and Jander, 2008; Baldwin, 2010).

Most inducible plant defenses are known to be regulated by the oxylipin signaling cascade and its immediate product, jasmonic acid (JA), which in its interactions with other phytohormones mediates the large transcriptional and metabolic reconfigurations in plants after attack from biotic aggressors (Verhage *et al.*, 2010). Herbivore attack induces a rapid release of polyunsaturated fatty acids (PUFAs; e.g. 18:3 α-linolenic acid and 18:2 α-linoleic acid) from chloroplastic membranes. α-Linolenic acid is enzymatically converted to 13*S*-hydroperoxyoctadecatrienoic acid (HPOT) by a specific lipoxygenase, which, in turn, is converted to oxophytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is transported to peroxisomes, where it is reduced by a peroxisomal OPDA reductase 3 (OPR3). Finally, JA is produced after three rounds of

β-oxidation by the enzymes acyl-CoA oxidase 1 (ACX1), multifunctional protein (MFP) and L-3-ketoacyl CoA-thiolase (KAT) (Schaller and Stintzi, 2009). A proportion of JA is conjugated with isoleucine (Ile) by the JAR enzyme(s) to produce bioactive (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile) in the cytosol (Staswick and Tiryaki, 2004; Kang *et al.*, 2006; Wang *et al.*, 2007; Fonseca *et al.*, 2009). Interestingly, JAR enzymes belong to the well-known GH3 family of acyl acid amidosynthases that conjugate amino acids to indole acetic acid (IAA) (Staswick *et al.*, 2005; Wang *et al.*, 2007; Westfall *et al.*, 2010), suggesting a parallel evolution of JA and IAA metabolic pathways.

JA-Ile associates with an F-box protein COI1 that, as part of SCF^{COI1}, forms the E3-ubiquitin ligase complex and leads to the degradation of JAZ repressors by the *26S* proteasome (Thines *et al.*, 2007). Degradation of the JAZ repressors releases MYC2 transcription factor(s) from repression and activates the transcription of genes involved in plant defense responses (Fonseca *et al.*, 2009; Memelink, 2009). The presence of bioactive JA-Ile, therefore, directly determines the duration of plant defense responses to biotic stresses that elicit JA signaling (Galis *et al.*, 2009).

After the transduction of initial signals into downstream defense responses, plants must reset their signaling cascades to sense new environmental cues. The COI1-dependent upregulation of JAZ repressors was proposed as a negative feedback loop that downregulates JA signaling (Thines *et al.*, 2007). Recently, hydroxylation of JA (Miersch *et al.*, 2008) and hydroxylation and carboxylation of JA-Ile (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Heitz *et al.*, 2012) was reported to be another efficient means of directly inactivating JA-Ile. Two cytochrome P450 enzymes, CYP94B3 (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Koo and Howe, 2012) and CYP94C1 (Heitz *et al.*, 2012), were identified and functionally annotated as JA-Ile hydroxylase and carboxylase, respectively. Although expected, reversible attenuation of JA-dependent plant defense signaling by direct cleavage of JA-Ile to JA has not been reported.

Conjugation of hormones and their hydrolysis are common means of modulating the activity of several plant hormones (Staswick, 2009). Auxins are inactivated by conjugation to amino acids, sugars and other small molecules by members of the GH3 family proteins (Bartel and Fink, 1995; Woodward and Bartel, 2005). Conversely, free IAA is released from the conjugates by enzymes belonging to the ILR1-like family of IAA amidohydrolases that, in *Arabidopsis thaliana*, comprise ILL1, ILL2, ILL3, ILL5, ILL6, ILR1 and IAR3 (Campanella *et al.*, 2003). Considering the common origin of JA- and IAA-conjugating GH3 class enzymes (Staswick *et al.*, 2005), IAA-amidohydrolases have long been considered as possible candidates for JA-Ile hydrolyzing enzymes. Previously, LeClere *et al.* (2002) expressed IAA-alanine resistant 3 (IAR3; AT1G51760) protein *in vitro* as a GST fusion protein, and demonstrated its hydrolytic activity against several IAA-amino acid conjugates. Although discussed, the activity of this protein against JA conjugates was not reported (Davies *et al.*, 1999; LeClere *et al.*, 2002). IAR3-like genes identified from *Brassica rapa* (Savic *et al.*, 2009) and *Triticum aestivum* (Campanella *et al.*, 2004) have also been shown to cleave IAA-amino acid conjugates *in vitro*.

In this study, we cloned a herbivory-regulated homologue of IAR3 from *Nicotiana attenuata* (named NaJIH1) and showed that it is an active hydrolase of JA-Ile *in vitro*. We demonstrated the contribution of JIH1 to the control of JA-Ile levels *in vivo*, and its direct role in attenuating defense responses against herbivores in nature and in the glasshouse.

RESULTS AND DISCUSSION

Nicotiana attenuata IAR3-like gene transcripts (*NaJIH1*) are strongly upregulated by herbivory

Herbivore attack is known to induce JA-signaling-mediated transcriptional reconfiguration of a large number of genes (Schmidt *et al.*, 2005; Balbi and Devoto, 2008; Woldemariam *et al.*, 2011). In previously published microarray data from *N. attenuata* with wounded leaves (WW) or with leaves treated with oral secretions from *M. sexta* (WOS) (Kim *et al.*, 2011), a transient induction of several JA biosynthetic genes was observed in local and systemic tissues (leaves and/or roots) (Figure S2). A similar induction profile of an *IAR3*-like gene (renamed to *jasmonoyl-L-isoleucine hydrolase 1*, *JIH1*; Figures S3 and S4) indicated a possible role of this gene in plant–insect interactions (Figure 1; confirmed by quantitative PCR with an independent set of samples, see Figure S1). In an earlier report, *A. thaliana IAR3* was identified as a wound-inducible, COI1-dependent gene named *JR3* (*jasmonate-responsive 3*) (Titarenko *et al.*, 1997), but it was grouped in the IAA amidohydrolase family (Bartel and Fink, 1995; Campanella *et al.*, 2003, 2004; Figure S4b) based on its initially examined *in vitro* activity against IAA conjugates (Bartel and Fink, 1995; Davies *et al.*, 1999; LeClere *et al.*, 2002; Campanella *et al.*, 2003, 2004).

Heterologously expressed *NaJIH1* hydrolyses JA-Ile and IAA-Ala

In previous reports, heterologously-expressed *A. thaliana* IAR3 protein hydrolyzed IAA-Ala and IAA-Gly with high efficiency. The detectable hydrolysis of other substrates such as IAA-Phe, IAA-Leu and IAA-Val, however, suggested a broad substrate specificity of this enzyme (Davies *et al.*, 1999). Because *NaJIH1* transcripts accumulated in response to simulated herbivory (Figure 1a,b), and *IAR3* showed wound-inducible expression in Arabidopsis (Titarenko *et al.*, 1997), we hypothesized that NaJIH1 might hydrolyze JA-Ile, the bioactive compound in JA signaling. Interestingly, *NaJIH1* showed stronger induction after wounding compared with WOS treatment in local leaves (Figures 1a and S1),

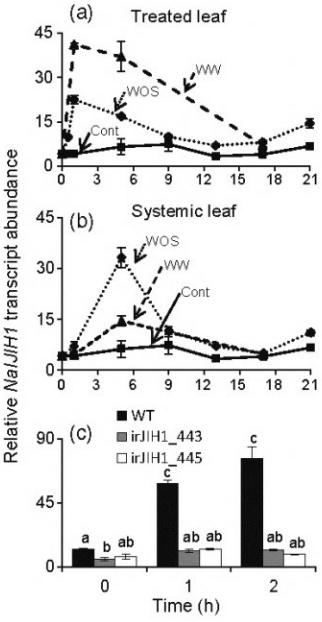

Figure 1. Transcript accumulation and silencing of the *JIH1* gene in *Nicotiana attenuata* plants.
Rosette leaves were left untreated (control), or were wounded (WW) or subjected to simulated herbivory (WOS), and mean ± SE relative transcript levels (n = 3) were determined by microarrays in (a) treated leaves and (b) systemic untreated leaves. (c) Relative mean ± SE transcript levels (n = 3) in two independent *JIH1*-silenced lines (irJIH1-443 and irJIH1-445) were determined by qPCR before, and 1 and 2 h after WOS treatment; the different letters indicate statistically significant differences among samples, determined by ANOVA ($P < 0.05$).

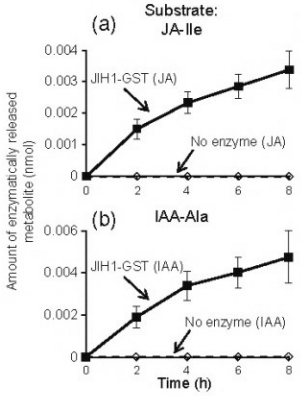

Figure 2. *In vitro* enzymatic activity of heterologously expressed JIH1.
JIH1 was expressed *in vitro*, purified and incubated for 8 h with JA-Ile (a) or IAA-Ala (b) to test its hydrolytic activity. Samples were taken from the reaction tubes every 2 h and the level (nmol) of products (JA or IAA) were measured by LC-MS^3 using known concentrations of D_2-JA and [$^{13}C_6$]IAA standards. Control reactions were run without the enzyme.

which was inversely correlated with the typical higher accumulation of JA-Ile found in WOS-induced *N. attenuata* leaves (Wu and Baldwin, 2009).

As a direct test of this hypothesis, we expressed the *N*-terminally truncated form of NaJIH1 (Figure S3) as a GST-tagged fusion protein, and examined its hydrolytic activity against JA-Ile and IAA-Ala *in vitro* (pH 7.5, 37°C and Mn^{2+}, which was determined as the optimal enzyme co-factor). Under these conditions, NaJIH1 hydrolyzed both JA-Ile and IAA-Ala and released free JA and IAA (Figure 2a and b), respectively. Even when JA-Ile and IAA-Ala were supplied as a mixture, the enzyme cleaved both substrates (Figure S5a). When tested against other available JA-amino acid conjugates, the recombinant NaJIH1 enzyme hydrolyzed JA-Val, JA-Met and JA-Glu. In particular, JA-Val cleavage was consistent with the patterns of accumulation of this metabolite in *NaJIH1*-silenced plants (Figure S5b and S6). However, the enzyme showed no detectable hydrolytic activity against IAA-Asp, an auxin conjugate recently reported to play an important role in plant–pathogen interactions (Gonzalez-Lamothe *et al.*, 2012).

irJIH1 plants accumulate more JA-Ile after simulated herbivory

To test which activity of NaJIH1 observed *in vitro* is reflected *in vivo*, we generated transgenic plants with reduced *JIH1* transcript levels using RNA interference (RNAi), and chose two homozygous (T_2), single insert-harboring lines (irJIH1-443 and irJIH1-445) for further analysis. Both irJIH1 lines had significantly reduced *JIH1* transcript levels compared with wild-type (WT) plants before WOS induction (ANOVA, $F_{2,6} = 6.66$, $P = 0.05$) and after 1 h (ANOVA, $F_{2,6} = 323.59$, $P < 0.0001$) or 2 h (ANOVA, $F_{2,6} = 65.34$, $P < 0.0001$) after WOS treatment (Figure 1c).

After WOS induction, irJIH1 plants accumulated significantly more JA-Ile compared with WT plants 1 h after WOS treatment (ANOVA, $F_{2,4} = 20.24$, $P = 0.008$; Figure 3a). The increase in JA-Ile levels in irJIH1 plants did not result from transcriptional activation of *JAR* genes in *N. attenuata* or from the increased transcript levels of *threonine deaminase* (*TD*) involved in isoleucine biosynthesis in irJIH plants (Figure S5c). Also, the higher JA-Ile accumulation in irJIH1 plants was not associated with a lower level of JA, as might be expected (Figure 3f). This comes as no surprise given that after simulated herbivory, only a small fraction of the JA burst (<15%) is converted to JA-Ile in *N. attenuata*. Hence, the contribution of the JIH1-mediated release of JA to the total JA burst may not be readily detectable against the background of a large and very dynamic pool of JA. Alternatively, a compensatory response through positive feedback of higher JA-Ile levels on JA biosynthesis could have contributed to the total pool of free JA, thus eliminating the expected differences between irJIH1 plants and WT. It

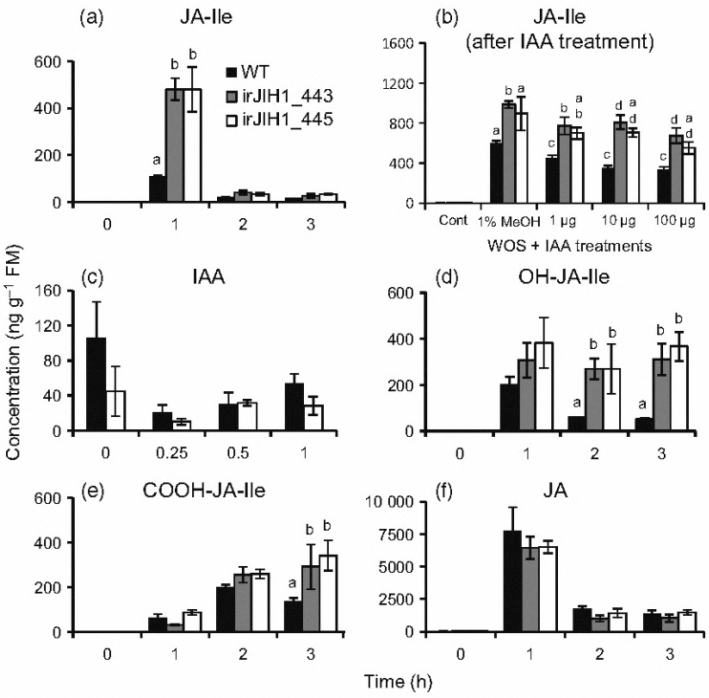

Figure 3. Accumulation of herbivory-induced jasmonates in WOS-induced wild-type (WT) and irJIH1 plants.
(a) Following WOS treatment, irJIH1 plants accumulated significantly more JA-Ile than did WT plants. (b) The exogenous application of IAA did not suppress the increased accumulation of JA-Ile in irJIH1 compared with WT plants, and (c) no significant difference was observed in the levels of endogenous IAA in irJIH1 and WT plants after WOS treatments. At later time points, irJIH1 plants accumulated significantly higher levels of OH-JA-Ile (d) and COOH-JA-Ile (e) than did WT plants; but the level of JA was not significantly different (f). Different letters indicate statistically significant differences (ANOVA; $P < 0.05$).

has been shown that Arabidopsis CYP94B3 mutant plants defective in JA-Ile hydroxylation, and therefore with elevated JA-Ile content, contained more JA (Kitaoka *et al.*, 2011). However, these differences were only observed 3 h after wounding, a relatively late time point at which JA-Ile levels tend to decline to near basal levels in wounded Arabidopsis leaves (Heitz *et al.*, 2012). Unfortunately, no data on JA accumulation have been provided in two other reports demonstrating the function of the CYP94B3 enzyme (Koo *et al.*, 2011; Heitz *et al.*, 2012).

In contrast to JA-Ile, the levels of IAA were not significantly different between WT and irJIH1 plants at early time points following WOS induction (Figure 3c; 0 h, ANOVA, $F_{1,7} = 1.37$, $P = 0.28$; 0.25 h, ANOVA, $F_{1,6} = 0.33$, $P = 0.58$; 0.5 h, ANOVA, $F_{1,6} = 0.027$, $P = 0.87$; 1 h, ANOVA, $F_{1,8} = 2.69$, $P = 0.13$), suggesting that the primary effect of NaJIH1 is on JA-Ile rather than IAA-Ala metabolism. To further test this inference, we re-examined whether the JA-Ile phenotype in irJIH1 plants could result from local changes in IAA content that might be missed in the analysis of samples of entire homogenized leaves (see Experimental procedures). We induced leaves of WT and irJIH1 plants with WOS, and sprayed 1 mL of 1, 10 or 100 µg mL^{-1} IAA to simulate the potential release of IAA by NaJIH1 during simulated herbivory. When we determined the accumulation of JA-Ile in the leaves after 1 h (i.e. when plants not treated with IAA showed significantly different JA-Ile levels; Figure 3a), JA-Ile levels always remained significantly higher in irJIH1 than in WT plants (0 h, ANOVA, $F_{2,9} = 0.670$, $P = 0.535$; WOS, 1% methanol, ANOVA, $F_{2,9} = 4.834$, $P = 0.03$; 1 µg mL^{-1}, ANOVA, $F_{2,7} = 9.104$, $P = 0.01$; 10 µg mL^{-1}, ANOVA, $F_{2,9} = 24.404$, $P = 0.0002$; 100 µg mL^{-1} IAA, ANOVA, $F_{2,9} = 8.902$, $P = 0.007$), suggesting that the observed differences in WOS-induced JA-Ile levels among WT and irJIH1 plants were independent of their IAA content (Figure 3b).

JA and JA-Ile metabolism in irJIH1 plants

The catabolism of JA-Ile into OH-JA-Ile and COOH-JA-Ile has been recently described as a mechanism to downregulate JA signaling (Kitaoka *et al.*, 2011; Koo *et al.*, 2011; Heitz *et al.*, 2012). We reasoned that, if JA-Ile hydrolysis functions as another independent switch in JA signaling, irJIH1 plants should accumulate more OH-JA-Ile and COOH-JA-Ile than WT plants, to compensate for the lack of NaJIH1-mediated hydrolysis. Indeed, 3 h after WOS, irJIH1 plants accumulated significantly more OH-JA-Ile (ANOVA, $F_{2,5} = 10.86$, $P = 0.015$) and COOH-JA-Ile (ANOVA, $F_{2,5} = 5.595$, $P = 0.05$) compared with WT plants (Figure 3d,e). As the same result was obtained in another independent field experiment with empty vector (EV) and irJIH1 plants after multiple WOS leaf treatments (Figure S7; OH-JA-Ile, ANOVA, $F_{1,12} = 9.26$, $P = 0.01$), we conclude that hydroxylation and hydrolysis of JA-Ile are two equally important means of attenuating the JA-Ile burst in plants, a conclusion consistent with the

higher accumulation of JA-Ile in CYP94B3 mutant plants deficient in JA-Ile hydroxylation (Kitaoka *et al.*, 2011; Koo *et al.*, 2011). The increase in OH-JA-Ile levels in irJIH1 plants was, most likely, not regulated at the transcriptional level because the transcripts of a tentative *CYP94B3* gene homologue from *N. attenuata* were not differentially regulated in WT and irJIH1 plants (Figure S5c).

Silencing of *NaJIH1* suppresses the performance of herbivores

To investigate whether the silencing of *NaJIH1* and the higher levels of JA-Ile in these plants directly affected the performance of feeding herbivores, *M. sexta* (a specialist) and *Spodoptera littoralis* (a generalist) caterpillars were fed on WT and irJIH1 plants, and the mass gain of herbivores was determined. Compared with caterpillars fed on WT plants, both the specialist (ANOVA, $F_{2,52} = 9.47$, $P = 0.0003$) and the generalist (ANOVA, $F_{2,54} = 8.154$, $P = 0.0008$) herbivores gained significantly less mass when reared on irJIH1 plants (Figure 4). Previously, *M. sexta* caterpillars performed better on *N. attenuata* plants deficient in the production of JA-Ile (irJAR4/6), whereas exogenous supplementation of JA-Ile reduced the performance of these caterpillars (Kang *et al.*, 2006; Wang *et al.*, 2008), consistent with the irJIH1 phenotype.

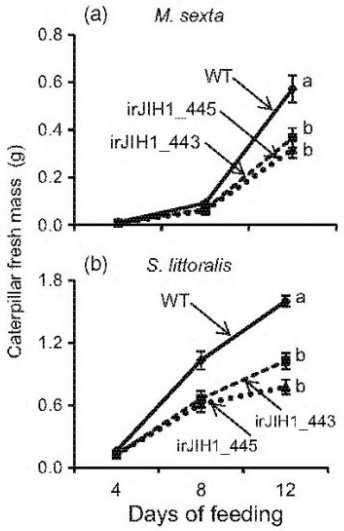

Figure 4. Performance of caterpillars on wild-type (WT) and irJIH1 plants. Caterpillars of the specialist (*Manduca sexta*) and generalist (*Spodoptera littoralis*) herbivores were allowed to feed on fully elongated leaves of WT and irJIH1 plants for 12 days, and their masses were recorded every fourth day ($n = 19$). Caterpillars of both *M. sexta* (a) and *S. littoralis* (b) gained significantly less mass (ANOVA; $P < 0.001$) when fed on irJIH1 plants than on WT *Nicotiana attenuata* plants. Different letters indicate significant statistical differences.

JIH1 attenuates multiple herbivore-induced direct defenses

Plants accumulate a number of JA-mediated secondary metabolites that directly suppress the performance of caterpillars (Kessler and Baldwin, 2004; Wang *et al.*, 2007; Heiling *et al.*, 2010). To investigate the role of NaJIH1 in the regulation of secondary metabolism, we compared the accumulation of several herbivory-induced defense metabolites in WT and irJIH1 plants grown in the glasshouse, and found that 24 h after WOS induction irJIH1 plants accumulated significantly higher levels of nicotine (ANOVA, $F_{2,6} = 9.31$, $P = 0.01$), 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs; ANOVA, $F_{2,6} = 8.91$, $P = 0.01$) and protease inhibitors (PIs; ANOVA, $F_{2,11} = 4.02$, $P = 0.04$) than WT plants (Figure 5). When we examined the WOS-induced accumulation of individual HGL-DTGs in irJIH1 plants, we found that they accumulated significantly more mono- and di-malonylated HGL-DTGs than did wild-type plants (Figure 5d). Because protease inhibitors (Zavala *et al.*, 2004) and HGL-DTGs (Heiling *et al.*, 2010) play very important roles in the defense of *N. attenuata* plants against herbivores, the over-accumulation of these metabolites in irJIH1 plants is sufficient to explain the reduced performance of caterpillars on irJIH1 plants.

Performance of irJIH1 plants in nature

Taking advantage of irJIH1 plants and their limited ability to regulate active jasmonate levels after WOS treatment, we decided to examine the ecological importance of this regulation in nature and planted EV and irJIH1 plants in a field plot at the field station in Santa Clara (UT, USA) in 2011. After establishment, the plants were WOS-induced to determine the robustness of the NaJIH1-silencing phenotype in nature. Whereas EV plants accumulated progressively more JA-Ile and its catabolites after repeated simulated herbivory (see Experimental procedures), irJIH1 contained significantly more JA-Ile 4 h after the first induction and following the third WOS treatment (ANOVA; $F_{1,12} = 6.01$, $P = 0.03$) (Figure S7), showing that JIH1-mediated hydrolysis of JA-Ile, similar to glasshouse conditions, was required to attenuate the JA-Ile burst in nature.

We also determined the accumulation of defense metabolites in the field-grown EV and irJIH1 plants after WOS treatment. As expected, irJIH1 plants contained significantly more HGL-DTGs (ANOVA, $F_{1,8} = 9.81$, $P = 0.01$) and protease inhibitors (PIs; ANOVA, $F_{1,6} = 6.48$, $P = 0.04$) than did EV plants. When we examined the accumulation of individual HGL-DTGs, irJIH1 plants accumulated significantly more mono- and di-malonylated HGL-DTGs than EV plants (Figure S8).

NaJIH1 attenuates indirect defenses in *Nicotiana attenuata*

When attacked by herbivores, plants release a blend of volatile organic compounds (VOCs) to attract predators, parasitoids or pathogens of the attacking herbivores (Pare

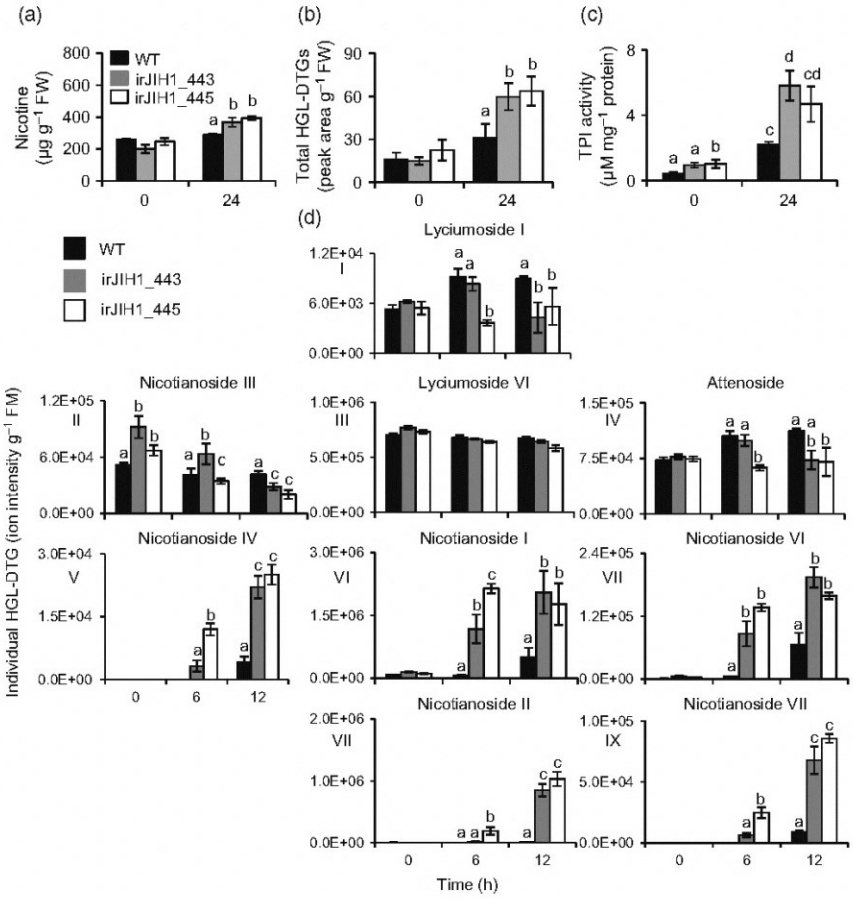

Figure 5. Accumulation of WOS-induced secondary metabolites in glasshouse-grown WT and irJIH1 plants.
Fully elongated leaves of glasshouse-grown WT and irJIH1 plants were WOS-induced and analyzed for the accumulation of defense secondary metabolites (n = 3). irJIH1 plants accumulated significantly higher levels of nicotine (a), total HGL-DTGs (b) and TPIs (c) than did WT plants (ANOVA; $P < 0.05$). (d) The analysis of individual HGL-DTGs by LC-MS^2 (d) revealed that irJIH1 plants had more mono-malonylated (V, VI and VII) and dimalonylated (VIII and IX) HGL-DTGs than did WT plants (ANOVA, $P < 0.01$). Statistically significant differences are indicated by different letters.

and Tumlinson, 1999; Baldwin, 2010). In *N. attenuata*, the predator *Geocoris pallens* is attracted by the volatile cues released by attacked plants, and feeds on *M. sexta* eggs and larvae (Kessler and Baldwin, 2004). To evaluate whether *JIH1* contributed to the regulation of indirect defense responses in *N. attenuata* in a natural ecological setting, we attached *M. sexta* eggs to the underside of leaves of control or WOS-treated EV and irJIH1 plants in the field, and quantified the eggs predated upon by *G. pallens*. On control EV and irJIH1 plants, 15.7 and 17.6% of the eggs were predated upon, respectively. However, 24 h after WOS treatment, the percentage predation on EV and irJIH1 plants increased to 31.6 and 52.9%, respectively (Figure 6a). Following simulated herbivory, irJIH1 plants experienced a higher rate of egg predation than EV plants. Previously, *N. attenuata* plants silenced in the activity of WRKY3/6 transcription factors were less attractive to predators compared with EV plants in the field. Notably, irWRKY plants are impaired in their responses to repeated elicitations, and show lower accumulations of JA and JA-Ile, and consequently less TPIs and HGL-DTGs, and lower emissions of volatile compounds from WOS-induced leaves in the field (Skibbe *et al.*, 2008).

To evaluate if differential egg predation was associated with altered levels of herbivory-elicited emission of VOCs in irJIH1 plants, we analyzed the WOS-induced emission of VOCs from glasshouse-grown plants (Kessler and Baldwin, 2004; Gaquerel *et al.*, 2009). Consistent with observed predation data in the field, irJIH1 plants emitted significantly more trans-α-bergamontene (ANOVA, $F_{1,15} = 8.66$, $P = 0.01$), caryophyllene (ANOVA, $F_{1,22} = 7.60$, $P = 0.01$), α-duprezianene

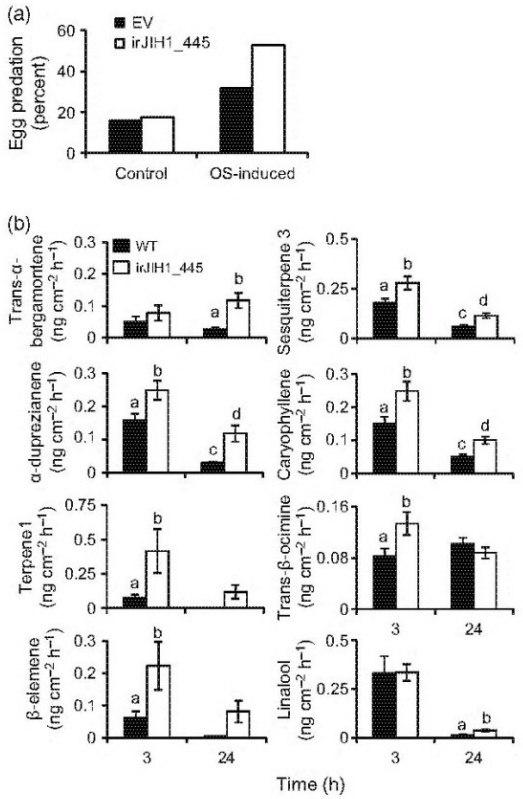

Figure 6. Egg predation and herbivore-induced emission of volatile organic compounds.
(a) One *Manduca sexta* egg was attached per plant on the underside of the leaves of EV and irJIH1 field-grown plants (n = 19), and the percentage of eggs predated by *Geocoris pallens* was measured on control and WOS-induced plants. After WOS induction, eggs on irJIH1 plants experienced a higher rate of predation than did WT plants. (b) WT and irJIH1 plants grown in the glasshouse (Jena Bioscience, http://www.jenabioscience.com) were induced by WOS and the emitted volatiles were trapped for 3 or 24 h after WOS and analyzed by GC-MS (n = 12). Statistically significant differences (ANOVA; $P < 0.05$) are indicated by different letters.

(ANOVA, $F_{1,15} = 8.66$, $P = 0.01$), *trans*-β-ocimine (ANOVA, $F_{1,22} = 5.75$, $P = 0.02$), β-elemene (ANOVA, $F_{1,22} = 4.41$, $P = 0.04$) and linalool (ANOVA, $F_{1,15} = 16.1$, $P = 0.01$) than did WT plants (Figure 6b), consistent with a role of NaJIH1 (and JA-Ile) in the regulation of herbivory-induced VOC emissions.

Conclusions and perspectives

Focusing on the attack from a single herbivore, and not listening to other warning signals from the environment, could be very harmful for plants in their natural environments. The ability of plants to reset JA signaling (Figure 7) after an initial attack is likely to be important in helping plants tailor their defense responses when repeatedly attacked, or attacked by other aggressors that also elicit JA signaling. In addition, excessive production of defense metabolites is known to be costly for plants (Brown, 1988; Baldwin, 1998), and hence the tight control over defense responses optimizes the fitness of competing plants (Ito and Sakai, 2009). It will be interesting to compare how irJIH1 plants perform in direct competition with other plants in order to address additional roles of rapid JA-Ile catabolism in plants under natural stress conditions.

EXPERIMENTAL PROCEDURES

Plant growth and treatments

The seed germination, growth conditions and *Agrobacterium*-mediated transformation were previously described in Krügel *et al.* (2002). Selected *NaJIH1* cDNA fragment (277 bp; Figure S3) was used to generate transgenic plants with suppressed *NaJIH1* expression by RNAi using pSOL8 transformation vector (Bubner *et al.*, 2006). To simulate herbivory, fully expanded (+1) leaves were wounded with a serrated fabric pattern wheel and 20 μL of either de-ionized water (WW) or diluted *M. sexta* oral secretions (WOS; diluted in water 1:5, v/v) were applied to the wounds (control samples remained untreated). For herbivore bioassays, wild-type and irJIH1 plants were maintained in the glasshouse until rosette stage, when freshly hatched neonates of the specialist herbivore (*M. sexta*) were placed on fully elongated (+1) leaves. Neonates of the generalist herbivore *S. littoralis* were first fed for 6 days on an artificial diet before they were transferred to plants. Caterpillars were allowed to continuously feed for 12 days, whereas their mass was determined every 4 days.

Field experiments

Field experiments were conducted at the Lytle Ranch Preserve research station (Santa Clara, UT, USA). Seeds were imported (APHIS number, 10-004-105m) and released (APHIS number, 06-242-3r-a3) into the field station following Animal and Plant Health Inspection Service (APHIS) regulations. Germination and growth conditions in the field were as described previously (Kessler *et al.*, 2008). irJIH1 and EV plants were planted in randomized pairs and grown until the early elongation stage in the field plot. To determine jasmonate accumulation after multiple elicitations, EV and irJIH1 plants were WOS-treated every hour for 3 h (by making one row of puncture wounds on both sides of the lamina every hour, and applying either water or OS). Samples were collected at the fourth hour and stored on dry ice. To estimate predation rates in the field, one *M. sexta* egg per plant was glued with a non-toxic glue to the underside of the leaves of 19 pairs of EV and irJIH1 plants, and the percentage of predated eggs by *G. pallens* (Heteroptera, Geocoridae) was determined visually after 24 h. The adjacent leaves were either left un-induced or induced by WOS to stimulate the release of volatiles.

Transcript abundances

Total RNA was extracted from deep-frozen leaf material using TRIzol reagent (Invitrogen, http://www.invitrogen.com). After treatment of total RNA with DNase (RQ1 RNase-Free DNase; Promega, http://www.promega.com), cDNA was synthesized using oligo $(dT)_{18}$ and Superscript II reverse transcriptase (Invitrogen). All qRT-PCR experiments were performed on Mx3005P Multiplex qPCR (Stratagene, now Agilent Technologies, http://www.genomics.agilent.com) with qPCR core kit for SYBR Green I (Eurogentec, http://www.eurogentec.com). Transcript abundances were normalized

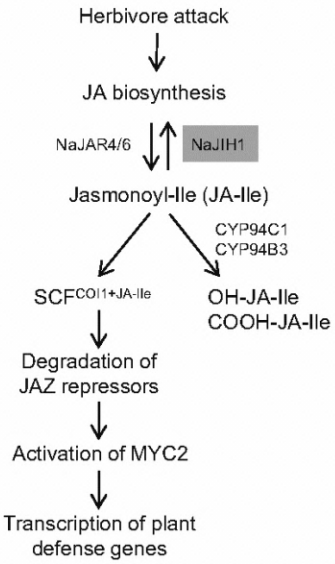

Figure 7. A proposed model for JA-mediated defense responses in *Nicotiana attenuata* plants.
To reset the JA-signaling cascade after herbivore attack and/or wounding, plants use two equally important pathways of inactivating the bioactive signaling compound, JA-Ile: (i) hydroxylation/carboxylation of JA-Ile by the cytochrome p450 enzymes; and (ii) hydrolysis by the newly identified JIH1 enzyme.

using *N. attenuata* elongation factor-1α (EF-1α) as the internal reference. The primer pairs used in qPCRs are listed in Table S1.

Phytohormone analyses

For phytohormone analyses, leaves of rosette WT and irJIH1 plants were WOS induced, collected and flash frozen in liquid nitrogen (or dry ice in the field). About 200 mg powder prepared in liquid nitrogen was homogenized in 1 mL ethyl acetate spiked with 200 ng mL^{-1} of D_2-JA and 40 ng mL^{-1} D_6-ABA, D_4-SA and JA-[$^{13}C_6$]Ile internal standards. The homogenate was centrifuged for 20 min (16 100 ***g***, 4°C) and the supernatants were transferred into new tubes. The pellets were re-extracted with 0.5 mL ethyl acetate, centrifuged as above, and combined supernatants were dried in a vacuum concentrator (Eppendorf, http://www.eppendorf.com). After dissolving the residue in 0.5 mL 70% methanol in water (v/v) with centrifugation for 10 min (16 100 ***g***, 4°C), cleared supernatants (10 μL) were analyzed on Varian 1200L Triple-Quadrupole-LC-MS (Varian, http://www.varian.com) using a ProntoSIL® column (C18; 5 μm, 50 × 2 mm; Bischoff, http://www.bischoff-chrom.com) attached to a pre-column (C18; 4 × 2 mm; Phenomenex, http://www.phenomenex.com). The mobile phase, consisting of solvent A (0.05% formic acid in water) and solvent B (methanol), was used in a gradient mode with times/concentrations (min/%B) of 0:00/5, 1:02/5, 2:30/5, 5:30/98, 10:30/98, 11:30/5, 15:00/5, and with a flow, time/flow (min/mL), of 0:00/0.4, 1:02/0.2, 2:30/0.2, 5:30/0.2, 10:30/0.4, 11:30/0.4 and 15:00/0.4. The MS was operated in negative ionization mode using multiple reactions monitoring (MRM). The molecular ions, $[M–H]^-$, generated for JA, JA-Ile, OH-JA-Ile, COOH-JA-Ile, D_2-JA and JA-$^{13}C_6$-Ile were, respectively, at *m/z* 209.0, 322.0, 338.0, 352.0, 213.0 and 328.0, and were fragmented under CE 12.0 V (for JA and D_2-JA) or 19.0 V (for JA-Ile, OH-JA-Ile, COOH-JA-Ile and JA-$^{13}C_6$-Ile), generating daughter ions at *m/z* 59.0, 130.0, 130.0, 130.0, 59.0 and 136.0, respectively. The ratios of the ion intensities of endogenous compounds and internal standards were used for quantification after normalizing them with the fresh mass of the samples. Synthesis of the JA-amino acid conjugates used was as described in Wang *et al.* (2007), and the same instrumental parameters were used to estimate the content of these metabolites in plants. The molecular ions were detected in a negative mode at *m/z* 308.0, 340.0 and 337.0 for JA-Val, JA-Met and JA-Glu, respectively, fragmented at CE 19.0 V, yielding the respective daughter ions at *m/z* 116.0, 148.0 and 145.0. D_2-JA was used for relative normalization of the contents.

Secondary metabolite analysis

For secondary metabolite analyses (nicotine, total 17-hydroxygeranyllinalool diterpene glycosides, i.e. HGL-DTGs, individual HGL-DTGs and caffeoylputrescine), samples were flash-frozen in liquid nitrogen (or dry ice in the field) and 100 mg of ground powder was extracted and analyzed by HPLC equipped with a photodiode array detector (nicotine, total HGL-DTGs, caffeoylputrescine), as described in Onkokesung *et al.* (2012), or LC-MS^3 (individual HGL-DTGs), as described in Heiling *et al.* (2010). The proteinase inhibitor activity in plant extracts was quantified by radial diffusion assay as described previously (Jongsma *et al.*, 1993) after measuring the protein concentrations by standard Bradford assay.

Herbivore-induced volatile organic compound emissions

To compare herbivore-induced VOC emissions, fully expanded (+1) leaves of elongated, glasshouse-grown WT and irJIH1 plants were treated with WOS. Induced leaves were immediately clip-caged to trap the volatiles for 3 h, when the volatile traps were replaced and VOCs were collected for an additional 24 h. The trapping and analysis of VOCs were performed as described in Wu *et al.* (2008).

Cloning, heterologous expression and purification of *NaJIH1* protein

The truncated *NaJIH1* gene lacking its first 23 *N*-terminal amino acids was PCR amplified and cloned between *Bam*HI/*Not*I sites of pGEX-4T-3 expression vector (GST Gene Fusion system; GE Healthcare, http://www.gehealthcare.com) using specific adaptor primers (Table S1). A sequence-verified expression clone of pGEX-JIH1 vector was transformed into BL-21 (DE3) pLysS strains of *Escherichia coli* (Novagen, now EMD Millipore, http://www.emdmillipore.com), and the production of the JIH1-GST fusion protein was induced in LB media containing ampicillin (100 μg mL^{-1}) at 28°C by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Carl Roth, http://www.carlroth.com) to cells at an OD of 0.6–0.8. Protein accumulation was carried out for 24 h at 28°C, cultures were centrifuged at 922 ***g*** for 10 min and pellets were stored at −80°C until protein purification using GST SpinTrap™ columns (GE Healthcare), following the manufacturer's protocols.

Enzyme activity assays

Previously, Arabidopsis IAR3 protein hydrolyzed IAA-Ala in TRIS buffer (pH 7.5 and 8.0) in the presence of Mn^{2+} co-factor (LeClere *et al.*, 2002). Similar conditions were adopted to test the activity of recombinant NaJIH1-GST against JA-Ile and IAA-Ala in 100-μL enzyme reactions: 50 mM TRIS buffer, 1 mM dithiothreitol, 1 mM $MnCl_2$, 40 μL of purified enzyme and 2 μM IAA-Ala or 2 μM JA-Ile (or both substrates). Control reactions were set without enzyme to

monitor non-enzymatic hydrolysis. To test the optimum temperature and co-factor preference, reactions were incubated at 25 or 37°C in the presence of selected bivalent co-factors (Mn^{2+}, Mg^{2+}, Ca^{2+} and Co^{2+}). To terminate reactions, 100 μL of stop solution (99 : 1; v/v, methanol : acetic acid) spiked with equimolar concentrations of D_2-JA or phenyl-$^{13}C_6$-indole-3-acetic acid as internal standards was added to each 100-μL reaction. After brief vortexing and centrifugation (3 min, 13 000 ***g***, 4°C), 10 μL of clear supernatant was injected onto a ProntoSIL® column (C18; 5 μm, 50 × 2 mm; Bischoff) attached to a pre-column (C18; 4 × 2 mm, Phenomenex) on Varian 1200L Triple-Quadrupol-MS (Varian). The MS was run in negative MRM mode with the same gradient used for phytohormone analysis. The molecular ions, $[M\text{-}H]^-$, for JA, D_2-JA, IAA, [$^{13}C_6$]IAA, JA-Ile and IAA-Ala were at *m/z* 209.0, 213.0, 174.0, 179.0, 322.0 and 245.0, respectively. The molecular ions were fragmented at CE 10.0, 9.5, 12.0, 12.0, 15.0 and 19.0 V, yielding respective daughter ions at *m/z* 59.0, 59.0, 130.0, 136.0, 130.0 and 88.0. The intensities of the daughter ions were normalized against that of labeled internal standards.

Extraction and quantification of auxin

Control and WOS-treated WT and irJIH1 leaves were finely ground in liquid nitrogen and 1 g of the powder was extracted overnight in 10 mL of 100% methanol containing 2.5 mM diethyldithiocarbamic acid (Sigma-Aldrich, http://www.sigmaaldrich.com) and 50 ng phenyl-[$^{13}C_6$]IAA (Cambridge Isotope Laboratories, http://www.isotope.com). Extracts were centrifuged for 30 min (3000 ***g***, 4°C), and supernatants were transferred to new tubes. Pellets were re-extracted as before for 30 min in pure methanol, centrifuged and supernatants were combined. The concentration of extracts was adjusted to 50% methanol (v/v) by adding distilled water, and samples were purified by sequentially passing through Supelco Supelclean LC-18 SPE columns (Sigma-Aldrich) and were then adsorbed to activated DEAE Sephadex A25 columns (GE Healthcare) that were pre-equilibrated with 50% methanol. After adsorption of the IAA, columns were rinsed with 50 mL of 50% (v/v) methanol and IAA was eluted by 6% (v/v) formic acid into new Supelco Supelclean LC-18 SPE columns coupled underneath. After briefly drying the columns with a syringe filled with air, IAA was eluted from the column with 5 mL of diethylether (the water phase retained in the samples was immediately removed). The organic phase was evaporated under a stream of nitrogen, and the residue was dissolved in 1.5 mL of 100% methanol. Samples were dried under vacuum, dissolved in 70% methanol, centrifuged for 30 min (16 000 ***g***, 4°C) and measured on Varian 1200 L Triple-Quadrupol-MS (Varian) by injecting 10 μL of the supernatant onto a Prodigy column [3 μm, ODS(3), 100 Å, 150 × 2 mm; Phenomenex] attached to a pre-column (C18, 4 × 2 mm; Phenomenex). The mobile phase consisted of solvent A (0.05% acetic acid) and solvent B (acetonitrile), used in a gradient mode with time/concentration (min/% B) compositions of 0:00/20, 1:30/20, 6:00/97, 17:00/97, 18:00/20 and 25:00/20, with a flow, time/flow (min/mL), of 0:00/0.2, 1:30/0.2, 6:00/0.2, 17:00/0.3, 18:00/0.3 and 25:00/0.2. The MS was run in negative MRM mode and the molecular ions for IAA and phenyl-[$^{13}C_6$]IAA detected, respectively, at *m/z* 174.0 and 180.0. The daughter ions were generated at collision energy (CE) 10.0 V and detected at *m/z* 130.0 and 136.0, respectively. The ratios of the signal intensities of the daughter ions were normalized by the fresh mass used for extraction to quantify the WOS-induced auxin content.

Exogenous IAA treatments

To test the effect of exogenous IAA application on WOS-induced JA-Ile accumulation, fully expanded leaves of elongated *N. attenuata* plants were treated by WOS and immediately sprayed with 1 mL of 1 μg mL^{-1}, 10 μg mL^{-1} and 100 μg mL^{-1} IAA (Sigma-Aldrich). Samples were collected 1 h after treatment and the levels of JA-Ile were analyzed as before.

Statistical analysis

All statistical analyses were performed using STATVIEW 5.0 (SAS Institute, http://www.sas.com). We used an alpha level of 0.05 for all statistical tests.

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SUPPORTING INFORMATION

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

Figure S1. WW or WOS-induced transcript abundance of *NaJIH1* in treated leaves and roots of WT *Nicotiana attenuata* plants.

Figure S2. Expression of JA biosynthetic genes in WW or WOS-induced leaves of WT *Nicotiana attenuata* plants, as determined by cDNA microarrays.

Figure S3. Nucleotide and amino acid sequences of the *Nicotiana attenuata* JIH1.

Figure S4. Phylogeny of JIH1 and ILR-like hydrolases.

Figure S5. Enzymatic activity of JIH1 against other substrates.

Figure S6. WOS-induced *in vivo* accumulation of other JA-amino acid conjugates in WT and irJIH1 plants.

Figure S7. Accumulation of jasmonates in empty vector and irJIH1 plants after multiple elicitations.

Figure S8. Accumulation of herbivory-induced defense secondary metabolites in field-grown empty vector and irJIH1 plants.

Figure S9. Accumulation of other jasmonates after exogenous IAA application.

Figure S10. Volatile organic compounds not differentially regulated in irJIH1 plants.

Table S1. List of the primers used in this study.

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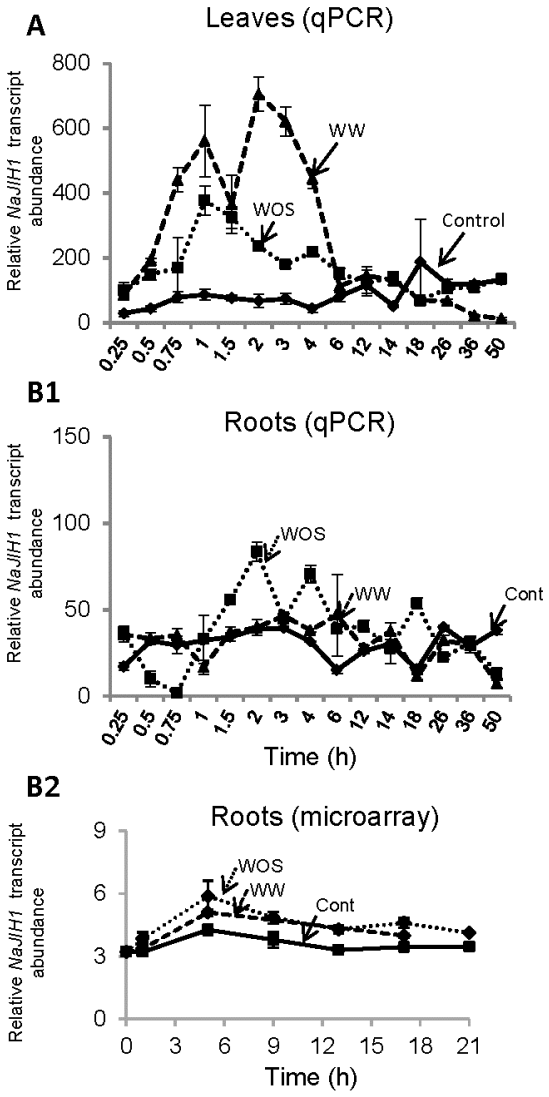

Figure S1. WW or WOS-induced transcript abundance of *NaJIH1* in treated leaves and roots of WT *N. attenuata* plants.

Five fully elongated leaves of WT *N. attenuata* plants were elicited by wounding (WW) or wounding plus *M. sexta* OS application (WOS) and pooled before RNA extraction and cDNA synthesis. Mean ± SE transcript abundances of *NaJIH1* (technical replicates *n=3*) were determined by qPCR for treated leaves (A) and roots (B1) or by microarrays for roots (B2; biological replicates *n=3*).

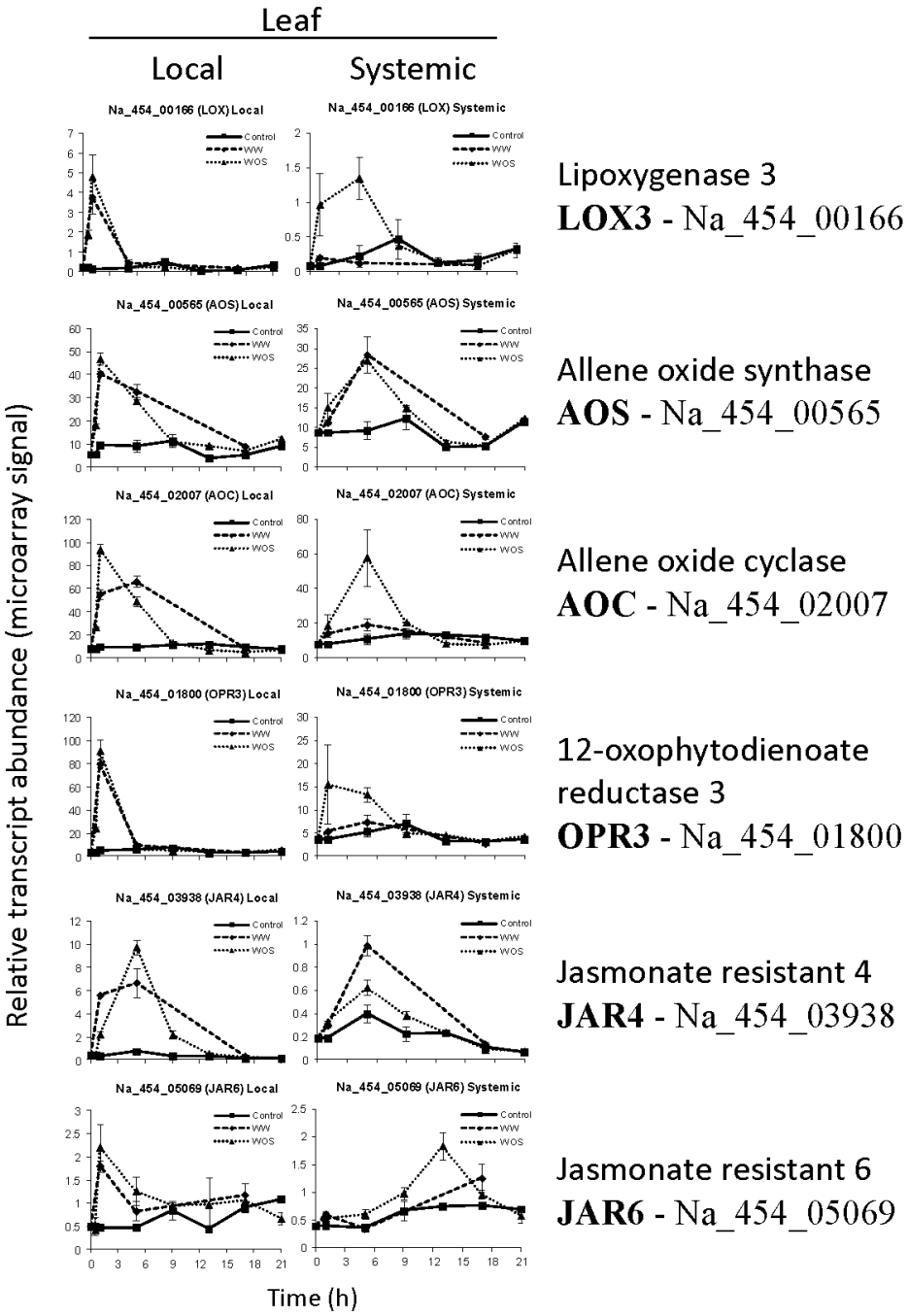

Figure S2. Expression of JA biosynthetic genes in WW or WOS-induced leaves of WT *N. attenuata* plants as determined by cDNA microarrays.

After WW or WOS treatments of fully elongated leaves of WT *N. attenuata* plants ($n = 3$), RNA extraction and cDNA synthesis were performed with pooled samples and the relative transcript accumulation (mean ± SE) of JA biosynthetic genes were determined by microarray in treated and systemic leaves.

A

>NaJIH1_full length

ATGGATTTCTCCAGATGGGTTTTCTTGATTTTGATTTTTGTTTCATTTTCTGCCATAC
CCATTTGGTCAGACTCTTCATTATCAGAAATTCCTATTAATTTCCTCAATTTTGCAAAGA
AAGCTGAGGTTTTTGATTGGATTGTGGGGGTTAGGAGAAGGATACATGAGAATCCTGAG
CTGGGATATGAAGAATTTGAGACCAGTAAGATTATAAGGGAAGAATTGGATAAATTGGG
GATTTCATACAAATACCCTTTTGCTACTACTGGTATTGTTGGTTTTGTTGGTTCAGGAAAA
TCCCCTTTTGTTGCAATCAGAGCTGATATGGATGCTCTCCCTATGCAGGAAATGGTGGAC
TGGGAGCACAAAAGTAAAAATGCTGGAAAAATGCATGCATGTGGACATGATGCTCATGT
TGCAATGCTTCTCGGTGCTGCAAAGATTCTTCAAGAACATCGAGACATTTTGAAGGGAAC
GGTTGCTCTTGTTTTTCAACCAGCAGAGGAGGGAGGTGGTGGGGCCAAGAAAATGATAG
ATGCTGGAGCACTAGAAAACATAGAATCAATATTTGGTCTGCATGTCAATCCCCAGTTTC
CTTTGGGTAAAGTTTCTTCAAGGCCTGGACCTTTTTTGGCTGGAAGTGGTTTTTTTGAAGC
TGTAATTAGTGGAAAAGGAGGGCATGCCGCTATTCCACAACATTCGATAGACCCAATTCT
GGCAGCATCAAATGTAATTGTCAGCTTACAACATCTTGTTTCCCGAGAGGCTGATCCTCT
GGATTCGCAGGTAGTCACAGTTGCTAAATTCCAAGGAGGTGGTGCATTTAACGTTATTCC
AGACTCTGTTACCATCGGTGGCACTTTTCGGGCCTTTTCAAAGGAGAGCTTTCAGCAGCT
TAGGCAGCGAATTGAGGAGGTTATTGTTGGGCAAGCTGCTGTACAGAG**ATGCAATGCAA**
CTGTGGATTTTCTTACAAAAGAGAAACCCTTCTTCCCTCCAACCGTGAACGATAAAA
ACTTGCACAAACACTTCCAGAGAGTTGCAGGTGATATGCTTGGTAACGATCATGTAA
AAGACATGGAACCGCTAATGGGATCAGAGGATTTTGCGTTTTACCAAGAGGTTATTC
CTGGTTACTTCTACCTACTCGGTATGCAGGATGAAACTAATGAAAAACTTGTTTCAG
TACATTCACCTTATTTTAAAATCAACGAAGAAGCACTTCCTATCGGTGCTGCACTTCAA
GCATCTTTGGCTATCAGATATCTTCTCGAAGCACAATCACAAGTTCCTTCGTCAAGTATA
AGTGATCATCACGATGAATTGT**TAA**

B

>NaJIH1

MDFSRWVFLILIFVSFSAIPIWSDSSLSEIPINFLNFAKKAEVFDWIVGVRRRIHENPELGYEEF
ETSKIIREELDKLGISYKYPFATTGIVGFVGSGKSPFVAIRADMDALPMQEMVDWEHKSKNA
GKMHACGHDAHVAMLLGAAKILQEHRDILKGTVALVFQPAEEGGGGAKKMIDAGALENIE
SIFGLHVNPQFPLGKVSSRPGPFLAGSGFFEAVISGKGGHAAIPQHSIDPILAASNVIVSLQHLV
SREADPLDSQVVTVAKFQGGGAFNVIPDSVTIGGTFRAFSKESFQQLRQRIEEVIVGQAAVQR
CNATVDFLTKEKPFFPPTVNDKNLHKHFQRVAGDMLGNDHVKDMEPLMGSEDFAFYQEVIP
GYFYLLGMQDETNEKLVSVHSPYFKINEEALPIGAALQASLAIR
YLLEAQSQVPSSSISDH**HDEL***

Figure S3. Nucleotide and amino acid sequences of the *Nicotiana attenuata* JIH1.

(A) Sequence-verified, full-length coding sequence of the *NaJIH1* gene (stop codon is highlighted in green bold). The predicted signal peptide sequence that was removed from *in vitro*–expressed protein is indicated in red bold; 277 bp-long underlined sequence was introduced as an inverted repeat into an RNAi silencing construct. (B) Amino acid sequence of the full-length NaJIH1 protein; consensus tetrapeptide sequence (HDEL) known to function as an ER localization and retention signal is located at the C-terminus of the NaJIH1 the sequence (underlined). Predicted signal peptide at the N-terminus of the protein is highlighted in red bold.

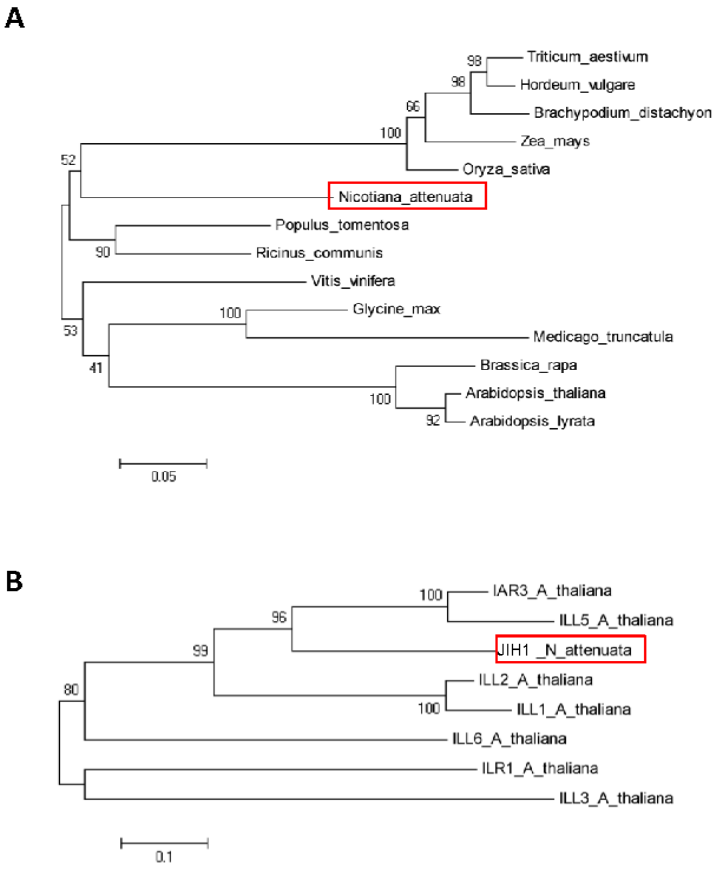

Figure S4. Phylogeny of JIH1 and ILR-like hydrolases.

(A) IAR3 sequences from several plant species were BLAST-retrieved from NCBI using the tBLASTX program. After aligning the sequences using the CLUSTAL W package, Maximum Likelihood trees were re-constructed and tested by bootstraping in MEGA5. (B) NaJIH1 was compared with other members of the *A. thaliana* ILR1-like IAA amidohydrolases (sequence retrieval, alignment and phylogeny reconstruction was performed as described in A).

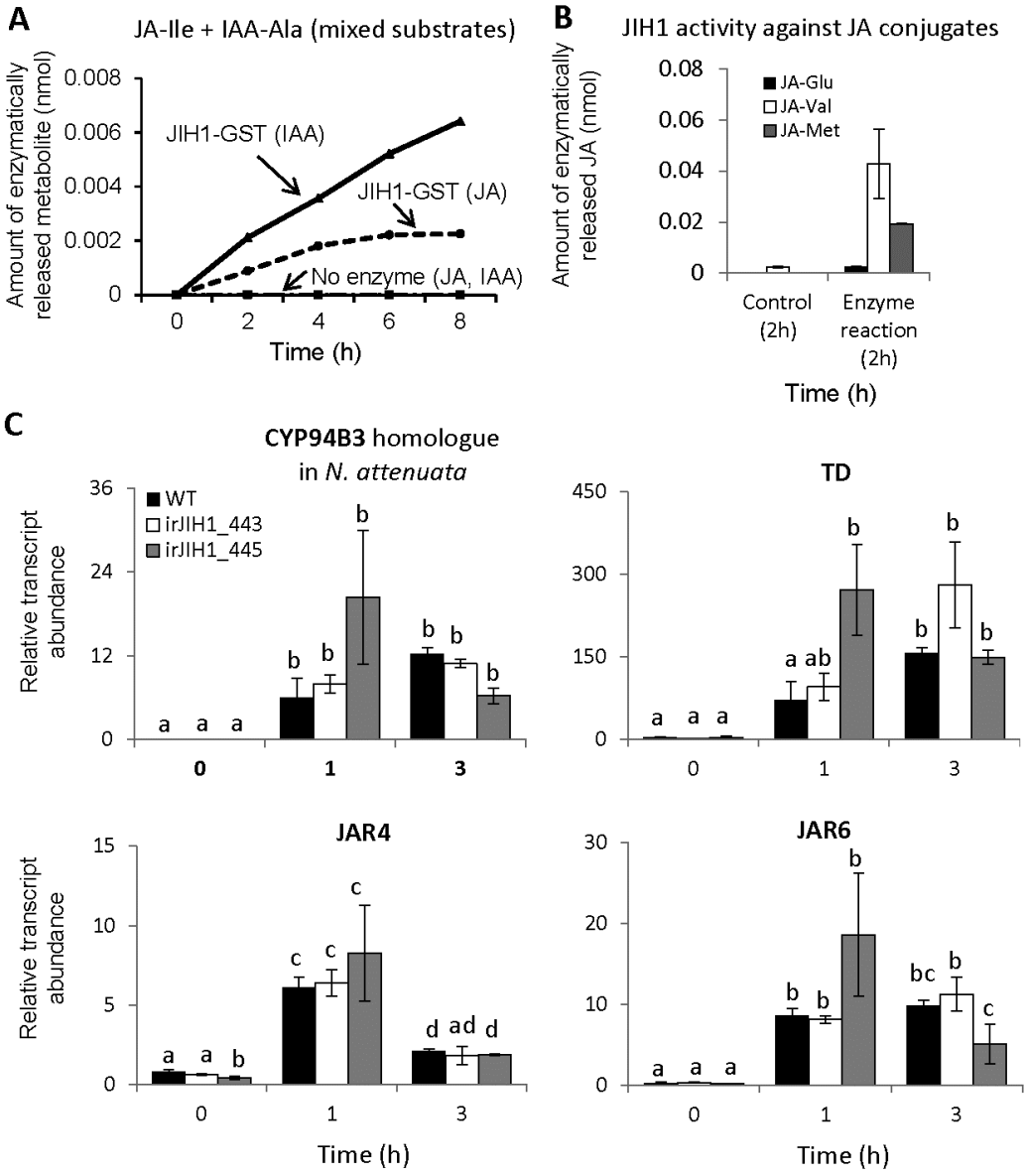

Figure S5. Enzymatic activity of JIH1 against other substrates.

The activity of JIH1 was tested against (A) equimolar mixture of JA-Ile and IAA-Ala (B) JA-Glu, JA-Met and JA-Val using Mn^{2+} as a co-factor. In controls, no significant amounts of products were detected while JA and IAA were released from the respective conjugates in the presence of recombinant *Na*JIH1-GST protein. Quantification of the released JA/IAA was performed using known amounts of D_2-JA/$^{13}C_6$-IAA standards. (C) Relative transcript abundance of the putative *N. attenuata* homologue of the *A. thaliana CYP94B3* gene, and threonine deaminase (TD), jasmonic acid resistant 4 (JAR4) and JAR6 from *N. attenuata* in WOS-induced leaves of WT and irJIH1-silenced plants. Statistical differences are described with different letters (ANOVA; $P < 0.05$).

e5

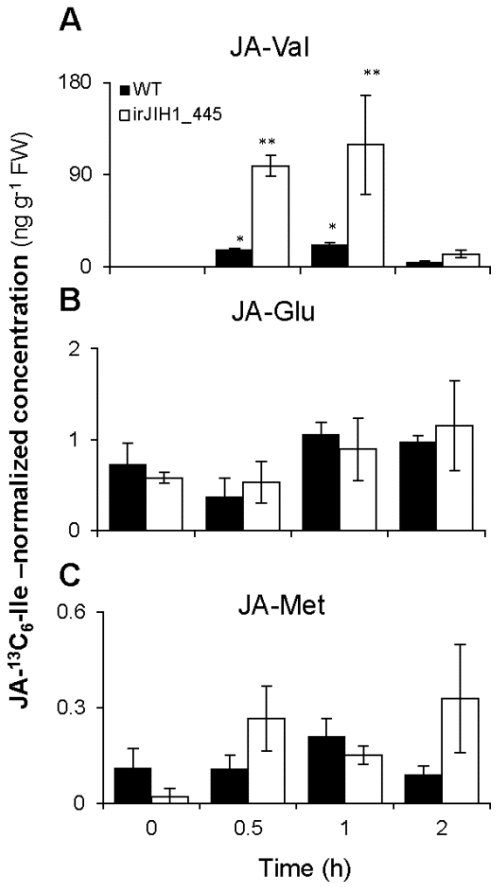

Figure S6. WOS-induced *in vivo* accumulation of other JA-amino acid conjugates in WT and irJIH1 plants.

Fully elongated leaves of WT and irJIH1 plants (*n =3*) were WOS-induced and the levels of JA-Val (A), JA-Glu (B) and JA-Met (C) were measured by LC-MS^3. irJIH1 plants accumulated significantly higher amounts of JA-Val than did WT plants (A), while the levels of other conjugates (B and C) were not significantly different (ANOVA, $P < 0.05$). The relative amounts of other JA conjugates were normalized using the $^{13}C_6$-labeled JA-Ile internal standard.

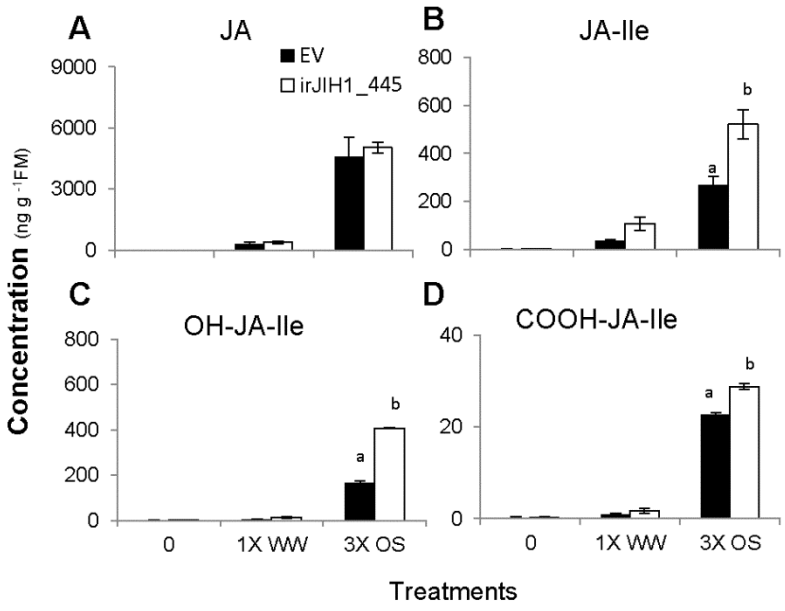

Figure S7. Accumulation of jasmonates in EV and irJIH1 plants after multiple elicitations.

EV and irJIH1 plants were grown in the field (Santa Clara, Utah, USA) and fully elongated leaves were elicited either once by WW (1X WW) or three times by WOS (3X OS) every h. Samples ($n = 7$) were collected and jasmonates were analyzed by LC-MS^3. Following multiple elicitation (3X WOS), irJIH1 plants showed significantly higher accumulations of JA-Ile (B) and OH-JA-Ile (C) than did WT plants (ANOVA; $P < 0.05$).

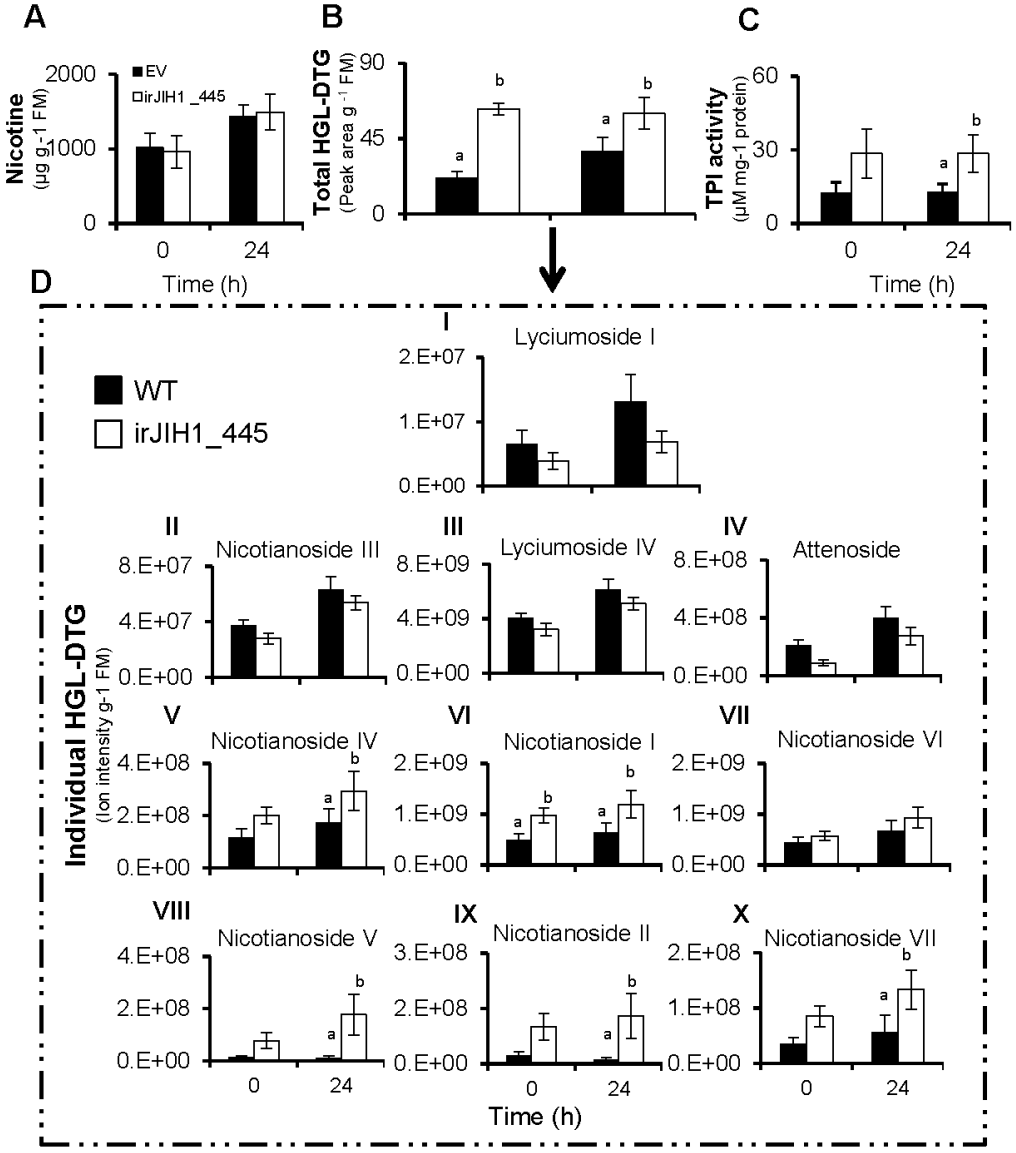

Figure S8. Accumulation of herbivory-induced defense secondary metabolites in field-grown EV and irJIH1 plants.

Field-grown (UT, USA) EV and irJIH1 ($n = 6$) plants were treated by WOS. Samples were collected after 24 h to analyze the accumulation of defense secondary metabolites. irJIH1 plants accumulated significantly higher levels of (ANOVA, $P < 0.01$) HGL-DTGs (B) and TPI (C) than did EV plants. When individual HGL-DTGs were analyzed by LC-MS^3 (D), irJIH1 plants were found to have more mono-malonylated (V and VI) and dimalonylated (VII, IX and X) HGL-DTGs than did EV plants (ANOVA, $P < 0.01$). Significant differences are indicated by different letters. 8

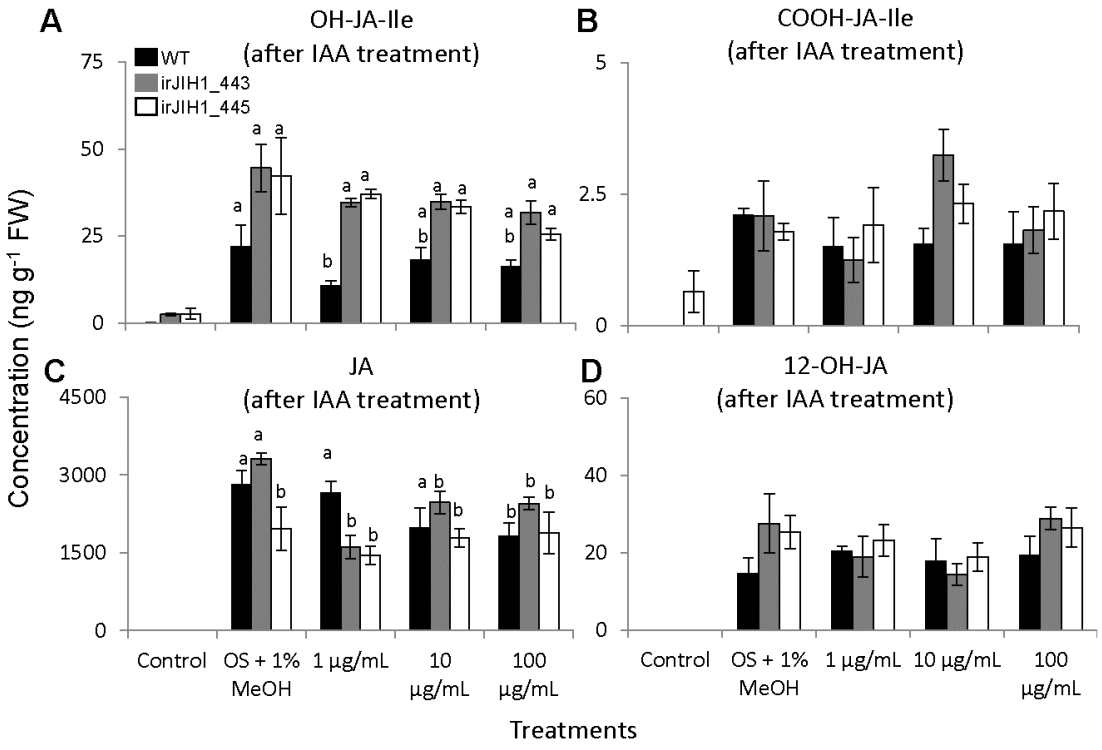

Figure S9. Accumulation of other jasmonates after exogenous IAA application.

WT and irJIH1 plants (*n* = *5*) were wounded by a pattern wheel and treated with OS that was supplemented with varying concentrations of IAA. After 1 h, samples were collected and the effect of exogenous IAA application on the endogenous accumulation of JA-Ile (in Figure 3) and other jasmonates in WOS-induced leaves was examined: (A) OH-JA-Ile, (B) COOH-JA-Ile, (C) JA and (D) 12-OH-JA.

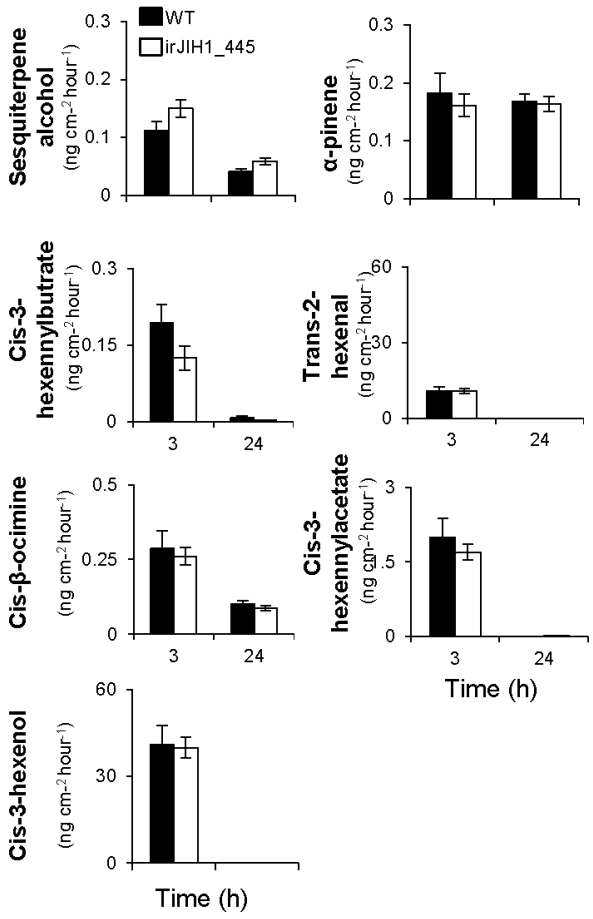

Figure S10. Volatile organic compounds not differentially regulated in irJIH1 plants.

WT and irJIH1 plants (*n = 12*) were induced by WOS treatments and the volatile organic compounds emitted in the first 3 h, and in the following 24 h after elicitation were analyzed by GC-MS. Volatile compounds listed in this figure were not significantly different in irJIH1 plants compared to WT plants (ANOVA; $P < 0.05$); volatiles that were released at significantly different amounts are shown in Figure 6.

Table S1. List of primers.

Primer	Primer sequence
JIH1 FWD (qPCR)	5’-GAGAAACCCTTCTTCCCTCCA-3’
JIH1 RVS (qPCR)	5’-TCATCCTGCATACCGAGTAGG-3’
pGEX-JIH1-noSP_FWD_BamHI	5'-CTCACTCACTGGATCCGACTCTTCATTATCAGAAATTCC-3‘
pGEX-JIH1-UNIV-RVS_NotI	5'-ATATATATATGCGGCCGCTTACAATTCATCGTGATGATC-3'
NaCYP94B3_FWD	5’-CTGGAGGTTTGTTCCTAAGGATCCA-3’
NaCYP94B3_RVS	5’-AATCCTCACCAAGAAACCACCCTTC-3’
NaTD_FWD1	5’-TAAGCCATTTGATGGGAGGC-3’
NaTD_RVS1	5’-TCTCCCTGTCCACGATAATGGAA-3’
NaJAR4_FWD1	5’-ATGCCAGTCGGTCTAACTGAA-3’
NaJAR4_RVS1	5’-TGCCATTGTGGAATCCTTTTAT-3’
NaJAR6_FWD1	5’-AGAATTTGCTTGCTCAATGCCCA-3’
NaJAR6_RVS1	5’-TGGAGTAAACGTTAACCCGAAA-3’

Chapter 4:

Transcriptional regulation of plant inducible defenses against herbivores: a mini-review

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REVIEW ARTICLE

Transcriptional regulation of plant inducible defenses against herbivores: a mini-review

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Inducible plant defenses against herbivores are controlled by a transient burst of jasmonic acid (JA) and its conversion to the active hormone (3R,7S)-jasmonoyl-L-isoleucine (JA-Ile). JA-Ile shows high affinity for binding to the COI1 protein complex with JAZ repressor protein(s), a multi component JA-Ile receptor, promoting hormone-dependent ubiquitination and degradation of JAZ transcriptional repressors. Degradation of JAZ proteins in *Arabidopsis* leads to the release of a bHLH transcription factor, MYC2, which functions as a master regulator of JA-dependent defense responses. Because the activity of the MYC2 coincides with the presence of active jasmonate in cells, it is unlikely that MYC2, alone, regulates prolonged transcriptional responses of genes encoding enzymes required for the accumulation of defense metabolites. In this review, we focus on MYC2 and a specific group of MYC2-regulated 'secondary' transcription factors as critical components of the JA signal transduction pathway that controls inducible chemical defense responses in plants.

Keywords: bHLH; defense; herbivores; jasmonic acid (JA); MYC2; transcription factor

Introduction

In a world of complex ecological and trophic interactions, the sedentary lifestyle of plants exposes them to a complex array of biotic and abiotic selection pressures. Survival under these stressful conditions requires prompt and adequate responses that can circumvent the pressures without compromising the overall fitness of plants (Heil and Baldwin 2002). Herbivore attack is one of the most severe biotic stresses that plants have endured in their long evolutionary history. Plants evolved various defense strategies against herbivores including constitutive defensive structures such as thorns, trichomes, and tough cuticles, reviewed in Hanley et al. (2007). In contrast to mechanical defenses, direct chemical defenses can be either constitutive or inducible expressed (Kessler and Baldwin 2002), as exemplified by the production and accumulation patterns of anti-digestive – or toxic secondary metabolites. In addition, attacked plants release a blend of volatile organic compounds to attract natural predators or parasitoids of the attacking herbivores as a form of indirect defense (Baldwin 2010). Production and deployment of chemical defense is often costly for plants (Mckey 1974; Heil and Baldwin 2002) utilizing resources that could otherwise be used for growth or reproduction. Hence, the activation of plant defenses must be fine-tuned and titrated with the need for protection.

Phytohormone-mediated responses to stress

Herbivores of different feeding guilds attack plants and activate a complex web of defense signaling pathways (Voelckel and Baldwin 2004; Bodenhausen and Reymond 2007). Jasmonic acid (JA), ethylene (ET), salicylic acid (SA), and abscisic acid (ABA), with their synergistic and/or antagonistic cross-talks, mediate the majority of plant defenses against biotic and abiotic stress (Bari and Jones 2009). JA has been repeatedly shown to be the most important mediator of plant-herbivore interactions (Katsir et al. 2008; Koo and Howe 2009; Rasmann and Agrawal 2009). For example, *Nicotiana attenuata* plants deficient in production of JA (*ir-lox3*) or JA perception (*ir-coi1*) have been shown to be highly vulnerable to the natural herbivore community when transplanted in their natural habitat in Great Basin desert, USA (Halitschke and Baldwin 2003; Paschold et al. 2007). While the central role of JA in plant defense against herbivores is well established, the next important task in understanding plant-herbivore interactions is to uncover how the attack signals are recognized by plants, and how these signals are translated into downstream defense responses.

Early plant responses to herbivore attack

Herbivore attack is usually associated with wounding of plant tissues and direct contact of cells with oral secretions of the herbivores (Howe and Jander 2008). Plant-specific green leaf volatiles (GLVs) are immediately released from the wounded tissues (Turlings et al. 1998). These 'attack-associated' cues are perceived by plants as markers of either imminent or apparent attack (Hilker and Meiners 2010). The exact mechanisms of how the herbivore-associated

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molecular patterns (HAMPSs) are perceived is not yet clear; however, some of the active components in oral and oviposition secretions have already been identified and characterized (Bonaventure and Baldwin 2010).

Perception of herbivory is followed by a rapid Ca^{2+}-influx, membrane depolarization, generation of reactive oxygen and nitrogen species, and activation of mitogen associated protein kinases (MAPKs). In *N. attenuata*, activation of MAPK cascade results in rapid phosphorylation of two critical wound and SA induced protein kinases, WIPK, and SIPK, respectively (Wu and Baldwin 2009). SIPK is proposed to regulate the activity of chloroplastic GLA1 phospholipase (Kallenbach et al. 2010) which releases polyunsaturated fatty acids (PUFAs) from the plastidial membranes. The PUFAs (e.g. 18:3 linolenic acid) are then channeled into the synthesis of JA (for review on JA biosynthesis see Wasternack 2007). In many plant species, JA/JA-Ile burst start immediately after herbivore attack, with the accumulation attaining maximum levels in a few hours. For example, in *N. attenuata* maximum JA accumulations are attained in leaves one hour after simulated herbivore attack (Stork et al. 2009). Similar trends were reported in plants belonging to *Solanaceae*, *Brassicaceae*, *Poaceae*, and *Salicaceae* families (Pauwels et al. 2009; Wasternack and Kombrink 2010). The increase in both JA and JA-Ile is, however, transient as JA and JA-Ile levels in *N. attenuata* decrease to the pre-induced level few hours after elicitation (Halitschke et al. 2001; Stork et al. 2009). This presents an interesting scenario in which plants respond to initial attack and quickly reset their perception and signal transduction systems, preparing themselves for the perception of the next elicitation event. This may apply particularly to cases of discontinuous feeding by herbivores, allowing plants to monitor activity, and possibly, the size or numbers of attacking herbivores.

Rapid metabolism of JA

Owing to a transient nature of the JA burst, JA is rapidly metabolized to various forms, some of which are considered to be activated, transport, storage, or inactive forms of the hormone (Wasternack 2007). JA methylation at carbon 1 by JA carboxyl methyl transferase (JMT) forms a volatile methyl jasmonate (MeJA); JA hydroxylation at carbon 11 or 12 (by yet unknown enzyme) forms 11- or 12-hydroxy JA, which can be further sulfonated by hydroxyjasmonate sulfotransferases into a probable storage forms (Swiatek et al. 2004; Miersch et al. 2008). JA can also be enzymatically conjugated into sugars (forming JA-glucose), ACC (forming JA-ACC) or amino acids. Members of the JAR family of enzymes are responsible for biosynthesis of the JA-amino acid-conjugates, including (3R,7S)-jasmonoyl-L-isoleucine (JA-Ile), which is the major biologically active molecule perceived by the COI1-JAZ receptor complex (Wasternack 2007; Fonseca et al. 2009; Avanci et al. 2010; Wasternack and Kombrink 2010).

JA perception machinery

The translation of the initial JA and JA-Ile increase into downstream defense responses requires specific perception and maintenance mechanisms. The process starts when JA-Ile binds to the coronatine insensitive 1 (COI1) protein that associates with the JAZ repressor protein(s) to form an active receptor for JA-Ile. COI1 is an F-box protein that associates with the SKP-Cullin-F-box (SCF) complex, also known as E3 ubiquitin ligase (Figure 1). In the presence of JA-Ile, E3 ligase specifically recognizes JAZ repressors via COI1-JAZ interaction and mediates ubiquitination and consequent degradation of the JAZ repressors by the 26S proteasome. Degradation of the JAZ repressors releases their target MYC2 transcription factor (TF), and possibly other TFs, which in turn activates defense-related transcriptional reprogramming. Therefore, MYC2 protein plays a key role in translating the JA burst to downstream defense responses. It is believed that in the pre-induced state, JAZ proteins partially or completely repress MYC2 transcriptional activity (Figure 1). Interestingly, JA (possibly via MYC2) activates transcription of multiple JAZ repressors (Thines et al. 2007; Chico et al. 2008), and therefore triggers the attenuation of its own signaling at very early levels of signal transduction. Recently, substantial information on the perception of JA signal, the function of JAZ repressors and the structure of JA-Ile receptor complex has been obtained and thoroughly reviewed (Chini et al. 2007; Wasternack 2007; Chico et al. 2008; Chini et al. 2009; Memelink 2009; Avanci et al. 2010; Pauwels et al. 2010).

MYC2 plays a central role in transcriptional reprogramming of plant cells

MYC2 (also known as JAI1, JIN1, RD22BP1, RAP-1, or ZBF1) belongs to the basic Helix-Loop-Helix (bHLH) superfamily of TFs. TFs of this superfamily are known to regulate cellular processes ranging from cell proliferation and differentiation, neurogenesis, and heart development in animals to controlling light signaling, production of flavonoids, and other secondary metabolites in plants. bHLH TFs are characterized by two conserved motifs (1) the basic region, a stretch of about 15 basic and largely hydrophobic amino acids, that is used for DNA binding and (2) the HLH domain composed of two conserved alpha helices separated by a less conserved loop. The HLH domain is required for hetero- and / or homodimer protein formation (Heim et al. 2003; Toledo-Ortiz et al. 2003; Carretero-Paulet et al. 2010).

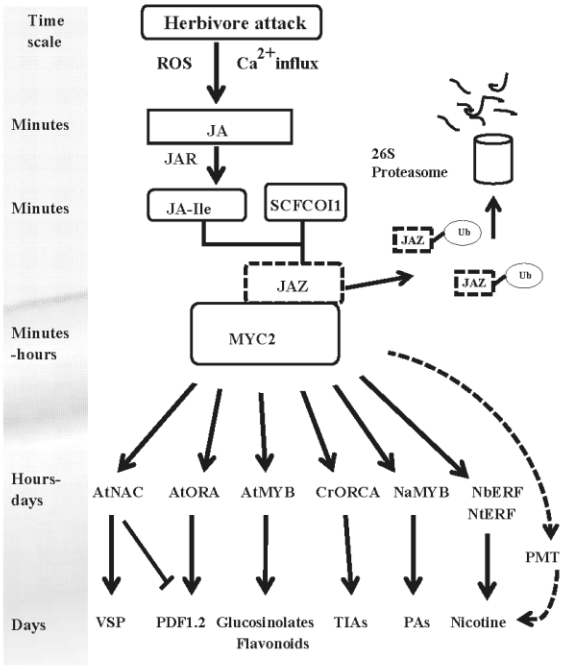

Figure 1. Proposed model of herbivore-induced JA-signaling cascade. Herbivore attack induces JA and JA-Ile bursts. JA-Ile accumulation mediates the SCF^{COI1}-JAZ interaction resulting in degradation of the JAZ repressors and the release of the MYC2 TF. MYC2 is proposed to regulate the transcription of defense-related genes directly (dotted lines) or indirectly through secondary TFs (solid lines) that mediate transcription of defense-related genes and accumulation of defense metabolites. VSP, vegetative storage protein; PDF1.2, plant defensin gene 1.2; TIAs, terpenoid indole alkaloids; PAs, phenolamides; PMT, putrescine N-methyltransferase.

Most TFs in bHLH superfamily have a DNA binding affinity. The MYC2 protein is shown to bind the G-box consensus hexanucleotide sequence, 5'-CACGTG-3', or its variants. The G-box consensus sequence or other G-Box-like sequences are found in many defense-related genes and transcription factors like the promoters of potato proteinase inhibitor 2 (PIN2) (Kim et al. 1992), Arabidopsis VSP1 (Guerineau et al. 2003), tobacco putrescine-N-methyltransferase (PMT1a) (Xu and Timko 2004), and the octadecanoid-derivative responsive AP2 domain genes (Endt et al. 2007). As expected from their general defense-related function, MYC2-like TFs are found in highly conserved forms in many plant species (Heim et al. 2003; Toledo-Ortiz et al. 2003; Li et al. 2006; Dombrecht et al. 2007; Carretero-Paulet et al. 2010; Todd et al. 2010). Moreover, wounding, MeJA or herbivory have been reported to induce transient expression of MYC2 genes in *Arabidopsis thaliana* (Lorenzo et al. 2004), *Catharanthus roseus* (Chatel et al. 2003), *Medicago truncatula* (Naoumkina et al. 2008), *Nicotiana benthamiana* (Todd et al. 2010) and *Nicotiana tabacum* (Shoji et al. 2008). Interestingly, three MYC2-like bHLH TFs have been reported in *N. benthamiana* that either positively (NbbHLH1 and NbbHLH2) or negatively (NbbHLH3) regulate nicotine biosynthesis (Todd et al. 2010).

How can plants shift from transient to prolonged defense responses?

Inferring from the mechanisms involved in jasmonate biosynthesis, degradation and perception, the wound- or herbivore-mediated activation of MYC2 protein should, more or less, coincide with the JA and JA-Ile bursts, starting few minutes after attack, reaching the peak level in about an hour and returning to the pre-induced levels in few hours. However, the accumulation of many, if not all, transcripts required for biosynthesis of defense-related secondary metabolites controlled by MYC2 lasts for many hours to days. Considering the fact that plant defense responses to herbivory last for long time periods, and in comparison to the transient nature of the MYC2 expression, it is tempting to speculate that MYC2 regulates certain defense-related secondary transcription factors which, in turn, regulate the defense metabolite

accumulation (Figure 1; Dombrecht et al. 2007). The following examples serve to elaborate this point further.

Identification of MYC2-dependent suite of secondary transcription factors

In *Arabidopsis thaliana*, AtMYC2 positively regulates flavonoid biosynthesis and alleviates oxidative stress. It also enhances herbivore resistance against the generalist herbivore, *Helicoverpa armigera*. Transcripts of many defense genes (for example, PDF1.2, CHI/PR3, HEL/PR4, TAT, and VSP) as well as several TFs (for example MYB34, MYB75, MYB51, WRKY33, ERF1, ERF4) were reported to have been differentially regulated (up- or down-regulated) in *Atmyc2*-silenced plants, suggesting that MYC2 may use other TFs as secondary messengers to regulate target genes. Consistent with this argument and as mentioned above, the MYC2 binding site (the G-Box or one of its variants) is found on promoters of these transcription factors and genes (Dombrecht et al. 2007).

Gigolashvili et al. (2007) reported the involvement of members of the subgroup 12 R2R3-MYB type TFs in the synthesis of indolic and aliphatic glucosinolates. Amongst them, MYB29, MYB34, MYB51 are wound- or MeJA-inducible TFs that are controlled by AtMYC2. These MYB TFs may be examples of secondary transcription factors in the JA signaling cascade.

A regulatory association between MYB transcription factors and defense metabolites was also shown in non-Arabidopsis plant models. For example, Galis et al. (2006) identified NtMYBJS1 as a MeJA-inducible MYB transcription factor by using large-scale microarray analysis of MeJA-elicited tobacco BY2 cells. NtMYBJS1 regulates the phenylpropanoid-polyamine conjugates (phenolamides, PAs) biosynthesis in tobacco cells. Its over-expression results in selective activation of genes involved in the phenylpropanoid biosynthetic pathway (PAL A, PAL B, 4CL, and C3H) without affecting the expression of genes involved in the synthesis of nicotine or other metabolites. Silencing (antisense) of the NtMYBJS1 resulted in reduced transcript accumulation of the PAL A, PAL B, 4CL1, and 4CL2. A functional homologue of NtMYBJS1 (named NaMYB8) was cloned in *N. attenuata* plants (Kaur et al. 2010). The authors showed that the transcripts of NaMYB8 are transiently induced by mechanical wounding. Interestingly, NaMYB8 transcript accumulation was further amplified by application of oral secretion from the specialist herbivore, *Manduca sexta,* to the wounds. Efficient silencing of NaMYB8 by RNA interference in *N. attenuata* made plants unable to accumulate the PAs, caffeoylputrescine and dicaffeoylspermidine, and rendered the transgenic plants more susceptible for herbivore attack (Kaur et al. 2010). Because the expression of NaMYB8 depends on NaMYC2 in *N. attenuata* (unpublished data Ivan Galis, Melkamu G. Woldemariam, and Ian T. Baldwin), NaMYB8 can be positioned between MYC2 and defense genes as a typical secondary TF in the JA signaling cascade.

In *N. benthamiana*, silencing (by VIGS) of two AtMYC2 homologues, NbbHLH1, and NbbHLH2, resulted in reduction of both the constitutive and MeJA-induced nicotine accumulation in leaves. VIGS silencing of ethylene response factor 1 (NbERF1) and a homeobox domain like TF (NbHB1) also reduced MeJA-induced nicotine levels. Interestingly, PMT promoters in tobacco contain both the G-Box (MYC2 binding site) and the GCC box (AP2/ERF binding site) (Todd et al. 2010). It remains therefore unclear whether these bHLHs exert their regulatory effects at the level of transcription factors or genes, or both. Considering the MYC2 function in Arabidopsis, we propose that bHLH1 and bHLH2, either separately or in concert, regulate the activity of ERF TFs which, in turn, regulate the promoters through their GCC motif.

The involvement of the MeJA-inducible group IX tobacco ERFs (closely related to ORCA2 and ORCA3 of *C. roseus*) in the regulation of nicotine biosynthetic genes was recently demonstrated in low nicotine-containing *nic1|nic2* mutant plants of *N. tabacum* (Shoji et al. 2010). This report emphasizes the importance of ERF family TFs in regulation of alkaloid biosynthesis and accumulation in tobacco. Two other members of the AP2/ERF domain transcription family, NtORC1 and NtJAP, were also shown to positively regulate the activity of the PMT promoters in tobacco (Goossens et al. 2003). Memelink (2009) proposed an MeJA inducible homologue of AtMYC2 (named NtMYC2 in tobacco) to regulate the activities of NtORC1 and NtJAP1. This brief summary is consistent with the view that alkaloid biosynthesis and accumulation in tobacco is under complex regulation which involves MYC2-like genes as one of the major regulatory nodes. Terpenoid indole alkaloids (TIAs) occur as defense metabolites against pathogens and UV exposure in *C. roseus* plants. The octadecanoid-responsive *C. roseus* AP2/ERF TFs, ORCA2, and ORCA3, are JA-inducible TFs that bind to the GCC-like box on jasmonate and elicitor responsive elements (JERE) in the promoter of the strictosidine synthase (STR) gene. STR is a key biosynthetic gene that is directly controlled by ORCA TFs (van der Fits and Memelink 2000, 2001). It is possible to propose that the JA-inducible ORCA TFs could be regulated by the master MYC2 TFs in regulating TIA biosynthetic genes. In *A. thaliana*, ORA59 (octadecanoid-responsive Arabidopsis AP2/ERF 59), a member of the Arabidopsis AP2/ERF family, is rapidly induced by ET and MeJA, contributing to the activation of many JA- and ET-inducible genes. Although the MeJA-inducibility of

ORA59 is COI1- and EIN2.1- dependent, the direct involvement of AtMYC2 remains unknown (Pre et al. 2008).

In Arabidopsis, ANAC019 and ANAC055 are MeJA inducible TFs belonging to the NAC family of proteins. The COI1- and MYC2- dependent induction of these genes by MeJA starts as early as 15 minutes and is maintained for six hours after induction. A double knockout of ANAC019 and ANAC055 reduced expression of AtMYC2-regulated VSP2 and LOX2, whereas their ectopic over-expression increased their respective expression. Moreover, overexpression of ANAC019 partially rescued *Atmyc2* mutation. It is suggested that ANAC019 and ANAC055 act downstream of the AtMYC2 regulator (Bu et al. 2008). If the latter is true, these NAC TFs would play the roles of secondary TFs that directly control the expression of VSP2 and LOX2 upon initial activation by AtMYC2.

Conclusions

The high level of inter-species sequence and functional-conservation of MYC2, together with the universal presence of its DNA binding sites in a large number of genes and transcription factors mediating defense processes, indicates that MYC2 is a master regulator of plant defense responses that directly regulates defense genes or indirectly regulates transcription factors that in turn control these genes.

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Chapter 5:

NaMYC2 transcription factor regulates a subset of plant defense responses in *Nicotiana attenuata.*

Running title: MYC2 regulates plant defenses in *N. attenuata*

NaMYC2 transcription factor regulates a subset of plant defense responses in *Nicotiana attenuata.*

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Summary

Background: To survive herbivore attack, plants have evolved potent mechanisms of mechanical or chemical defense that are either constitutively present or inducible after herbivore attack. Due to the costs of defense deployment, plants often regulate their biosynthesis using various transcription factors (TFs). MYC2 regulators belong to the bHLH family of transcription factors that are involved in many aspects of plant defense and development. In this study, we identified a novel MYC2 TF from *N. attenuata* and characterized its regulatory function using a combination of molecular, analytic and ecological methods.

Results: The transcript and targeted metabolite analyses demonstrated that NaMYC2 is mainly involved in the regulation of the biosynthesis of nicotine and phenolamides in *N. attenuata*. In addition, using broadly-targeted metabolite analysis, we identified a number of other metabolite features that were regulated by NaMYC2, which, after full annotation, are expected to broaden our understanding of plant defense regulation. Unlike previous reports, the biosynthesis of jasmonates and some JA-/NaCOI1-dependent metabolites (e.g. HGL-DTGs) were not regulated by NaMYC2, suggesting the involvement of other independent regulators. No significant differences were observed in the performance of *M. sexta* on *MYC2*-silenced plants, consistent with the well-known ability of this specialist insect to tolerate nicotine.

Conclusion: By regulating the biosynthesis of inducible defense compounds, NaMYC2 enhances plant resistance against herbivores and contributes to plant fitness; however, multiple JA/ NaCOI1-dependent mechanisms (perhaps involving other MYCs) that regulate defense responses are likely to exist in *N. attenuata*. The considerable variation observed amongst different plant families in the responses regulated by jasmonate signaling highlights the

sophistication with which plants craft highly specific and fine-tuned responses against the herbivores that attack them.

Background

In their natural habitats, plants are exposed to a number of abiotic (e.g. drought, ultra-violet radiation, salinity) and biotic (e.g. herbivore and/or pathogen attack, competition) stresses which strongly undermine their Darwinian fitness. To cope with herbivory, plants have evolved intricate defense mechanisms that include mechanical barriers, trichomes, thorns, latex, waxes, and a toxic- / anti-nutritive chemical arsenal deployed either constitutively (e.g. nicotine, glucosinolates) or following herbivore attack (e.g. hydrogeranyllinalool-diterpene glycosides (HGL-DTGs), phenolamides, trypsin protease inhibitors) [1-3]. In addition, and in concert with these direct defenses, plants recruit predators or parasitoids of the attackers using informative volatile organic compounds or nutritional rewards [4-6]. However, the costs of defense responses [2, 7, 8] necessitate the development of stringent regulatory mechanisms and several families of plant transcription factors (TFs) (e.g. ERF, bZIP, MYB, bHLH and WRKY) have been shown to regulate plant defense against biotic and abiotic stresses [9-11]. Many of these transcription factors are co-induced in response to different stresses suggesting the existence of complex interaction [12-14].

In many plant species, the role of phytohormones in coordinating the development of defense responses has clearly been shown, frequently with cross-talk among them to achieve intricately fine-tuned response outcomes [15-18]. Specifically, the jasmonate signaling pathway plays a critical role in mediating defense responses against herbivores [19-21]. In response to herbivore attack, GLA1 enzymes release 18:3 α-linolenic acid (α-LeA) from chloroplast membranes. α-LeA is subsequently converted to oxophytodienoic acid (OPDA) in the

chloroplasts by lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) enzymes. OPDA is transported to peroxisomes and oxidized by OPDA reductase (OPR3) forming jasmonic acid (JA). In the cytosol, JA is conjugated to isoleucine by JAR enzymes that produce the bioactive jasmonate, (+)-*7-iso*-jasmonoyl-L-isoleucine (JA-Ile) [22, 23]. JA-Ile associates with the SCF^{COI1} complex, presumably to ubiquinate JAZ repressors and tag them for degradation by the 26S proteasome. In the absence of stressful conditions, MYC2 is repressed by the JAZ repressors, which recruit TOPLESS (TPL) as a co-repressor either directly through the EAR (Ethylene Response Factor-Associated Amphifilic Repression) motif or using the EAR motif of the NINJA (Novel Interactor of JAZ) protein [24, 25]. Degradation of JAZ proteins releases the MYC2 transcription factor from repression and allows it to mediate the reconfiguration of downstream transcriptional processes [11, 24, 26-28].

MYC2 is a member of the basic Helix Loop Helix (bHLH) family of transcription factors (TFs) [29, 30] that are characterized by structurally and functionally conserved domains in many plant species. One of these conserved domains, the basic (b) region, is used to bind to variants of the G-box hexamer (5'-CACNTG-3') found on the promoters of MYC2-regulated genes. The HLH and ZIP domains are used for homo-/hetero-dimerization, while the JID (JAZ Interacting Domain) domain is used to interact with JAZ proteins [11, 28, 29, 31-34].

MYC2 transcription factors participate in the regulation of many JA-dependent physiological processes: defense against herbivores/pathogens, drought tolerance, circadian clock, light signaling and root growth [11, 35-39]. Guo *et al.* [40], in a proteomic study that involved mock- or MeJA-treated wild type and myc2 plants, recently identified 27 differentially regulated, JA-inducible and MYC2 dependent proteins involved in glucosinolate metabolism

(22%), stress and defense (33%), photosynthesis (22.2%), carbohydrate metabolism (7.4%), protein folding and degradation (11.1%), highlighting the very diverse roles of MYC2.

N. attenuata is a wild tobacco species native to the Great Basin Desert in Utah (USA) which our group has developed into an ecological plant model. The defense responses of this species against its specialist herbivore, *Manduca sexta,* are well studied, and include the production of potent secondary metabolites: nicotine, HGL-DTGs, phenolamides and protease inhibitors [10, 41-47]. In this study, we identified a putative MYC2 transcription factor in *N. attenuata* (NaMYC2) and characterized its role in defense response regulation using reverse genetic, transcriptomic and untargeted/ targeted metabolomic approaches. Our transcriptomic and metabolomic data indicate a strong involvement of NaMYC2 in nicotine accumulation. However, silencing this gene had only a limited effect on the accumulation of other plant defense metabolites which strongly implicates the involvement of multiple independent and /or redundant transcriptional regulators in defense signaling of *N. attenuata* plants.

Results and Discussion

NaMYC2 *transcripts are induced after herbivory*

Herbivore attack induces a transient reconfiguration of plants' transcriptome, which translates into a reconfiguration of the metabolome [48-51]. In previous studies, the function of MYC2 TFs (Figure 1) in plant defense regulation was demonstrated; however, the detailed regulatory mechanisms differ amongst different plant species [11, 31, 37, 52]. In wild type *N. attenuata* plants, transcripts of *MYC2* were transiently up-regulated after treatment with wounding (WW) or simulated herbivory (WOS) in both treated and untreated systemic leaves (Figure 2*A* and 2*B*), strongly suggesting the involvement of NaMYC2 TF in defense regulation

in *N. attenuata* [53]. Hence, to determine the function(s) of MYC2 in *N. attenuata*, we used a reverse genetic approach to knock down the accumulation of *NaMYC2* transcripts (by **V**irus **I**nduced **G**ene **S**ilencing, VIGS) and characterized the transformed plants after verifying the efficiency of the VIGS procedure. Compared to empty vector (EV; transformation control) plants, a significant reduction was observed in *NaMYC2* transcript accumulation in MYC2-VIGS plants before (ANOVA, $F_{1,6}$=339.22, P=0.0001) or 1 h (ANOVA, $F_{1,8}$=418.72, P=0.0001) or 3 h (ANOVA, $F_{1,3}$=42.41, P=0.007) after WOS induction (Figure 2*C*). In subsequent experiments, we used the silenced plants to determine the regulatory roles of MYC2 in plant defense in *N. attenuata*.

Targeted analysis of secondary metabolite accumulation in MYC2-VIGS plants

Nicotine, phenolamides, hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) and phenolic compounds are among the potent, JA-dependent anti-herbivore compounds in *N. attenuata* [2, 10, 54, 55]. Their JA-dependent pattern of accumulation suggests that the biosynthesis of these compounds might be regulated by NaMYC2. To test this hypothesis, we used a targeted metabolomic approach and measured the accumulation of the following compounds in untreated control and WOS-treated (24, 48 and 72 h) EV and MYC2-VIGS plants.

Nicotine

Nicotine is among the most prominent chemical defense compounds in *N. attenuata* [56] and most of the genes involved in its biosynthesis have been identified [52, 57]. Nicotine is synthesized in roots and transported to leaves. To test if MYC2 regulates herbivore-induced biosynthesis of nicotine in *N. attenuata*, we measured the accumulation of nicotine in untreated or WOS-treated EV and MYC2-VIGS plants on HPLC-PDA. We found that compared to EV

plants, the accumulation of nicotine was significantly lower before (ANOVA, $F_{1,7}$=6.94, *P*=0.03) or 24 h (ANOVA, $F_{1,7}$=10.06, *P*=0.01), 48 h (ANOVA, $F_{1,8}$=17.53, *P*=0.003) and 72 h (ANOVA, $F_{1,8}$=28.81, *P*=0.0007) after WOS treatment in MYC2-VIGS plants (Figure 3). Similar results were observed in another independent VIGS experiment (Figure S1*A, B*) demonstrating that nicotine biosynthesis is strongly regulated by the MYC2 TF in *N. attenuata*. In addition to nicotine, we found MYC2-specific differences in the accumulations of two other alkaloids, anatabine and cotinine, as determined by a more selective and sensitive LC-TOF/MS method (Figure S1*C, D*). Interestingly, while the ion intensities of anatabine and nicotine reduced in MYC2-VIGS leaves, cotinine accumulation increased.

Overall, our results are consistent with previous reports which demonstrated regulation of jasmonate-induced nicotine/ alkaloid biosynthesis by MYC2 TFs. Recently, in *N. tabacum* Bright Yellow (BY-2) cells that were transformed with an inverted-repeat (ir)NtMYC2a/2b construct, the accumulation of nicotine and anatabine was significantly less than untransformed controls [58]. The NtMYC2 protein was also shown to regulate nicotine biosynthesis either by directly binding to the promoters of nicotine biosynthetic genes in roots or activating NtERF189 which, in turn, activates genes involved in nicotine biosynthesis [52]. In *N. benthamiana*, VIGS of two bHLH transcription factors (named NbbHLH1 and NbbHLH2) as well as NbERF1 and NbHB1 decreased MeJA-induced accumulation of nicotine [59]. These results demonstrate both the regulatory functions of MYC2 and the involvement of a network of transcription factors in the regulation of nicotine biosynthesis. However, the functions of the tobacco *MYC2* genes were not examined in the context of natural herbivore feeding; neither were the influence of these *MYC2* genes on the accumulations of other tobacco defense metabolites (e.g. phenolamides, HGL-DTGs, etc.) studied. From the phylogenetic relationship of MYC/bHLH TFs in *N.*

attenuata, N. tabaccum and *N. benthamiana* (Figure 1) and our results, the presence of additional MYC TFs in *N. attenuata* is a reasonable prediction. Identification and characterization of these putative TFs might be helpful to fully understand the biosynthesis and ecological consequences of nicotine/alkaloid biosynthesis. Moreover, identification of such a regulator would complement the partial regulatory function of NaMYC2 in the control of different classes of *N. attenuata* defense metabolites, as demonstrated in the next sections.

Phenolamides

Recently, regulation of the biosynthesis of phenolamides by NaMYB8 TF and its ecological relevance were reported in *N. attenuata* [10, 47]. Considering a previous report in *A. thaliana* which indicated regulation of MYB TFs by AtMYC2 [11] and our microarray data which identified a MYB TF among the NaMYC2-regulated genes (Table S1), we reasoned that, in *N. attenuata*, NaMYB8 or the genes it regulates might be regulated by NaMYC2. To test this possibility, we treated EV and MYC2-VIGS plants by WOS and measured the relative transcript abundances of NaMYB8 and downstream genes involved in phenolamide biosynthesis. Transcript accumulations of these genes did not differ between EV and MYC2-VIGS plants in untreated plants (0 h); however, 1 h after WOS treatment, a significant reduction was observed in transcript accumulation of *NaMYB8* (ANOVA, $F_{1,6} = 9.81$, $P = 0.02$), *NaPAL* (ANOVA, $F_{1,6} = 17.14$, $P = 0.006$), *NaAT1* (ANOVA, $F_{1,6} = 16.00$, $P = 0.007$), *NaDH29* (ANOVA, $F_{1,6} = 28.25$, $P = 0.001$) and *NaCV86* (ANOVA, $F_{1,6} = 6.66$, $P = 0.04$) in MYC2-VIGS plants (Figure 4). Our data and the previously demonstrated regulation of *NaAT1*, *NaDH29*, *NaCV86* by *NaMYB8* [47] point to the possibility that NaMYC2 controls phenolamide biosynthesis by regulating the expression of *NaMYB8*.

Next, we measured the WOS-induced accumulation of caffeoylputrescine, dicaffeoylspermidine, chlorogenic acid and rutin in EV and MYC2-VIGS plants to test if the accumulation of these compounds followed the observed NaMYC2-dependent transcript accumulation patterns. Surprisingly, we found very few significant differences between EV and MYC2-VIGS samples (Figure 5), which was also confirmed in an independent VIGS experiment (Figure S2*A* to *D*). The disconnect between the transcript and metabolite accumulation data, though explainable, was a surprise. In both VIGS experiments, due to time required for the efficient spread of silencing (as demonstrated by progress of bleaching in PDS positive controls), the samples used to extract secondary metabolites were collected when the plants were at the transition into the flowering stage. At late elongated/flowering stage, the inducible character of phenolamide accumulation is known to cease, although transcript accumulation at this stage was not previously investigated [10]. Thus, due to the "constitutive" nature of phenolamide accumulation at later stages of plant development, their biosynthesis may not be strongly influenced by NaMYC2. Alternatively, even though the transcription of the biosynthetic enzymes remains inducible at later stages of development (Figure 4), translation/ post-translational modifications of the enzymes might not occur or the necessary substrates, such as phenylpropanoids and polyamines, could be diverted to other important functions in flowering plants. Finally, it is possible that our ability to detect MYC2-dependent differences was masked because of the plants' response to the VIGS process (i.e. virus infection that may induce phenolamide biosynthesis) or that the level of silencing was not simply sufficient to affect phenolamide biosynthesis. Therefore, the disconnect between transcript and metabolite data could be explained by the dynamic and/or synergistic regulation of phenolamide biosynthesis; developmentally and by herbivore/ pathogen attack. We predict that NaMYC2 is likely involved

in the regulation of phenolamide biosynthesis in younger plants. However, this could not be tested in the current experimental setup (using MYC2-VIGS plants) and would require the generation of stably transformed plants.

Total hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) and TPI levels

HGL-DTGs are JA-dependent metabolites with well-demonstrated roles in plant defense against herbivores in *N. attenuata* [55, 60-62]. To determine if herbivore-induced accumulation of HGL-DTGs was regulated by MYC2 in *N. attenuata*, we treated EV and MYC2-VIGS plants with WOS, extracted metabolites and analyzed total HGL-DTGs by HPLC-PDA. Interestingly, we found no significant difference in accumulation of total HGL-DTGs in control plants or plants treated with WOS for 24, 48 and 72 h (Figure 6*A* and Figure S2*E*), indicating that MYC2 may not be involved in regulating the biosynthesis of this class of compounds. We used a radial diffusion assay [63] to compared the WOS-induced TPI activity between EV and MYC2-VIGS plants and found that, although TPI activity levels were significantly reduced 24 h after WOS treatment, the levels were higher in MYC2-VIGS plants prior induction; and this did not correlate with *MYC2* expression (Figure 6*B*).

Taken together and considering the JA-/COI1-dependency of HGL-DTG and TPI accumulation in *N. attenuata* [64], the biosynthesis of HGL-DTGs and TPIs in *N. attenuata* is likely regulated by a JA-dependent, but NaMYC2-independent mechanism. Alternatively, the function and/or synergism of an independent *MYC2* gene in *N. attenuata* can explain the partial function of NaMYC2.

NaMYC2 and regulation of herbivory-induced phytohormone accumulation

In *A. thaliana*, MYC2 regulates genes involved in the biosynthesis of phytohormones and contributes to the feedback loop in jasmonate biosynthesis. MYC2 also regulates its own transcription, presumably to further enhance jasmonate responses [11, 38]. Hence, we asked if NaMYC2 contributed to the biosynthesis or metabolism of phytohormones in *N. attenuata*, and to address this question, we measured the accumulation of jasmonates in untreated and WOS-treated EV and MYC2-VIGS plants in two independent VIGS experiments. In general, no consistent, MYC2-dependent differences were observed in the accumulation of JA, OH-JA, JA-Ile, OH-JA-Ile and COOH-JA-Ile among EV and MYC2-VIGS plants; neither did we detect consistent differences in the accumulations of ABA and SA (Figure 7, Figure S3). In agreement with these observations and unlike in *A. thaliana* [11], we did not find significant changes in transcript accumulation of any of the genes involved in the biosynthesis/metabolism of these phytohormones in our microarray data (Table S1). From these observations, we conclude that, in *N. attenuata*, MYC2 does not regulate the biosynthesis and/or metabolism of jasmonates, ABA or SA.

Performance of the specialist herbivore on MYC2-VIGS plants

As a key regulator of plant defense responses, we asked if the performance of the specialist herbivore, *M. sexta* was affected by *MYC2* silencing. Consequently, we fed neonates (n = 20) of *M. sexta* on EV and MYC2-VIGS plants for 13 d measuring their masses every 4 d. At all measurement times, we observed no significant difference in the mass gained by caterpillars when fed on EV or MYC2-VIGS plants (Figure 8). This is consistent with the observation that in MYC2-VIGS plants, significant changes were observed only in the accumulation of nicotine, a

metabolite to which neonates of *M. sexta* are very tolerant. In contrast, in a manner that was also consistent with the patterns of metabolite accumulation in irCOI1 plants, neonates of *M. sexta* fed on COI1-silenced plants gained significantly more mass compared to those fed on WT plants [64]. The question is, hence, whether there are other JA/COI1-in/dependent MYC2 (or other) TFs that regulate other defense metabolites of *N. attenuata* that are particularly important for the performance of *M. sexta* larvae.

Large scale transcriptomic and metabolomic analysis of MYC2-silenced leaves

The role of MYC2 TFs in orchestrating plant defense and developmental processes in several plant species were previously reviewed [35, 65, 66]. As master regulators, MYC2 TFs may either directly regulate the genes responsible for defense metabolite biosynthesis or regulate their regulators [11, 65]. To provide information for further work, we used unbiased approaches and compared herbivore-induced (WOS) changes in the transcriptome and metabolome of EV and MYC2-VIGS *N. attenuata* plants.

NaMYC2 regulated transcriptome of N. attenuata

For transcriptomic analysis, we treated EV and MYC2-VIGS plants with WOS for 1 h and compared their respective induced transcriptome using microarrays. This approach, although unable to discover late induced metabolic genes could reveal the intermediate regulators and TFs downstream of NaMYC2. We normalized and $\log_2$-transformed the raw data, identified genes whose expressions were significantly altered in MYC2-VIGS plants (using Significance Analysis of Microarrays (SAM) package) and annotated them by Blast2Go. Compared to EV plants, the expressions of 47 genes were significantly (fold change of 2 or more) altered in MYC2-VIGS plants (Table S1). When we grouped the regulated genes according to TAIR (The Arabidopsis

Information Resource) functional annotation scheme, the genes were found to be involved in diverse physiological processes: regulation of transcription (20.45%), amino acid metabolism (11.3%), secondary metabolism (4.5%), biotic stress (6.8%), development (6.8%), transport (9.1%), post-translational modification (4.5%) and protein degradation (6.8%) (Figure 9, Table S1). Specifically, several key regulators of plant defense responses, transcription factors (WRKY, MYB) or signaling components (calmodulin or calcium binding proteins) were among those identified by the microarray analysis. Close inspection of MYC2-regulated genes in *N. attenuata* identified additional early induced genes involved in defense against herbivores (terpene synthases and proteinase inhibitors) or pathogens (PR proteins) (Table S1). Our data is in general agreement with the report in *A. thaliana* in which the regulatory role of AtMYC2 on a spectrum of physiological processes was shown: from herbivore/pathogen defense to hormone biosynthesis; from primary and/or secondary metabolism [11, 67] to photomorphogenic development [32, 68].

Silencing of NaMYC2 *significantly affects the* N. attenuata *metabolome*

Do MYC2-mediated changes in the herbivore-induced transcriptome translate into a wider spectrum of defense secondary metabolites, apart from alkaloids already demonstrated by targeted analytical approach? We used an unbiased metabolomic profiling approach by HPLC/ESI-TOF-MS and analyzed metabolites extracted from leaves of EV and MYC2-VIGS plants that were continuously attacked (4 d) by neonates of *M. sexta*. The raw data were normalized, $\log_2$-transformed and preprocessed using XCMS and CAMERA packages as described in the materials and methods section. To visualize the direction of the total variability in our samples without taking the class labels into consideration, we used an unsupervised approach (Principal Component Analysis, PCA) and observed that EV and MYC2-VIGS

samples were separated to two clusters by PCA suggesting genotype-specific differences at the level of metabolites (Figure 10*A*). The features that contributed strongly to PC1 (which explains 51.5% of the total variability) and PC2 (which explains 25.5% of the total variability) are depicted in the loading plot (Figure 10*B*). When we screened for metabolic features that differed among the genotypes (fold changes of 2 or more), we identified 897 features; 741 of which differed significantly (t-test threshold of 0.05 or less) between EV and MYC2-VIGS plants (Table S2). The overall pattern of regulation can be visualized from the heat map (Figure 10*C*) generated on Metaboanalyst 2.0 using the significant metabolic features (Ward clustering algorithm and Pearson distance measures). In total, 712 metabolite features that met both fold change and t-test thresholds (2-fold or more, $P < 0.05$, respectively) were identified and the most important features were plotted on the volcano plot (indicated by the purple dots) (Figure 10*D*, Table S2). Some of these features (m/z 163.123, 132.082, 163.039) were previously annotated as molecular fragments of metabolites involved in plant defense against herbivores in *N. attenuata* [47, 69], though identification and annotation of the remaining features remain as significant challenge for future experiments. Overall, our metabolomic analysis demonstrates the importance of MYC2 in the regulation of plant's metabolome. When these metabolomic features are identified and annotated, it will be possible to precisely map the regulatory role of MYC2 on plant defense and developmental responses [40].

Conclusions

In many plant species, attack from herbivores elicits a cascade of complex transcriptional and metabolic responses that improve plant defense. The effectiveness of plant defense depends on the efficiency by which the timing and duration of responses are regulated. In this study, we identified a MYC2 TF in *N. attenuata* and characterized its regulatory role using transcriptomic

and metabolomic approaches. Transcriptionally, we showed that the expressions of many genes, including transcription factors, involved in plant development or defense responses were affected when MYC2 was silenced in *N. attenuata*. This was supported by the metabolomic data which identified a large number of differentially regulated molecular features following the silencing. Most importantly, as was previously reported in *N. tabacum* and *N. benthamiana*, we showed that NaMYC2 regulates the *in planta* accumulation of nicotine in *N. attenuata* leaves. The fact that MYC2 did not strongly affect the accumulation of other JA-dependent metabolites, HGL-DTGs and proteinase inhibitors, suggests that another MYC TF is likely involved in the process.

Despite the considerable conservation of the basic components of plant defense responses among different plant species, substantial variations exist in the responses outcomes which highlights between species differences in downstream regulatory fine-tuning [31, 70]. For example, in contrast to the considerable similarity among members of the genus *Nicotiana* in the regulation of nicotine biosynthesis by MYC2 [52, 58, 59] (Figure 1), silencing MYC2 in *N. attenuata* did not have the exact same effects as reported in *A. thaliana*; we did not observe a role of MYC2 either in a positive feedback loop activating JA biosynthesis or in a negative feedback involving suppression of the jasmonate response through the activation of JAZ repressors [11, 71].

In addition, not all JA-dependent defense metabolites (e.g. HGL-DTGs) were regulated by MYC2 in *N. attenuata*. In fact, when compared against the diversity of defense metabolites in *N. attenuata*, the regulatory function of MYC2 is quite limited. This rather limited role suggests that other members of the bHLH family of transcription factors might be involved in the regulation of defense responses not regulated by MYC2. The recent identification of additional MYC2 TFs in *A. thaliana* [36, 37], *N. tabacum* [58] and *N. benthamiana* [59] with overlapping

or distinct functions support this conjecture. Identification and characterization of other MYC2 TFs in *N. attenuata* might offer a more complete picture of how JA signaling regulates JA-regulated defense responses.

Considering the high degree of conservation in the binding site of MYC2 TFs in different species [29, 31], we believe future research in determining the binding sites of these TFs will be critical to understanding their function. Once the binding sites are identified, genes or other transcription factors that respond to herbivory, disease, environmental stress or development and are MYC2-dependent can readily be identified. It would be interesting to identify the interacting partners of MYC2 TFs in *N. attenuata* and characterize the mechanisms of interaction to understand how the signaling components evolved. In *A. thaliana*, transcriptional regulation by MYC2 requires interactions with important regulatory elements including members of the mediator complex proteins (e.g. MED25), chromatin-opening proteins like General Control Non-repressible5 (GCN5), members of the histone acetyl transferase family and SPLAYED (SYD) [35, 72, 73]. Identification and characterization of homologues of these components in *N. attenuata* might test the generality of the signaling processes across different plant families.

Methods

Plant growth and treatments

N. attenuata seeds that were collected from its native habitat in Great Basin desert, Utah (USA) and inbred for 31 generations were used for the experiments. Seed germination and plant growth conditions were described in Krügel *et al*. [74]. To experimentally simulate herbivory, we wounded fully expanded leaves of EV and MYC2-VIGS (*n*=5) *N. attenuata* plants with a serrated fabric pattern wheel and the wounds were treated with 20 µL of diluted (1:5, v/v in

water) *M. sexta* oral secretions (WOS), while controls were collected from untreated plants. To evaluate performance of the specialist herbivore (*M. sexta*) on transformed plants, freshly hatched neonates were fed on EV and transformed plants (n = 20) and their masses were measured every 4d.

Virus Induced Gene Silencing (VIGS)

Virus **I**nduced **G**ene **S**ilencing (VIGS) system, described in Saedler and Baldwin [75], was used to transiently silence MYC2 transcription factor. Briefly, we amplified ~250 bp fragment of the *N. attenuata MYC2* using specific primers (Table S3), cloned them into the P^{TV00} vector. We verified the clone by sequencing and transformed GV3101 strain of *Agrobacterium tumefaciens* with either untransformed plasmid (P^{TV00}, control) or plasmids harboring the inserts ($p^{TV\text{-}MYC2}$) and incubated them at 26°C for two days. On the day of infiltration, overnight cultures of all constructs and p^{BINTRA} and p^{TVPDS} were inoculated into YEP media containing antibiotics (Kanamycin 50 mg/L) and incubated (28°C) for 5 h. When the cultures attained an OD of 0.6 to 0.8, we centrifuged them (1125*g*, 4°C for 5 min), resuspended the pellets in an equimolar mix (5 mM) of MgCl2 and MES and prepared a 1:1 mix of each construct with the helper strain p^{BINTRA}. Using 1 mL syringes, we infiltrated the suspension into five leaves of 25 d old *N. attenuata* plants, covered them with plastic and left them in a dark chamber for 2 d. The plants were kept in the growth chamber under 16 h/day, 8 h/night light regime at 22°C. We monitored the spread of silencing using control plants infiltrated with the p^{TVPDS} construct which induced leaf bleaching, while the efficiency of silencing was determined by measuring transcript abundances using qRT-PCR.

Microarray analysis

We treated fully elongated leaves of EV and MYC2-VIGS plants (n = 3) with WOS for 1h, collected and ground the leaves in liquid nitrogen and extracted RNA for the microarray analysis as described in Gillardoni *et al*. [76]. After hybridization and array processing, we normalized (with the 75th percentile of the respective columns) and $\log_2$-transformed the raw expression values obtained from the "gProcessedSignal" column and processed them using Significance of Microarrays (SAM; http://www-stat.stanford.edu/~tibs/SAM/) package on Excel (Microsoft). For the analysis, we set the minimum fold change, delta and median FDR (%) values to 2, 0.69 and 15.8 (%) respectively. Genes that differed significantly in comparison to EV plants were annotated using Blast2Go [77] and grouped according to TAIR classification.

Transcript abundance measurement

We extracted total RNA from frozen leaf material of untreated or WOS-treated EV and MYC2-VIGS plants (n = 5) using TRIzol reagent (Invitrogen) as recommended by the manufacturer. We treated the total RNA with DNAse (RQ1 RNase-Free DNase; Promega) before synthesizing cDNA using oligo $(dT)_{18}$ and Superscript II reverse transcriptase (Invitrogen). Transcript abundances were measured on Mx3005P Multiplex qPCR (Stratagene) with qPCR core kit for SYBR Green I (Eurogentec). We normalized gene expression values obtained with the expression values of *N. attenuata elongation factor-1α* (*EF-1α*). The primers used for qRT-PCR are listed in Table S3.

Phytohormone analyses

Fully-expanded leaves of EV and MYC2-VIGS plants (n = 5) were treated with WOS for 1 h or 2 h, collected and ground in liquid nitrogen and stored at -80^{o}C until use. We homogenized about 200 mg powder in 1 mL ethyl acetate (containing 200 ng/mL D_2-JA and 40 ng/mL D_6-ABA, D_4-SA and JA-$^{13}C_6$-Ile internal standards), centrifuged for 20 min (16,100g, 4^{o}C) and transferred the

supernatants into new tubes. After re-extracting the pellets with 0.5 mL ethyl acetate and combining the supernatants, we evaporated the ethyl acetate on a vacuum concentrator (Eppendorf) and resuspended the residue in 0.5 mL 70% methanol in water (v/v). Then, we centrifuged the re-suspended samples for 10 min (16,100g, 4^{o}C) and analyzed the supernatant (10 µL) on Varian 1200L Triple-Quadrupole-LC-MS (Varian) using a ProntoSIL® column (C18; 5µm, 50 x 2 mm; Bischoff) attached to a precolumn (C18; 4 x 2 mm, Phenomenex). Detail measurement conditions are described in Woldemariam *et al.* [62].

Secondary metabolite analysis

To undertake targeted defense secondary metabolite (nicotine, total 17-hydroxygeranyllinalool diterpene glycosides [HGL-DTGs], caffeoylputrescine, dicaffeoylspermidine, chlorogenic acid and rutin) analysis, we treated leaves of EV and MYC2-VIGS (n = 5) plants with WOS for 24, 48 or 72 h, collected and ground the samples in liquid nitrogen. Control samples were collected without treatment. About 100 mg powder was extracted and analyzed on HPLC equipped with a photodiode array detector as previously described in Onkokesung *et al*. [78].

Untargeted metabolomic analysis

To undertake an unbiased metabolomic analysis, metabolites were extracted from leaves (n = 3) of EV and *MYC2* silenced *N. attenuata* plants fed on for 4 d by neonates of *M. sexta* and analyzed on an HPLC 1100 Series system (Agilent, Palo Alto, USA) coupled to a MicroToF mass spectrometer (Bruker Daltonik, Bremen, Germany). The optimized analytic procedures are described in Gaquerel *et al*. [69]. Briefly, peak picking, peak detection and RT corrections were performed by XCMS (and CAMERA) package using the following parameters: centWave method; ppm = 20; snthresh =10; peakwidth = between 5 and 18 s; minfrac=0.5; minsamp=1;

bw=10; mzwid=0.01; sleep=0.001). To fill missing features, we used the FillPeaks function from XCMS. We exported the pre-processed data to Excel, filtered those features with RTs < 60 seconds and m/z < 80 and analyzed the processed data on Metaboanalyst 2.0 following the procedure described before [79].

Statistical analysis

We used STATVIEW (version 5.0; SAS Institute, Cary, NC, USA) software to perform statistical analyses with alpha level of 0.05 for all statistical tests.

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Competing interests

The authors verify that there are no competing interests.

Authors' contributions

MGW (designed experiments; conducted experiments; analyzed data; wrote manuscript); STD (conducted experiments); YO (conducted experiments); EG (designed experiments; analyzed data); ITB (designed experiments; wrote manuscript; provided financial support); IG (designed experiments; wrote manuscript).

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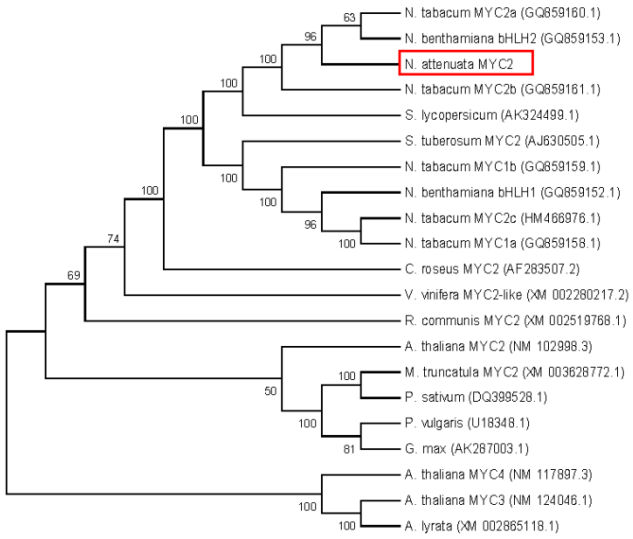

Figure 1. Phylogeny of MYC2 transcription factors.
Sequences with high similarity to the *N. attenuata* MYC2 were retrieved from NCBI by Blast. Sequence alignment and phylogeny reconstruction were performed on MEGA5 using CLUSTAL W and Maximum Likelihood packages, respectively. The consensus tree generated was tested by bootstrapping (1000 times).

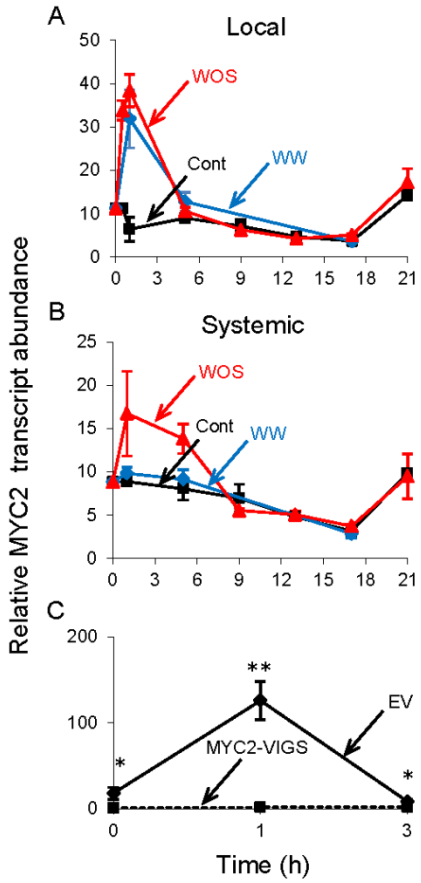

Figure 2. Transcript abundance and silencing efficiency of MYC2 transcription factor in *N. attenuata*.
Rosette stage leaves (*n*=3) of wild type *N. attenuata* plants were treated with WW (blue line) or WOS (red line) or left untreated (black) and transcript abundances (mean ± SE) of MYC2 TF were measured by microarrays in (A) treated and (B) untreated systemic leaves (data were extracted from a previously published microarray dataset by Kim *et al.* [53]). (C) Using Virus Induced Gene silencing (VIGS), the accumulation of *MYC2* transcripts were knocked down and the efficiency of silencing was determined by measuring the relative *MYC2* transcript abundances (mean ± SE; *n*=5) in control and WOS-induced EV (solid line) and MYC2-VIGS (dashed) plants by qRT-PCR. Asterisks indicate statistically significant differences (ANOVA, $P < 0.05$).

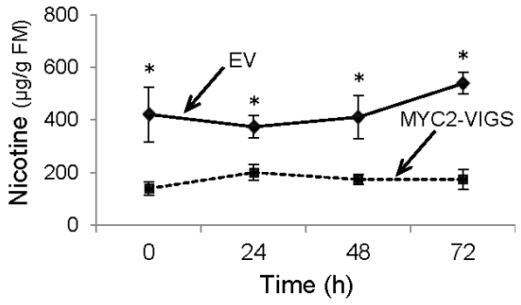

Figure 3. Accumulation of nicotine in EV and MYC2-VIGS plants.
Metabolites were extracted from leaves (*n*=5) of EV- and MYC2-VIGS plants which were collected before or 24 h, 48 h or 72 h after WOS treatment and the average (mean ± SE) accumulation of nicotine was analyzed by HPLC-PDA. Asterisks indicate significant statistical (ANOVA, $P < 0.05$) differences.

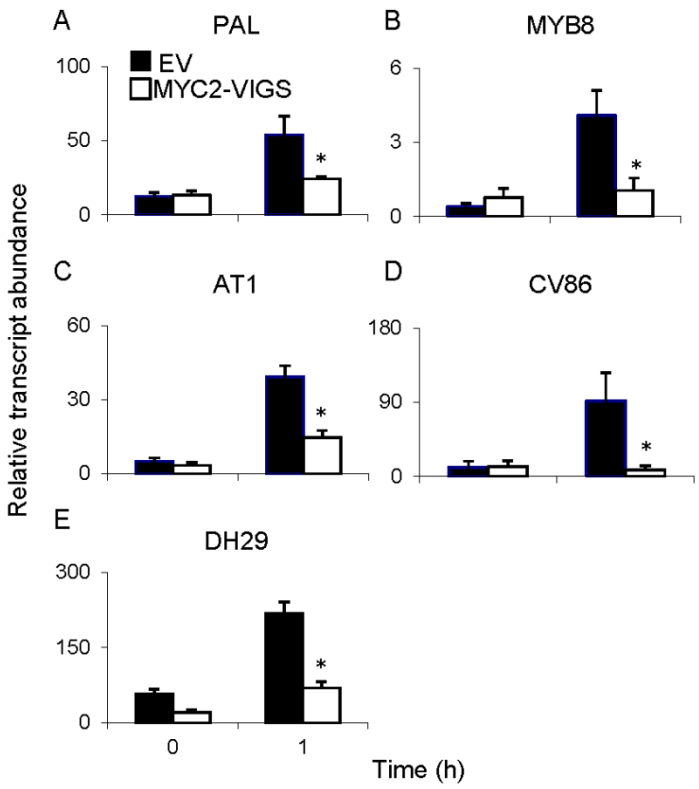

Figure 4. Transcript accumulation of selected genes involved in phenolamide biosynthesis in EV and MYC2-VIGS *N. attenuata* plants.
Transcript abundance (mean ± SE; n=5) of genes involved in JA-dependent phenolamide biosynthesis was determined in WOS-induced EV and MYC2-VIGS plants. Quantification was performed by qRT-PCR using the house-keeping gene, *elongation factor* (*EF-1α*), for normalization. One h after WOS-treatment, significant reductions (ANOVA, $P < 0.05$, indicated by asterisks) were observed in transcript accumulation of *PAL* (A), *MYB8* (B), *AT1* (C), *CV86* (D) and *DH29* (E) in MYC2-VIGS plants.

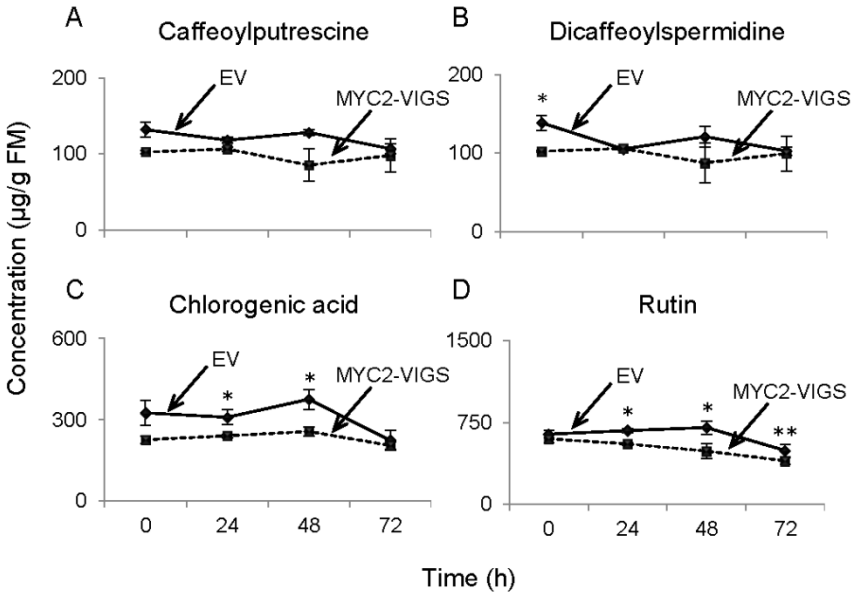

Figure 5. Targeted analysis of phenolamide accumulation in EV and MYC2-VIGS plants.

Following the extraction of metabolites from leaves (n=5) of EV- and MYC2-VIGS plants that were collected before or 24 h, 48 h or 72 h after WOS treatment, the average (mean ± SE) accumulations of caffeoylputrescine (A), dicaffeoylspermidine (B), chlorogenic acid (C) and rutin (D) were analyzed by HPLC-PDA. Asterisks indicate significant (ANOVA, $P < 0.05$) statistical differences.

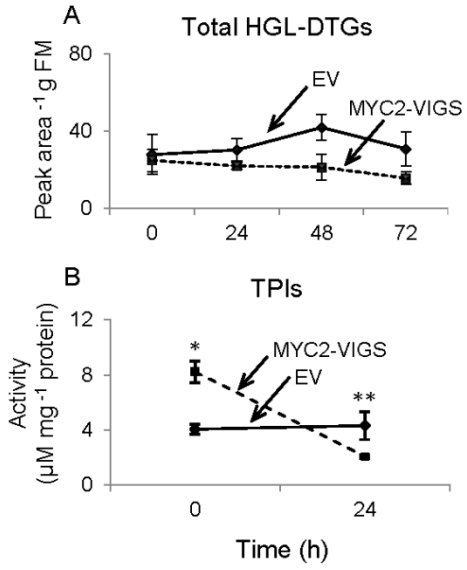

Figure 6. WOS-induced accumulation of HGL-DTGs and TPI activity in EV and MYC2-VIGS plants.

Leaves (*n*=5) of EV- and MYC2-VIGS plants were treated with WOS for 24 h, 48 h or 72 h or left untreated (0 h) and collected to extract and analyze accumulation (mean ± SE) of total HGL-DTGs (A) on HPLC-PDA. Using un-induced (0 h) and WOS-induced (24 h) samples from the same experiment, TPI activity (B) was determined using a radial diffusion assay. Asterisks indicate significant statistical differences.

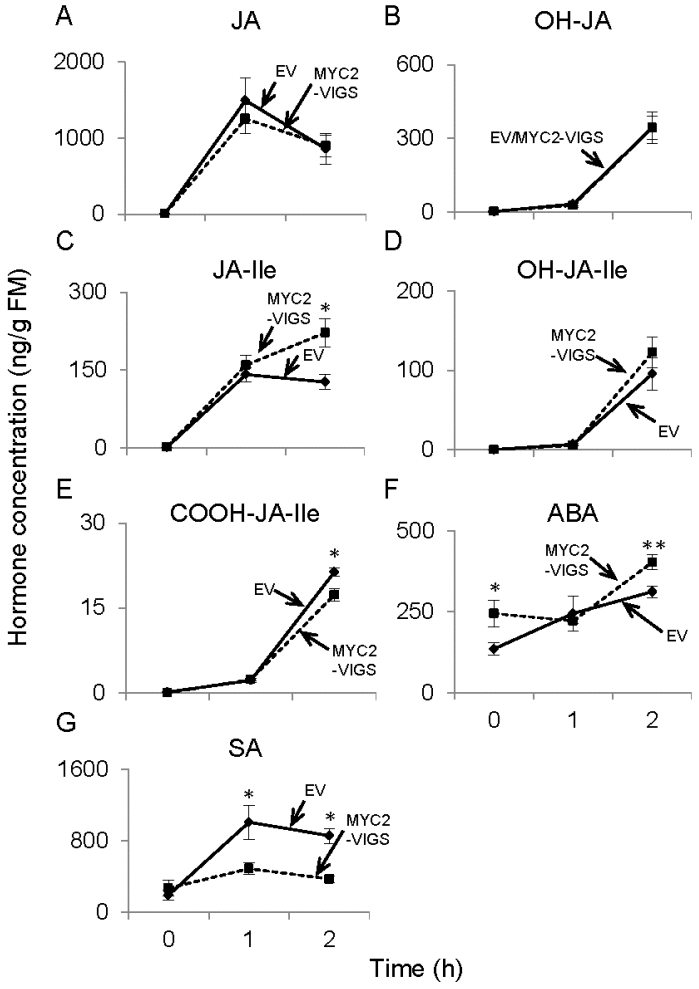

Figure 7. Herbivore-induced accumulation of phytohormones in EV and MYC2-VIGS plants.

Fully elongated leaves (n=5) of EV and MYC2-VIGS plants were collected before or 1 h or 2 h after WOS-treatment for phytohormone extraction. Extracts were analyzed on LC-MS^3 and the levels (mean ± SE) of JA (A), OH-JA (B), JA-Ile (C), OH-JA-Ile (D), COOH-JA-Ile (E), ABA (F) and SA (G) were determined. Different letters indicate statistically significant differences.

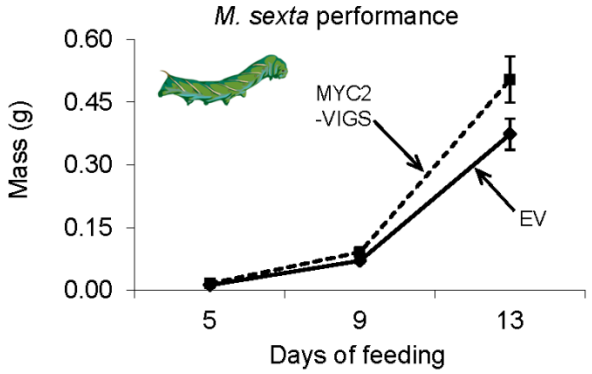

Figure 8. Performance of *M. sexta* on EV- and MYC2-VIGS plants.
Neonates (*n*=20) of the specialist herbivore, *M. sexta,* were fed on EV and MYC2-VIGS *N. attenuata* plants for 13 d and their masses (mean ± SE) were determined on the 5th, 9th and 13th day to compare their relative growth performance. No significant differences were observed in mass gained among caterpillars fed on EV or MYC2-VIGS plants.

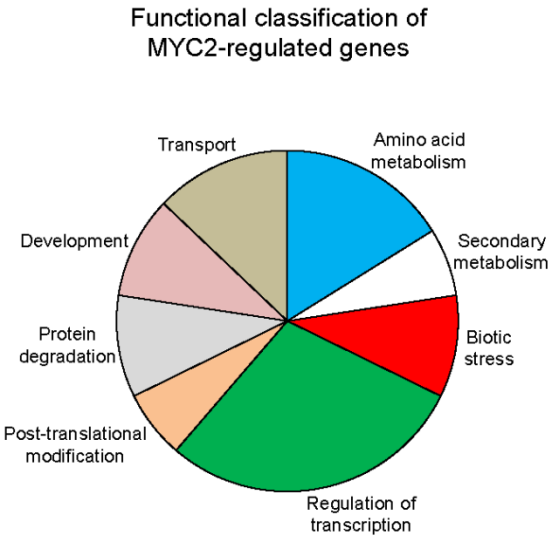

Figure 9. Transcriptional regulation by MYC2 transcription factors.
Rosette leaves (*n*=3) were collected from WOS-induced (1 h) EV and MYC2-VIGS *N. attenuata* plants for microarray analysis. After pre-processing the raw data, genes whose expression changed significantly among the genotypes were identified using Significance Analysis of Microarrays (SAM) package and functional annotation was performed on Blast2Go. Pie chart depicts the function categories of MYC2-regulated genes in *N. attenuata*.

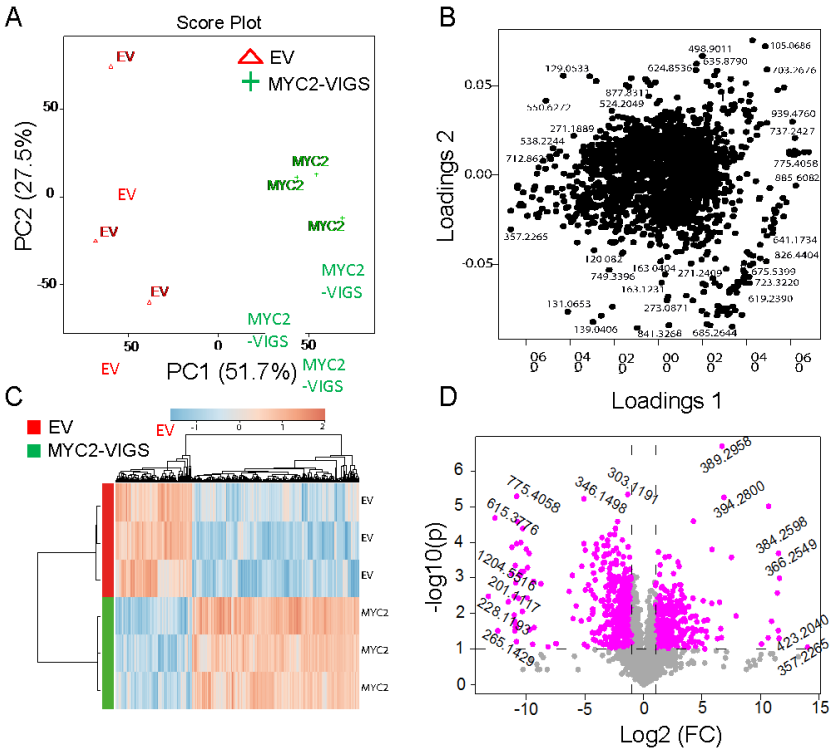

Figure 10. Broadly targeted metabolomic analysis of herbivore-induced EV and MYC2-VIGS plants.

Leaves (*n*=3) were collected from caterpillar-attacked (4 d) EV and MYC2-VIGS plants and used for untargeted metabolomic analysis with an HPLC-TOF-MS. Raw data were pre-processed by XCMS and CAMERA packages and a PCA plot (A) was generated based on the molecular features that differed significantly (fold change > 2, P < 0.05) among the indicated genotypes. Principal component 1 (PC 1) explains 51.7% of the variance while PC 2 explains 24.5%. The contribution of the molecular features to the PCA clusters is shown by the loading plot (B) while the volcano plot (D) depicts important features with a fold change and t-test threshold of 2 and 0.1, respectively. (C) A heat map depicts the expression of regulated molecular features in EV and MYC2-silenced plants.

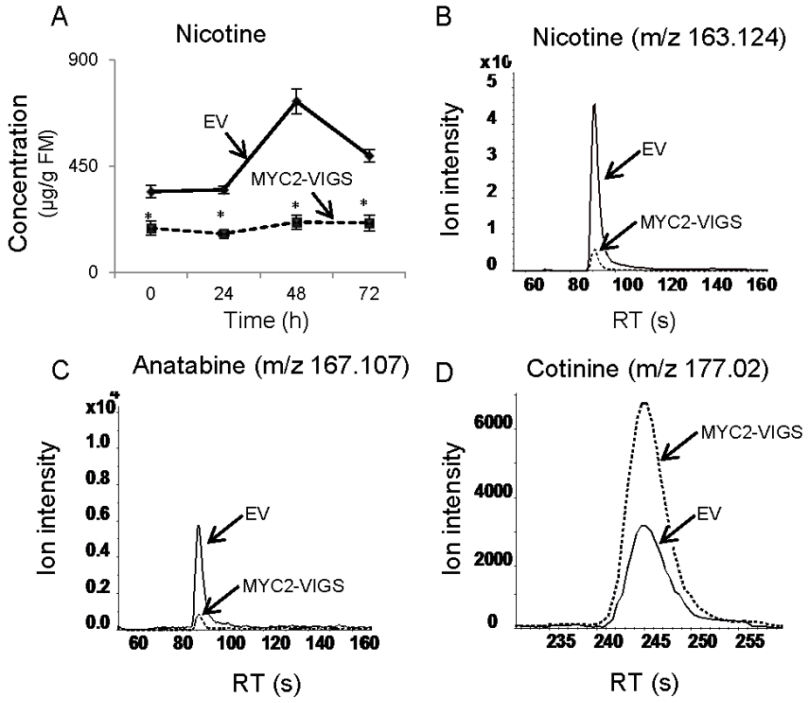

Figure S1. Targeted and untargeted analysis of nicotine and related alkaloids in EV and MYC2-VIGS plants. (A) Control and WOS-treated leaves (n = 5) of EV and MYC2-VIGS plants were collected and used to analyze the accumulation (mean ± SE) of nicotine. Statistically significant differences are indicated by asterisk. (B) to (D). Leaves (n = 3) of EV and MYC2-VIGS plants that were attacked by neonates of *M. sexta* for 4 d were collected to extract metabolites which were subsequently analyzed by HPLC-TOF-MS. The extracted ion chromatographs (EIC) for nicotine (B), anatabine (C) and cotinine (D) were overlaid to compare the regulation of alkaloid biosynthesis by MYC2.

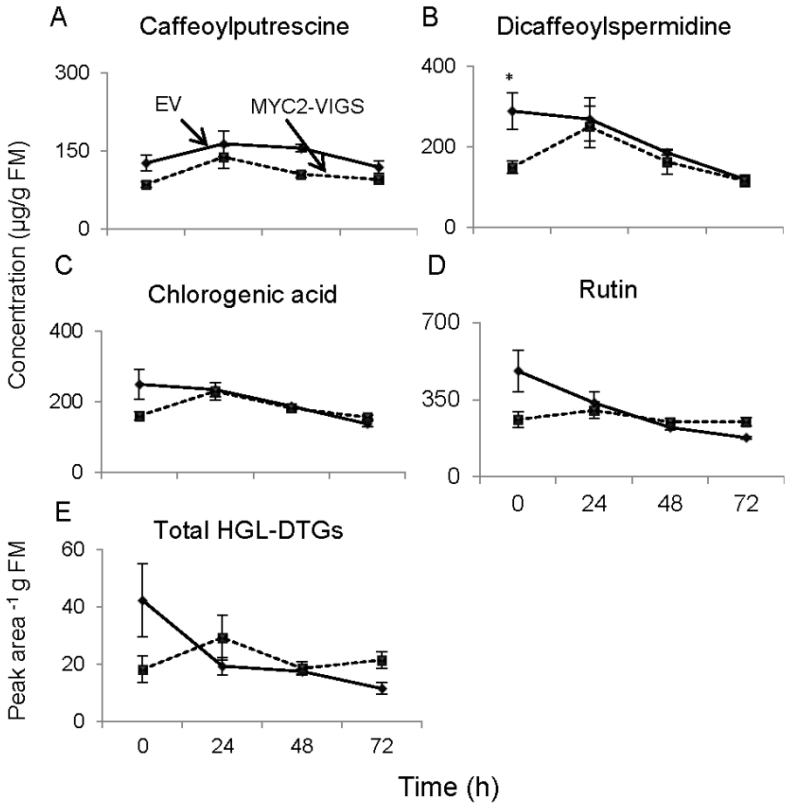

Figure S2. Secondary metabolites accumulation in EV and MYC2-VIGS plants before (0 h) or 24 h, 48 h and 72 h hours after WOS treatment. Control and WOS-treated leaves (n = 3) of EV and MYC2-VIGS plants were collected and used to analyze the accumulation (mean ± SE) of caffeoylputrescine (A), dicaffeoylspermidine (B), chlorogenic acid (C), rutin (D) and total HGL-DTGs (E) on HPLC-PDA.

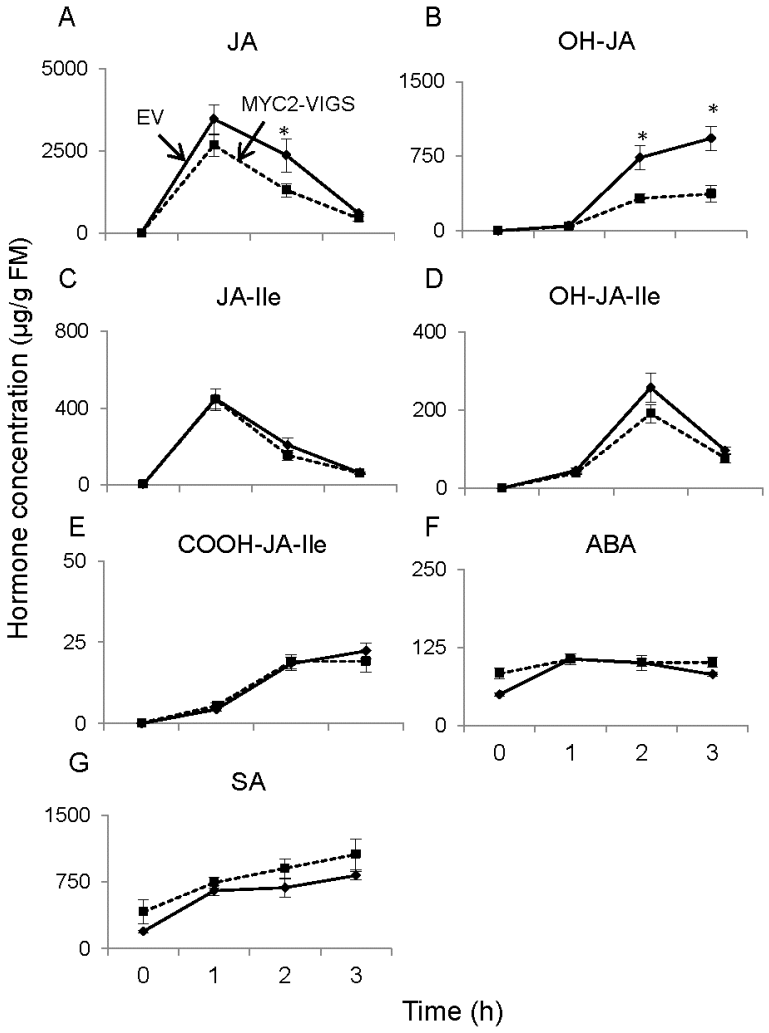

Figure S3. Accumulation of phytohormones in untreated and WOS-treated EV and MYC2-VIGS plants. Fully elongated leaves of EV and MYC2-VIGS plants were treated with WOS after 1, 2 , and 3 h or collected without treatment and the accumulation of JA (A), OH-JA (B), JA-Ile (C), OH-JA-Ile (D), COOH-JA-Ile (E), ABA (F) and SA (G) was measured on LC-MS^3. Differences that are statistically significant are indicated by asterisk ($P < 0.05$).

Table S1. Differentially regulated genes by NaMYC2 transcription factor in *N. attenuata.*

a. List of down-regulated genes in MYC2-VIGS plants

Probe ID	Fold change	BIN CODE	TAIR No	Name	Description
Na_454_35716	2.31	10.6	at5g62150	cell wall.degradation	peptidoglycan-binding LysM domain-containing protein
Na_454_11932	3.60	13.1.1.1.1	at5g17330	amino acid metabolism	GAD; calmodulin binding / glutamate decarboxylase
Na_454_38855	4.52	13.1.4.4.1	at1g74040	amino acid metabolism	IMS1, MAML-3, IPMS2 | IMS1 (2-ISOPROPYLMALATE SYNTHASE 1)
Na_454_02419	3.82	13.2.3.1.1	at3g16150	amino acid metabolism.degradation	L-asparaginase
Na_454_34394	3.75	13.2.3.1.1	at3g16150	amino acid metabolism.degradation	L-asparaginase, putative / L-asparagine amidohydrolase
Na_454_33674	4.58	13.2.4.4	at3g45300	amino acid metabolism.degradation	IVD (ISOVALERYL-COA-DEHYDROGENASE
Na_454_16864	2.69	16.1.5	at2g24210	secondary metabolism.isoprenoids.terpenoids	TPS10 (terpene synthase 10 ; (E-beta-ocimene synthase/ myrcene synthase)
Na_454_41485	9.09	16.1.5	at3g25810	secondary metabolism.isoprenoids.terpenoids	myrcene/ocimene synthase, putative
Na_454_26468	2.34	20.1.3.1	at4g02600	stress.biotic.signalling.MLO-like	ATMLO1 | MLO1; calmodulin binding
Na_454_11776	4.18	20.1.7	at1g69550	stress.biotic.PR-proteins	disease resistance protein (TIR-NBS class , putative)
Na_454_19854	2.13	20.1.7.6.1	at1g73325	stress.biotic.PR-proteins.proteinase inhibitors	trypsin and protease inhibitor family protein / Kunitz family protein
Na_454_40969	5.09	23.2	at5g18860	nucleotide metabolism.degradation	inosine-uridine preferring nucleoside hydrolase family protein
Na_454_12178	2.35	25.5	at2g38660	C1-metabolism	tetrahydrofolate dehydrogenase/cyclohydrolase
Na_454_23010	2.49	26.2	at4g38040	misc.UDP glucosyl and glucoronyl transferases	exostosin family protein
Na_454_35151	2.48	27.3.11	at5g22890	RNA.regulation of transcription.C2H2	zinc finger (C2H2 type family protein)
Na_454_36990	2.47	27.3.11	at1g10480	RNA.regulation of transcription.C2H2 zinc finger family	ZFP5 (ZINC FINGER PROTEIN 5
Na_454_13204	2.24	27.3.14	at3g05690	RNA.regulation of transcription.CCAAT box binding factor	NF-YA2 | NF-YA2 (NUCLEAR FACTOR Y, SUBUNIT A2 ; transcription factor)
Na_454_34042	4.27	27.3.25	at1g69560	RNA.regulation of transcription.MYB domain	MYB105 (myb domain protein 105
Na_454_39997	3.28	27.3.25	at1g69560	RNA.regulation of transcription.MYB domain	AtMYB105 (myb domain protein 105; DNA binding / transcription factor)
Na_454_28012	2.63	27.3.32	at1g80840	RNA.regulation of transcription.WRKY domain	WRKY40; transcription factor
Na_454_37824	4.03	27.3.32	at1g80840	RNA.regulation of transcription.WRKY domain	WRKY40, ATWRKY40 | WRKY40; transcription factor
Na_454_22020	4.04	27.3.57	at5g46910	RNA.regulation of transcription.JUMONJI family	transcription factor jumonji (jmj) family protein
Na_454_32324	3.44	27.3.62	at5g08630	RNA.regulation of transcription.Nucleosome/chromatin	DDT domain-containing protein
Na_454_36282	2.89	28.99	at3g13610	DNA.unspecified	oxidoreductase, 2OG-Fe(II oxygenase family protein)
Na_454_03136	5.81	29.4	at4g24730	protein.postranslational modification	calcineurin-like phosphoesterase family protein
Na_454_00400	4.18	29.4	at1g32640	protein.postranslational modification	ATMYC2, RD22BP1, JAI1, JIN1, MYC2, ZBF1 |transcription factor
Na_454_16548	3.29	29.5	at5g09640	protein.degradation	SNG2, SCPL19; serine-type carboxypeptidase/ sinapoyltransferase
Na_454_21861	2.36	29.5.5	at2g22980	protein.degradation.serine protease	SCPL13, Serine-type carboxypeptidase
Na_454_19373	2.67	29.5.7	at2g45040	protein.degradation.metalloprotease	matrix metalloproteinase
Na_454_40155	2.55	30.11	at4g15090	signalling.light	FAR1 (FAR-RED IMPAIRED RESPONSE 1 ; transcription factor)
Na_454_41435	2.31	30.2.17	at1g70520	signalling.receptor kinases.DUF 26	protein kinase family protein
Na_454_27831	2.11	30.3	at4g20780	signalling.calcium	calcium-binding protein, putative

Na_454_35735	2.58	33.1	at4g37070	development.storage proteins	PLP1, PLA IVA | patatin, putative
Na_454_14791	3.64	33.1	at3g63200	development.storage proteins	PLP9, PLA IIIB | PLP9 (PATATIN-LIKE PROTEIN 9
Na_454_33785	5.05	33.99	at5g16750	development.unspecified	TOZ (TORMOZEMBRYO DEFECTIVE
Na_454_41066	2.12	34.12	at1g05300	transport.metal	ZIP5; cation transmembrane transporter
Na_454_30201	2.39	34.2	at3g19930	transporter.sugars	ATSTP4 | STP4 (SUGAR TRANSPORTER 4
Na_454_31068	2.71	34.9	at1g12480	transport.metabolite transporters	OZS1, SLAC1, RCD3, CDI3 | OZS1 (OZONE-SENSITIVE 1 ; transporter)
Na_454_26329	3.86	35.1	at1g07850	not assigned.no ontology	transferase, transferring glycosyl groups
Na_454_14501	3.59	35.2	at5g24740	not assigned.unknown	protein localization

b. List of up-regulated genes in MYC2-VIGS plants

Probe ID	Fold change	BIN CODE	TAIR no	Name	Description
Na_454_36276	5.42	34.16	at1g17840	ABC transporters and multidrug resistance systems	WBC11 (WHITE-BROWN HOMOLOG COMPLEX PROTEIN 11)

Table S2. **List of primers used for the experiments**

Primer	FWD sequence (5'->3')	RVS sequence (5'->3')
VIGS_MYC2	ATATATATGGGCCCTGAAGAGAAATACAGTAAATGG	TGTGTGTGGTCGACGAGATCTAGTATTGGTTTCACA
NaPAL	TGCATACGCTGATGAC	TGGAAGATAGAGCTGTTCGC
NaAT1	TCACAAGGTTCACTTGTGGCTCTG	GCATTTGCCTTGAGTTTGCCTAGG
NaMYB8	AACCTCAAGAAACTCAGGACATACAA	GATGAATGTGTGACCAAATTTTCC
NaCV86	ATCAAATAGCTGAAGATGTC	CCAACAAAGTAGTGCTGTACT
NaDH29	GGCGGGCATTAATTCGTGCTTC	CCAAAAATGATTTGCAAGGTC

Chapter 6: Discussion

In the last decade, important discoveries were made that ushered our understanding of the jasmonate signaling, its components, its functions in plant defense and development and some mechanisms of regulation. Many studies that spanned over different families of plants and herbivores informed us of the level of overall conservation of the jasmonate-mediated responses across different plant families, while alerting us of some variations. The bioactive jasmonate, its receptor complex and the repressors of the jasmonate signaling were identified and a mechanism of degradation of the repressors that releases the transcriptional regulators of downstream processes was proposed (Anderson *et al.* 2005, Chini*, et al.* 2007, Fernandez-Calvo*, et al.* 2011, Fonseca*, et al.* 2009, Goossens *et al.* 2003, Kombrink 2012, Mosblech *et al.* 2011, Pauwels *et al.* 2010, Pauwels and Goossens 2011, Sheard*, et al.* 2010, Thines*, et al.* 2007, VanDoorn*, et al.* 2011, Wasternack and Kombrink 2010).

There are, however, still open questions that need to be addressed. The initial process by which plants recognize herbivore attack, the sources of the elicitors of defense responses (whether they are plant- or insect-driven) and the modalities of the cross-talk among the different phytohormone signaling pathways are yet to be determined. Considering the heterogeneity of the plant habitat, our knowledge about the mechanisms by which plants achieve specific response or the receptors, ligands or interactions that mediate these processes is scanty.

For this work, we honed in on the following general research questions: How do *N. attenuata* plants regulate defense responses against herbivores? What is the role of MYC2 TF in *N. attenuata*? How do plants attenuate the jasmonate burst? How do these regulatory processes affect the ecological interactions of this species with its herbivore community? To answer these

and related questions, we used the molecular, ecological and analytical platforms developed in our group to understand the ecological interactions between *N. attenuata* and its natural herbivores.

In this thesis, two regulatory mechanisms that control jasmonate-mediated defense responses of *N. attenuata* against herbivore attack were described. First, we identified a novel herbivore-inducible hydrolase in *N. attenuata* (named as jasmonoyl-L-isoleucine hydrolase 1, JIH1) that regulated herbivore-induced JA-Ile levels and JA-dependent direct and indirect defenses. Our findings about this novel regulator were reported in manuscript I. Second, we identified an herbivore-induced MYC transcription factor in *N. attenuata* (NaMYC2) that mediated jasmonate-dependent defense metabolite accumulation. Manuscript II reviewed transcriptional regulation of defense responses taking MYC2 as a focal point, while manuscript III reported the findings of the second regulatory mechanism.

To illustrate the function of jasmonoyl-l-Ile hydrolase 1 (JIH1) in *N. attenuata*, a reverse genetic approach (based on RNAi silencing technique) was used to knock down its expression. The study showed that this hydrolase attenuates the herbivore-induced JA-Ile burst *in vivo*. Consequently, the silenced irJIH1 plants accumulated more direct (e.g. nicotine, HGL-DTGs, TPIs) and indirect (e.g. trans-α-bergamotene, α-duprezianene) defenses than wild type plants and were more protected from herbivory. These results signify the ecological relevance of JIH1-dependent defense attenuation process in mediating the interactions of this plant with its herbivore community.

The importance of this mechanism was also highlighted in the field where irJIH1 plants were transplanted into their native habitat, treated with continuous WOS induction and the

accumulations of JA-Ile and its metabolites were measured. With every induction, irJIH1 plants accumulated more JA-Ile than wild type plants, and later, channeled the excess JA-Ile into its inactive catabolic products, OH-JA-Ile and COOH-JA-Ile. This provided enough evidence to conclude that hydrolysis (by JIH1) and hydro-/carboxylation by the recently identified cytochrome p450 enzymes (CYP94B3 and CYP94C1) (Heitz*, et al.* 2012, Kitaoka*, et al.* 2011, Koo*, et al.* 2011) are complementary pathways that plants use to attenuate the JA-Ile burst. By regulating the expression of JIH1, plants regulate the duration of the jasmonate burst that, in turn, regulates the direct and indirect defenses.

The close homologue of JIH1 in *A. thaliana*, indole acetic acid alanine resistant 3 (IAR3), belongs to the ILR1 family of hydrolases. Hydrolases in the ILR1 family release free IAA from its amino acid-conjugated forms. As amino acid conjugates of IAA are often inactive, activation of IAA requires hydrolysis of these conjugates by these enzymes. Specifically, IAR3 is annotated as an IAA-Ala hydrolase (Bartel and Fink 1995, Davies *et al.* 1999, LeClere *et al.* 2002, Rampey *et al.* 2004, Savic *et al.* 2009, Schuller and Ludwig-Muller 2006). Pursuant to this, the heterologously expressed JIH1 was enzymatically active against both JA-Ile and amino acid conjugates of IAA *in vitro*. These findings point to the possibility in which JIH1 may mediate the functional cross-talk between IAA and JA signaling during herbivory. Examples of such cross-talks between JA and IAA signaling pathways are already reported (Grunewald *et al.* 2009, Vanstraelen and Benkova 2012). However, compared to wild type plants, no significant differences were found in the herbivore-induced accumulation of IAA (but JA-Ile) in irJIH1 plants. From this observation and the specific induction of JIH1 transcripts in response to herbivory, it is possible to predict that the *in vivo* activity of JIH1 might be contextually determined: by herbivory, development or both. And, to achieve these context-dependent

responses, plants may be required to compartmentalize the activity of JIH1 temporally or spatially. In other words, plants could either produce the enzyme only when it is needed or store the enzyme in inactive forms till the need arises.

Consistent to these predictions, we found a variant of the endoplasmic reticulum localization signal sequence (HDEL) at the C-terminus of the full length sequence of JIH1. In plants, variants of this signal sequence (HDEL or KDEL) function in targeting (and retaining) newly produced proteins to the ER. So, the HDEL sequence of JIH1 proteins likely targets nascent JIH1 proteins to the ER and retains them until they are needed. It seems that cleavage of this signal peptide may be required to release the hydrolase out of the ER into places where its functions are needed (Gomord *et al.* 1997, Napier *et al.* 1992, Pagny *et al.* 2003). Testing this hypothesis requires protein localization studies with the full length or a truncated form JIH1 protein lacking the signal peptide (HDEL).

There is a very fascinating possibility by which plants could compartmentalize the functions of IAR3/ JIH1 into different parts of the plant and harness the benefits of this bifunctional enzyme. This could allow plants to use the enzyme to mediate defense processes in one tissue and development processes in the other or respond to different stress conditions in different tissues. In a recent study, Kinoshita *et al*., (2012) exposed hydroponically grown *A. thaliana* plants to osmotic stress and used deep sequencing to identify miRNA-mRNA pairs that respond to the stress. Interestingly, as a new target of the miR167a, they identified IAR3 and showed the inverse relationship in the expression patterns of miR167a and IAR3 in response to osmotic stress. More interestingly, plants that expressed cleavage resistant forms of IAR3 developed more lateral root and were more tolerant of osmotic stress indicating a new role of IAR3 in regulation of developmental processes. Considering the high level of conservation of the

miR167a in different species, it is rather reasonable to predict the generality of this process in several plant families.

Our work is in agreement with the general notion that the basic framework of the jasmonate signaling and response is largely conserved among plants of different families. Homologues of the enzymes for JA/ JA-Ile biosynthesis (e.g. LOX, AOX, AOC), the components of JA-Ile perception (e.g. COI1) and the transcriptional regulators (e.g. MYC2, MYB, WRKY) are characterized in different plant species (Boter*, et al.* 2004, Heim *et al.* 2003, Katsir*, et al.* 2008, Wang *et al.* 2008, Wang *et al.* 2005, Wasternack *et al.* 2006, Xie*, et al.* 1998, Zhao*, et al.* 2013, Zhao *et al.* 2011). However, over the course of their evolutionary history components of plant defense responses must have diverged; variations are observed in the functions of downstream regulatory elements. An example could illustrate this point: in both *N. attenuata* and *A. thaliana*, similar numbers of JAZ repressors are reported with fairly similar sequences that may predict functional homology. However, distinct differences are reported in the functions of some JAZ proteins between the two species (Oh *et al.* 2012). In another case, transgenic *Solanum nigrum* plants that had reduced SnLOX3, SnCOI1 and SnJAR4 expressions were generated to study the jasmonate signaling and defense responses in this wild Solanaceous species. Again, the study indicated a variation of the jasmonate signaling cascade in this species (VanDoorn*, et al.* 2011). Interestingly, natural variations were observed in induced JA and JA-Ile accumulation among wild populations of *N. attenuata* in the field site in Utah, USA (Kallenbach *et al.* 2012).

As members of the highly conserved bHLH family of TFs, MYC transcription factors share considerable similarities with each other. The similarities in their sequence and conservation of important regulatory domains, as is the general case for bHLH members,

indicate functional homology (Carretero-Paulet *et al.* 2010, Heim*, et al.* 2003, Pires and Dolan 2010). We have a case supporting this suggestion. In *N. attenuata*, similar to *N. tabacum* and *N. benthamiana*, MYC2 regulated the accumulation of nicotine indicating the functional conservation of MYC2 TFs. In *N. tabacum*, MYC2 interacted with ERF or homeodomain TFs to regulate nicotine biosynthesis (Shoji and Hashimoto 2011, Shoji*, et al.* 2010, Shoji *et al.* 2008, Todd*, et al.* 2010). The generality of the later interactive co-regulation needs to be studied in other members of the genus *Nicotiana*.

On the other hand, there are differences in the regulatory role of MYC2 transcription factors between *A. thaliana* and *N. attenuata*. A transcriptomic study in *A. thaliana* plants indicated that in response to MeJA treatment, the expressions of many jasmonate biosynthesis genes were up-regulated by MYC2 indicating that MYC2 is a positive regulator of jasmonate biosynthesis. At the same time, the report indicated that MYC2 negatively regulates its own transcription (Dombrecht*, et al.* 2007, Fernandez-Calvo*, et al.* 2011, Lorenzo *et al.* 2004). No such MYC2-dependent transcriptional feedback control mechanisms were observed in *N. attenuata* hinting the variations in downstream processes.

When attacked by herbivores, *N. attenuata* plants produce potent direct chemical defense compounds: nicotine, HGL-DTGs, phenolamides and trypsin protease inhibitors (Heiling *et al.* 2010, Onkokesung*, et al.* 2012, Steppuhn*, et al.* 2004). However, NaMYC2 regulated a relatively small portion of the JA-dependent defense metabolites in *N. attenuata* that suggests that other JA-dependent transcriptional regulator (s) may be involved in the regulation of these classes of metabolites. An interesting pattern was observed in accumulation of phenolamides when EV and MYC2-VIGS *N. attenuata* plants were WOS-induced and the transcript accumulations of phenolamide biosynthetic genes were compared with the metabolite accumulation. Despite the

significant reduction in the transcript accumulations of phenolamide biosynthetic genes, no significant differences were observed in the accumulations of the metabolites between EV and MYC2-VIGS plants. One of the possible reasons forwarded was that the herbivore-inducibility of phenolamides decreased as plants started flowering. In agreement to this, in a recent report, the reduced inducibility of some metabolites was restored in flowering plants few days after the removal of the flowers (Diezel *et al.* 2011, Meldau*, et al.* 2012a) suggesting that the extent of protection that plants offer to their tissues is determined by the value that the tissues add to their overall fitness. How do plants know the values of their tissues? Could transcription factors (e.g. MYC2) be involved in the process of determining the values of tissues? The plant circadian clock might hold the answer to these questions.

In plants, defense responses are strictly regulated by the diurnal cycle (Kim *et al.* 2011), which means that plants have to synchronize their circadian clock with their defense response cascades. There are several interesting reports that demonstrate how plants' responses to pathogen- or herbivore-attack are influenced by the circadian clock (Bhardwaj *et al.* 2011, Goodspeed *et al.* 2012, Roden and Ingle 2009). Recently, a regulatory relationship has been described between TIME FOR COFFEE, a component of the plant circadian clock, and MYC2 that influences the diurnal defense responses (Shin *et al.* 2012). These observations allow us to reasonably propose a link between MYC2 regulation and optimal plant defense. We speculate that as master regulators of the jasmonate signaling cascade and multifunctional interactors (Cevik *et al.* 2012, Chen *et al.* 2011, Chen *et al.* 2012, Gangappa *et al.* 2010, Hong *et al.* 2012, Lackman *et al.* 2011, Wild *et al.* 2012), MYC2 TFs may be involved in setting the priorities among plant tissues, and hence, contribute to plant fitness. This tantalizing possibility needs further experimental support.

In conclusion, we described two novel regulatory mechanisms in *N. attenuata* that control plant defense responses against herbivores. A novel hydrolase and a new mechanism of attenuation of the jasmonate burst, and consequently defense responses, were identified in *N. attenuata*. The influences of the attenuation mechanisms on the ecological interaction of *N. attenuata* with its herbivore community were also demonstrated. Moreover, the roles of the *N. attenuata* MYC2 in transcriptional regulation of defense responses against herbivores and their consequences were described. In the process, the following interesting questions were identified:

- Why is the locally-induced transcript level of JIH1 lower in WOS compared to WW treatment?
- Why regulates defense responses not regulated by MYC2 in *N. attenuata*?
- What other mechanisms of attenuation of defense exist in plants?
- Are there additional compounds/mechanisms that plants use to inactivate the jasmonates?
- How do MYC2/ JIH1 transcription factors mediate cross-talk among different hormone signaling pathways?
- Are MYC2 TFs involved in the regulation of indirect defenses in *N. attenuata*?

Future research addressing these questions will help us identify the positions of these novel regulators on the regulatory map of plant defense and development.

Summary

The jasmonate signaling is one of the most studied hormone signaling pathways in plants that mediate important physiological processes. When plants are attacked by herbivores, they induce the biosynthesis of huge pools of jasmonic acid (JA) and the biologically active (+)-7-*iso*-jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is perceived by the receptor complex (SCF^{COI1}) and facilitates degradation of the repressors of the jasmonate signaling and activation of MYC2 transcription factors (TFs). MYC TFs, then, orchestrate downstream jasmonate responses. Few hours after the initial attack, the levels of JA and JA-Ile wanes to the basal level; however, no mechanism has been identified so far that attenuates defense responses by hydrolysis of JA-Ile.

Due of the cost of defense responses, plants regulate the duration and amount of responses. Here, we report two novel regulatory mechanisms that regulate herbivore-induced plant defense responses in *N. attenuata*. The first regulatory mechanism involves a hydrolase we identified (Jasmonoyl-L-isoleucine hydrolase 1, JIH1) in *N. attenuata*. JIH1 hydrolyses JA-Ile and attenuates the JA-Ile burst *in planta*; in a manner that mirrors the *in vitro* hydrolytic activity of heterologously expressed JIH. When JIH1 was silenced by inverted-repeat silencing, the transformed plants (irJIH1 plants) accumulated significantly more JA-Ile and direct/indirect defenses. The performances of a specialist (*Manduca sexta*) and generalist (*Spodoptera littoralis*) herbivores were significantly lower on irJIH1 plants compared to wild type plants. Our data signify the importance of this regulatory mechanism in plant defense regulation.

The second mechanism concerned a MYC transcription factor (NaMYC2) that we identified and its role in plant defense regulation in *N. attenuata*. We silenced MYC2 transcript level using virus induced gene silencing technique and characterized the transformed plants using

transcriptomic, metabolomics and ecological methods. We showed that the herbivore-inducible NaMYC2 transcription factor regulates the biosynthesis of nicotine and phenolamides in *N. attenuata*: defense compounds that shape the ecological interaction of this species in it native habitat. However, the accumulation of other secondary metabolites (e.g. HGL-DTGs, TPI) was not MYC2-dependent indicating that other JA-dependent regulatory mechanisms might be involved in the regulation of these classes of compounds.

We conclude that despite the overall conservation of the components of the jasmonate signaling cascade among plants of different families, variations exist that help each species to fine-tune its defense responses.

Zusammenfassung

Der Jasmonsäure-Signalweg ist einer der am meisten studierten Hormonsignalwege in Pflanzen, welcher vor allem wichtige physiologische Prozesse reguliert. Wenn Pflanzen von Fraßfeinden angegriffen werden, wird ein großer Pool von Jasmonsäure (JA) und dem biologisch aktiven (+)-7-*iso*-jasmonoyl-L-isoleucin (JA-Ile) induziert. JA-Ile bindet an den Rezeptorkomplex (SCF^{COI1}) und verursacht dadurch einen Abbau von Repressoren des Jasmonsäure Signalweges und die Aktivierung von MYC2 Transkriptionsfaktoren (TFs). MYC TFs regulieren die Jasmonsäure Antworten. Wenige Stunden nach der ersten Attacke, sinken die JA und JA-Ile Level wieder auf den Ausgangszustand; dennoch wurde bis heute kein Mechanismus zur Hydrolyse von JA-Ile identifiziert.

Auf Grund der hohen Kosten für Verteidigungsantworten, regulieren Pflanzen die Dauer und die Menge dieser Reaktionen. In dieser Arbeit berichte ich von zwei neuen Regulationsmechanismen in der Herbivor-induzierten Pflanzenverteidigung von *N. attenuata*.

Der erste Mechanismus involviert eine von mir identifizierte Hydrolase (Jasmonoyl-L-isoleucine hydrolase 1, JIH1) in *N. attenuata*. JIH1 hydrolysiert JA-Ile und verstärkt den JA-Ile Anstieg *in planta*; in einer ähnlichen Art und Weise wie die hydrolytische Aktivität von heterolog exprimierter JIH1 *in vitro*. Nach Ausschaltung von JIH1 mittels „inverted- repeat silencing“ zeigen die transformierten Pflanzen (irJIH1) einen starken Anstieg in JA-Ile und direkten/indirekten Verteidigungsantworten. Der Spezialist *Manduca sexta* und der Generalist *Spodoptera littoralis* wachsen sehr viel schlechter auf irJIH1 Pflanzen im Vergleich zu Wildtyp Pflanzen. Meine Daten bestärken die Wichtigkeit dieses Regulationsmechanismus in der Verteidigung von Pflanzen.

Der zweite Mechanismus zeigt die Funktion eines von mir identifizierten MYC Transkriptionsfaktors (NaMYC2) in der Verteidigungsregulation von *N. attenuata*. Mittels Virus induziertem Gen- „Silencing“ wurde das Transkript Level von MYC2 in der Pflanze gesenkt, diese Pflanzen wurden dann mit Hilfe von Transkriptions-, Metabolom- und Ökologischen Methoden untersucht. Ich konnte zeigen, dass der Herbivor-induzierte Transkriptionsfaktor NaMYC2 die Biosynthese von Nikotin und Phenolamiden in *N. attenuata* reguliert: beides sind Verteidigungskomponenten welche die Interaktion dieser Pflanzenart in ihrem natürlichen Lebensraum stark beeinflussen. Die Akkumulierung anderer sekundärer Stoffe (z. Bsp. HGL-DTGs, TPI) war nicht MYC2-abhängig, dies zeigt, dass wahrscheinlich noch andere JA-abhängige Mechanismen in die Regulierung dieser Komponenten involviert sind.

Unabhängig davon, dass die Elemente der Jasmonsäure Kaskade in verschiedensten Pflanzenfamilien konserviert sind, existieren also auch Variationen, die jeder einzelnen Art helfen, ihre Verteidigungsantworten an ihre ökologischen Bedürfnisse anzupassen.

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Curriculum Vitae

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List of Publications

Woldemariam, M., Baldwin, I. T., Galis, I. (2011). Transcriptional regulation of plant inducible defenses against herbivores: A mini-review. Journal of Plant Interactions, 6(2), 113-119.

Woldemariam, M., Onkokesung, N., Baldwin, I. T., Galis, I. (2012). Jasmonoyl-L-Isoleucine Hydrolase 1 (JIH1) regulates jasmonoyl-L-isoleucine levels and attenuates plant defenses against herbivores. The Plant Journal.

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Oral presentations

Woldemariam M. A novel hydrolase function of Indoleacetic acid alanine resistant 3 (IAR3) against JA-Ile: an important link to plant defense signaling. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2011.

Poster presentations

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Woldemariam M. INDOLEACETIC ACID ALANINE RESISTANT 3 (IAR3) is a jasmonoyl-L-isoleucine hydrolase that attenuates plant defense against herbivores in *Nicotiana attenuata*. 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2012.

Woldemariam M. The MYC2 - MYC2-like transcriptional factors in plant defense regulation. ICE Symposium, MPI for Chemical Ecology, Jena, DE, Sep 2011.

Woldemariam M. IAR3 as a potential JA-Ile hydrolase. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE, Feb 2010.

Eigenständigkeitserklärung

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

_____

Melkamu Gezahagne Woldemariam

Jena, den 16. Mai, 2013