# Multiple functions of Jasmonate ZIM domain (JAZ) proteins in *N. attenuata* plants: growth, development, and defense against herbivores

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# General Introduction

### *History of plant-herbivore interactions*

Dispersed fossils provide the earliest evidence for plant-(arthropod) herbivore interactions. Since the first land plants appeared ca. 450 million years ago, plant and herbivores formed one of the earliest terrestrial communities. That plants have been intensively consumed by herbivores since their arrival on land is shown in fossils with herbivory-damaged plant structures, e.g. spores, stems, leaves, roots and seeds (Labandeira, 1998; Wellman and Gray, 2000). Maximizing fitness – contributing as many grandchildren as possible to following generations – is the ecological goal of both plants and herbivores (and all other organisms); however, herbivore attacks increase plant mortality and decrease plant growth and reproduction, and plant defense mechanism increase herbivore mortality and decrease herbivore growth and reproduction. This antagonistic relationship has formed the basis for strong natural selection pressure and spurred the evolution of new species of both plants and herbivores. Herbivores have co-evolved with plants, overcome their defenses and even utilizing defense compounds, while plants have evolved to increase their resistance against herbivore attack (Ehrlich and Raven, 1964; Berenbaum and Zangerl, 1998)

However, not all plant-herbivore interactions are equal. Among plant species and populations, there are different types of herbivory and levels of damage can be limited by geographic availability, structure, mechanical characteristics (physical barriers such as trichomes, thorns, resine, etc.) and chemical components (toxic compounds, e.g. nicotine, glucosinolates, phenolic compounds, etc.) of plants (Ehrlich and Raven, 1964). Around 90% of herbivores are specialized to feed on a limited number of plants families (less than three different plant families) based on plant defense responses; these are known as specialist herbivores. Some herbivores can feed on a wide-range of plants and we usually refer to them as generalist herbivores (Futuyma and Gould, 1979; Meijden, 1996).

### *Plant defense responses*

Plants are frequently exposed to various abiotic and biotic stresses such as high light, water deficit, salinity stress, variable temperature, lack of nutrients, pathogen and

herbivore attack. To survive in a multitude of harmful environmental conditions, plants have evolved sophisticated defense systems which can be categorized into two major types: direct defense and indirect defense. These can be either of constitutive or induced character (Kessler and Baldwin, 2002; Wu and Baldwin, 2010; Meldau et al., 2012).

*Direct defense vs. indirect defense*

Direct defense is a local mechanism that negatively affects herbivore growth and reproduction. It is mainly represented by mechanical defenses (physical structures) and chemical defenses (Schardl and Chen, 2001; Kessler and Baldwin, 2002). Mechanical defenses can be defined as various morphological or anatomical trait that leads to a fitness advantage for the plant by directly deterring herbivore feeding, for example sharp prickles, spines and thorns (spinescences), trichomes, resins, lignins and silica (Fordyce and Agrawal, 2001; Hanley et al., 2007; Konno, 2011). Chemical defenses are secondary metabolites which increase plant fitness in the face of herbivory by deterring herbivore performance, such as protease inhibitors (PIs) that can affect insect digestive enzymes in the gut (Jongsma et al., 1995; Zavala et al., 2004b; Hartl et al., 2010), toxic compounds (e.g., glucosinolates, alkaloids, terpenoids, phenolics) which are directly poisonous to herbivores, and generally any compounds which lead to appetite suppression, stupor, or death in herbivores (Duffey and Stout, 1996; Pichersky and Lewinsohn, 2011; Mithöfer and Boland, 2012).

Indirect defenses do not directly affect herbivore performance, but attract natural enemies of herbivore such as predators and parasitoids that remove herbivores from plants (Halitschke et al., 2000; Kessler and Baldwin, 2002; Kessler and Heil, 2011). Release of volatile organic compounds (VOCs) from plants acts as a guide for natural enemies to reveal the location of feeding herbivores (Paschold et al., 2006; Dicke, 2009; Allmann and Baldwin, 2010). VOCs can also act as direct defenses by repelling ovipositing herbivores and thus avoiding infestation a single tobacco hornworm larvae requires several host plants to complete development (Kessler and Baldwin, 2001). Apart from VOCs, plants provide nutrients such as extrafloral nectar to attract natural enemies of herbivores (Heil et al., 2004; Choh et al., 2006).

*Constitutive defense vs. induced defense*

Plant defense responses can be categorized into two groups according to the timing of the presence of physical and chemical defensive compounds in plants. If these traits are expressed constantly, they are categorized as constitutive defenses. Defenses that are expressed only after herbivore attack are called induced (inducible) defense (Howe and Jander, 2008). Induced defense responses are very common compared to special cases of constitutive defense (Karban and Myers, 1989; Karban et al., 1997). For plants, it is important to minimize the cost of defenses because they have limited resources; in case of constitutive defense, (1) plants have to always allocate a certain amount of their resources to defense and consequently these resources are never available for growth and reproduction, and (2) plants have to produce many kinds of defensive metabolites to cope with unpredictable environment. In the case of induced defense responses, plants only need to allocate resources to defense when herbivore attack occurs. Although inducible defense mechanisms save on costs compared to constitutive defenses, induced defenses have one substantial disadvantage, which is the time lag between the first attack and the actual activation of the defense. In other words, plants have to cope with herbivores for hours or even days until defenses can be activated.

This has long been a matter of intense discussion: why are induced defenses found so commonly in the more than 110 studied plant-herbivore interactions? Some experimental evidence suggests that some metabolites toxic to herbivores can be also toxic to plants. To avoid this negative effect, plants constitutively express the inactive form of toxic compounds, and quickly activate them only after herbivore attack. Also, plants are in unpredictable environments; constitutive defense has no lag time, but is inflexible, whereas induced defenses allow plants to respond to different types of damage, in different locations within an individual plant. In addition, previous studies showed that plants can remember certain herbivory patterns and develop priming and/or vaccination strategies to quickly respond to herbivores (Baldwin, 1998; Agrawal, 1999; Kessler and Baldwin, 2002; Cipollini et al., 2003; Kessler and T. Baldwin, 2004; Voelckel and Baldwin, 2004; Zavala et al., 2004a; Frost et al., 2008; Steppuhn and Baldwin, 2008; Mithöfer and Boland, 2012). Interestingly, Karban and Baldwin (Karban and Baldwin, 1997) suggested that plants may have originally evolved only induced defense systems to respond to unpredictable

environmental variability, and constitutive defenses may have come later under particular conditions in some plants.

***Jasmonic acid: important phytohormone for plant growth, development and defense***

Jasmonic acid (JA) is an oxylipin (oxygenated fatty acid) synthesized from membrane-derived fatty acids (16:3 or 18:3) via the well-characterized octadecanoid pathway in chloroplasts and peroxisomes (reviewed in (Schaller and Stintzi, 2009)). JA can be metabolized to several other derivatives – collectively referred to as jasmonates – such as methylated JA (MeJA; methyl jasmonic acid) by methyltransferases or it can be conjugated to amino acids (JA-Ile, JA-Leu, JA-Val, etc.) by jasmonate resistant (JAR) enzymes. Jasmonate biosynthetic and metabolic pathways are highly conserved in mosses, fungi, gymnosperms and angiosperms.

Jasmonates are important plant hormones known to regulate plant growth, development and defense against abiotic and biotic stress. Processes influenced by jasmonate signaling include root growth (Staswick et al., 1992), trichome initiation (Li et al., 2004; Qi et al., 2011), fruit ripening (Pérez et al., 1997; Fan et al., 1998), anthocyanin accumulation (Shan et al., 2009), senescence (Parthier, 1990; He et al., 2002; Shan et al., 2011), pollen and flower development (McConn and Browse, 1996; Stintzi and Browse, 2000; Li et al., 2004; Mandaokar et al., 2006), and defense in response to wounding (Glauser et al., 2008), herbivore attack (Halitschke and Baldwin, 2003; Glazebrook, 2005; Zavala and Baldwin, 2006; Browse and Howe, 2008) and pathogen infection (Vijayan et al., 1998). These data suggest that jasmonates are the crucial natural integrators of plant defense and development (reviewed in (Wasternack, 2007; Balbi and Devoto, 2008; Howe and Jander, 2008)). To date, jasmonate-synthesis- or perception-deficient plants have been used to conduct many advanced functional studies of jasmonates, revealing their ubiquitous function in plants' defense and development.

***Negative regulators of Jasmonate signaling, Jasmonate ZIM domain proteins, and their interaction partners***

Jasmonate ZIM domain (JAZ) proteins belong to the previously characterized family of ZIM (Zinc-finger protein expressed in Inflorescence Meristem) proteins. They were identified as key regulators of jasmonate signaling that are ubiquitously found in

many plants species including *Arabidopsis*, rice, tomato, and tobacco plants (Chini et al., 2007; Thines et al., 2007; Shoji et al., 2008; Ye et al., 2009; Seo et al., 2011; Sun et al., 2011; Ismail et al., 2012; Oh et al., 2012). JAZ proteins are characterized by two highly conserved motifs, TIF[F/Y]XG (or its variant) (Shikata et al., 2004; Vanholme et al., 2007) and Jas (S-L-X(2)-F-X(2)-K-R-X(2)-R) (Yan et al., 2007; Melotto et al., 2008), required for the regulation of jasmonate-responsive genes. These conserved motifs are essential for functional jasmonate signaling: the TIFY motif is typically located in the N-terminal part of the protein and is involved in homo- and heteromeric interactions among JAZ proteins (Chini et al., 2009; Chung and Howe, 2009) as well as it interactions with the JAZ/co-repressor NINJA-TPL complex (Pauwels et al., 2010). The Jas motif is typically located in the C-terminus of JAZ proteins and is required for binding of several core- ($SCF^{COI1}$ complex, MYC2/3/4) and co-regulatory proteins (EIN3/EIL1, MYB21/24, TT8/GL3/EGL3 and DELLA) that induce or control downstream processes in jasmonate signaling (reviewed in (Browse and Wager, 2012)). The Jas motif has also been shown to contribute to the stability of JAZ proteins via a yet-unknown mechanism (Yan et al., 2007; Chung et al., 2009).

***JAZ-mediated jasmonate signalling***

Since the discovery of core components in jasmonate signalling: the CORNATINE INSENSITIVE1 (COI1) protein, an F-box protein acting as a receptor of jasmonates (Feys et al., 1994; Xie, 1998; Devoto et al., 2005; Katsir et al., 2008; Paschold et al., 2008; Yan et al., 2009; VanDoorn et al., 2011; Ye et al., 2012), Jasmonate ZIM domain (JAZ) proteins identified as negative regulators of jasmonate signaling (Chini et al., 2007; Thines et al., 2007; Shoji et al., 2008; Ye et al., 2009; Seo et al., 2011; Sun et al., 2011; Ismail et al., 2012; Oh et al., 2012) and the bioactive jasmonate (+)-7-iso-JA-L-Ile (JA-Ile) (Fonseca et al., 2009; Suza et al., 2010; Koo et al., 2011), knowledge of jasmonate-mediated plant responses is dramatically expanding. In the presence of the active hormone JA-Ile, JAZ proteins are degraded by the action of the $SCF^{COI1}$-E3 ubiquitin ligase complex and 26S proteasome, releasing jasmonate-induced transcription factors (e.g. MYC2/3/4) from repression, and triggering expression of jasmonate-dependent genes (reviewed in (Kazan and Manners, 2008)). While upstream jasmonate signaling pathways (JA-Ile-$SCF^{COI1}$ complex-JAZ interactions) apparently share many common features and retain a high

degree of conservation in higher plants, downstream jasmonate signaling is significantly more variable, with various kinds of interacting partners involved. Recently, significant progress has been made in the identification of co-regulators of the core complex (JA-Ile-$SCF^{COI1}$ complex-JAZ), target transcription factors, and proteins downstream of jasmonate signaling in plants.

*Co-regulators of JAZ proteins to repress downstream transcriptional events*

JAZ proteins are generally known to act as transcriptional repressors; however, it is still unclear how JAZ proteins repress transcription of target transcription factors at the molecular level. Recent studies have uncovered interacting proteins and small molecules:

(1) $InsP_5$ (inositol pentakisphosphate) was found via mutant screening to act as a co-receptor of COI1-JAZ by interacting with amino acid residues of COI1. The *Arabidopsis ipk1-1* mutant, which has more $InsP_5$, showed elevated expression of the wound- and jasmonate-induced genes *AtWRKY70* and *AtAOS*, stronger root growth inhibition and reduced performance of caterpillars compared to WT plants (Stevenson-Paulik et al., 2005; Sheard et al., 2010; Mosblech et al., 2011).

(2) JAZ proteins recruit a co-repressor, the TPL(TOPLESS)-NINJA(Novel Interactor of JAZ) complex, which contains the EAR (ERF-Associated Amphiphilic Repression) motif (LxLxLx or DLNxxP) well known to function in gene repression (Kazan, 2006; Szemenyei et al., 2008). These proteins therefore play a crucial role in repression of multiple jasmonate responsive genes and pathways in *Arabidopsis* (Pauwels et al., 2010). Interestingly, a subgroup of JAZ proteins (AtJAZ5, AtJAZ6, AtJAZ7 and especially, AtJAZ8) contains highly conserved EAR (LELRL) or EAR-like (DLNEPT) motifs, allowing the recruitment of TPL protein without NINJA assistance to repressed jasmonate signaling such as root growth through MYC2 in *Arabidopsis* (Shyu et al., 2012).

(3) DELLA proteins are important repressors of gibberellic acid (GA) signaling that promote growth and repress defense-related gene transcript accumulation, proposed to be essential for fully elicited jasmonate-mediated responses (Hou et al., 2010; Wild et al., 2012). DELLA proteins can directly interact with C-terminus of JAZ1 and JAZ8 proteins in *Arabidopsis* and they repressed interaction of JAZ and MYC2, which led to increased ability of MYC2 to activate defense-related gene expression and inhibit root growth.

Notably, DELLA proteins play an antagonistic role to the NINJA-TPL complex in JAZ-mediated jasmonate signaling.

*Transcription factor targets of JAZ repressors*

The bHLH transcription factor (TF) MYC2 was the first identified repression target of JAZ proteins in *Arabidopsis*. MYC2 is known to regulate the majority of jasmonate-responsive genes after wounding and herbivore attack, as well as jasmonate-mediated root growth inhibition (Boter et al., 2004; Lorenzo et al., 2004; Chung et al., 2009). MYC2-like genes have been identified in many other plant species: MYC2 regulates nicotine biosynthesis in *Nicotiana* spp. (Todd et al., 2010; Shoji and Hashimoto, 2011) and tolerance to drought stress in rice (Seo et al., 2011). In addition, bHLH-type TFs in *Arabidopsis* (AtMYC3 and AtMYC4) and *N. tabacum* (NtMYC2a, NtMYC2b and NtMYC2c) were identified; these new genes showed both redundant and specific functions in the regulation of jasmonate-responsive genes (Fernandez-Calvo et al., 2011; Niu et al., 2011; Zhang et al., 2012).

Recently, the R2R3-MYB transcription factors MYB21 and MYB24, and the WD-repeat/bHLH (GL3, EGL3, TT8)/MYB75 complex were identified as targets of JAZ proteins in *Arabidopsis*: MYB21, MYB24, and the WD-repeat/bHLH (GL3, EGL3, TT8)/MYB75 complex directly interact with the C-termini of JAZ proteins; MYB21 and MYB24 regulate male fertility in flowers by controlling pollen maturation, anther dehiscence, and filament elongation, and the WD-repeat/bHLH (GL3, EGL3, TT8)/MYB75 complex controls jasmonate-regulated anthocyanin accumulation and trichome initiation (Qi et al., 2011; Song et al., 2011).

Ethylene (ET) and jasmonates are also known to synergistically regulate plant development and defense responses (Robert-Seilaniantz et al., 2011). Recently, Zhu et al. (2011) demonstrated that AtJAZ directly interacts with EIN3/ EIL1 transcription factors, which are positive regulators of jasmonate/ET-responsive defense-related genes and jasmonate-induced root hair development, but not fertility or pigment metabolism (Zhu et al., 2011). They showed that AtJAZ directly interacts with EIN3/EIL1 transcription factors and represses their transcriptional activity together with the co-repressor HDR6 that modulates de-acetylation of histones.

*Diversity and specificity of JAZ proteins in* N. attenuata *plants*

**In chapter 3**, I identified 12 novel JAZ protein family members in the native tobacco *Nicotiana attenuata,* and conducted a functional study of NaJAZh protein in plant defense against herbivores and plant development, both in the glasshouse and in the native habitat of *N. attenuata* (Great Basin Desert, Utah, USA). We demonstrate that silencing *NaJAZh* deregulates a subset of inducible direct defense compounds: trypsin proteinase inhibitors (TPIs) and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs), and indirect defense compounds, volatile organic compounds (VOCs) such as GLVs and volatile terpenes. In contrast, silencing of *NaJAZh* suppressed the accumulation of nicotine in irJAZh transgenic plants. This shows an unexpected but very interesting crosstalk in JAZ regulation in tobacco plants. We suggest that control of nicotine biosynthesis may be separate from the other inducible defense metabolites in tobacco. In addition, we found that *NaJAZh*-silenced plants develop necrosis on their leaves which shows several hallmarks of programmed cell death. My work demonstrates the first clear example of jasmonate-regulated defense functions mediated by one particular *JAZ* gene.

**In chapter 5**, I described the function of NaJAZd in defense and flower development in *Nicotiana attenuata* plants. In contrast to NaJAZh, NaJAZd has only minor role(s) in plant defense against herbivores. We suggest that NaJAZd is a weak negative regulator (or redundant regulator) of nicotine biosynthesis or transport in *N. attenuata* plants. In contrast to defense responses, *NaJAZd* silencing strongly affected the lifetime production of seed capsules, which was associated with enhanced flower abscission in late flower developmental stages. This is likely due to the impact of NaJAZd on JA and JA-Ile as well as the transcript abundance of the master flower regulator *MYB305* in *N. attenuata* flowers. Our data suggest that NaJAZd counteracts flower abscission by regulating hormone levels and/or expression of the *NaMYB305* gene in *N. attenuata*, thus regulating seed production.

***Roles of R2R3-MYB transcription factors in plant***

The MYB protein family is one of the largest and most highly diverse gene families represented in all eukaryotes, and most MYB proteins function as transcription factors. MYB proteins are divided into different classes depending on the number of tandem repeats of 50 amino acids (R1, R2 and R3), and generally three and two repeats are found in

animals and plants (R2R3-MYB class), respectively (Rosinski and Atchley, 1998; Stracke et al., 2001). In the last decade, numerous functional studies of R2R3-MYB proteins in many plant species have been conducted, demonstrating their roles in various plant-specific processes: (1) **metabolism**: primary and secondary metabolites such as flavonoid (anthocyanin, tannin, phenylpropanoid, etc.) accumulation, cell wall construction (lignin biosynthesis, lignin, cellulose, and xylan deposition), and biosynthesis of glucosinolates; (2) **morphogenesis**: determination of cell fate and identity such as trichome and root hair initiation, extension, and patterning, control of petal shape, and cell differentiation; (3) **development**: developmental processes such as anther and pollen development, axillary meristem formation, side shoot formation, and lateral organ separation; and (4) **stress**: response to abiotic and biotic stresses such as hypersensitive cell death program against pathogen attack, systemic resistance against fungi and bacteria, regulation of stomata movements in response to drought stress and disease resistance, cold tolerance, and phosphate starvation (reviewed in (Dubos et al., 2010)).

Recently, the R2R3-type MYB305 transcription factor (homologue of AtMYB21/24 and PhEOBII) was identified and shown to function as a major flower regulator in petunia and tobacco plants. It controls phenylpropanoid volatile production in flowers, flower anthesis (opening), floral nectar production, and nectary maturation (Liu et al., 2009; Spitzer-Rimon et al., 2010; Colquhoun et al., 2011; Liu and Thornburg, 2012)

**In chapter 4**, we described the function of EOBII and MYB305 in petunia and *N. attenuata*, respectively. *EOBII* (*MYB305)*-silenced petunia and *N. attenuata* flowers failed to enter anthesis and showed premature senescence of closed buds, resulting in extreme difficulty for these silenced plants in sexual propagation. In this study, we proposed that PhEOBII (NaMYB305) has a dynamic function in flowers, especially in flower anthesis (opening) in both petunia and *N. attenuata*. We suggest that the function of PhEOBII (NaMYB305) is highly conserved in angiosperms.

### Nicotiana attenuata *as a model plant*

*Nicotiana attenuata* (*N. attenuata*; Solanaceae) is a wild tobacco species found as a summer annual native plant to Southwestern North America (Figure 1A). *N. attenuata* seeds germinate in nitrogen-rich soils after being exposed to smoke-related cue(s) in post-fire environments (Baldwin and Morse, 1994; Preston and Baldwin, 1999). As mentioned

above, maximal fitness is general goal for all plants. However, the synchronized post-fire germination behavior of *N. attenuata* creates intense intraspecific competition within populations, and these plants tend to allocate many of their resources to rapid growth and increased lifetime seed production. However, as a pioneer plant in a post-fire environment, *N. attenuata* is also exposed to unpredictable herbivore communities (Figure 1B-1F), for example the piercing-sucking herbivores *Tupiocoris notatus* (mirid) and *Empoasca* spp. (leafhopper), the chewing herbivores *Epitrix* spp. (flea beetles), *Spodoptera* spp. (armyworm) and larvae of the specialists *Manduca sexta* (tobacco hornworm) and *Manduca quinquemaculata* (tomato hornworm). Because *N. attenuata* is native to the Great Basin Desert of the southwestern USA, plants also experience high UV irradiance, high and variable , *N. attenuata* plants have to balance and strictly allocate their resources to growth, reproduction, and defense to adapt and survive in complex environments, leading to development of sophisticated strategies (Baldwin, 1998; Baldwin, 2001).

In summary, *N. attenuata* plants have synchronized post-fire germination behavior which produces low interspecific and high intraspecific competition, complex defense systems to make them highly successful in stressful environments, an unpredictable herbivore community, short generation time, and are self-compatible but opportunistic outcrossers. It is relatively easy to manipulate gene expression in *N. attenuata* via stable transformation and virus-induced gene silencing (VIGS). All these properties make *N. attenuata* an excellent a model plants for ecological and molecular studies of plant defense responses against herbivores in nature.

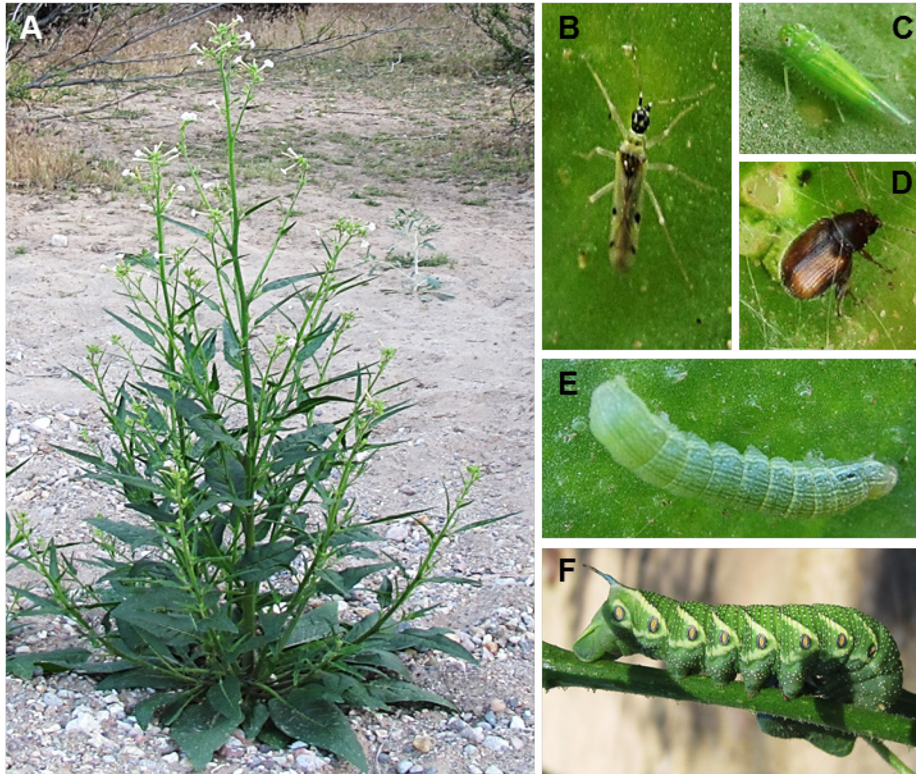


**Figure 1.** The ecological model plant *Nicotiana attenuata* and its native herbivores in the Great Basin Desert, Utah, USA. (A) *N. attenuata* is a wild annual tobacco species native to Southwestern North America. (B) - (F) native herbivores of *N. attenuata*: the piercing-sucking herbivores *Tupiocoris notatus* (mirid, B) and *Empoasca* sp. (leafhopper, C) and the chewing herbivores *Epitrix hirtipennis* (flea beetle, D), *Spodoptera* sp. (armyworm, E), and *Manduca quinquemaculata* (tomato hornworm, F; the tobacco hornworm *M. sexta* is also a native herbivore).

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# Overview of Manuscripts

## Manuscript I

**NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants**

Youngjoo Oh, Ian T. Baldwin, Ivan Galis
Published in *Plant Physiology* 2012, **159**(2):769-788

In manuscript I, I identified 12 novel putative Jasmonate ZIM domain (JAZ) proteins in the native tobacco, *Nicotiana attenuata*. Here I show that *NaJAZ* genes display temporal and spatial differences in their expression after simulated herbivory. Among these genes, I further characterized NaJAZh, which was highly induced after simulated herbivory using reverse genetic approaches. I show that NaJAZh negatively regulates a subset of direct (HGL-DTGs and TPIs) and indirect (volatiles) defense responses and also suppresses ROS (reactive oxygen species) accumulation in *N. attenuata*. This is the first experimental evidence that a single JAZ protein can regulate a specific part of jasmonate signaling in plants.

Youngjoo Oh designed and performed the experiments, analyzed the data and wrote the manuscript. Ian T. Baldwin designed the experiments, helped perform field experiments, and wrote the manuscript. Ivan Galis designed and coordinated the experiments and wrote the manuscript.

## Manuscript II

**EOBII controls flower opening by functioning as a general transcriptomic switch**

Thomas A. Colquhoun, Michael L. Schwieterman, Ashlyn E. Wedde, Bernardus C.J. Schimmel,Danielle M. Marciniak, Julian C. Verdonk, Joo Young Kim, Youngjoo Oh, Ivan Galis, Ian T.Baldwin and David G. Clark

Published in *Plant Physiology* 2011, **156**(2):974-984

In manuscript II, we characterized EOBII (a homologue of NaMYB305) and demonstrated that it controls a highly dynamic process fundamental to sexual reproduction in petunia, P. x hybrida ‘Mitchell Diploid’ (MD), and *Nicotiana attenuata* plants. Both petunia and *N. attenuata* plants strongly silenced in the expression of *EOBII* or *MYB305*, respectively, displayed non-opening and prematurely abscised flower phenotypes, which were partially recovered when *PhEOBII*- or *NaMYB305*-deficienct plants were made ethylene-insensitive through crossing with ethylene-insensitive transgenic line or when treated with an ethylene inhibitor, 1-MCP. This manuscript shows the functional conservation of PhEOBII (NaMYB305) in an angiosperm system. Although I only partially contributed to this manuscript, it allowed deeper interpretation and crucial progress in the understanding the function of the NaJAZd protein described in the next chapter, and was therefore included as part of my thesis dissertation.

This manuscript included two groups of collaborators: (1) researchers working with petunia in the University of Florida, Gainesville, U.S.A., and (2) researchers using *N. attenuata* in the Max Planck Institute for Chemical Ecology in Jena, Germany.

Thomas A. Colquhoun, Michael L. Schwieterman, Ashlyn E. Wedde, Bernardus C.J. Schimmel, Danielle M. Marciniak, Julian C. Verdonk and Joo Young Kim contributed to the research on EOBII in petunia. They designed, performed the experiments and wrote the part of the manuscript regarding the work on petunia. Youngjoo Oh, Ivan Galis, and Ian T. Baldwin contributed to the study of NaMYB305 (a homologue of PhEOBII) in *N. attenuata*. Youngjoo Oh designed and performed the experiments, analyzed data, and wrote the manuscript. Ivan Galis designed and coordinated the experiments and wrote the manuscript. Ian T. Baldwin designed the experiments and wrote the manuscript.

## Manuscript III

**Jasmonate ZIM-domain protein NaJAZd regulates floral jasmonic acid levels and counteracts flower abscission in *Nicotiana attenuata* plants**

Youngjoo Oh, Ian T. Baldwin, Ivan Galis

Submitted to *PLoS One* (22-Oct-2012)

In manuscript III, I characterized the functions of NaJAZd protein in both plant development and defense responses against herbivore attack. NaJAZd plays minor roles in plant defense responses and may possibly be involved in nicotine biosynthesis or transport together with an unknown co-regulator or redundant JAZ proteins. Apart from its function in defense responses, NaJAZd has an important role in the control of flower abscission in later stages of flower development, which eventually affects the lifetime seed capsule production in *N. attenuata*; this is most likely due to the regulation of the phytohormones jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels, and/or expression of *NaMYB305* gene in *N. attenuata* flowers. I provide a novel insight into the function of JAZ regulators in flower and seed development. This finding supports the widely discussed hypothesis of functional specialization of JAZ proteins in both defense and development in plants.

Youngjoo Oh designed and performed the experiments, analyzed the data and wrote the manuscript. Ian T. Baldwin designed the research and wrote the manuscript. Ivan Galis designed and coordinated the research and wrote the manuscript.

# NaJAZh Regulates a Subset of Defense Responses against Herbivores and Spontaneous Leaf Necrosis in *Nicotiana attenuata* Plants[C][W][OA]

**Youngjoo Oh, Ian T. Baldwin, and Ivan Gális[1]***

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The JASMONATE ZIM DOMAIN (JAZ) proteins function as negative regulators of jasmonic acid signaling in plants. We cloned 12 *JAZ* genes from native tobacco (*Nicotiana attenuata*), including nine novel *JAZ*s in tobacco, and examined their expression in plants that had leaves elicited by wounding or simulated herbivory. Most *JAZ* genes showed strong expression in the elicited leaves, but *NaJAZg* was mainly expressed in roots. Another novel herbivory-elicited gene, *NaJAZh*, was analyzed in detail. RNA interference suppression of this gene in inverted-repeat (ir)JAZh plants deregulated a specific branch of jasmonic acid-dependent direct and indirect defenses: irJAZh plants showed greater trypsin protease inhibitor activity, 17-hydroxygeranyllinalool diterpene glycosides accumulation, and emission of volatile organic compounds from leaves. Silencing of *NaJAZh* also revealed a novel cross talk in JAZ-regulated secondary metabolism, as irJAZh plants had significantly reduced nicotine levels. In addition, irJAZh spontaneously developed leaf necrosis during the transition to flowering. Because the lesions closely correlated with the elevated expression of programmed cell death genes and the accumulations of salicylic acid and hydrogen peroxide in the leaves, we propose a novel role of the NaJAZh protein as a repressor of necrosis and/or programmed cell death during plant development.

Jasmonic acid (JA) is an important plant signal that regulates the defense of plants against biotic stress. In addition, JA exerts control functions in plant development, such as root growth, senescence, pollen and flower development, tuber formation, and tendril coiling (for review, see Wasternack, 2007). JA rapidly accumulates in mechanically wounded tissues, after attack by herbivores or after infection of plants by necrotrophic pathogens (Farmer et al., 2003; Glazebrook, 2005; Browse and Howe, 2008; Glauser et al., 2008; Howe and Jander, 2008; Wu and Baldwin, 2010). It is produced from membrane lipids in a well-characterized octadecanoid pathway compartmentalized in two plant organelles, chloroplasts and peroxisomes (for review, see Schaller and Stintzi, 2009).

In response to JA, plants accumulate a variety of defense metabolites that reflect the extreme chemical diversity of terrestrial plants. For example, Arabidopsis (*Arabidopsis thaliana*) plants use glucosinolates (amino acid derivatives) for defense (Rask et al., 2000; Mewis et al., 2006; Shroff et al., 2008), whereas tobacco (*Nicotiana* spp.) plants produce nicotinic alkaloids to ward off attack from feeding herbivores (Baldwin et al., 1997; Wink and Roberts, 1998; Shoji et al., 2000; Steppuhn et al., 2004). In addition, most plants produce protease inhibitors in response to herbivory, which inhibit proteolysis and negatively affect the digestibility of ingested plant material in insect guts (Jongsma et al., 1994, 1995; Koiwa et al., 1997; Zavala et al., 2004a; Habib and Fazili, 2007; Hartl et al., 2010). Green leaf volatiles (GLVs) and volatile organic compounds (VOCs) constitute another important plant defense mechanism to attract predators of herbivores; this strategy is also known as indirect plant defense (Halitschke et al., 2000; Kessler and Baldwin, 2001; Baldwin et al., 2002; Allmann and Baldwin, 2010).

Despite the large diversity found in downstream JA-regulated defense responses, the main components of the JA signaling pathway are conserved between plant species. A central component in JA signaling is the CORONATINE INSENSITIVE1 (COI1) protein that was found and functionally analyzed in several plant species (Feys et al., 1994; Xie et al., 1998; Devoto et al., 2002, 2005; Li et al., 2004; Paschold et al., 2008). The F-box protein COI1, as part of the JA receptor complex, contributes to the binding of a bioactive JA derivative, (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile; Thines et al., 2007; Katsir et al., 2008; Fonseca et al., 2009). In the presence of JA-Ile, COI1 interacts with JASMONATE ZIM DOMAIN (JAZ) repressors that are subsequently ubiquitinated and degraded by the 26S proteasome (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007).

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Because the Jas domain in JAZ is known to bind MYC2-class transcription factors (TFs) that control the expression of a majority of JA-inducible genes (Boter et al., 2004; Lorenzo et al., 2004; Chini et al., 2007; Cheng et al., 2011, Fernández-Calvo et al., 2011; Niu et al., 2011; Shoji and Hashimoto, 2011; Zhang et al., 2012), the degradation of JAZ repressors by the $SCF^{COI1}$ complex leads to the active transcriptional status of JA-dependent genes. In contrast, the accumulation of JAZ proteins, which interact with an EAR domain-containing NINJA protein, represses JA-mediated responses, as EAR binds a strong plant transcriptional corepressor protein, TOPLESS (Pauwels et al., 2010).

The transcriptional activity of MYC2 and MYC2-like TFs, and several additional JA-regulated TFs, therefore, depends on their release from JAZ-imposed repression. The JAZ proteins, typically present as protein families in plants, contain two important functional domains, ZIM and Jas (for review, see Pauwels and Goossens, 2011). The ZIM domain (Shikata et al., 2004; Vanholme et al., 2007) with the TIF[F/Y]XG motif (or its variant), located in the N-terminal part of JAZ proteins, mediates the homomeric and heteromeric interactions between JAZ proteins (Chini et al., 2009; Chung and Howe, 2009) and the binding of the NINJA protein, a strong interactor of the TOPLESS corepressor mentioned above (Pauwels et al., 2010). The Jas domain (Yan et al., 2007) is required for the JAZ/COI1 interaction and the binding of MYC2 TFs. It is characterized by an S-L-X(2)-F-X(2)-K-R-X(2)-R core, delimited by a conserved N-terminal Pro and a C-terminal PY sequence. Two positively charged amino acid residues, Ala-205 and Ala-206, in the Jas domain of the AtJAZ1 protein were shown to be essential for the JAZ/COI1 interaction (Melotto et al., 2008).

Recently, it has been reported that Arabidopsis AtJAZ8 lacks a typical degradation sequence (degron) in its Jas motif that is required for the sealing of JA-Ile in the binding pocket at the COI1-JAZ interface (Sheard et al., 2010; Shyu et al., 2012). In contrast, AtJAZ8 contained an EAR sequence at the N terminus, suggesting that AtJAZ8 may be directly binding the TOPLESS corepressor and repressing TFs without the help of NINJA adaptor proteins (Shyu et al., 2012).

Since the identification of JAZ proteins, much effort has focused on the functional characterization of JAZ complexes, such as the binding of JA-Ile in the coreceptor structure (Melotto et al., 2008; Yan et al., 2009; Sheard et al., 2010), interactions among and splicing of individual JAZ proteins (Chini et al., 2009; Chung et al., 2009, 2010), and interaction of JAZs with various TFs in plants (for review, see Pauwels and Goossens, 2011). To demonstrate the regulatory function of JAZ proteins, Jas-truncated or alternatively spliced forms of JAZ proteins were efficiently used (Chini et al., 2007; Thines et al., 2007; Shoji et al., 2008; Chung et al., 2010). Such proteins may interfere with the degradation of other JAZ proteins, as previously shown for the *jai3-1* mutant using in vitro degradation assays (Chini et al., 2007), thus causing the wide-ranging dominant JA-insensitive phenotypes observed in transgenic or mutant plants, including male sterility and resistance to JA in root growth bioassays.

Despite current progress in the general understanding of JAZ proteins as repressors of JA signaling, the functions of individual JAZ proteins in plants remain largely unknown (see final section in Pauwels and Goossens, 2011). To extend the current JAZ repertoire in plants, we cloned 12 full-length *JAZ* genes from native tobacco (*Nicotiana attenuata*) and subsequently initiated a functional screen of JAZ proteins in this ecologically relevant plant model. Here, we show that silencing the *NaJAZh* gene results in a dramatic shift in the regulation of JA-dependent responses in *N. attenuata*. In addition, the NaJAZh-deficient plants developed spontaneous necrotic lesions on the leaves, a novel phenotype associated with JA signaling in plants.

## RESULTS

### Identification of Novel *N. attenuata* JAZ Proteins

We identified 12 JAZ proteins in *N. attenuata* based on the assembly of tobacco JAZ sequences available in public EST databases, focusing on the presence of characteristic conserved domains, ZIM and Jas, in these proteins. Primers flanking the coding regions of putatively assembled tobacco *JAZ* genes (Supplemental Table S3) were then used in PCR with leaf- and/or root-derived cDNAs from *N. attenuata*. The obtained PCR-amplified fragments were cloned and sequenced to yield 12 distinct *N. attenuata JAZ* genes named *NaJAZa*, *-b*, *-c*, *-d*, *-e*, *-f*, *-g*, *-h*, *-j*, *-k*, *-l*, and *-m* (which include nine novel *JAZ* genes in tobacco). In two cases, we retrieved cDNA clones of variable length that most likely belonged to the same *JAZ* gene (*NaJAZc.1*/*NaJAZc.2* and *NaJAZk.1*/*NaJAZk.2*; Supplemental Fig. S1), suggesting that alternative splicing may occur in the processing of specific *N. attenuata* JAZ transcripts.

When we performed a phylogenetic analysis of full-length NaJAZ proteins, together with JAZ proteins from Arabidopsis (AtJAZ1 to -12; Chung et al., 2008), tomato (*Solanum lycopersicum*; SlJAZ1 to -12; Sun et al., 2011), rice (*Oryza sativa*; OsJAZ1 to -12; Seo et al., 2011), and three already identified JAZ proteins from *Nicotiana tabacum* (NtJAZ1 to -3; Shoji et al., 2008), JAZ proteins clustered into five main subgroups (I–V; Fig. 1; Supplemental Text S1). Four of those contained the members from all plant species (I, III, IV, and V), whereas branch II did not contain any sequence from the monocot rice.

### *NaJAZ* Genes Are Differentially Expressed in Shoot and Root Tissues

Using a previously reported microarray data set (Kim et al., 2011), the expression of 10 individual *N. attenuata JAZ* genes was examined. The rosette leaves of *N. attenuata* plants were untreated, elicited with wounding (punctured with a fabric pattern wheel and

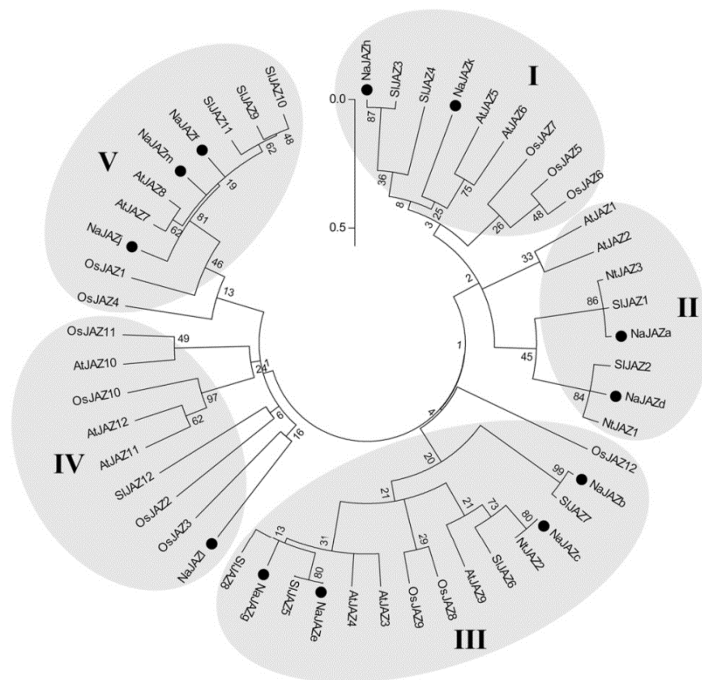


**Figure 1.** Phylogenetic tree of tobacco, rice, tomato, and Arabidopsis JAZ proteins. The phylogenetic tree was constructed using previously reported tobacco, rice, tomato, and Arabidopsis JAZ proteins and 12 JAZ protein sequences from *N. attenuata* identified in this paper. Protein sequences were aligned with ClustalW, Gonnet matrix gap penalty of 10, and extension penalty of 0.2. The phylogenetic tree was constructed from the alignment using MEGA 5.05 software using the maximum likelihood bootstrap method (1,000 replicates). Black circles indicate the positions of *N. attenuata* proteins in the phylogram.

supplied with water [W+W]), or treated with simulated herbivory (wounds treated with diluted oral secretions from *Manduca sexta* larvae [W+OS]). In addition to expression in the elicited leaf, we also examined expression in the young untreated leaves and roots to monitor systemic induction of *JAZ* genes. As shown in Figure 2, *NaJAZc*, *NaJAZe*, and *NaJAZk* genes were similarly expressed in the leaf and root tissues. In contrast, *NaJAZa*, *NaJAZb*, *NaJAZd*, *NaJAZf*, *NaJAZh*, and *NaJAZj* genes were strongly induced by wounding in the leaves but showed very limited expression in the roots (note the variable *y* axis scales in the graphs in Fig. 2). *NaJAZg* was mainly expressed in roots, showing very low and noninducible expression in leaves. Furthermore, some *N. attenuata JAZ* genes differed in their response to W+W and W+OS treatments. Whereas most *JAZ* genes were highly up-regulated by W+OS treatment in locally elicited and systemic leaves, *NaJAZc* was expressed constitutively across all conditions. Interestingly, *NaJAZe* was more induced by W+W compared with W+OS in the local leaves 1 h after elicitation, suggesting that elicitors in the oral secretion may have suppressed the wound-induced expression of this gene. These differential patterns of expression directly support the previously proposed functional specialization of *JAZ* genes in the defense and growth of plants (Howe and Jander, 2008).

**Silencing of NaJAZh Alters the Expression of Other *JAZ* Genes**

A subgroup I *NaJAZh* gene (Fig. 1) with transcripts strongly accumulated in response to W+OS in local and systemic leaves (Fig. 2) was selected for detailed functional analysis. This phylogenetic subgroup also contained Arabidopsis *AtJAZ5/6* genes, suggesting a possibly conserved function of these proteins. We transformed *N. attenuata* plants with the inverted-repeat (ir) RNA interference construct of NaJAZh (Supplemental Fig. S2) and suppressed its expression in planta. Three efficiently silenced lines (90%–95% reduction of transcripts in W+OS-elicited plants; Fig. 3A) that contained a single T-DNA insert (Supplemental Fig. S3) were selected for the functional analysis conducted in this paper (irJAZh-264, -267, and -368).

The expression of 11 *NaJAZ* genes was examined in control (untreated) and 1-h W+OS-elicited leaves of irJAZh plants by quantitative PCR (qPCR; Fig. 3B). *NaJAZg*, which could not be reliably detected by qPCR in the leaves, was analyzed together with *NaJAZh* in the roots of rosette leaf W+OS-induced plants (Fig. 3C). In addition to the strongly reduced accumulation of *NaJAZh* transcripts in both root and leaf tissues of irJAZh plants, compared with identically treated tissues of wild-type plants, we found a higher expression

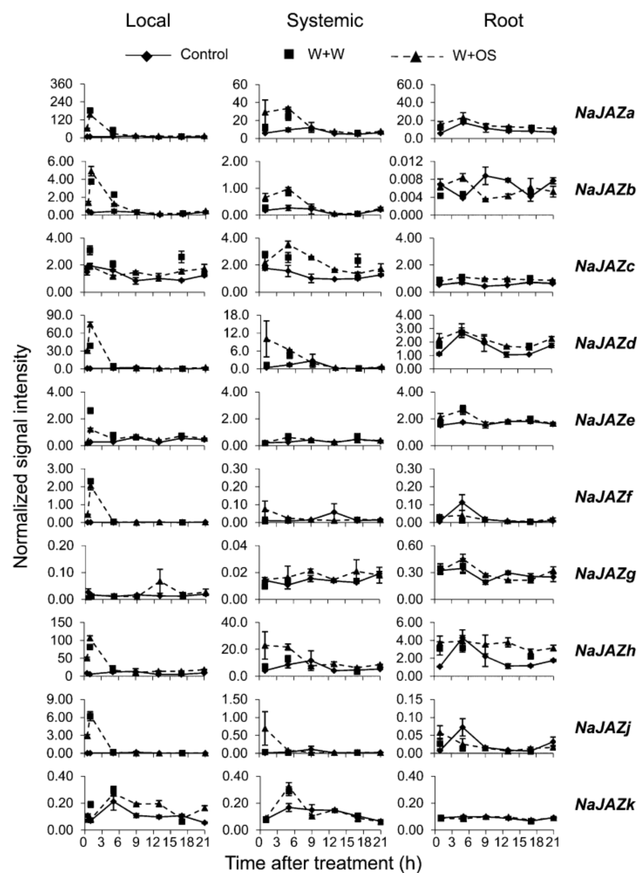


**Figure 2.** Basal and induced expression of individual *JAZ* genes in *N. attenuata*. The accumulation of *NaJAZ* transcripts in local treated leaves, systemic leaves, and roots was determined by microarrays ($n = 3$) after elicitation of the leaves with wounding (W+W) or simulated herbivory (W+OS); control plants remained untreated. Control and W+OS samples were harvested at 0, 1, 5, 9, 12, 17, and 21 h post elicitation; because samples from the W+W treatment were collected only at 0, 1, 5, and 17 h post elicitation, the time points of the W+W treatments are not connected with lines in the graphs.

of *NaJAZf* (approximately 2-fold) and moderately reduced expression of two additional genes, *NaJAZe* (approximately 1.8-fold) and *NaJAZm* (approximately 2-fold), in irJAZh tissues. However, the NaJAZh silencing construct did not share any obvious similarity with *NaJAZe* and *NaJAZm* genes, and there was no continuous sequence homology of 21 nucleotides (or longer) between *NaJAZe*, *NaJAZm*, and our inverted repeat construct (Supplemental Text S2) that would suggest the possibility of a cosilencing effect. In addition, the increased expression of *NaJAZf* could not be explained by cosilencing mechanisms. The expression of this gene 2 h after oral secretion elicitation of leaves, analyzed in an independent microarray experiment, was approximately five times higher in irJAZh compared with wild-type leaves (Supplemental Fig. S4). The reduction in *NaJAZe* transcripts was not observed in this experiment, possibly due to the analysis of plants at a later time point after elicitation (2 h) relative to the qPCR experiment (1 h; Fig. 3B). The *NaJAZm* probe was not on the microarray chip, and data for this gene could not be compared. Overall, consistent changes in *NaJAZf* expression in irJAZh plants support the existence of a mutual regulatory network among individual JAZ repressors in JA signaling, which is further supported by the presence of G-boxes that bind the MYC2 TF in promoter regions of most Arabidopsis *JAZ* genes (Chini et al., 2007).

#### NaJAZh Silencing Suppressed the Performance of a Specialist Herbivore without Changing JA-Ile Levels

To examine if W+OS-responsive NaJAZh could regulate defense against herbivores, we performed

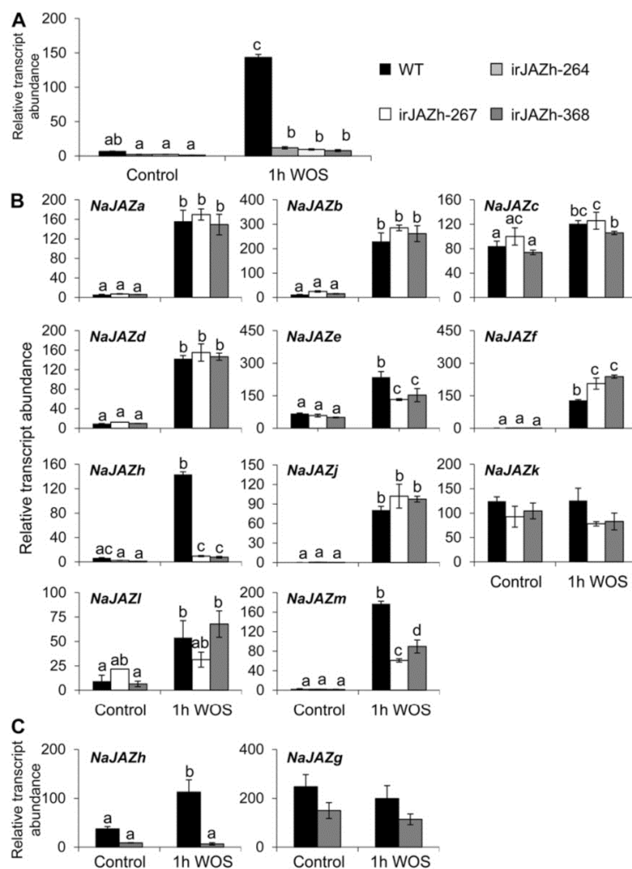


**Figure 3.** Silencing of the *NaJAZh* gene affects the expression of several other *NaJAZ* genes in *N. attenuata*. A, Transcript abundances of the *NaJAZh* gene were determined by real-time qPCR in three independent silenced irJAZh lines before and after elicitation with W+OS. B, Transcript abundances of other *NaJAZ* genes determined by qPCR in untreated (Control) and 1-h W+OS-elicited (1 h WOS) leaves of irJAZh plants. C, *NaJAZh* and *NaJAZg* expression in systemic roots of irJAZh plants. Bars in indicate *EF1a*-normalized relative transcript abundances ± SE ($n$ = 3). Different letters (a–d) indicate significant differences among the combination of genotypes (wild type [WT] versus independent irJAZh lines irJAZh-264, -267, and -368) and treatments determined by ANOVA ($P \leq 0.05$).

herbivore performance bioassays using the specialist herbivore *M. sexta* and irJAZh plants. Because JAZ proteins likely repress defense genes, their absence should result in strong constitutive defense responses in JAZ-silenced plants. To examine the behavior of young as well as older irJAZh plants (showing necrotic symptoms, as described later), we placed one *M. sexta* neonate per leaf on 20 and 10 replicates of rosette and flowering stage, respectively, irJAZ and wild-type plants. The mass of caterpillars was first determined at 5 d and then every 2 d until 13 d (Fig. 4). NaJAZh silencing strongly suppressed the performance of *M. sexta* specialist herbivores, both in young and older plants; however, the growth of larvae was retarded to a greater degree on mature irJAZh plants. These results suggested that irJAZh plants are better defended against specialist herbivores, presumably due to the high levels of defense metabolites in these plants.

Before analyzing the levels of direct defense metabolites, we determined if changes in upstream JA and JA-Ile accumulations and/or metabolism could be responsible for the extraordinary defense properties of irJAZh plants. The levels of JA, JA-Ile, abscisic acid (ABA), and salicylic acid (SA) were measured in W+OS-elicited leaves of rosette stage irJAZh-264, -267, and -368 plants and wild-type plants at 0, 1, 2, and 3 h after oral secretion elicitation (Fig. 5). Whereas the content of JA was significantly higher in irJAZh plants compared with wild-type plants at 1 h post elicitation, JA-Ile levels in irJAZh leaves did not differ from those in wild-type plants. Similar to JA-Ile, the levels of SA and ABA did not differ significantly between irJAZh and wild-type plants. Because JA-Ile is the active form of JA in the signaling process, we assumed that higher JA levels would not be directly responsible for the strongly elevated resistance of irJAZh plants.

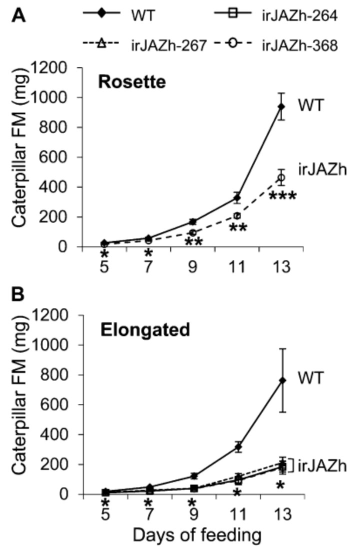


**Figure 4.** The performance of larvae of the specialist herbivore *M. sexta* is strongly suppressed on irJAZh plants. The performance of *M. sexta* larvae was observed on the wild type (WT) and NaJAZh-silenced lines at two stages of development. A, *M. sexta* neonates were placed on rosette-stage leaves of wild-type and irJAZh-368 plants. The mean fresh mass (FM) ± SE of irJAZh-368 caterpillars ($n$ = 20) was significantly smaller at all time points as determined by Student's $t$ test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). B, *M. sexta* performance ($n$ = 10) on early-flowering-stage wild-type plants and three independent JAZh-silenced lines (irJAZh-264, -267, and -368) that developed necrotic lesions during the experiment. Significant differences between genotypes were determined at each time point by ANOVA (* $P \leq 0.05$).

#### Silencing of NaJAZh Shows a Differential Effect on Direct Defenses in *N. attenuata*

To evaluate if the substantially elevated herbivore resistance of irJAZh plants was due to higher levels of toxic defense metabolites in these plants, we examined trypsin protease inhibitor (TPI) activity (Jongsma et al., 1994, 1995; Habib and Fazili, 2007) and the accumulation of the secondary metabolites nicotine (Shoji et al., 2000; Steppuhn et al., 2004) and 17-hydroxygeranyllinalool diterpene glycosides (DTGs; Jassbi et al., 2008; Heiling et al., 2010) in W+OS-elicited irJAZh and wild-type plants (Fig. 6). The basal levels of TPI activity in irJAZh plants were already six to 10 times higher compared with wild-type plants, and these levels remained approximately five times higher in 72-h W+OS-elicited irJAZh leaves (Fig. 6A). The pooled DTG levels in irJAZh plants, determined by HPLC, showed accumulation patterns similar to TPI activity (Fig. 6B). In particular, the constitutive levels of DTGs in uninduced irJAZh plants were dramatically higher compared with wild-type plants. Both constitutive and induced levels of nicotine in leaf tissues were surprisingly reduced in irJAZh compared with wild-type plants already at constitutive uninduced levels (Fig. 6C). When we checked the root expression of *N. attenuata* putrescine-*N*-methyltransferase (*NaPMT*) involved in nicotine biosynthesis, and nicotine levels in the roots, they were not significantly different between roots of uninduced wild-type and irJAZh plants (Supplemental Fig. S5). We also examined the levels of other potentially important defense-related secondary metabolites, including dicaffeoylspermidine (DCS) and caffeoylputrescine (CP) in irJAZh plants (Kaur et al., 2010). Whereas CP levels appeared higher at 72 h in W+OS-elicited irJAZh plants, DCS levels were not significantly different between wild-type and irJAZh plants (Supplemental Fig. S6).

A shift from core to dimalonylated DTGs after herbivore attack is a typical JA-regulated (and COI1-dependent) process in *N. attenuata* plants (Heiling et al., 2010). To see how DTGs are regulated in irJAZh plants, we measured DTG levels by a more sensitive, high resolution liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method (Fig. 7) that allowed relative quantification of individual DTGs subdivided into precursor, core, single-malonylated, and dimalonylated groups (listed in the putative order of biosynthesis; Heiling et al., 2010). Interestingly, the irJAZh plants showed very high basal levels of precursor and core DTGs (Fig. 7;

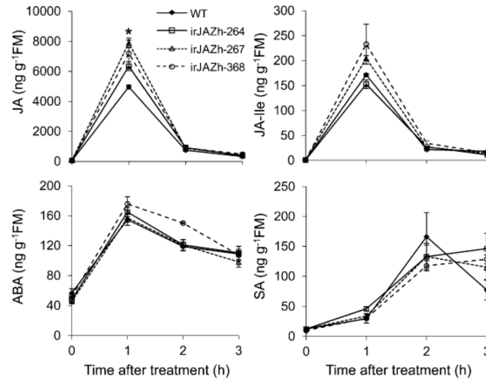


**Figure 5.** NaJAZh silencing does not alter JA-Ile levels induced by simulated herbivory. Rosette-stage plants (the wild type [WT] and NaJAZh-silenced lines irJAZh-264, -267, and -368) were treated with simulated herbivory (W+OS) and harvested before and 1, 2, and 3 h after elicitation. Mean ± SE levels of JA, JA-Ile, ABA, and SA ($n$ = 3) were determined by LC-ESI-MS/MS using internal deuterium-labeled hormone standards. JA levels were significantly higher at 1 h after elicitation in irJAZh lines, but other hormones showed no significant differences compared with wild-type plants. Asterisks indicate significant differences among the wild type and independent irJAZh lines determined by ANOVA (* $P \leq 0.05$). FM, Fresh mass.

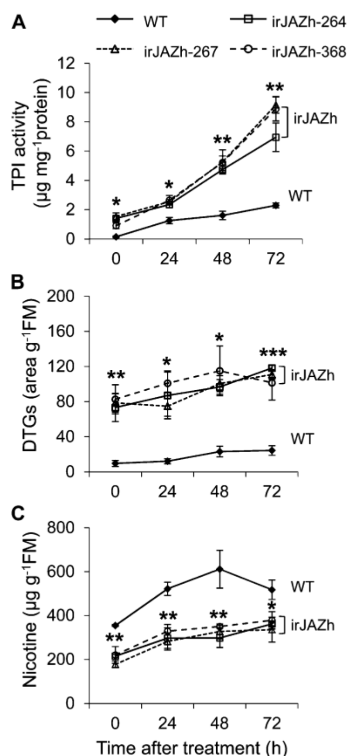


**Figure 6.** NaJAZh silencing enhanced constitutive and inducible levels of TPIs and DTGs but suppressed nicotine accumulation. Rosette-stage plants (the wild type [WT] and NaJAZh-silenced lines irJAZh-264, -267, and -368) were treated with simulated herbivory (W+OS), and treated leaves were harvested before and 24, 48, and 72 h after elicitation. Mean ± SE levels of TPIs determined by radial diffusion assay (A) and DTGs determined by HPLC (B) were significantly higher at each time point in irJAZh compared with wild-type plants. Mean ± SE levels of nicotine (C) determined by HPLC were significantly lower in irJAZh compared with wild-type plants at every time point (ANOVA; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; $n = 3$). There were no significant differences found in metabolite content when three independent irJAZh lines were compared. FM, Fresh mass.

0-h time point), which quickly declined after W+OS elicitation of the plants. Subsequently, W+OS treatment increased the single-malonylated and dimalonylated DTG levels, both in irJAZh and wild-type plants; however, the levels in irJAZh were dramatically higher. These results suggest that although irJAZh plants can constitutively accumulate a large amount of DTG precursors, another JA-dependent signal(s) may be required for their conversion to malonylated and dimalonylated forms during herbivore attack.

To further examine the role of NaJAZh in the regulation of secondary metabolism under real herbivory attack, we analyzed the leaves from wild-type and irJAZh plants that were exposed to *M. sexta* feeding for 5 d. The accumulation of total DTGs and nicotine was consistent with the previous W+OS elicitation results, showing significantly higher levels of DTGs and lower nicotine levels in herbivore-attacked leaves of irJAZh compared with wild-type plants (Supplemental Fig. S7).

#### irJAZh Plants Release More Volatile Compounds

The releases of GLVs and VOCs are typical herbivory-induced responses in *N. attenuata* plants (Halitschke et al., 2000; Kessler and Baldwin, 2001; Baldwin et al., 2002; Paschold et al., 2006). To determine if *NaJAZh* also regulates volatile emissions, we collected GLVs and VOCs released from the leaves of irJAZh and wild-type plants over 24 h, both before and after W+OS elicitation (Fig. 8). The irJAZh plants released more GLVs (e.g. cis-3-hexenylacetate and cis-3-hexenylbutyrate); however, the most remarkable differences were found in terpenoid emissions (VOCs) of these plants. For example, the sesquiterpenes trans-$\alpha$-bergamotene and trans-$\beta$-farnesene were strongly elevated, especially in the case of W+OS-elicited irJAZh plants. These data suggest that NaJAZh, apart from regulating the accumulation of direct defense metabolites like TPIs and DTGs, also regulates the volatile emissions that are known to function as an indirect defense against herbivores in *N. attenuata* plants.

#### Global Transcriptional Changes Associated with NaJAZh Silencing in *N. attenuata*

As we have shown above, NaJAZh silencing strongly altered defensive metabolite profiles in *N. attenuata* leaves. To gain more insight into the mechanisms (and genes) involved in defense against herbivores, we compared global gene expression by microarrays using irJAZh-368 and wild-type leaf samples 2 h after W+OS elicitation. NaJAZh silencing altered the expression of a large number of genes (188 out of 43,503 microarray probes were at least 3-fold up-regulated [97 genes] or down-regulated [91 genes] in irJAZh plants compared with wild-type plants). As predicted from the strong defense profile of irJAZh plants, a number of the 58 functionally annotated up-regulated genes could be assigned to volatile emission or defense-related functional categories (Supplemental Table S1). For example, the six-domain trypsin inhibitor precursor (3.9-fold), 5-epi-aristolochene synthase (5.2-fold), and $\gamma$-thionin-defensin-like protein (30.1-fold) were strongly up-regulated after W+OS treatment in irJAZh compared with wild-type plants. Furthermore, the primary metabolism and signal transduction genes (nitrate transporter, 5.2-fold; *A. thaliana* chlorophyllase2, 3.3-fold; basic helix-loop-helix family protein, 4.6-fold) were among the strongly regulated targets of the NaJAZh repressor. Similar to previous qPCR analysis (Fig. 3), NaJAZh silencing modulated the expression of several

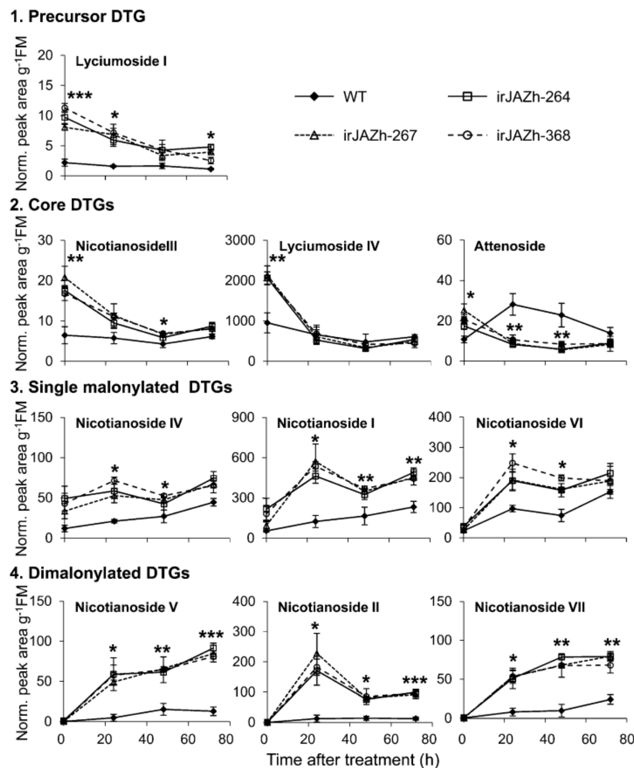


**Figure 7.** NaJAZh silencing strongly affects the accumulation and structural modification of DTGs in transgenic plants. Rosette-stage plants in the glasshouse were treated with simulated herbivory (W+OS) and harvested before and 24, 48, and 72 h after elicitation. Mean ± SE relative amounts of individual DTGs were determined by LC-ESI-MS/MS. The malonylated DTGs (rows 3 and 4) strongly accumulated after treatment in all three independent irJAZh lines (irJAZh-264, -267, and -368) compared with wild-type (WT) plants, whereas core (row 2) and precursor (row 1) DTGs were highest in the irJAZh lines before treatment (constitutive levels at 0 h). Asterisks indicate significant differences among the wild type and three independent irJAZh lines determined by ANOVA (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; $n = 3$). There were no significant differences found in metabolite content among three independent irJAZh lines. FM, Fresh mass.

other *JAZ* genes (*NaJAZf*, up 4.6-fold; *NaJAZj*, up 3.6-fold; Supplemental Fig. S4).

NaJAZh silencing also resulted in the repression of 52 functionally annotated genes in irJAZh plants compared with wild-type plants (greater than 3-fold change; Supplemental Table S2). As expected, *NaJAZh* was among the strongly suppressed genes (3.4-fold). Interestingly, NaJAZh silencing resulted in the down-regulation of genes from similar categories, as already found in the up-regulated gene list (e.g. monoterpene synthase 2, 8.3-fold), consistent with a complex metabolic reconfiguration and possible redirection of metabolic fluxes within the same metabolic pathways during defense (e.g. monoterpene versus sesquiterpene biosynthesis).

#### NaJAZh Silencing-Induced Leaf Necrosis at Late Developmental Stages

NaJAZh silencing did not affect the growth and development of *N. attenuata* plants until the elongation stage, when the strongly silenced irJAZh lines displayed spontaneous necrotic lesions on the leaves. This necrotic phenotype was observed in five independent irJAZh transgenic lines, therefore excluding the possibility that it was the consequence of the random insertion of a NaJAZh-silencing construct into another independent gene in the *N. attenuata* genome.

The symptoms first appeared as small necrotic spots on cotyledons that gradually spread to older leaves, after the development of the leaves (Fig. 9). Late in development, all leaves but not flowers or seed capsules of irJAZh plants were typically engulfed by the necrosis in strongly silenced irJAZh lines (Fig. 9). To a large extent, the degree of necrosis was dependent on the transgenic line used in the experiment (with the strongest symptoms observed in irJAZh-368), and the necrotic symptoms were completely lost when the irJAZh-267 plants (showing strong necrosis in irJAZh homozygous plants) were crossed with the wild type to create a hemizygous cross (Supplemental Fig. S8). We interpret these results to mean that a low threshold level of *NaJAZh* expression can possibly counteract the development of necrotic symptoms. Although not addressed in this work, the hypothesis that accumulations

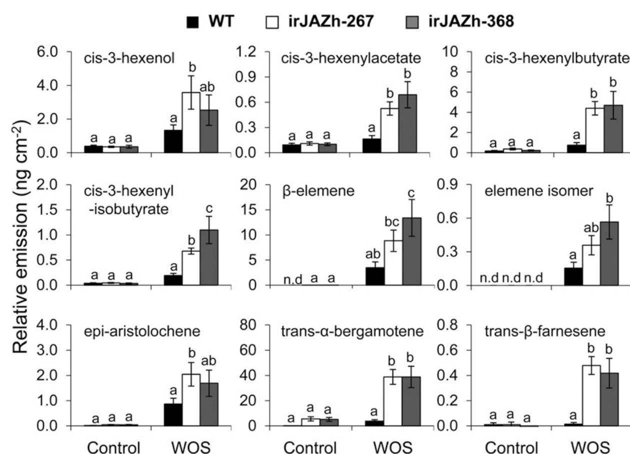


**Figure 8.** NaJAZh-silenced plants emit higher amounts of VOCs and GLVs. Volatiles were collected from the head space of wild-type (WT) and irJAZh (irJAZh-267 and -368) leaves for 24 h after connecting the volatile trap units to locally treated leaves 3 h after W+OS elicitation; control plants were collected in parallel but remained untreated. Samples were analyzed by gas chromatography-mass spectrometry with tetraline as an internal standard. Bars indicate normalized relative emissions of volatiles per $cm^2$ of leaf area ± SE ($n$ = 5). Different letters (a–c) indicate statistically significant differences in emissions among genotypes (the wild type versus two independent irJAZh lines) and treatments determined by ANOVA ($P \leq 0.05$). n.d, Not detected.

of some toxic intermediate in nicotine biosynthesis, shown to be impaired in irJAZh plants, could be responsible for the lesions in older leaves should be thoroughly examined.

### Reactive Oxygen Species Accumulation in Wounded Leaves

Reactive oxygen species (ROS) are common markers of programmed cell death (PCD) and necrosis in plants (Allan and Fluhr, 1997; Desikan et al., 1998; Beers and McDowell, 2001; Houot et al., 2001; Mittler et al., 2004; Cheeseman, 2007). To determine if the necrotic lesions in irJAZh plants could be due to the activation of ectopic PCD in irJAZh plants, we first analyzed the hydrogen peroxide ($H_2O_2$) levels in leaves of two independent NaJAZh-silenced lines (irJAZh-267 and -368) and compared them with wild-type $H_2O_2$ levels. We used presymptomatic unwounded leaves as well as puncture-wounded leaves detached from mature plants and stained them with the $H_2O_2$-sensitive indicator diaminobenzidine (DAB); the leaves were floated on staining solution overnight in darkness and destained the next day in a clearing solution to visualize the brown precipitate of oxidized DAB (Fig. 10A). A stronger DAB staining in the unwounded leaves of irJAZh compared with wild-type plants was readily observed. Although the wound-induced accumulation of $H_2O_2$ appeared as dark brown circles around puncture wounds on the leaves, these circles were significantly more intense in irJAZh compared with wild-type leaves, suggesting that irJAZh plants are subjected to strong oxidative stress due to the high ectopic accumulation of $H_2O_2$ in their leaves.

Because DAB staining requires a long incubation of the leaves in the staining solution, which may have caused some artifacts in observed $H_2O_2$ accumulation patterns, we also quantified the $H_2O_2$ accumulation in wounded leaves using the more sensitive Amplex Red kit, which allows more precise determination of the immediate levels of $H_2O_2$ (Fig. 10B). In addition to significantly higher basal levels of $H_2O_2$ found in the irJAZh leaves (Fig. 10B; 0 h), we detected a strong burst of $H_2O_2$ after wounding that occurred only in irJAZh plants. We conclude that the NaJAZh protein is essential for the suppression of both basal and wound-induced ROS in mature *N. attenuata* leaves.

### Gene Expression during Necrosis in irJAZh Leaves

To further examine the possible mechanisms involved in leaf necrosis in irJAZh plants, we measured the expression of *NaJAZh* and three known PCD markers in tobacco (harpin-induced1 [Hin1], hypersensitivity-related203 [Hsr203], and *N. attenuata* vacuolar processing enzyme361 [NaVPE361]) using a time-resolved kinetic of leaf samples from irJAZh and wild-type plants (Fig. 10C). In this experiment, we first labeled one rosette leaf growing at the −1 node (one position younger than the leaf undergoing the source-sink transition) of 34-d-old irJAZh and wild-type plants, well before the development of necrotic symptoms on irJAZh plants was observed. Subsequently, we kept collecting sets of labeled leaves in 2-d intervals until necrotic lesions appeared on labeled leaves of irJAZh plants. As expected, irJAZh plants showed low expression of *NaJAZh* throughout the experiment, consistent with the stable silencing genotype of these plants. Interestingly, whereas wild-type plants showed a relatively constant expression of the *NaJAZh* gene, the transcript levels suddenly increased at 50 d post germination, when the full necrosis phenotype was observed in

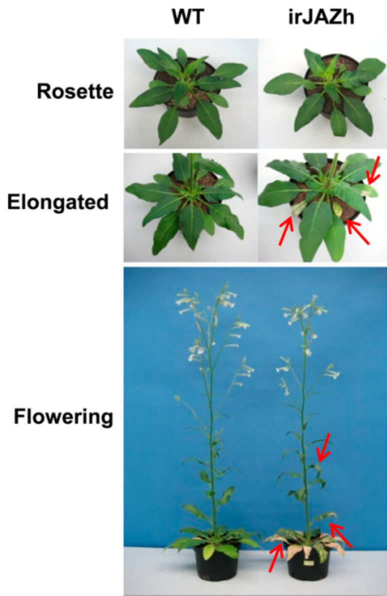


**Figure 9.** Elongated and mature irJAZh plants develop spontaneous necrosis on leaves. Necrotic spots on leaves of irJAZh plants (indicated by red arrows) appeared in a strict developmental sequence as irJAZh plants started to elongate and transitioned into the flowering stage of growth. Symptoms were first detected on cotyledons of irJAZh plants and gradually spread to the next developed but still fully green leaves. Necrotic lesions were not detected on flowers or capsules of the irJAZh plants. In contrast to irJAZh plants, wild-type (WT) plants developed natural senescence that was characterized by yellow color of the old leaves and necrosis of the yellow senescent leaves in the final stages of development.

irJAZh leaves (Fig. 10, C and D; the arrows depict the first day when the labeled irJAZh leaves showed visible necrotic spots on their lamina). At the same time, the expression of the PCD marker genes *Hin1*, *Hsr203*, and *NaVPE361* spiked in irJAZh plants but not in wild-type plants. Because *VPE* genes are involved in the vacuole collapse that triggers hypersensitive cell death in plants (Hatsugai et al., 2004; Hara-Nishimura et al., 2005), NaJAZh, directly or indirectly, contributed to the suppression of cell death in mature *N. attenuata* leaves.

Next, we examined if the necrotic symptoms could be associated with the unbalanced phytohormone levels in irJAZh leaves, using the same set of samples used in gene expression analysis. Although JA and ABA levels were fairly normal in irJAZh plants, we found a strong increase in SA levels that coincided with the development of necrosis on irJAZh leaves (Fig. 10D). However, it was not clear if the accumulation of SA was the cause or consequence of PCD in irJAZh leaves. As necrotic symptoms on irJAZh plants always spread in a strictly ontogenetic order, affecting the oldest leaves found on the plant, the possibility of a senescence-related origin of PCD in irJAZh plants seemed very likely, a hypothesis examined next.

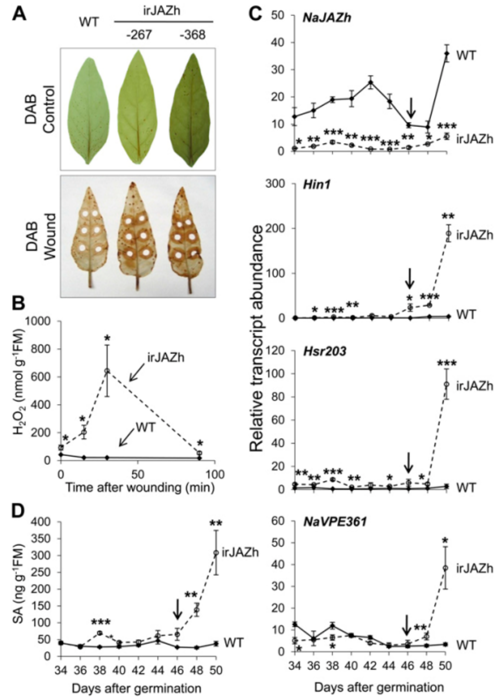


**Figure 10.** irJAZh plants accumulate ROS and express PCD markers during leaf necrosis. A, For DAB staining, leaves were detached from plants and either punched with a cork borer to wound (bottom panel) or leaves remained unwounded (top panel). The entire leaf was floated in DAB staining solution overnight in the dark and destained to visualize the brown DAB precipitate. B, For the Amplex Red assay, leaf extracts prepared from wounded leaves of irJAZh-368 and wild-type (WT) plants were incubated with Amplex Red reagent. Amplex signal intensity was determined by measuring sample fluorescence at excitation/emission = 530/590 nm. Mean ± SE levels of $H_2O_2$ ($n$ = 3) were calculated using $H_2O_2$ external calibration curves, and significant differences between wild-type and irJAZh plants were determined by Student's $t$ test at every measured time point (* $P \leq 0.05$). C, Mean relative transcript abundances ± SE of *NaJAZh* and PCD marker genes (*Hin1*, *Hsr203*, and *VPE361*) in developing *N. attenuata* plants (34–50 d post germination; $n$ = 4). D, SA levels determined by LC-ESI-MS/MS in developing *N. attenuata* plants (34–50 d post germination; $n$ = 4). Arrows in C and D indicate the date of the first appearance of necrotic symptoms of a leaf that during early rosette-stage growth (34 d post germination) occupied a node one position younger than the source-sink transition leaf (−1). Asterisks in C and D indicate significant differences between wild-type and irJAZh plants determined by Student's $t$ test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). FM, Fresh mass.

**Necrotic Lesion Symptoms in irJAZh Plants Are Suppressed by High Nitrogen Availability in Soil**

If the necrosis was a result of exaggerated natural senescence in irJAZh plants, we assumed that a delay in senescence should slow or even prevent the development of necrosis on irJAZh leaves. Therefore, we planted irJAZh and wild-type plants in soil as before and supplied (in watering solution) one group of the plants with 50 mL of 20 mM ammonium nitrate solution every 2 d to delay the senescence process in these plants. As expected, the treatment resulted in dark green leaves in the nitrate-supplied group of both irJAZh and wild-type genotypes (Fig. 11A), and greening of irJAZh plants strongly suppressed the appearance of necrotic symptoms observed on 45-d-old irJAZh plants, when the control group of irJAZh plants displayed a clear necrotic phenotype. In later stages of development, although necrosis also developed on nitrate-supplied irJAZh plants, it was significantly milder compared with plants grown under a normal glasshouse fertilization regime. Interestingly, watering with nitrate solution applied to mature elongated plants in soil could stop the spread of already initiated necrosis on the irJAZh leaves, leading to recovery and normal growth of the leaves. Rejuvenation of irJAZh plants was capable of counteracting the leaf necrosis, suggesting that an out-of-control senescence process could be responsible for the necrotic phenotype of irJAZh plants. When we measured the chlorophyll content in selected rosette leaves starting from 35 d post germination until the development of necrosis on irJAZh leaves (approximately 50 d post germination; Fig. 11B), the chlorophyll content started to decline in both wild-type and irJAZh plants just before the first necrotic symptoms in irJAZh plants. We assume that plants may initiate the natural senescence process at this point, which is tempered by the function of the *NaJAZh* gene in wild-type plants.

**Performance and Metabolism of NaJAZh-Silenced Plants in Nature**

Lastly, we wanted to know if the unbalanced defense of irJAZh plants could influence the performance of these plants when grown in the native habitat of *N. attenuata* in the Great Basin Desert of Utah. Although the silencing efficiency and the main metabolic features of irJAZh plants were preserved in the field (Supplemental Figs. S9–S12), damage inflicted by native herbivores on irJAZh plants was not significantly different from the empty vector (EV) plants that were planted in a paired size-matched design with the irJAZh plants in the field (Supplemental Fig. S13). Similar to "naive" glasshouse plants (Fig. 7), field-grown irJAZh plants also accumulated much more of the core, single-malonylated, and dimalonylated DTGs, but not the DTG precursor lyciumoside I. This suggests that exposure to natural herbivory in the field may have

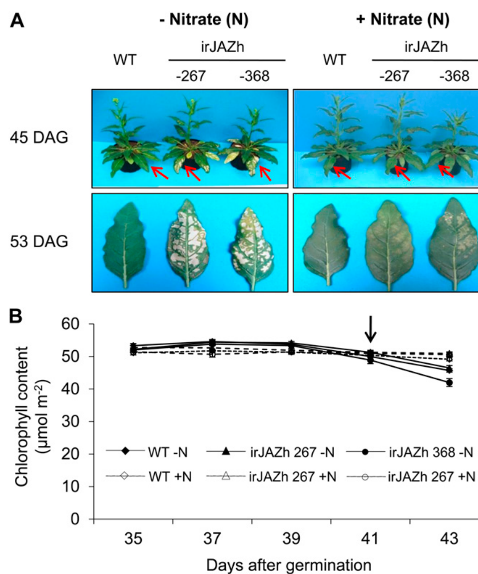


**Figure 11.** Rejuvenation of irJAZh plants delays the development of necrosis on the leaves. A, The extent of necrotic lesions was documented in wild-type (WT) and irJAZh (irJAZh-267 and -368) plants at 45 and 53 d post germination. Plants were cultivated either under the normal glasshouse fertilization regime (Peters Allrounder and Borax; left panel) or under high nitrogen supply rates (supplied with an additional 50 mL of 20 mM $NH_4NO_3$ every 2 d starting at 30 d post germination [DAG]; right panel). Arrows indicate the leaf that during early rosette-stage growth (34 d post germination) occupied a node one position younger than the source-sink transition leaf (−1) and was used to measure chlorophyll contents. B, Mean ± SE chlorophyll content ($n$ = 5) in wild-type and irJAZh (irJAZh-267 and -368) leaves determined between 35 and 43 d post germination. The arrow indicates the date of the first visible necrotic symptoms on the labeled leaf. Chlorophyll content started to decline when the first necrotic symptoms appeared on irJAZh plants grown under the normal glasshouse fertilization regime; compared with 39 d, the chlorophyll content at 41 d post germination significantly decreased in all plants grown under the normal fertilization regime (determined by Student's $t$ test; $P \leq 0.01$). [See online article for color version of this figure.]

shifted the DTG pools in irJAZh plants toward modified forms on account of precursor lyciumoside I (Supplemental Fig. S11). We also observed some differences in volatile profiles of irJAZh plants under field and glasshouse conditions. Whereas the trans-$\alpha$-bergamotene content was still substantially elevated as in glasshouse irJAZh plants (Fig. 8), the field plants did not release elevated amounts of trans-$\beta$-farnesene (Supplemental Fig. S12). $H_2O_2$ accumulation in the leaves was still higher in irJAZh compared with EV plants (Supplemental Fig. S14), but necrotic lesions never developed under field conditions (Supplemental Fig. S15). We assume that the levels of JAZh silencing

determined in the field (Supplemental Fig. S9) were below the threshold levels required for tissue damage and lesion formation. Alternatively, field plants that were grown under approximately twice the photosynthetically active radiation levels of glasshouse-grown plants may have activated stronger antioxidant protection that restricted necrosis in irJAZh plants.

## DISCUSSION

Since the recent discovery of JAZ proteins as repressors of JA signaling, functional characterization of *JAZ* genes has mainly been performed in Arabidopsis. However, the phenotypes associated with the silencing of individual *JAZ* genes in Arabidopsis have rarely been reported (for review, see Pauwels and Goossens, 2011). When we silenced a newly identified *JAZh* gene in *N. attenuata*, a strong derepression of direct (TPIs and DTGs) and indirect (volatile emissions such as terpenoids) defense responses was observed, whereas the accumulation of another defense metabolite, nicotine, was reduced. In addition, irJAZh plants displayed an unexpected necrotic lesion phenotype during late growth and maturation. Although the silencing of NaJAZh strongly deregulated defense responses in *N. attenuata*, it still remains to be determined which of the phenotypes observed in irJAZh plants are directly controlled by the NaJAZh repressor and which result from the regulatory function of JAZh over the other JAZ proteins in *N. attenuata*.

### Identification and Expression of *JAZ* Genes in *N. attenuata*

Seven novel JA-inducible genes containing the ZIM domain, previously implicated in the regulation of transcription in Arabidopsis (Shikata et al., 2004), were identified by microarrays of methyl jasmonate (MeJA)-treated plants and, as a consequence, were classified as a novel JAZ protein family (Thines et al., 2007). Using a sequence homology search, five additional JAZ proteins were identified in the Arabidopsis genome, based on the presence of characteristic structural motifs, ZIM and Jas (Thines et al., 2007, Yan et al., 2007). In this report, using a similar strategy, we found 12 putative *JAZ* genes in *N. attenuata* that complement three previously identified proteins, JAZ1, JAZ2, and JAZ3, from *N. tabacum* (Shoji et al., 2008). All *NaJAZ* genes were expressed in aboveground and/or belowground tissues after W+W or W+OS treatment (Fig. 2) or even without treatment. Our results, summarized in Figure 2, are consistent with previous studies showing that the expression of most *JAZ* genes is inducible by local wounding and/or herbivore feeding (Chung et al., 2008; Shoji et al., 2008; Koo et al., 2009), which propagates to systemic uninduced leaves, similar to *AtJAZ5* and *AtJAZ7* genes (Koo et al., 2009).

Alternative splicing of *JAZ* genes was proposed to play an important role in JAZ/COI1 interactions and JA signaling in Arabidopsis (Chung and Howe, 2009; Chung et al., 2010). Two differentially spliced forms of the AtJAZ10 protein show differential binding affinity to $SCF^{COI1}$, suggesting that alternative splicing of *JAZ* genes may be contributing to the fine-tuning of JA-dependent responses. During the cloning of *N. attenuata JAZ* genes, we also retrieved cDNA clones with variable lengths in at least two cases, *NaJAZc* and *NaJAZk*. While NaJAZc proteins differed in the presence of an internal 32-amino acid sequence (NaJAZc.1 and NaJAZc.2), NaJAZk occurred in two forms, one that completely lacked the Jas motif (NaJAZk.2) and another that contained an incomplete Jas sequence (NaJAZk.1; Supplemental Fig. S1). Further analysis of alternatively spliced forms of tobacco JAZ proteins and completion of the tobacco genome will be required to understand the function of these modifications in the regulation of JA signaling.

### NaJAZh Controls a Subset of Direct and Indirect Defense Responses

Because JAZ proteins repress JA-dependent responses, plants lacking JAZ function should be displaying stronger and constitutive accumulation of anthocyanins or should have altered pollen development, senescence, and root responses (Mandaokar et al., 2006; Balbi and Devoto, 2008; Howe and Jander, 2008; Browse, 2009; Reinbothe et al., 2009; Shan et al., 2009; Qi et al., 2011; Song et al., 2011). When Thines et al. (2007) examined the effect of several single JAZ knockouts or ectopically overexpressed *JAZ* genes in Arabidopsis, no strong phenotypic changes were observed in these lines, revealing a functional redundancy of JAZ proteins in plants. In contrast, Jas domain-truncated (dominant negative) forms of JAZ proteins produced several of the expected phenotypes, including male sterility and insensitivity to JA in root inhibition assays (Chini et al., 2007; Thines et al., 2007; Chung and Howe, 2009). A higher sensitivity to JA resulting in stronger suppression of root growth was also observed in transgenic lines with reduced *AtJAZ1* (Grunewald et al., 2009) and *AtJAZ10* (Yan et al., 2007; Demianski et al., 2012) expression. In addition, a knockdown line of AtJAZ10 became more susceptible to *Pseudomonas syringae* DC3000 infection (Demianski et al., 2012).

Previously, a dominant negative form of NtJAZ1 and NtJAZ3 proteins, equivalents of NaJAZd and NaJAZa, respectively, repressed the MeJA-induced accumulation of nicotine and related alkaloids in tobacco hairy roots and cell cultures (Shoji et al., 2008); however, no other defense metabolites were reported in these experiments. In this report, irJAZh transgenics silenced in the expression of a single *JAZ* gene in *N. attenuata* accumulated and released abnormally high levels of constitutive direct (and indirect) defense metabolites, which was further amplified by oral secretion elicitation. While irJAZh plants were deficient in

nicotine accumulation, cross talk among individual JAZ-regulated defense responses in tobacco was revealed. The reduced nicotine contents in irJAZh plants and the generally negative role of JAZ in nicotine accumulation (Shoji et al., 2008) suggests a branched regulation of JA-mediated defense metabolites in tobacco controlled by separate JAZ proteins.

#### Cross Talk in the JAZ Regulatory Network

NaJAZh silencing resulted in profound changes in direct and indirect defense profiles of *N. attenuata* plants. We first suspected that such a broad phenotype could be the result of nonspecific cross-silencing of other *JAZ* genes by the irJAZh construct. Nevertheless, we rechecked the expression of *NaJAZ* genes in wild-type and NaJAZh-silenced plants (Fig. 3B; determined by qPCR). Two genes, *NaJAZe* and *NaJAZm*, showed reduced gene expression, together with *NaJAZh* at 1 h after W+OS elicitation. However, no other *JAZ* gene except for *NaJAZh* was found to be significantly reduced in irJAZh plants at 2 h after W+OS treatment, which was determined by an independent microarray approach (Supplemental Fig. S4). In addition, we observed the induction of *NaJAZf* expression at 1 h after W+OS in irJAZh plants, which persisted even at 2 h after W+OS elicitation. Therefore, it is very likely that NaJAZh silencing directly or indirectly affected the expression of other *JAZ* genes, which was not the result of a cross-silencing effect. In Arabidopsis, promoter regions of many *JAZ* genes contain G-box sequences, a known target sequence of MYC2 proteins (Chini et al., 2007), which provides a possible mechanism for cross talk and the mutual regulation of *JAZ* genes in JA signaling. Subsequently, some of the observed phenotypes associated with NaJAZh silencing could be attributed to the function of other *JAZ* genes that were deregulated by silencing of NaJAZh.

The results of our transcriptional studies were further supported by the reduction of nicotine levels in irJAZh plants. Because nicotine is synthesized in the roots and subsequently transported to leaves through the xylem stream (Baldwin et al., 1997; Wink and Roberts, 1998), we assume that nicotine biosynthesis is regulated independently from defense metabolites that are directly expressed in the leaves (TPI, DTGs, and volatiles). Recently, increased root expression of *AtJAZ1*, *AtJAZ2*, and *AtJAZ9* genes was reported in Arabidopsis after local wounding (Hasegawa et al., 2011; Sogabe et al., 2011). Possibly, a subset of JAZ proteins may have a specific function in the root tissues. Because the *NaJAZf* gene was up-regulated after W+OS induction in irJAZh plants, we speculate that this gene could be involved in the control of the biosynthesis and/or transport of nicotine in tobacco plants. The most likely targets of this JAZ would be the root-expressed *MYC2*-like genes in tobacco and/or AP2/ERF TFs reported as positive regulators of nicotine biosynthesis in tobacco (Shoji et al., 2010; Shoji and Hashimoto 2011; Zhang et al., 2012).

#### The irJAZh Phenotype Is Robust But Not Beneficial for Field-Grown Plants

A number of previous studies demonstrated that plants use inducible defense as a resource-saving strategy (Cipollini and Bergelson, 2001; Strauss et al., 2002; Cipollini et al., 2003; Zavala et al., 2004b). However, the time delays required to accumulate defensive quantities of metabolites could be a significant drawback of the inducible deployment of defenses. Because irJAZh plants expressed high constitutive and stronger inducible defenses compared with wild-type plants, we were curious whether these "super-defenders" would perform better in the native habitat of *N. attenuata* in the Great Basin Desert, an environment characterized by its extremely variable and stressful conditions. However, in two field seasons, we did not observe any evidence for higher anti-herbivore defense in irJAZh plants that were planted together with EV control plants in the field plot (Supplemental Fig. S13). This suggests that, under natural conditions, constitutively higher TPI and DTG levels did not provide any real advantage to irJAZh plants compared with EV plants (mainly dependent on their inducible defenses). In other words, the Utah ecotype of *N. attenuata* used in this study may have already been equipped with the most efficient defense system, and the time lags associated with defense deployment do not significantly compromise the plant's ability to defend against herbivores in nature. In addition, several alternative hypotheses need to be examined, such as whether the lower nicotine levels in irJAZh plants or the priming of EV plants by natural herbivores in the field (Kessler and Baldwin, 2004; Steppuhn et al., 2004; Frost et al., 2008) could be responsible for the equal performance of EV and irJAZh plants under natural conditions.

#### NaJAZh and Plant Development

As already introduced in this paper, jasmonate signaling regulates several non-defense-related plant responses. In *N. attenuata*, JAZh silencing had no visible effects on plant growth and/or development until elongation and the transition to flowering. Although flowers developed normally and seeds were produced as in wild-type plants, strongly silenced glasshouse-cultivated irJAZh plants developed visible necrotic lesions on their leaves (Fig. 9). This process resembled the PCD in plants that occurs during the hypersensitive response induced by pathogens; however, in irJAZh, it occurred spontaneously. PCD is closely associated with the accumulation of ROS in plant cells during the hypersensitive response (Allan and Fluhr, 1997; Desikan et al., 1998; Houot et al., 2001; Mittler et al., 2004; Cheeseman, 2007; Quan et al., 2008), which is known to inhibit the growth of biotrophic pathogens in plants. PCD also occurs in response to wounding, ozone and UV exposure, and cold and high-light stress (Pennell and Lamb, 1997; Buckner et al., 2000; Rao

et al., 2000a, 2000b; Beers and McDowell, 2001; Pasqualini et al., 2003). In addition, PCD is associated with plant growth and development; it occurs during senescence, pollen development, and vascular tissue differentiation (Wang et al., 1996; Pennell and Lamb, 1997; Calderon-Urrea and Dellaporta, 1999; Buckner et al., 2000; Wu and Cheun, 2000; Lee and Chen, 2002).

In our experiments, the *NaJAZh* gene appeared to be directly (or indirectly) responsible for the ectopic accumulation of $H_2O_2$ in *N. attenuata* leaves, which correlated with the elevated expression of several PCD marker genes in irJAZh plants. Previously, it has been shown that MeJA treatment induces ROS production in plants (Orozco-Cárdenas et al., 2001; Hung and Kao, 2007; Reinbothe et al., 2009), and it was shown that *Atrboh* (a respiratory burst oxidase homolog) D and F genes were required for COI1-dependent $H_2O_2$ production in Arabidopsis leaves treated with MeJA (Maruta et al., 2011). In particular, $H_2O_2$ accumulation was essential for the induction of JA-dependent genes such as *vegetative storage protein1* (*VSP1*) and Arabidopsis NAC domain-containing transcription factors *ANAC019* and *ANAC055*, suggesting that JA-controlled ROS is playing an active role as a secondary messenger in various physiological and defense-related processes in plants. Therefore, if NaJAZh works as an actual suppressor of ROS, some of the NaJAZh-silencing phenotypes could be attributed to the ectopic ROS accumulation that occurred even in the absence of JA in irJAZh plants.

From our data, we surmise that NaJAZh exerts a controlling function over the senescence process in mature tobacco leaves, possibly by controlling ROS levels in these leaves. A number of previous studies suggested that JA signaling intersects with senescence in plants (Weidhase et al., 1987; Parthier, 1990; He et al., 2002; Kong et al., 2006; Balbi and Devoto, 2008, Shan et al., 2011). Therefore, in the absence of a NaJAZh repressor, the leaves could enter a premature and/or exaggerated senescence process, as observed in irJAZh plants. However, the role of JA in the senescence process was recently questioned in the literature, by showing that JA-induced senescence differs from natural age-related senescence processes. Because none of the known JA signaling and biosynthetic mutants in Arabidopsis showed obvious senescence-related phenotypes (Schommer et al., 2008; Seltmann et al., 2010), additional research is required to understand the exact role of endogenous JAs and JAZ proteins in the regulation of this developmental process.

## CONCLUSION

In this paper, we show that a single *JAZ* gene controls a suite of JA-dependent defense responses in a native tobacco (Fig. 12). Furthermore, our results point to a novel type of cross talk among individual JAZ proteins, which is demonstrated by the positive regulatory function of a NaJAZh repressor in nicotine accumulation in *N. attenuata* plants. Finally, a novel role for a JAZ protein in controlling ROS levels is demonstrated. However, despite current progress and the analysis of individual *JAZ* genes, much work is still required to fully understand the complex regulatory network of JAZ proteins. In particular, the identity of downstream TFs interacting with, and directly repressed by, JAZ repressors needs to be identified in order to connect JAZ proteins and downstream physiological responses occurring during stress exposure and/or development in plants.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

The 31st inbred generation of *Nicotiana attenuata* seeds (originally collected in Utah) was used for all experiments and the generation of transgenic plants. Seeds were germinated as described previously by Krügel et al. (2002), and approximately 10 d after germination, the seedlings were transferred into Teku plastic pots (Pöppelmann; http://www.poeppelmann.com) containing peat-based substrate (Tonsubstrat; Klasmann-Deilmann; http://www.klasmann-deilmann.com). The plantlets were maintained in the growth chamber under

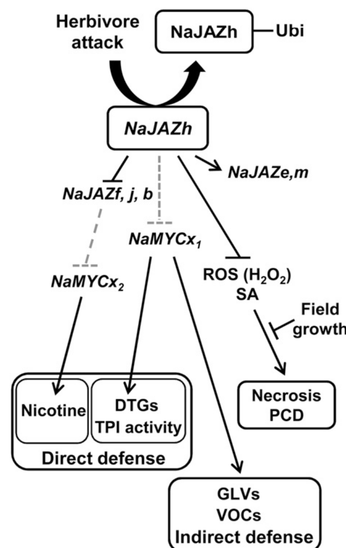


**Figure 12.** Summary of JAZh function in *N. attenuata* plants. NaJAZh is a major repressor of JA-dependent defense responses, including direct (DTGs and TPI activity) and indirect (GLVs and VOCs) defenses. It is proposed that NaJAZh regulates nicotine accumulation via interaction with other *JAZ* genes in *N. attenuata*. Furthermore, NaJAZh is required for direct or indirect repression of ROS, SA, and PCD during plant development in the glasshouse; necrosis (PCD) is prevented by an unknown environmental factor in the field. The black lines indicate interactions established in this paper; gray dashed lines show predicted components of the JAZ signaling pathways.

a 16-h-light/8-h-dark regime at 26°C. After an additional 10 to 12 d, the plants were transplanted into individual 1-L pots with the same substrate and maintained in the glasshouse (16 h of natural daylight supplemented by Philips Master Sun-T PIA Agro 400-W or 600-W sodium lights, temperature of 23°C–25°C, and 45%–55% relative humidity/8 h of dark, 19°C–23°C, 45%–55% relative humidity). The plants were supplied daily with water containing nutrients (0.5 g $L^{-1}$ Peters Allrounder fertilizer [Scotts] containing nitrate, potassium, kalium, and magnesium + 0.1 g $L^{-1}$ Borax; nutrient levels were adjusted weekly by checking the conductivity of the watering solution and resupplying nutrients) using an automatic glasshouse watering system. Unless stated otherwise, experiments were conducted with transient leaves (i.e. leaves undergoing the source-sink transition; node −1) of approximately 35-d-old rosette-stage *N. attenuata* plants.

The release of transgenic plants was carried under Animal and Plant Health Inspection Service notification 06-242-3r-a3, and the seeds were imported to the United States under permit number 10-004-105m. For field releases, we germinated the seeds on Gamborg's B5 medium as described previously; approximately 15 d after germination, the seedlings were transferred to prehydrated 50-mm peat pellets (Jiffy 703; http://www.jiffypot.com), and the seedlings were gradually adapted to the high light and low relative humidity of the habitat over a 2-week-period. Finally, preadapted rosette-stage plants were transplanted into a field plot and watered daily for approximately 2 weeks until the roots had established and the plants were able to grow without water supplementation.

## Plant Transformation

To generate irJAZh plants, we cloned a 240-bp fragment of the *NaJAZh* gene (Supplemental Fig. S2A) as an inverted repeat into the pSOL8 transformation vector containing a hygromycin (*hptII*) resistance gene as selection maker (Supplemental Fig. S2B). *N. attenuata* plants were transformed using the LBA4404 strain of *Agrobacterium tumefaciens* and the transformation method described by Krügel et al. (2002). Homozygous transgenic lines were selected by screening of T2 generation seeds that showed hygromycin resistance, and T-DNA insertions were confirmed by Southern-blot hybridization using genomic DNA from selected lines and a $^{32}$P-labeled PCR fragment of the *hptII* gene as hybridization probe (Supplemental Fig. S3). Real-time qPCR was used to select the best silenced single-insert-containing transgenic lines, irJAZh-264, irJAZh-267, and irJAZh-368, which were used in further experiments.

## Cloning of *N. attenuata JAZ* Genes

To clone *N. attenuata JAZ* genes, we first searched public EST databases using two conserved ZIM and Jas motifs of *JAZ* genes known from other plant species, including Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*), to obtain a pool of tobacco-specific JAZ-related EST sequences. The sequences were then assembled into putative tobacco *JAZ* genes using the publicly available CAP3 Sequence Assembly Program (http://deepc2.psi.iastate.edu/aat/cap/cap.html). Obtained assemblies were reblasted against the EST database to extend the initial JAZ sequences overlapping with Jas and ZIM domains. By repeating the process, we obtained 12 putative tobacco JAZ assemblies, presumably representing individual *JAZ* genes in tobacco, and designed 12 pairs of primers outside putative coding sequences to clone the corresponding full-length sequences from *N. attenuata*. Because the non-*N. attenuata* sequences were used for initial primer design (mainly *N. tabacum* and *Nicotiana benthamiana*), if necessary, the primers were redesigned until a clear PCR product of estimated length was amplified from the *N. attenuata* cDNA template in reverse transcription-PCR. Final and successful sets of primers are provided in Supplemental Table S3. In specific cases, a variation of the standard protocol was used; nine of the *N. attenuata JAZ* genes were cloned by direct PCR, whereas the three remaining genes (*JAZd*, *JAZl*, and *JAZm*) were cloned by the 3′ RACE method (3′ RACE System for RACE kit; Invitrogen). All genes were cloned from *N. attenuata* leaf cDNA samples, except for *NaJAZg*, which was only amplified when *N. attenuata* root cDNA was used. All 12 PCR products were cloned into pJET1.2/blunt Cloning Vector (Fermentas) using T4 ligase (Invitrogen) and transformed to *Escherichia coli* TOP10 electro-competent cells by a standard electroporation method. Plasmids containing the correct size of inserts were sequenced after isolating plasmids by the miniprep method (Nucleospin Extract II). At least three or more independent clones were fully sequenced for each type of insert.

## Sequence Alignments and Construction of a Phylogenetic Tree

The translated putative protein full-length sequences of *NaJAZ* genes were aligned with other JAZ proteins using the ClustalW program, and a phylogenetic tree was built using bioinformatics software MEGA 5.05 (www.megasoftware.net) and the maximum likelihood bootstrap method (1,000 replicates).

## Real-Time qPCR

Total RNA was extracted from approximately 100 mg of frozen leaf tissues with Trizol reagent as recommended by the manufacturer (Invitrogen). The RQ1 RNase-Free DNase was used to treat RNA following the manufacturer's instructions (Promega). The remaining DNase was removed by phenol extraction and precipitated with the addition of 3 M sodium acetate (pH 5.2) and pure ethanol. The cDNA was prepared from 1 $\mu$g of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo(dT) primer (Fermentas). Real-time qPCR was conducted with synthesized cDNA using the core reagent kit for SYBR Green I (Eurogentec) and gene-specific primer pairs (Supplemental Tables S4 and S5) using Mx3005P PCR cycler (Stratagene). Relative gene expression was calculated from calibration curves obtained by an analysis of a dilution series of cDNA samples, and the values were normalized by the expression of the tobacco housekeeping gene *NtEF*$\alpha$*1* (for *N. tabacum* elongation factor $\alpha$-1). All reactions were performed using the following qPCR conditions: initial denaturation step of 95°C for 30 s, followed by 40 cycles each of 95°C for 30 s, 58°C for 30s, and 72°C for 1 min, followed by melting curve analysis of PCR products.

## Herbivore Bioassays in the Glasshouse

Specialist herbivore *Manduca sexta* performance was conducted with the wild type and two independent irJAZh lines (irJAZh-267 and -368) during the 13-d time interval. Plants were grown in randomized spatial order on the same table in the glasshouse until the rosette stage (approximately 30 d after germination) or the elongated stage (close to flowering; approximately 40 d after germination). One freshly hatched *M. sexta* neonate was placed on the rosette leaf of each plant. The larval fresh mass was measured on days 5, 7, 9, 11, and 13 after initial feeding.

## Phytohormone Analysis

Approximately 100 mg of frozen leaf material was homogenized with two steel balls in a Genogrider 2000 (SPEX Certi Prep) at 1,200 strokes $min^{-1}$, 30 s after freezing the samples and cooling plastic racks in liquid nitrogen. Phytohormones (JA, JA-Ile, SA, and ABA) were extracted by vortexing for 10 min after the addition of ethyl acetate spiked with internal standard: 200 ng of [$^2H_2$]JA and 40 ng each of JA-[$^{13}C_6$]Ile, [$^2H_4$]SA, and [$^2H_6$]ABA. The extracted samples were centrifuged at 16,100$g$ at 4°C for 15 min, and the upper organic phases were transferred into clean tubes. Samples were evaporated to near dryness in a vacuum concentrator (Eppendorf) under reduced pressure. Hormone extracts were reconstituted in 500 $\mu$L of 70% (v/v) methanol:water for analysis with the Varian 1200 LC-ESI-MS/MS system as described by Gilardoni et al. (2011). The phytohormones were detected in negative ESI mode. Molecular ions $[M-H]^-$ at mass-to-charge ratio ($m/z$) 209, 322, 137, and 263 (213, 328, 141, and 269), generated from endogenous JA, JA-Ile, SA, and ABA (or their internal standards), were fragmented under 12-, 19-, 15- and 9-V collision energy, respectively. The ratios of ion intensities of the respective product ions and internal standards, $m/z$ 59 and 63, $m/z$ 130 and 136, $m/z$ 93 and 97, and $m/z$ 153 and 159, were used to quantify endogenous JA, JA-Ile, SA, and ABA, respectively. The resulting amounts of hormones were divided by the fresh mass of plant material used for the extraction of each sample.

## Analysis of Secondary Metabolites by HPLC

The samples were extracted for a shared analysis of secondary metabolites by HPLC and individual DTGs by LC-ESI-MS/MS with the extraction method described by Heiling et al. (2010). Approximately 100 mg of frozen leaf material was homogenized with two steel balls by Genogrider 2000 (SPEX Certi Prep) at 1,200 strokes $min^{-1}$, 45 s, twice with 1 mL of buffer A (60% solution 1; 2.3 mL $L^{-1}$ acetic acid, 3.41 g $L^{-1}$ ammonium acetate adjusted to pH 4.8, 1 M

$NH_4OH$, and 40% [v/v] methanol) spiked with 120 ng of glycyrrhizinic acid as an internal standard for the determination of individual DTGs. Supernatants were collected after 20 min of centrifugation at 16,100*g* at 4°C. One microliter of particle-free supernatant (after additional centrifugation) was analyzed by HPLC (Agilent-HPLC 1100 series) using a chromatographic column (Chromolith FastGradient RP18e, 50 × 2 mm; Merck) connected to a precolumn (Gemini NX RP18, 3 $\mu$m, 2 × 4.6 mm; Phenomenex) with the column oven set at 40°C. Separated samples were detected with photodiode array and evaporative light-scattering detectors (Varian). The mobile phase, comprising solvent A (0.1% formic acid and 0.1% ammonium hydroxide solution in water [pH 3.5]) and solvent B (methanol), was used in a gradient mode (time/concentration [min/%] for A: 0:00/100, 0.50/100, 6.50/20, 10:00/20, 15:00/100) with a flow rate of 0.8 mL $min^{-1}$. Under these conditions, nicotine eluted at a retention time of 0.5 min (detected by UV $A_{260}$) and CP, chlorogenic acid (CGA), and DCS eluted at retention times of 2.6, 3.0, and 3.9 min, respectively (detected at 320 nm). Rutin eluted at a retention time of 4.7 min and was detected at 360 nm. The DTG peak pool eluting between retention times of 7.0 and 8.5 min was detected by evaporative light-scattering detection. The peak areas were integrated using Chromeleon chromatographic software (version 6.8; Dionex), and the amount of metabolites in plant tissue was calculated using an external dilution series of standard mixtures of nicotine, CGA, and rutin. CP and DCS contents were estimated based on the external CGA calibrations and expressed as CGA equivalents in the figures.

### Analysis of Individual DTGs by the LC-ESI-MS/MS Method

Samples were prepared as described above for the secondary metabolite analysis by HPLC. Before analysis of individual DTGs, particle-free extracts were first diluted (1:50) with buffer B (10× diluted buffer A with 40% [v/v] methanol:water), and 10 $\mu$L of diluted extract was analyzed by the Varian 1200 LC-ESI-MS/MS system as described previously by Heiling et al. (2010).

### TPI Activity Assay

Total protein fractions were extracted from approximately 100 mg of frozen leaf materials as described by Jongsma et al. (1994) with 300 $\mu$L of cold extraction buffer (0.1 M TRIS-HCl, pH 7.6, 5% polyvinylpolypyrrolidone, 2 mg $mL^{-1}$ phenylthiourea, 5 mg $mL^{-1}$ diethyldithiocarbamate, and 0.05 M $Na_2EDTA$). TPI activity in plant extracts was determined by the radial diffusion assay as described by Jongsma et al. (1993). Quantification of TPI activity in each sample was conducted using a standard soybean trypsin inhibitor (Sigma-Aldrich) calibration curve located on the same plate and normalized with total protein concentrations in each plant extract.

### Volatile Collection and Analysis

A single leaf growing at node −1 was enclosed immediately after W+OS elicitation in a 50-mL plastic container connected to self-packed Porapak Q filters (20 mg of Porapak [Sigma-Aldrich] packed with silanized glass wool and Teflon tubing in the column bodies [Analytical Research Systems, Inc., Gainesville, FL] as described by Halitschke et al. [2000]). The ambient air was pulled from the trapping container through the tubing connected to the Porapak Q filter by pressurized air at 2 to 3 bar (using a Venturi aspirator to create vacuum perpendicular to the direction of air flow). The air flow was adjusted to 200 to 300 mL $min^{-1}$ with individual valves attached to a custom-made manifold, and the air flow of each outlet was kept constant over the entire trapping period of 24 h. After trapping, Porapak Q filters were stored at −20°C until elution of volatiles with 250 $\mu$L of dichloromethane spiked with 320 ng of tetraline internal standard (Sigma-Aldrich) into a gas chromatography vial containing a glass insert for small volume samples. The samples were analyzed by the CP-3800 GC Varian Saturn 4000 ion-trap mass spectrometer (Varian) connected to a nonpolar ZB5 column (30-m × 0.25-mm i.d., 0.25-$\mu$m film thickness; Phenomenex). One microliter of samples was injected by a CP-8400 autoinjector (Varian) onto the column in splitless mode; the injector was returned to a 1:70 split ratio 0.5 min after injection through the end of each run. The gas chromatograph was programmed as follows: injector held at 250°C, initial column temperature at 40°C held for 5 min, then ramped at 5°C $min^{-1}$ to 185°C, finally at 30°C $min^{-1}$ to 300°C, and held for 0.17 min. Helium carrier gas was used, and the column flow was set to 1 mL $min^{-1}$. Eluted compounds from the gas chromatograph column were transferred to a Varian Saturn 4000 ion-trap mass spectrometer for analysis. The mass spectrometer was programmed as follows: transfer line at 250°C, trap temperature of 110°C, ion source temperature of 200°C, manifold temperature of 50°C, and scan range from 40 to 300 *m/z* at 1.02 spectra $s^{-1}$ as described by Schuman et al. (2009). Individual volatile compound peaks were quantified by peak areas using Mass Spectrometer Workstation software (Varian) and normalized by the peak area of the internal standard (tetraline) in each sample. The identification of compounds was conducted by gas chromatography, and retention times and mass spectra were compared with mass spectra database libraries, Wiley version 6 and the National Institute of Standards and Technology.

For volatile trapping in the field, we used single-use charcoal traps (ORBO-32 standard; Supelco) connected to self-made ozone scrubbers that contained eight-ply of 65-mm-diameter $MnO_2$-coated copper gauze to prevent the oxidation of terpenes and GLVs by ozone. A vacuum pump (DAA-V114-GB; Gast) powered by a car battery pulled air through the containers in the field. Treated plants were covered with open-ended transparent plastic cups to enhance the collection efficiency of released volatiles after elicitation of the leaves with W+OS (control plants remained untreated).

### Microarray Analysis

Three biological replicates of node −1 leaves from wild-type and irJAZh-368 rosette-stage plants 2 h after elicitation with W+OS were used for the microarray analysis. Total RNA was extracted as described by Kistner and Matamoros (2005), and cDNA preparation and hybridization were performed as described by Kallenbach et al. (2011). Agilent platform GPL13527 (sample series GSE33681) deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) was used for hybridizations. Raw microarray data were processed by SAM software version 3.11 (Significance Analysis of Microarrays; Stanford University; Tusher et al., 2001) after 75% percentile normalization and $\log_2$ transformation of the raw signal output values. Changes in gene expression were considered significant when fold changes (irJAZh versus the wild type) were larger than 3.0 or smaller than 0.33 and the false discovery rate was less than 4.82%.

### $H_2O_2$ Measurements

$H_2O_2$ in the leaves was determined by semiquantitative DAB staining and by quantitative Amplex Red assays. For DAB staining, the leaves were incubated overnight in 1 mg $mL^{-1}$ DAB solution in the dark at room temperature. To clear the stained leaves, we incubated them for 5 min in a 95°C water bath with a prewarmed lactic acid:glycerol:ethanol (1:1:3) mixture and repeated this procedure three to four times. The leaves were then transferred to a glycerol: ethanol (1:1) mixture and incubated overnight. $H_2O_2$ accumulation was determined as brown DAB precipitate on leaves.

For quantitative $H_2O_2$ measurements, we used the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). $H_2O_2$ was extracted from approximately 100 mg of liquid nitrogen-frozen leaf material that was stored under the same conditions. Liquid nitrogen-ground leaves were incubated on ice for 10 min after mixing with approximately 50 mg of activated charcoal and 200 $\mu$L of 25 mM HCl extraction buffer. The supernatants were transferred to clean tubes after 20 min of centrifugation at 16,100*g* and 4°C. The supernatants were used for $H_2O_2$ determination with the Amplex Red kit as follows: 5 $\mu$L of each sample was mixed with 45 $\mu$L of reaction buffer and 50 $\mu$L of working solution containing Amplex Red reagent and horseradish peroxidase on 96-well plates and incubated in the dark for 30 min. $H_2O_2$ content was determined from the amount of oxidized Amplex reagent with a TECAN Infinite M200 plate reader (Tecan) in fluorescence mode (excitation/emission = 530/590 nm. The concentrations of $H_2O_2$ in the samples were calculated from external $H_2O_2$ calibration curves measured on the same 96-well plate together with the samples.

### Chlorophyll Measurements

Chlorophyll content was determined in wild-type and irJAZh plants that were supplied with or remained without extra nitrate (50 mL of 20 mM $NH_4NO_3$ every 2 d) from 34 to 50 d after germination using a chlorophyll meter (SPAD-502; Minolta). Averaged values of chlorophyll measured in three different areas of the leaf were calculated.

## Field Bioassays

The field experiments were performed in the field plot at the Lytle Ranch Preserve, Utah. Fifteen pairs of EV and irJAZh plants were planted and grown in the field plot. One rosette leaf of each EV and irJAZh plant was treated with W+OS and harvested for the analysis of secondary metabolites, phytohormone, and gene expression at designated time intervals. Damage of plants by natural herbivores was determined by estimating the percentage of leaf area that was damaged by each herbivore relative to the total leaf area of the plant (Noctuidae larvae, *Spodoptera* spp.; flea beetles, *Epitrix* species; mirids, *Tupiocoris notatus*). A representative measurement conducted on June 2, 2011, is shown in Supplemental Figure S11.

## Statistical Analysis

Data were analyzed with StatView 5.0 software (SAS Institute) using appropriate methods (e.g. Student's *t* test for pair comparisons and ANOVA followed by Fisher's protected least significant differences for multiple samples).

Accession numbers are as follows: NtEF$\alpha$1, D63396; *Arabidopsis thaliana* JAZ (AtJAZ): AtJAZ1 (At1g19180), AtJAZ2 (At1g74950), AtJAZ3 (At3g17860), AtJAZ4 (At1g48500), AtJAZ5 (At1g17380), AtJAZ6 (At1g72450), AtJAZ7 (At2g34600), AtJAZ8 (At1g30135), AtJAZ9 (At1g70700), AtJAZ10 (At5g13220), AtJAZ11 (At3g43440), AtJAZ12 (At5g20900); *Oryza sativa* JAZ (OsJAZ): OsJAZ1 (AK061602), OsJAZ2 (AK073589), OsJAZ3 (AK070649), OsJAZ4 (AK120087), OsJAZ5 (AK061842), OsJAZ6 (AK065604), OsJAZ7 (AK108738), OsJAZ8 (AK065170), OsJAZ9 (AK103459), OsJAZ10 (AK059441), OsJAZ11 (AK107750), OsJAZ12 (AK107003); *Solanum lycopersicum* JAZ (SlJAZ): SlJAZ1 (Solyc07g042170), SlJAZ2 (Solyc12g009220), SlJAZ3 (Solyc03g122190), SlJAZ4 (Solyc12g049400), SlJAZ5 (Solyc03g118540), SlJAZ6 (Solyc01g005440), SlJAZ7 (Solyc11g011030), SlJAZ8 (Solyc06g068930), SlJAZ9 (Solyc08g036640), SlJAZ10 (Solyc08g036620), SlJAZ11 (Solyc08g036660), SlJAZ12 (Solyc01g009740); *Nicotiana tabacum* JAZ (NtJAZ): NtJAZ1 (AB433896), NtJAZ2 (AB433897), NtJAZ3 (AB433898); and *Nicotiana attenuata* JAZ (NaJAZ): NaJAZa (JQ172758), NaJAZb (JQ172759), NaJAZc.1 (JQ172760), NaJAZc.2 (JQ172761), NaJAZd (JQ172762), NaJAZe (JQ172763), NaJAZf (JQ172764), NaJAZg (JQ172765), NaJAZh (JQ172766), NaJAZj (JQ172767), NaJAZk.1 (JQ172768), NaJAZk.2 (JQ172769), NaJAZl (JQ172770), NaJAZm (JQ172771).

## Supplemental Data

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

**Supplemental Figure S1.** Differential splicing of *NaJAZ* genes.

**Supplemental Figure S2.** NaJAZh sequence and vector used for plant transformation.

**Supplemental Figure S3.** Southern blot and number of T-DNA insertions in genomes of stable transgenic irJAZh lines.

**Supplemental Figure S4.** Transcript levels of *NaJAZ* genes in irJAZh plants determined by microarrays.

**Supplemental Figure S5.** Nicotine levels and *NaPMT* gene expression in roots.

**Supplemental Figure S6.** Content of secondary metabolites in wild-type and irJAZh plants in the glasshouse.

**Supplemental Figure S7.** Secondary metabolite levels in *M. sexta*-fed leaves from wild-type and irJAZh plants in the glasshouse.

**Supplemental Figure S8.** Necrotic lesion symptoms of irJAZh plants were lost after crossing of homozygous irJAZh-267 with wild-type plants.

**Supplemental Figure S9.** Silencing efficiency of *NaJAZh* in field-grown EV and irJAZh plants.

**Supplemental Figure S10.** Secondary metabolite levels determined in field-grown plants.

**Supplemental Figure S11.** Individual DTGs determined in EV and NaJAZh-silenced plants grown in the native habitat in the Great Basin Desert.

**Supplemental Figure S12.** Volatile emission rates of EV and irJAZh plants grown in the native habitat in the Great Basin Desert.

**Supplemental Figure S13.** Herbivore damage inflicted by the native herbivore community to EV and NaJAZh-silenced plants grown in *N. attenuata*'s native habitat in the Great Basin Desert.

**Supplemental Figure S14.** DAB staining in the leaves from field-grown EV and irJAZh plants.

**Supplemental Figure S15.** Leaves of irJAZh and EV plants grown in the native habitat of the Great Basin Desert.

**Supplemental Table S1.** Up-regulated genes in irJAZh plants compared with wild-type plants by microarray.

**Supplemental Table S2.** Down-regulated genes in irJAZh plants compared with wild-type plants by microarray.

**Supplemental Table S3.** Primer sequences used for cloning of *JAZ* genes from *N. attenuata*.

**Supplemental Table S4.** Primer sequences used in real-time qPCR of *NaJAZ* genes.

**Supplemental Table S5.** Primer sequences used in real-time qPCR experiments.

**Supplemental Text S1.** Protein sequence alignment used to build the phylogenetic tree in Figure 1.

**Supplemental Text S2.** Nucleotide sequence alignment of *NaJAZ* genes that are regulated by silencing of *NaJAZh* against the sequence of an inverted repeat construct used in irJAZh plants.

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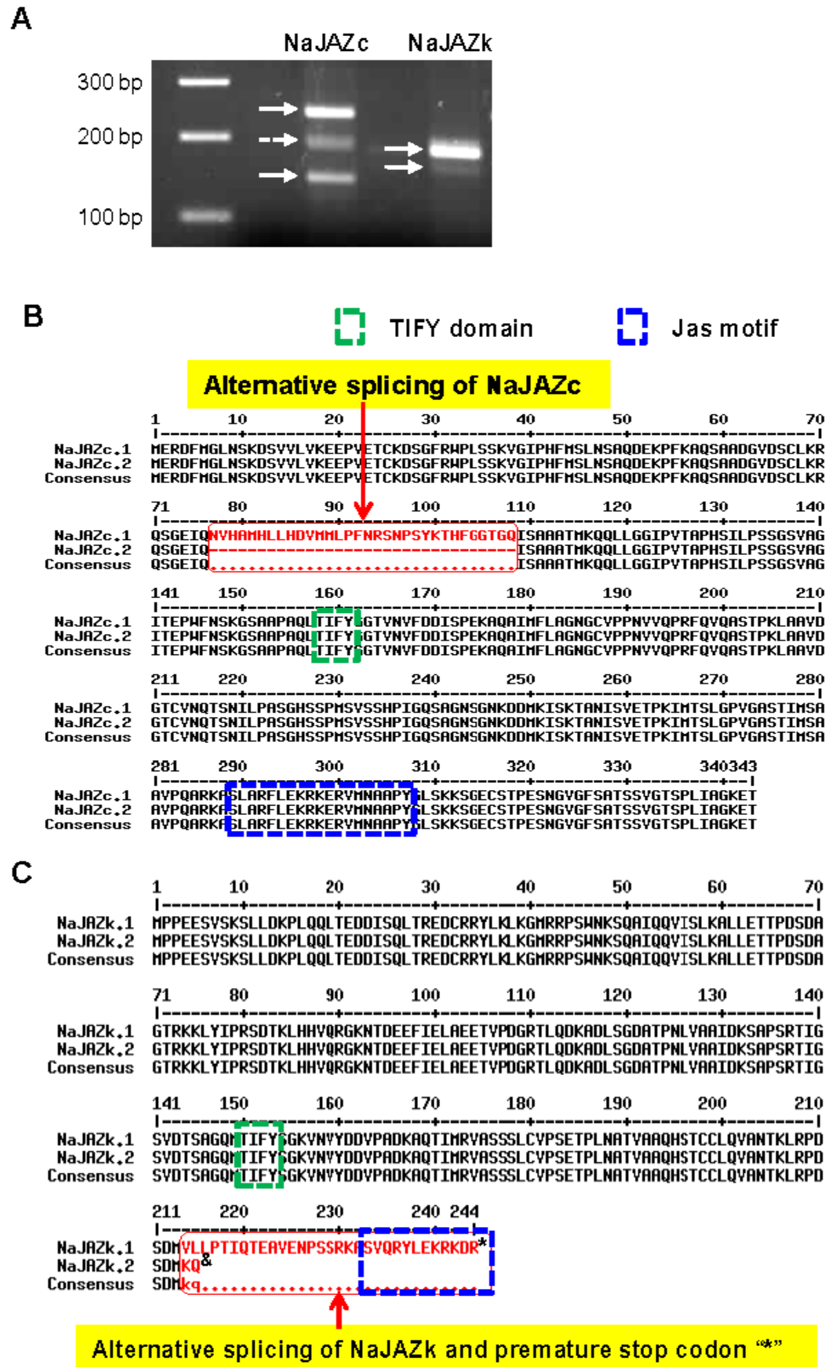


**Supplemental Figure S1.** Differential splicing of *NaJAZ* genes.

**Supplemental Figure S1** (continued)

(A) Primers outside differential sequences of NaJAZc and NaJAZk genes were designed to show the existence and relative ratios of alternatively spliced cDNA products. PCR products of differential length spanning the alternatively spliced regions of *NaJAZc* and *NaJAZk* genes were obtained in PCR reactions using cDNA templates of *N. attenuata* leaf samples. NaJAZc showed three bands of approximately the same intensity; NaJAZk produced two bands with a much stronger signal detected in a larger PCR product. (B) and (C) depict protein sequence alignments of the alternatively spliced forms of NaJAZc (NaJAZc.1, NaJAZc.2) and NaJAZk (NaJAZk.1 and NaJAZk.2) found during the cloning and sequencing of *N. attenuata* genes. NaJAZc.2 has 32 amino acid missing upstream of TIFY domain compared to NaJAZc.1 (B); NaJAZk.1 contains only a partial Jas motif (*) while NaJAZk.2 lacks complete Jas domain ($^{\&}$).

**A**

ATTTTCTTGTGATTTTTAAAACATGTCAAATTCGCAAAATTCTTTTGACGGCGGCAGAAGGG
CCGGAAAAGCGCCGGAGAGATCGAATTTCGTGCAGACTTGTAATTTATTGAGTCAGTTTATT
AAAGGAAAAGCTACTATTAGAGATCTGAATCTCGGAATTGCTGGAAAATCTGAAATCTCAGG
TAAAAGTGATGTTACAGAAGCTGCAACTATGGATTTATTGACAATTATGGAAAACCCCTCAA
TTGAAACTAAAGAACAAGAACAAAAATCCATAGATCCCGTTCGTCAGAGTGCTGTAACAGAA
TCTTCTAGAGATATGGAGGTGGCCGTAAATGAGCCCAGCACGAGCAAAGAGGCACCAAAAGA
GCCTAAGGCAGCACAATTGACTATGTTCTATGATGGTAAAGTGATAGTATTTGATGATTTTC
CAGCTGACAAAGCTAGAGCAGTAATGTTATTGGCTAGTAAAGGATGCCCTCAGAGTTCATTT
GGCACTTTTCATACTACAACCATCGACAAAATTAACACATCTGCTACTGCTGCTGCCACAGC
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AACAAAAGCAGCAGCAAATTCATGTTTCTTATAGTAAAAGTGACCAACTCAAGCCAGGGTAT
AATTCTGCTACGCCGCAAGTACTGCAGCAGCAGCTAGTCCATGTTTCTAGTACTAGTAAAAC
TGATCAGCTTAAGCCAGTATCAACTTCTTCTGCGTCGCAAAAACAGCAGGAGCAACATCAGC
AAACGCAGTCACAGACACCTGGAACTAGCAGCTCTGAGCTACCTATTGCAAGAAGATCATCA
CTACATAGGTTTCTTGAGAAGAGGAAAGATAGGGCAACGGCTAGAGCGCCATACCAAGTTGT
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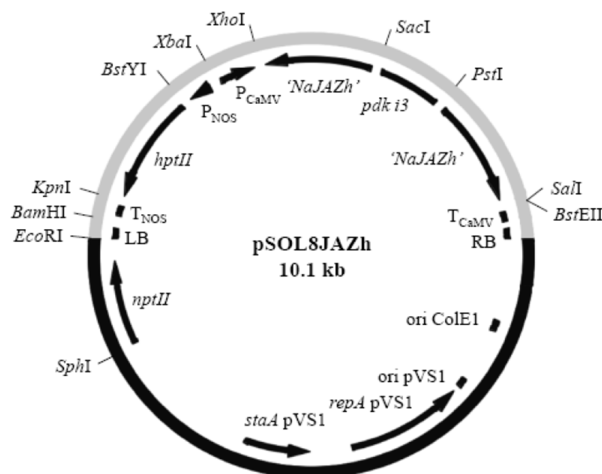
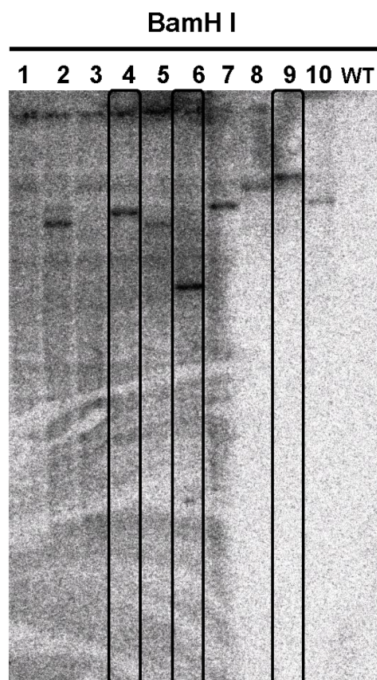
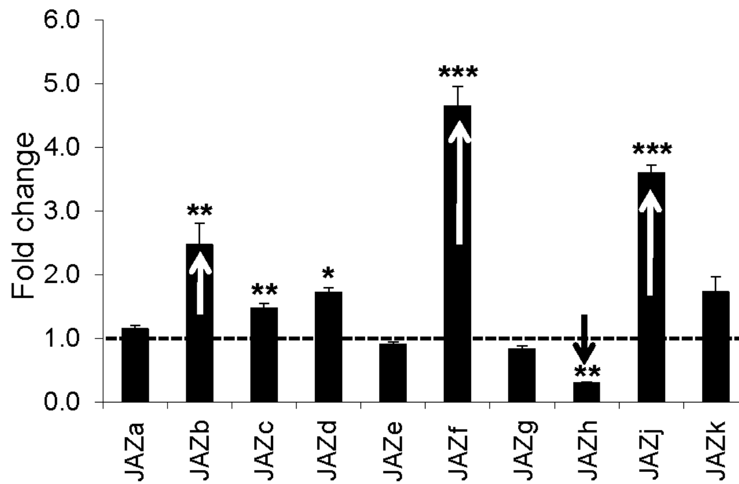
**B**



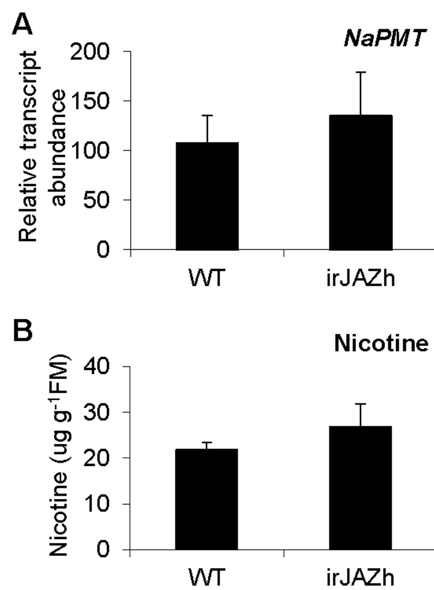

**Supplemental Figure S2.** NaJAZh sequence and vector used for plant transformation. (A) Red letters show a 240bp region in the 3'UTR of *NaJAZh* that was used for gene silencing. (B) The pSOL8JAZH vector used for *Agrobacterium tumefaciens*-mediated transformation of *N. attenuata* plants.



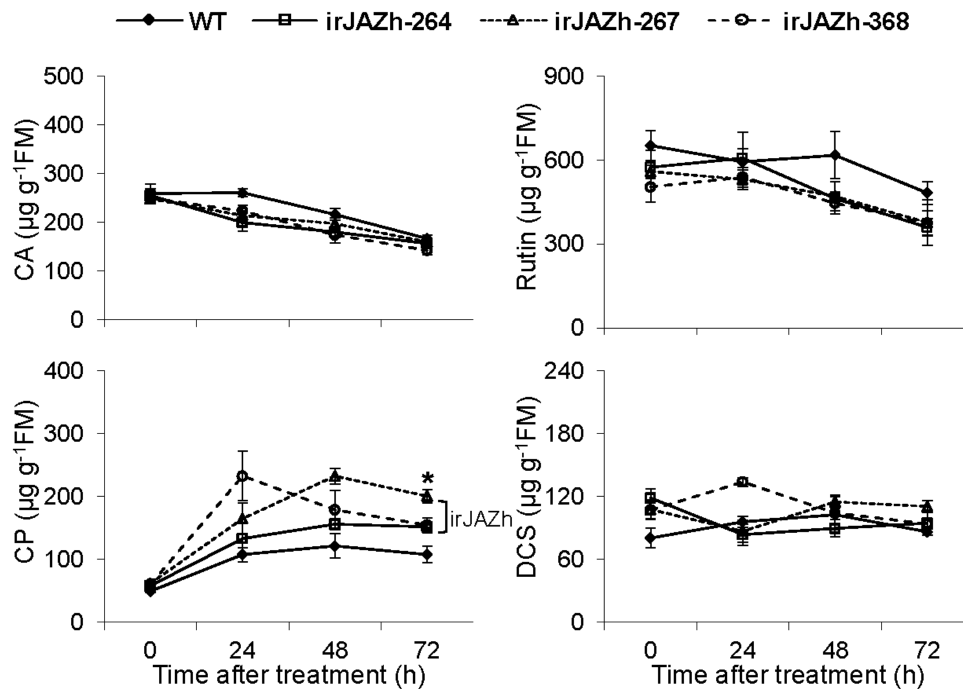

**Supplemental Figure S3.** Southern blot and number of T-DNA insertions in genomes of stable transgenic irJAZh lines. DNA gel blot of genomic DNA digested with BamHI enzyme from 10 independent irJAZh lines, hybridized with a probe coding for the hygromycin resistance gene located between right and left T-DNA borders of the transformation vector pSOL8JAZH. The black boxes indicate single insertion lines that were selected for further experiments: irJAZh-264 (loaded sample #4), -267 (sample #6) and -368 (sample #9).



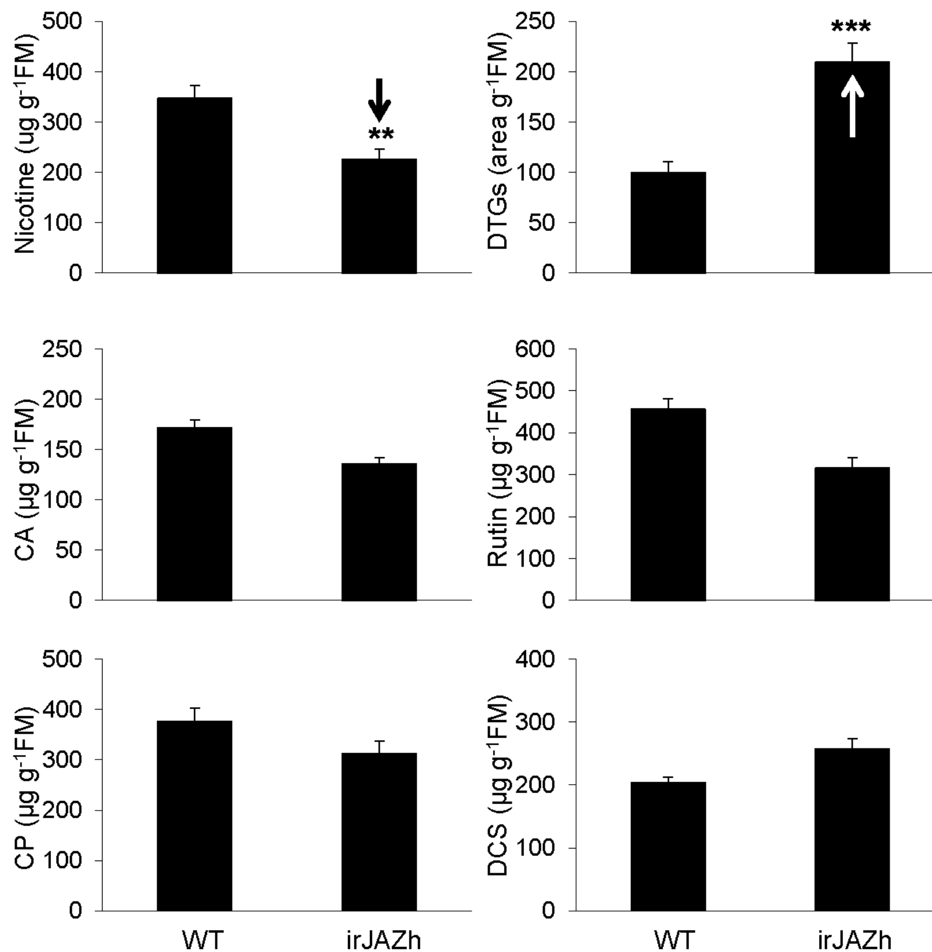

**Supplemental Figure S4**. Transcript levels of *NaJAZ* genes in irJAZh plants determined by microarrays. Fold changes (irJAZh-368/WT) of 10 *JAZ* genes in irJAZh leaves were determined by microarrays at 2 h after induction of the plants with simulated herbivory (W+OS); bars indicate fold changes ± SE of 75 percentile-normalized microarrays signals (*n=3*) in a single color (Cy-3) hybridization setup. Asterisks indicate significant differences between WT and irJAZh plants after W+OS treatment determined by Student's t-test (*P≤0.05, **P≤ 0.01, ***P ≤ 0.001). *NaJAZl* and *NaJAZm* were not represented on the current version of the Agilent GPL13527 microarray platform and their expression is not shown.



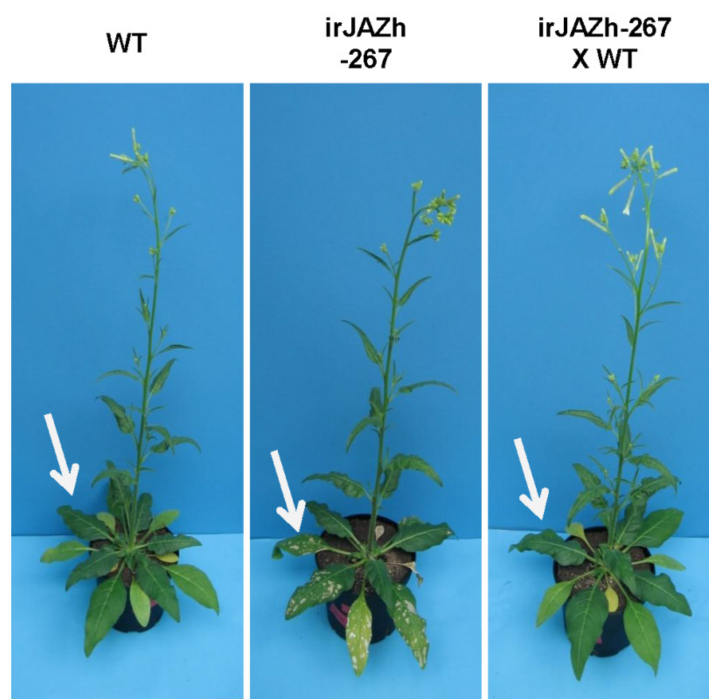

**Supplemental Figure S5.** Nicotine levels and *NaPMT* gene expression in roots. (A) Expression levels of *N. attenuata* putrescine-N-methyltransferase (*NaPMT*) involved in nicotine biosynthesis and (B) nicotine content in roots of hydroponically-grown untreated irJAZh and WT plants (*n=4*). Transcript abundances of *NaPMT* were determined by qPCR; nicotine content was determined by HPLC-PDA. No statistically significant differences were found between irJAZh and WT plants.



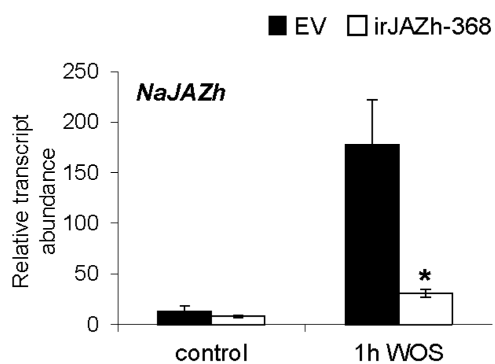

**Supplemental Figure S6.** Content of secondary metabolites in WT and irJAZh plants in the glasshouse. Rosette stage plants (WT; NaJAZh-silenced lines: irJAZh-264, -267 and -368) were treated with simulated herbivory (W+OS) and treated leaves were harvested before and 24, 48, and 72 h after elicitation. Mean ± SE levels of chlorogenic acid (CA), rutin, caffeoylputrescine (CP) and dicaffeoylspermidine (DCS) were determined by HPLC coupled to PDA (Photo Diode Array) detector (*n*=3). FM, fresh mass.



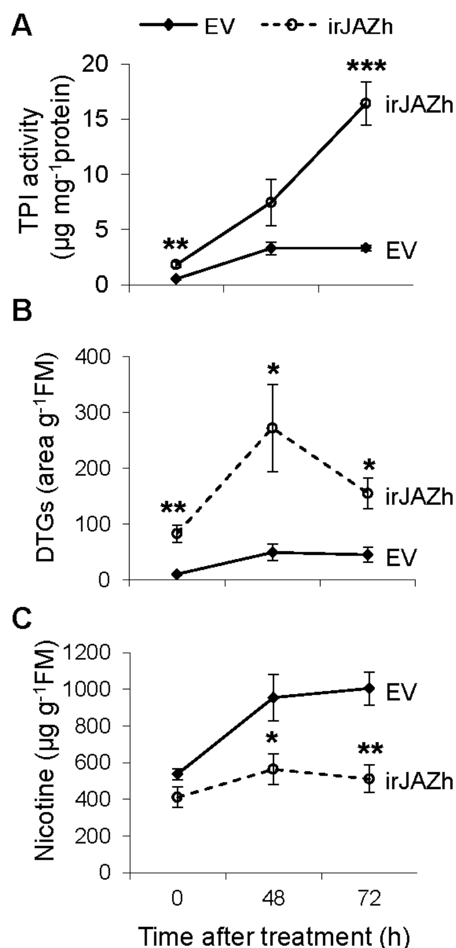

**Supplemental Figure S7.** Secondary metabolite levels in *M. sexta*-fed leaves from WT and irJAZh plants in the glasshouse. *M. sexta* neonates were placed on the leaf in the same position of each rosette plant. The leaves were then covered with transparent clip-cages to avoid caterpillar movement on the plant. After 5 days, *M. sexta* fed-leaves were harvested and analyzed for secondary metabolites by HPLC coupled to a combination of PDA/ELSD detectors; bars indicate level of secondary metabolites ± SE (*n=5*). Asterisks indicate statistically significant differences between WT and irJAZh plants after W+OS treatment determined by Student's t-test (**$P \leq 0.01$, ***$P \leq 0.001$). FM, fresh mass.



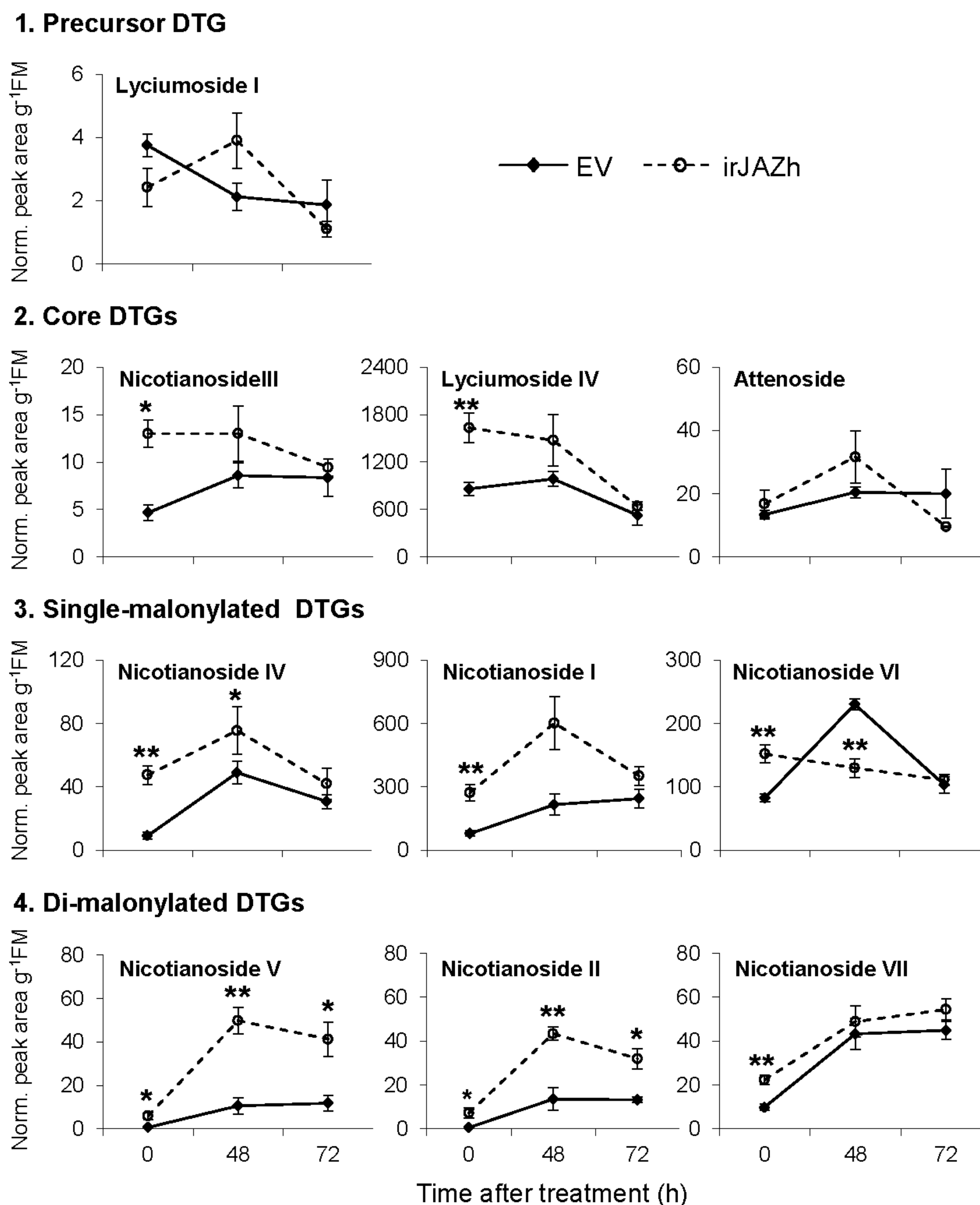

**Supplemental Figure S8.** Necrotic lesion symptoms of irJAZh plants were lost after crossing of homozygous irJAZh-267 with WT plants. The hemizygous irJAZh plants (right) do not show necrosis on their leaves compared to homozygous irJAZh-267 parents (middle) and resemble to WT plants (left); pictures of all plants were taken at 47 days after germination.



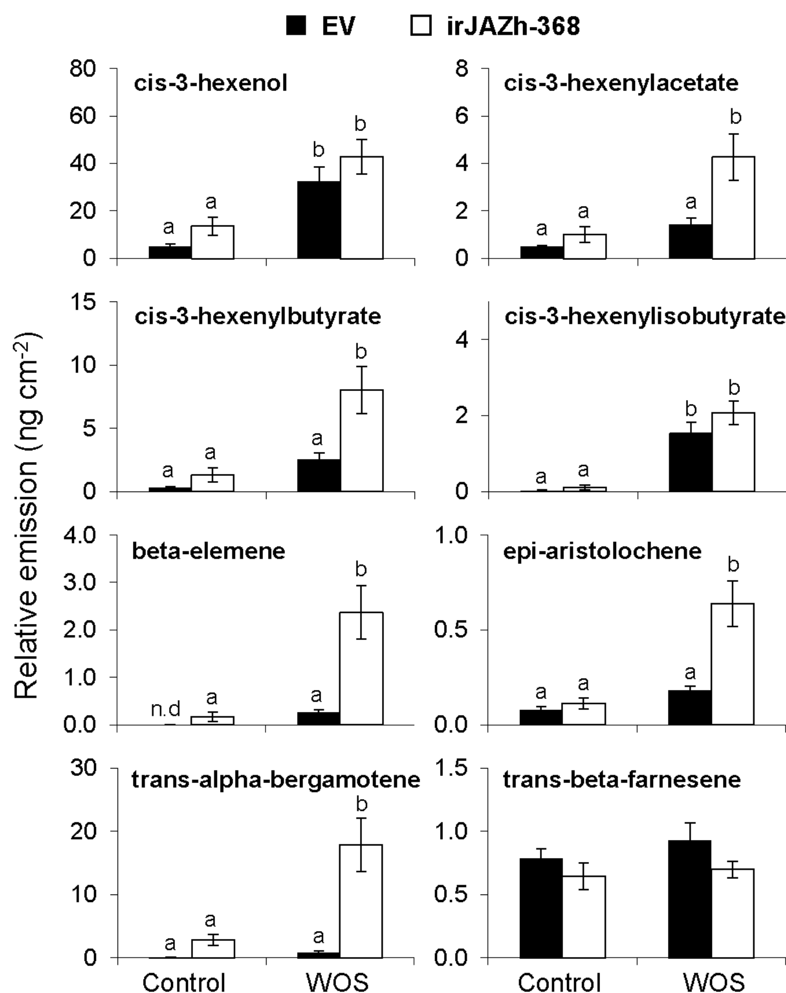

**Supplemental Figure S9.** Silencing efficiency of *NaJAZh* in field-grown EV and irJAZh plants. Transcript abundances of *NaJAZh* gene were determined by quantitative real time PCR (qPCR) in untreated (control) and1 h W+OS-elicited (W+OS) leaves of EV and irJAZh plants (*n=5*). Asterisks indicate significant differences between EV and irJAZh plants determined by Student's t-tests (*P≤0.05).




**Supplemental Figure S10.** Secondary metabolite levels determined in field-grown plants. (A) The activity of trypsin protease inhibitors (TPIs), content of (B) diterpene glycosides (DTGs) and (C) nicotine were determined in W+OS-elicited EV (empty vector) and NaJAZh-silenced plants in the native habitat in Great Basin Desert, Santa Clara, Utah, USA. Mean ± SE levels of TPI activity and DTG accumulation in irJAZh plants (*n=5*) were significantly higher compared to their levels in EV plants planted in a paired design in the same habitat. The levels of nicotine in irJAZh plants were significantly lower compared to EV plants (*n=5*). Asterisks indicate significant differences between EV and irJAZh plants determined by Student's t-tests at each individual time point of measurement (*P≤0.05, **P≤ 0.01, ***P ≤ 0.001). FM, fresh mass.

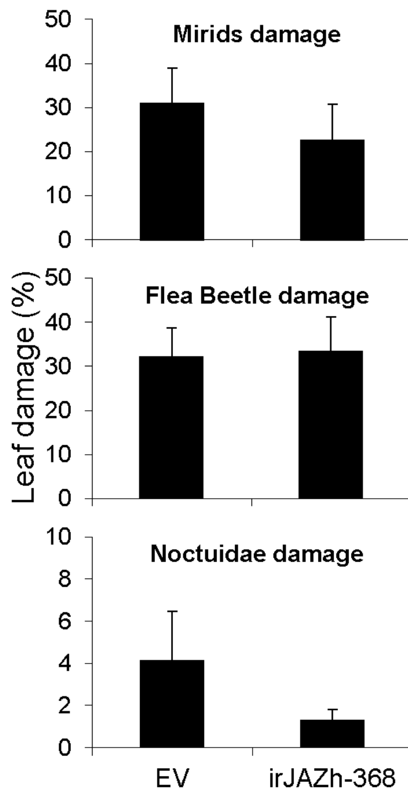



**Supplemental Figure S11**. Individual DTGs determined in EV and NaJAZh-silenced plants grown in the native habitat in Great Basin Desert, Santa Clara, Utah, USA. Mean ± SE relative levels of individual DTGs (*n=5*) measured in the leaves in native environment were consistently higher in W+OS-elicited irJAZh plants compared to EV plants (measured by LC-ESI-MS/MS). Asterisks indicate significant differences between EV and irJAZh plants determined by Student's t-test at each individual time point of measurement (*P≤0.05, **P≤ 0.01, ***P ≤ 0.001). FM, fresh mass.



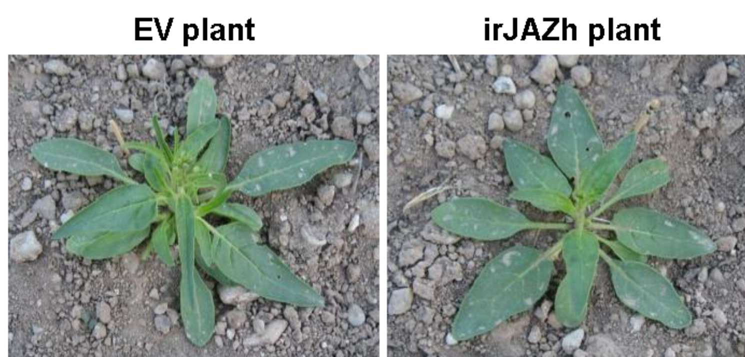

**Supplemental Figure S12.** Volatile emission rates of EV and irJAZh plants grown in the native habitat in Great Basin Desert, Santa Clara, Utah, USA. irJAZh plants emitted larger amounts of volatile organic compounds (VOCs) and green leaf volatiles (GLVs) in native environment: VOCs and GLVs were determined by GC-MS after 24 h field trapping of volatiles from locally treated leaf (W+OS) using charcoal filters with an open-flow trapping system that pulled air from the headspace of the leaf through the trap with a vacuum pump connected to a car battery.

**Supplemental Figure S10.** (continued)
irJAZh plants emitted significantly larger amounts of the sesquiterpenes, beta-elemene, epi-aristolochene and trans-alpha-bergamotene, but similar amounts of trans-beta-farnesene as determined by GC-MS. Bars indicate the emission rates of volatiles ± SE that were normalized to the internal standard, tetralin, added at the beginning of extraction process (*n=9*). Asterisks indicate significant differences between EV and irJAZh plants determined by Student's t-test (*$P \leq 0.05$, **$P \leq 0.01$).




**Supplemental Figure S13.** Herbivore damage inflicted by the native herbivore community to EV and NaJAZh-silenced plants grown in *N. attenuata*'s native habitat in Great Basin Desert, Santa Clara, Utah, USA. EV and irJAZh-368 plants were planted in a size-matched paired- design in the field plot and natural herbivore damage was scored throughout the 2011 field season. Mirid damage was determined as the % of chlorotic damaged area of the total plant canopy caused by cell-damaging feeding of *Tupiocoris notatus* mirid bugs; flea beetle damage as the % of leaf canopy damaged by the small feeding holes that characterize flea beetle feeding; and Noctuidae damage as the % of leaf canopy lost due to leaf consumption by these Lepidopteran larvae.

**Supplemental Figure S14.** Diaminobenzidine (DAB) staining in the leaves from field-grown EV and irJAZh plants. NaJAZh-silencing induced higher accumulations of hydrogen peroxide in the leaves of field grown plants. Leaves were subjected to a standardized mechanical wound by puncturing leaves with a cork-borer immediately prior to placing detached leaves in the DAB solution. irJAZh plants showed darker brown circles around punctured areas; however the EV leaf staining was relatively stronger compared to a similar experiments conducted in the glasshouse (compare with Figure 10 in Text). Despite high levels of staining, neither EV nor irJAZh plants developed necrotic symptoms in the field (see Supplemental Figure S13 online).




**Supplemental Figure S15.** Leaves of irJAZh and EV plants grown in native habitat of Great Basin Desert, Utah (USA). Despite high levels of DAB staining, neither EV nor irJAZh plants developed necrotic phenotype in the field. We planted 15 and 18 size-matched pairs of irJAZh and EV plants in the field in 2010 and 2011, respectively. Most of the plants were maintained in the field until the early flowering stage or later developmental stage (with periodically removed flowers); however, none of the leaves at any developmental stage of field-grown irJAZh plants showed necrosis.

**Supplemental Table S1** Up-regulated genes in irJAZh plants compared to WT plants by microarray.

| Classification | Fold Change | Bincode (TAIR) | Annotation | Probe number | ACC number |
|---|---|---|---|---|---|
| **Cell wall. Degradation** | 3.29 | 10.6.2 | Glycosyl hydrolase family 3 protein | CUST_146896_PI422650789 | NP_196618 |
| **Lipid metabolism** | 4.80 | 11.8.7 | Putative trans-2-enoyl-CoA reductase | CUST_75436_PI422650789 | NP_566881 |
| | 4.42 | 11.8.7 | Putative trans-2-enoyl-CoA reductase | CUST_33708_PI422650789 | NP_566881 |
| | 4.32 | 11.9.2.1 | Lipase class 3 family protein | CUST_160928_PI422650789 | NP_565701 |
| | 4.13 | 11.8.8 | Squalene synthase | CUST_163980_PI422650789 | AAM27472 |
| | 3.81 | 11.3.7 | Gamma-tocopherol methyltransferase | CUST_112272_PI422650789 | ABE41795 |
| | 3.75 | 11.1.15 | Acyl-[acyl-carrier-protein] desaturase | CUST_41928_PI422650789 | P46253 |
| | 3.57 | 11.9.2.1 | Glycerolipase A1 | CUST_160212_PI422650789 | ACZ57767 |
| | 3.33 | 11.8.1 | Sphingolipid delta-8 desaturase | CUST_80400_PI422650789 | ABO31111 |
| **Amino acid metabolism** | 3.36 | 13.1.7.4 | Cyclase | CUST_99572_PI422650789 | AAS46038 |
| **Secondary metabolism** | 5.22 | 16.1.5 | 5-epi-aristolochene synthase | CUST_46640_PI422650789 | AAO85555 |
| | 4.01 | 16.1.5 | 5-epi-aristolochene synthase (EAS) | CUST_34180_PI422650789 | Q40577 |
| | 3.86 | 16.1.5 | Plastid 1,8-cineol synthase precursor | CUST_12108_PI422650789 | ABP88782 |
| | 3.54 | 16.1.5 | 5-epi-aristolochene synthase 34 | CUST_173668_PI422650789 | AAP05761 |
| **Hormone metabolism** | 7.68 | 17.8.1 | SAMT(salicylic acid methyltransferase) | CUST_33280_PI422650789 | AAW66850 |
| | 3.35 | 17.1.1.1.11 | Short chain alcohol dehydrogenase | CUST_42160_PI422650789 | CAA11153 |
| | 3.34 | 17.5.2 | Ethylene responsive element binding factor 1 | CUST_115772_PI422650789 | BAA32418 |
| **Tetrapyrrole synthesis** | 3.25 | 19.99 | ATCLH2; chlorophyllase | CUST_94136_PI422650789 | NP_199199 |
| **Stress** | 30.80 | 20.1 | Gamma-thionin (Plant defensins) | CUST_57516_PI422650789 | AAS13436 |
| | 15.27 | 20.1 | Gamma-thionin (Plant defensins) | CUST_8140_PI422650789 | AAS13436 |
| | 4.58 | 20.1.7.6.1 | Kunitz trypsin inhibitor | CUST_14168_PI422650789 | ACL12055 |
| | 3.88 | 20.1.7.6.1 | 6-domain trypsin inhibitor precursor | CUST_9024_PI422650789 | AAQ56588 |
| | 3.78 | 20.1 | Proteinase inhibitor I-B (PI-IB) | CUST_11524_PI422650789 | Q03199 |
| | 3.47 | 20.1.7.6.1 | Kunitz trypsin inhibitor | CUST_79416_PI422650789 | ACL12055 |
| | 3.35 | 20.1.7.6.1 | Miraculin-like protein | CUST_8752_PI422650789 | ADK62529 |
| **Miscellaneous enzyme families** | 8.13 | 26.10 | CYP71D5v3 | CUST_103900_PI422650789 | ABC69398 |
| | 4.59 | 26.10 | CYP71D47v1 | CUST_102916_PI422650789 | ABC69394 |
| | 4.02 | 26.10 | Cytochrome P-450-like protein | CUST_143016_PI422650789 | BAB10537 |
| | 3.70 | 26.8 | FAD-binding domain-containing protein | CUST_127228_PI422650789 | NP_193818 |
| | 3.42 | 26.2 | UDP-glucoronosyl/UDP-glucosyl transferase family protein | CUST_43496_PI422650789 | NP_180576 |
| | 3.21 | 26.28 | GDSL-motif lipase/hydrolase family protein | CUST_64232_PI422650789 | NP_563774 |

| | | | | | |
|---|---|---|---|---|---|
| **RNA. Regulation of transcription** | 5.13 | 27.3.27 | ATNAC2 (Arabidopsis NAC domain containing protein 2) | CUST_14684_PI422650789 | NP_188170 |
| | 4.55 | 27.3.6 | Basic helix-loop-helix (bHLH) family protein | CUST_157324_PI422650789 | NP_567195 |
| | 3.25 | 27.3.5 | ARR4 (Arabidospsis regulation regulator 4) | CUST_56432_PI422650789 | NP_172517 |
| **DNA.synthesis** | 3.75 | 28.1.3 | Histone H1 | CUST_32040_PI422650789 | AAC41651 |
| **Protein. Degradation** | 6.07 | 29.5.9 | AAA-type ATPase family protein | CUST_76132_PI422650789 | NP_200556 |
| | 5.53 | 29.5 | Putative metallocarboxypeptidase inhibitor precursor | CUST_139476_PI422650789 | BAJ25782 |
| | 5.03 | 29.5 | Putative metallocarboxypeptidase inhibitor precursor | CUST_31104_PI422650789 | BAJ25782 |
| | 4.50 | 29.5.11.4.3.2 | F-box family protein | CUST_86652_PI422650789 | NP_566277 |
| | 3.69 | 29.5.11.4.2 | RING/U-box domain-containing protein | CUST_21448_PI422650789 | AAG43550 |
| | 3.41 | 29.5 | Putative metallocarboxypeptidase inhibitor precursor | CUST_40216_PI422650789 | BAJ25781 |
| | 3.19 | 29.5.9 | AAA-type ATPase family protein | CUST_78344_PI422650789 | NP_849972 |
| **Cell.organisation** | 4.22 | 31.1 | AtPP2-A11 (Phloem protein 2-A11) | CUST_73344_PI422650789 | NP_176497 |
| **Development** | 9.16 | 33.99 | Tumor-related protein | CUST_16488_PI422650789 | BAA05479 |
| | 6.07 | 33.1 | Patatin homolog | CUST_168304_PI422650789 | AAB08428 |
| | 4.01 | 33.1 | Patatin | CUST_1460_PI422650789 | AAB08428 |
| | 3.63 | 33.1 | Patatin homolog | CUST_81764_PI422650789 | AAB08428 |
| | 3.50 | 33.1 | Patatin homolog | CUST_142932_PI422650789 | AAB08427 |
| | 3.27 | 33.99 | Transducin family protein / WD-40 repeat family protein | CUST_170740_PI422650789 | NP_190535 |
| **Transport** | 6.61 | 34.9 | Mitochondrial carrier protein | CUST_124384_PI422650789 | ABO36621 |
| | 5.69 | 34.16 | ATP-binding cassette transporter, putative | CUST_87856_PI422650789 | XP_002525837 |
| | 5.24 | 34.4 | Nitrate transporter | CUST_171856_PI422650789 | BAC56915 |
| | 4.83 | 34.16 | WBC11 (White-Brown Complex homolog protein 11) | CUST_163156_PI422650789 | NP_173226 |
| **Not assigned** | 5.84 | 35.1 | Phosphorylase family protein | CUST_71848_PI422650789 | NP_567699 |
| | 4.77 | 35.1 | FAD-binding domain-containing protein | CUST_37768_PI422650789 | NP_196715 |
| | 4.59 | 35.2 | JAZ8 (Jsmonate ZIM domain protein 8) | CUST_80752_PI422650789 | NP_564349 |
| | 3.59 | 35.2 | JAZ3 (Jasmonate ZIM-domain protein 3) | CUST_52900_PI422650789 | BAG68657 |
| | 3.39 | 35.1.40 | Glycine-rich protein | CUST_69052_PI422650789 | BAA95941 |

* Microarray data is processed by SAM after 75% percentile normalization (FDR=4.82).

* Up-regulated genes were determined by a greater than 3-fold induction of normalized signals in their expression ratio (irJAZh / WT) of 2h-WOS treated leaves. The values are the average ratio of 3 biological replicates of the microarrays.

* All changes in gene expression were statistically significant by t-test ($P < 0.05$)

* Gene annotation is processed by Blast X (E-value < 1e-5)

* Classification of genes is based on GO classification from TAIR (http://www.arabidopsis.org).

**Supplemental Table S2** Down-regulated genes in irJAZh plants compared to WT plants by microarray.

| Calssification | Fold Change | Bincode (TAIR) | Annotation | Probe number | ACC number |
|---|---|---|---|---|---|
| **Photosynthesis** | 3.03 | 1.1.2.2 | Photosystem I P700 apoprotein A2 | CUST_32628_PI422650789 | ABB90040 |
| | 3.33 | 1.2.2 | Glycolate oxidase | CUST_6068_PI422650789 | ADM26718 |
| **Major Carbohydrates** | 3.03 | 2.2.2.1 | Alpha-amylase | CUST_12856_PI422650789 | ACZ26470 |
| **Lipid metabolism** | 10.00 | 11.3.1 | Acyltransferase-like protein | CUST_12976_PI422650789 | BAD93693 |
| | 6.25 | 11.3.1 | Acyltransferase-like protein | CUST_134584_PI422650789 | BAD93693 |
| **Metal handling** | 4.00 | 15.3 | Vacuolar iron transporter-like protein | CUST_26436_PI422650789 | NP_173538 |
| | 3.03 | 15.1 | Ferric-chelate reductase | CUST_64152_PI422650789 | AAP46144 |
| | 4.17 | 15.2 | Metallothionein-like protein type 2 | CUST_144048_PI422650789 | CAC12823 |
| **Secondary metabolism** | 3.45 | 16.1.5 | Sesquiterpene synthase | CUST_166968_PI422650789 | NP_001239041 |
| | 12.50 | 16.1.5 | Plastid 1,8-cineol synthase precursor | CUST_44188_PI422650789 | ABP88782 |
| | 12.50 | 16.1.5 | Plastid 1,8-cineol synthase precursor | CUST_20468_PI422650789 | ABP88782 |
| | 9.09 | 16.1.5 | Plastid 1,8-cineol synthase precursor | CUST_14120_PI422650789 | ABP88782 |
| | 8.33 | 16.1.5 | Monoterpene synthase 2 | CUST_165928_PI422650789 | AAX69064 |
| | 7.14 | 16.8.3 | Terpene cyclase/mutase-related | CUST_11104_PI422650789 | NP_195062 |
| **Hormone metabolism** | 3.33 | 17.2.3 | SAUR-like auxin-responsive protein | CUST_59948_PI422650789 | NP_187035 |
| | 3.23 | 17.4.1 | Cytokinin-O-glucosyltransferase 2 | CUST_173124_PI422650789 | NP_173656 |
| | 4.17 | 17.5.2 | ERF transcription factor 5 | CUST_116820_PI422650789 | AAU81956 |
| **Stress** | 3.57 | 20.2.3 | Early dehydration inducible protein | CUST_55152_PI422650789 | AAR26237 |
| | 3.57 | 20.2.3 | Salt responsive protein 1 | CUST_45184_PI422650789 | ACG50003 |
| | 3.23 | 20.1.7 | CC-NBS-LRR putative disease resistance protein | CUST_167984_PI422650789 | ACB70404 |
| | 3.13 | 20.2 | Pathogenesis-related protein | CUST_15548_PI422650789 | BAD15090 |
| **Miscellaneous enzyme families** | 3.85 | 26.10 | Elicitor-inducible cytochrome P450 | CUST_25520_PI422650789 | AAK62342 |
| | 2.94 | 26.10 | Elicitor-inducible cytochrome P450 | CUST_96052_PI422650789 | AAK62343 |
| | 3.85 | 26.22 | Short-chain dehydrogenase/reductase family protein | CUST_122964_PI422650789 | XP_002869743 |
| | 4.55 | 26.3.2 | Beta-galactosidase STBG5 | CUST_18760_PI422650789 | ADO34790 |
| | 3.57 | 26.3.2 | Beta-galactosidase STBG5 | CUST_888_PI422650789 | ADO34790 |
| | 3.85 | 26.10 | CYP71D49v2 | CUST_119780_PI422650789 | ABC69401 |
| **RNA. regulation of transcription** | 2.94 | 27.3.11 | Zinc finger protein 4 | CUST_73116_PI422650789 | NP_176788 |
| | 4.76 | 27.3.22 | Class 2 knotted1-like protein | CUST_139276_PI422650789 | BAF95776 |
| | 3.33 | 27.3.35 | bZIP transcription factor BZI-2 | CUST_107720_PI422650789 | AAK92213 |
| | 4.17 | 27.3.6 | Basic helix-loop-helix (bHLH) family protein | CUST_56184_PI422650789 | NP_181549 |

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|---|---|---|---|---|---|
| | 3.33 | 27.3.6 | Transcription factor style2.1 | CUST_33216_PI422650789 | ABX82930 |
| | 3.33 | 27.1.19 | Ribonuclease H-like protein | CUST_79632_PI422650789 | NP_172471 |
| | 4.55 | 27.1.19 | RNase H family protein | CUST_121612_PI422650789 | ABI34372 |
| | 3.23 | 27.3 | Transcription factor style2.1 | CUST_163752_PI422650789 | ABX82930 |
| **Protein. degradation** | 3.85 | 29.5 | Class S F-box protein | CUST_152204_PI422650789 | ABR18786 |
| **Signaling** | 3.03 | 30.5 | Transducin/WD40 domain-containing protein | CUST_21460_PI422650789 | NP_199823 |
| | 3.03 | 30.3 | Calcineurin B-like protein | CUST_107040_PI422650789 | ABQ23353 |
| **Development** | 3.23 | 33.3 | Squamosa promoter-binding-like protein 12 | CUST_109112_PI422650789 | NP_191562 |
| | 4.00 | 33.99 | Nodulin MtN21 family protein | CUST_29984_PI422650789 | NP_181622 |
| | 4.00 | 33.99 | Senescence-associated protein-related | CUST_6080_PI422650789 | NP_564160 |
| | 3.03 | 33.99 | Fruitfull-like MADS-box protein | CUST_42876_PI422650789 | ABF82231 |
| | 2.94 | 33.99 | NAC domain protein | CUST_81900_PI422650789 | AAU43923 |
| | 4.00 | 33.99 | Putative gag polyprotein, identical | CUST_81856_PI422650789 | AAT39964 |
| | 4.55 | 33.99 | Ripening regulated protein DDTFR18 | CUST_97944_PI422650789 | AAG49032 |
| **Transport** | 3.13 | 34.4 | Nitrate transporter (NTL1) | CUST_131240_PI422650789 | AAG52554 |
| | 3.45 | 34.12 | Metal transporter | CUST_159740_PI422650789 | AAP21819 |
| **Not assigned** | 3.13 | 35.1 | Oxidoreductase family protei | CUST_25856_PI422650789 | NP_193468 |
| | 4.00 | 35.2 | Transducin family protein / WD-40 repeat family protein | CUST_54908_PI422650789 | NP_196176 |
| | 3.33 | 35.2 | Jasmonate ZIM-domain protein 1 | CUST_40368_PI422650789 | BAG68655 |
| | 3.03 | 35.2 | GC-rich sequence DNA-binding factor | CUST_122872_PI422650789 | NP_196472 |
| | 3.85 | 35.2 | Serine-threonine protein kinase, plant-type, putative | CUST_171656_PI422650789 | XP_002532616 |

* Microarray data is processed by SAM after 75% percentile normalization (FDR=4.82).
* Down-regulated genes were determined by a greater than 3-fold repression of normalized signals in their expression ratio (irJAZh / WT) of 2h-WOS treated leaves. The values are the average ratio of 3 biological replicates of the microarrays.
* All changes in gene expression were statistically significant by t-test ($P < 0.05$)
* Gene annotation is processed by Blast X (E-value < 1e-5)
* Classification of genes is based on GO classification from TAIR (http://www.arabidopsis.org).

**Supplemental Text S1.** Protein sequence alignment used to build phylogenetic tree in Figure1.

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AtJAZ1    1 ---------- ---------- ---------- ----MSS-SM ECSEFVGSRR -----
AtJAZ10   1 ---------- ---------- ----MSKATI ELDFLGLEKK Q--TNNAPKP K----
AtJAZ11   1 ---------- ---------- ---------- ----MAEVNG D--FPVP--- -----
AtJAZ12   1 ---------- ---------- ---------- ----MTKVKD EPRASVEGGC -----
AtJAZ2    1 ---------- ---------- ---------- ----MSSFSA ECWDFSG--- -----
AtJAZ3    1 ---------- ---------- ---------M ERDFLGLGSK NSPITVKEET SESSR
AtJAZ4    1 ---------- ---------- ---------M ERDFLGLGSK LSPITVKEET NE---
AtJAZ5    1 ---------- ---------- ---------- ---MSSSNEN AKAQAPEK-- -----
AtJAZ6    1 ---------- ---------- ---------- ---MSTG--- ---QAPEK-- -----
AtJAZ7    1 ---------- ---------- ---------- ----MIIIIK NCDKPLLN-- -----
AtJAZ8    1 ---------- ---------- ---------- ---------- ---------- -----
AtJAZ9    1 ---------- ---------- ---------M ERDFLGLSDK QYLSNNVKHE VN---
NaJAZa    1 ---------- ---------- ---------- ---MASSEIV DSGKFSAG-- -----
NaJAZb    1 ---------- ---------- ----MDSSII EIDFMDLNSR P-QSEMAKQQ TK---
NaJAZc    1 ---------- ---------- ---------- ----MGLNSK D--SVVLVKE EP---
NaJAZd    1 ---------- ---------- ---------- ---MGLSEIV DSGKVTGQ-- -----
NaJAZe    1 ---------- ---------- ---------- ----MGLTHH VKQEVIEEHI DP---
NaJAZf    1 ---------- ---------- ---------- ---------- ---------- -----
NaJAZg    1 ---------- ---------- ---------M ERDFMGLA-- VKQEIPEEQP TD---
NaJAZh    1 ---------- ---------- ---------- ---MSNSQNS FDGGRRAG-- -----
NaJAZj    1 ---------- ---------- ---------- ---------- ---------- -----
NaJAZk    1 ---------- --------MP PEESVSKSLL DKPLQQLTED DISQLTREDC RRYLK
NaJAZl    1 ---------- ---------- ---------- --MYCSSKVA NNFLKIEKFN -----
NaJAZm    1 ---------- ---------- ---------- ---------- ---------- -----
NtJAZ1    1 ---------- ---------- ---------- ---MGSSEIV DSGKVSGQ-- -----
NtJAZ2    1 ---------- ---------- ---------M ERDFMGLNSK D--SVVVVKE EP---
NtJAZ3    1 ---------- ---------- ---------- ---MASSEIV DSGRFAAAAA -----
OsJAZ1    1 ---------- ---------- ---------- ---------- ---MAAAG-- -----
OsJAZ10   1 ---------- ---------- ---------- ---------- ---------- -----
OsJAZ11   1 ---------- ---------- ---MSTRAPV ELDFLGLRAA A--ADADDRH AK---
OsJAZ12   1 ---------- ---------- ---------- ---------- ---------- -----
OsJAZ2    1 ---------- ---------- --------MA GSSEQQLVAN AAATTVAG-- -----
OsJAZ3    1 ---------- ---------- ---------- ---------- --MASTDP-- -----
OsJAZ4    1 ---------- ---------- ---------- ---------- ---MAMEG-- -----
OsJAZ5    1 ---------- ---------- ---------- ---------M ASAKSGER-- -----
OsJAZ6    1 ---------- ---------- ---------- ---------- ---------- -----
OsJAZ7    1 ---------- ---------- ---------- ----MAGRAT ATATAAGK-- -----
OsJAZ8    1 ---------- ---------- -------MER DFLGAIGKDE EQRRHAEERK ESDYF
OsJAZ9    1 ---------- ---------- -------MQW QFP-ATKVGA ASSAFMSFRS -----
SlJAZ1    1 ---------- ---------- ---------- ---MASSEIV DSGRFAGQ-- -----
SlJAZ10   1 ---------- ---------- ---------- ---------- ---------- -----
SlJAZ11   1 ---------- ---------- ---------- ---------- ---------- -----
SlJAZ12   1 MSSGTDNVIG NTHPYEILKN ETTNIVTLSF ICDEIVEIFN VSRDKAEEIL KFADM
SlJAZ2    1 ---------- ---------- ---------- ---MGSSENM DSGKVTGQ-- -----
SlJAZ3    1 ---------- ---------- ---------- ---MSN---L CDARRRNGNG -----
SlJAZ4    1 ---------- ---------- ---------- ---MSN---- ---RQLCS-- -----
SlJAZ5    1 ---------- ---------- ---------M ERDFMGLT-- VKQEVLEEPI DP---
SlJAZ6    1 ---------- ---------- ---------M ERDFMGLNIK D--SLLVVKD EP---
SlJAZ7    1 ---------- ---------- ----MDS-RM EIDFMDLNSK PKLSEMEKQH KK---
SlJAZ8    1 ---------- ---------- ---------M HWSYS----- -----NKAHP -----
SlJAZ9    1 ---------- ---------- ---------- ---------- ---------- -----
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AtJAZ1    16 -FTGK VSGMKWPFS- LADLATHHEH TFFQNYKSTP IVS------- ----------
AtJAZ10   26 ----- -------QYH LSLKDQ-ENI IINNNNNNKP KIG------- ----------
AtJAZ11   12 -SFAD ---MRRNCNL ELT-LMPPS- -------ISD NFSS------ ----------
AtJAZ12   17 -GVAD ------FDSL ASTGLVTITT -TEAVDSSHR TYSDVTQN-- ----------
AtJAZ2    14 ----R ------LSAT DGVDAGLKRQ PGELQ----- ---------- ----------
AtJAZ3    27 DSAPN KPSFSQTCSR LSQYLK-ENG SFGDLS-LGM ACKP-DVNGT LGN-------
AtJAZ4    24 DSAPS -----FQKFL DRRRSFRDIQ G-AISKIDPE IIK------- ----------
AtJAZ5    16 ----- GTG-SVSAGL DLLVERSIHE ARSTEPDAST ------QLTI IFG-------
AtJAZ6    10 ----- GDGGAAEIGG TGSVEKSINE VRSTEIQTAE PTVPPNQLTI FFG-------
AtJAZ7    15 ---FK KPSFSQTCTR LSRYLK-EKG SFGDLS-LGM TCKP-DVN-- -GG-------
AtJAZ8     1 ----- RG-MNWSFSN KVSASSSQFL SFRPTQEDRH RKSGNYHLPH SGSFMPSSV-
AtJAZ9    24 --DDA RGMMDWSFSS KVGSGP-QFL SFGTSQQETR VNTVNDHLLS S---------
NaJAZa    16 --GGQ -SDFTRRCSL LSRYLK-EKG SFGNID-LGL YRKPDSSLAL PG--------
NaJAZb    28 ----- -SNFSQRCSL LSRYLK-EKG SFGNIN-MGL ARKSD--LEL AG--------
NaJAZc    17 --VET EMEMQTKCDL ELR-LLTSS- -------YDS DFH------- ----------
NaJAZd    16 ----- -MKLQQNCDL ELR-LFPTS- -------YDS DSSD------ ----------
NaJAZe    19 --APL VEERGLSTKA AREWGKSKVF ATSSFMPSSD FQE------- ----------
NaJAZf     1 ----- KSHFSQTCNL LSQYLKEKKG SFGDLS-LGI HR-------- ----------
NaJAZg    22 --PAM ASGMKWPFSS MANLATQAES RFFQNYNSSP IVS------- ----------
NaJAZh    16 -KAPE CKDSGFRWPL SSKVGIPHFM SLNSAQDEKP FKA------- ----------
NaJAZj     1 ----- KSQFSQTCNL LSQFLK-KKG SFGDLNNLGI YR-SFEPTGN Q---------
NaJAZk    38 LKGMR RSSAMQWSFS NNISTHPQYL SFKGAQEDRL KTG------- ----------
NaJAZl    19 ----K ---MRRNCNL ELR-LVPPCV SVS----PKD CTTT------ ----------
NaJAZm     1 ----- ARISAILQRS FSNKALPQYL SFKNAQGNTP KTG------- ----------
NtJAZ1    16 ----- RSNFVQTCNL LSQFIK-GKA TIRDLN-LGI AGKSEISGKS D---------
NtJAZ2    22 --VET ----RKNCNL ELR-LMPPSF SFS----PKS CTT------- ----------
NtJAZ3    18 --GGQ RPSWNKSQAI QQVISLKALL ETTPDSDAGT RKKLYIPRSD TKLHHVQ---
OsJAZ1     6 ----S NFDYQKQINE SNLKGIGNNG SHRRMSALEL AILP------ ----------
OsJAZ10    1 ----- ---MKHRIGL QLFPLLSSS- -------SSS EFH------- ----------
OsJAZ11   28 ----- KSQFSQTCNL SSQFLK-KKG SFGDLNNLGI YR-SFEPTGN Q---------
OsJAZ12    1 ----- CKDSGFRWQL SSKVGIPHFM SLSSAQDEKP TKA------- ----------
OsJAZ2    21 ----N KSNFSQTCNL LSQYLKEKKG SFGDLS-LGI HR-------- ----------
OsJAZ3     7 ----M SSRFAVTCGL LSQYMRER-- --QQPQ---- --PPVTVL-- ----------
OsJAZ4     6 ----R ---------- ---------- -MSSPPPNPS ------QLTI FYG-------
OsJAZ5    10 ----G ----SGGSSA SSSSSIRGME TSAIARIGPH LLR------- ----------
OsJAZ6     1 ----- ---MPPPAAV ASLTLQVPGG ---------- ---------- ----------
OsJAZ7    15 ----D GSRFAVTCGL LRQYMKEH-- --SGSNGGGG FLPAVTAMS- ----------
OsJAZ8    29 GAGGG TRRFAVACGV LSQYVKAN-- --SSQP---- -----STAA- ----------
OsJAZ9    23 SAAAA SRRFAVACGV LSQYVRAE-- --QKMAAAAG AAPARAVTT- ----------
SlJAZ1    16 ----- SSSFAMACSL LSRYVRQN-- -GAAAGELGL GIRGEADAN- ----------
SlJAZ10    1 ----- -----MACSL LSRYVRQN-- -GAAAAELGL GIRGEGEAPR AAPA------
SlJAZ11    1 ----- RSSFAVTCSL LSQFLKEKKG GGGGLQGLGL GLRPAPAAPP AAGAGGAFRP
SlJAZ12   56 SKVVN AAAAAMDWSF ASRAALMSFR SSSSAAAAAA REETRELAFP HFSALDGAKM
SlJAZ2    16 ----- REEDPKEAAV FDRFSLSGFR PPPRPSPGDA FDGAAAMKQR QFG-FNGRQQ
SlJAZ3    15 -KAPE KSHFSHTCNL LSQYLKEKKG SLGDLS-LDM HRNFDS---- ----------
SlJAZ4     9 -LDSE ---MRRKCNL ELT-ISP--- --------SN ---------- ----------
SlJAZ5    22 --APL ---MRRNCNL EFR-LMPPSL STFSPNICSN NNTS------ ----------
SlJAZ6    22 --VES DCSSKKPLGL ETQSQGKGKE KLSTARRNSQ LRFLEKRKER ICDVLENG--
SlJAZ7    28 ----- KSQFSQTCNL LSQFLK-KKG SVGDLNNLGI YKTTFESTGS QQT-------
SlJAZ8    12 ----- RSSFVQTCNL LSQFIK-GKA TIRDLN-LGI AGQPEAAGK- ----------
SlJAZ9     1 ----- KSHLMNTCNL LTQFFN-GKA NINDLN-LTI SNNGEAKAS- ----------
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AtJAZ1    52  ---------- -----ATTTT TTMNLLPMIE KSSDSSSSSS S--------- ----
AtJAZ10   51  ---------- -------TET ATMDLLTVME KPSIDLTK-- ---------- ----
AtJAZ11   38  ---------- ---------- ATKDLLTNME ELSTKTTE-- ---------- ----
AtJAZ12   52  ---------- ---------- ---------- ---------- ---------- ----
AtJAZ2    34  ---------- ---------- ---------- ---------- ---------- ----
AtJAZ3    72  ---------- ------INSK NSSLNNYKST IDPQYF---- ---------- ----
AtJAZ4    56  ---------- ------FESL ASAGLVTITT TTELFDTIHR PYT------- ----
AtJAZ5    52  ---------- --------KN CTTE-DQQ-- LENKQ----- ---------- ----
AtJAZ6    53  ---------- ---------- ---------- -LITTVNQLP GAGALV---- ----
AtJAZ7    54  ---------- ----MKQVLG ---------- ---------- ---------- ----
AtJAZ8    49  ---------- -----SRQPT TTMSLFPCEA SNMDSMV--- ---------- ----
AtJAZ9    67  ---------- -------SLL ASTGNNSDSS AKSRSV---- ---------- ----
NaJAZa    58  ---------- -------GSC RVFNGVPAQK VQEIIR---- ---------- ----
NaJAZb    65  ---------- -------GSV TVFDGLPSEK VQEILR---- ---------- ----
NaJAZc    44  ---------- -----SRQP- TMMNLFPCEA SGMDSSAG-- ---------- ----
NaJAZd    40  ---------- ------ADVY DSTRKAPYSS VQGVRMFPNS NQHEETN-AV SMSM
NaJAZe    55  ---------- -------AAM DQNQRTYFSS LQEDRVFPGS SQQDQTTITV SMSE
NaJAZf    32  ---------- -----KFDPP GKQNAMHKAG HSK------- ---------- ----
NaJAZg    58  ---------- -----KFDLK GQQNVIKKVE TSETRPFKLI Q--------- ----
NaJAZh    53  ---------- ---------S SLDESSSSEI SQPKQE---- ---------- ----
NaJAZj    40  ---------- --------TT SVVESTSSGN PQPNEE---- ---------- ----
NaJAZk    76  ---------- ------AKAF PGAYQWGSVS AANVF----- ---------- ----
NaJAZl    46  ---------- -------AGT TTMDLFPMIE KSGES----- ---------- ----
NaJAZm    34  ---------- ------SNSK SSPLKSYKLT TK-------- ---------- ----
NtJAZ1    55  ---------- ------QSAA DGVDSCLKRQ SGEIQNVHAM HLLHDVMMLP FNRS
NtJAZ2    49  ---------- -------TTT TTMNLLPMIE KSGDS----- ---------- ----
NtJAZ3    68  ---------- ------FDSL ASTGLVTITT -TEAVDSSHR PYSGVTQNNM MLEK
OsJAZ1    41  ---------- --------PY FSMR--DNQG TEEKQ----- ---------- ----
OsJAZ10   23  ---------- ------FDSL ASAGLVTITT SHEAVDSNYR PYTAVTQKNL MLEK
OsJAZ11   67  ---------- ------VTEA ATMDLLTIME NPSIETKE-- ---------- ----
OsJAZ12   34  ---------- --------PY FSMNREDKEN TEEKE----- ---------- ----
OsJAZ2    53  ---------- -------RGK NTDEEFIELA EETVPDGR-- ---------- ----
OsJAZ3    36  ---------- --------GI KHDTQLPVLQ REKSE----- ---------- ----
OsJAZ4    23  ---------- ---------- SSDHSVACSS TR-------- ---------- ----
OsJAZ5    40  ---------- -------TTT TTMNLLPMIE KSGDS----- ---------- ----
OsJAZ6    18  ---------- ------LSSA DGVDSCLKRQ SGEIQ----- ---------- ----
OsJAZ7    51  ---------- -------AGT TTMDLLPMIE KFGES----- ---------- ----
OsJAZ8    60  ---------- ---------- ---EAVAEEE EEED------ ---------- ----
OsJAZ9    63  ---------- -------GSV CVYDSVPPEK AQAIML---- ---------- ----
SlJAZ1    52  ---------- -------RVI AAAGP----- ---------- ---------- ----
SlJAZ10   37  -------TMS --------AH DVTSLATSPR TMAVPG---- ---------- ----
SlJAZ11   51  -------PPT ---------L MTGGADAEEE APEV------ ---------- ----
SlJAZ12  111  QQASHVLARQ ---------P VAQGVSGLMA AAAA------ ---------- ----
SlJAZ2    65  ------YAAA ---------L SLMPGAEVVV EEEE------ ---------- ----
SlJAZ3    54  ---------- -------KGK ETMELFPQNS GFG------- ---------- ----
SlJAZ4    28  ---------- LLPGEAERKK ETMELFPQSA GFGQ------ ---------- ----
SlJAZ5    55  ---------- TMNLLSGLDA PAVEVEPNTA ETAA------ ---------- ----
SlJAZ6    73  ---------- KSFGAESHGI PQYAAAAAVH GAHRGQPPHV LNGARVIPAS ----
SlJAZ7    70  ---------- AQHGHREQGV DSYGVAAPHH FPSPSPSPRH P--------- ----
SlJAZ8    49  ---------- -------AGS TTMDLLPMIE KSGE------ ---------- ----
SlJAZ9    38  ---------- ---------- LLME-DKR-- LENEQ----- ---------- ----
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AtJAZ1    78 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ10   72 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ11   56 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ12   52 ------ ---------- LPLIKAPA-- ---------- -------DQQ SDESASEAA
AtJAZ2    34 ------ -ILIFAELLK MFRVQSSPNL PNAVGAGGGA FKQPPFAMGN AVAGSTVGV
AtJAZ3    92 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ4    83 ------ --TQFGAHHV ---------- ---------- ---------- ---------
AtJAZ5    66 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ6    68 ------ --------VS ---------- ---------- ---------- ---------
AtJAZ7    60 ------ ----GIPVTA VTDVIPPTKS Q--------- --SNVETFSG GRFWEKQYP
AtJAZ8    71 ------ ---------- ---------- ---------- ---------- ---------
AtJAZ9    86 ------ ---------- ---------- ---------- ---------- ---------
NaJAZa    77 ------ ---------- ---------- ---------- ---------- ---------
NaJAZb    84 ------ ---------- PISAVPSSSI V--------- -------VGT TDLRGAP--
NaJAZc    66 ------ ---------- PHSMLPSRGS V--------- -------AGT TEPWFNS--
NaJAZd    77 PGFQSH HYAPGGRSFM ---------- ---------- ---------- ---------
NaJAZe    92 P----- -------NYI ---------- ---------- ---------- ---------
NaJAZf    50 ------ ---------- ---------- ---------- ---------- ---------
NaJAZg    84 ------ ---------- ---------- ---------- ---------- ---------
NaJAZh    70 ------ ---------- ---------- ---------- ---------- ---------
NaJAZj    58 ------ ---------- NNNNNSQPLV ---------- ---------- ---------
NaJAZk    95 ------ ---------- NSFINHQHLG ---------- ---------- ---------
NaJAZl    64 ------ ---------- ---------- ---------- ---------- ---------
NaJAZm    50 ------ ---------- ---------- ---------- ---------- ---------
NtJAZ1    93 NPSYKT HFGGTGQISA ---------- ---------- ---------- ---------
NtJAZ2    67 ------ ---------- ---------- ---------- ---------- ---------
NtJAZ3   105 QGGTHY TSTTFSPHHY ----RRCQFG ---------- ---------- ---------
OsJAZ1    56 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ10   61 QGITNY TMTTYPPHKI ---------- ---------- ---------- ---------
OsJAZ11   89 ------ ---------- AATMKQQLLG ---------- ---------- ---------
OsJAZ12   51 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ2    74 ------ ---------- DAHSVHRSHG VRVLPVSNLA NQISVSMTMP GHKSFVS--
OsJAZ3    53 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ4    35 ------ ---------- GTNSVQQSHE VRVLPVANQT HQISVSTRNM HGRQPLISP
OsJAZ5    58 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ6    37 ------ -------ISA ---------- ---------- ---------- ---------
OsJAZ7    69 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ8    71 ------ ---------- ---------- ---------- ---------- ---------
OsJAZ9    82 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ1    60 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ10   58 ------ ---------- AATMKQQLLG ---------- ---------- ---------
SlJAZ11   69 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ12  136 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ2    84 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ3    70 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ4    52 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ5    79 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ6   113 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ7   101 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ8    66 ------ ---------- ---------- ---------- ---------- ---------
SlJAZ9    50 ------ ---------- ---------- ---------- ---------- ---------
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AtJAZ1    78 - ---------- --VPFGHANP --EVGEEE-- ---------- -------AGP AT-
AtJAZ10   72 - ---------- ------LVQK AAAVKETP-- ---------- -------DAR EQE
AtJAZ11   56 - ---------- ---------- AITADSAA-- ---------- -------DAR EQE
AtJAZ12   72 - -------GEK AQQLTIFYGG SVVVFDDFP- ---------- AEKAGELMKL AGS
AtJAZ2    82 Y G-TRDMPKAK AAQLTIFYAG KVVVIDRCT- ---------- PAMAAELMRF ASA
AtJAZ3    92 - ---------- ---VETNPQK MLWVHSLPNV -----AGGSP YRNQSFSVGN SVA
AtJAZ4    91 - ---------- ------EEHK SMNLFPQG-- ---------- G------MKA ESE
AtJAZ5    66 - ---------- ------QDQK ---------- ---------- ---------- ---
AtJAZ6    70 - ---------- SEPLTIFYNG KVVVFENFP- ---------- STKVKDLLQI VST
AtJAZ7    94 R LNSCLKNGII IAPLTICYDG SVNVFNNVS- ---------- PEKAQELMFL ASR
AtJAZ8    71 - ---------- ---------- PMNLFPQE-- ---------- FDFSKEQSTK KTE
AtJAZ9    86 - ---------- ---------- SVDLV----T T----ESSRE KEAAVNEPST SKE
NaJAZa    77 - ---------- ---------- LIDHVP---- ---------- -KSAINKASG SKE
NaJAZb   105 - -----KTPPG PAQLTIFYGG KLV-VSHVT- ---------- DLQAKAIIYL ASR
NaJAZc    87 - -----KGSAA PAQLTIFYGG TSVSFNIFS- ---------- -DKVANILEF AEK
NaJAZd    93 - ---------- ------QDVK -RGTFPLLAK T--------- -------STY DSR
NaJAZe    96 - ---------- ------PSTP -----PTRNG V--------- -------VGT TEL
NaJAZf    50 - ---------- --------IA ---------- ---------- ---------- ---
NaJAZg    84 - ---------- --------IA SVCVYDNVS- ---------- PEKAQAIMLL AGN
NaJAZh    70 - ---------- -----QEDIK MVNVFEDIS- ---------- PEKAQAIMFL AGH
NaJAZj    68 - ---------- ---GVPIMAP PTNLFPRQ-- ---------- PSFSSSSSSL PKE
NaJAZk   105 - ---------- ---GSPIMAP REDQPQIPIS P--------- -------VHA SLA
NaJAZl    64 - ---------- -------GEP FAGKQTKN-- ---------- -------VTG IN-
NaJAZm    50 - ---------- ---KFSIGEA AKAMETKN-- ---------- -------STS ISP
NtJAZ1   109 - ---------- ---------- PKTMFPRQ-- ---------- SSFSSSSSSG TKE
NtJAZ2    67 - ---------- ---------- PISILPPPGS I--------- -------VGT TDI
NtJAZ3   127 - ---------- ----GAFQNA PVSVFPAP-- ---------- ---------- TTI
OsJAZ1    56 - ---------- ------NPQK STSSGGKVK- ---------- -----DVADL SES
OsJAZ10   77 - ---------- ---------- STSTEDKAIY ---------- ----IDLSEP AKV
OsJAZ11   99 - ---------- ----GIPVTA ---------- ---------- ---------- ---
OsJAZ12   51 - ---------- ---AEKKSQK ---------- ---------- ---------- ---
OsJAZ2   111 - ---------- -PLGQNPVAS TPLLLGGSVP L--------- -------PTH PSL
OsJAZ3    53 - ---------- ---------- PMNLFPQ--- ---------- -------TEA KSE
OsJAZ4    74 A GQNLISIINQ NPARGAQISS ---------- ---------- ---------- DSR
OsJAZ5    58 - ---------- ------QEQK PHSILPSSGS V--------- -------AGI TEP
OsJAZ6    40 - ---------- ---------- PMNLFPQE-- ---------- V-----ISTT KSE
OsJAZ7    69 - ---------- ------TLQD PISAVPTNSA V--------- -------VGT TDL
OsJAZ8    71 - ---------- ---------- ---------- ---------- ---------- ---
OsJAZ9    82 - ---------- ---------- SISILPNRNG V--------- -------VGT TEL
SlJAZ1    60 - ---------- ---AEKNSQK SIDPVRQSAV T----ESSRD MEVAVNEPST SKE
SlJAZ10   68 - ---------- ----GIPVTA ---------- ---------- ---------- ---
SlJAZ11   69 - ---------- ------NPQK KADLSGDATP N--------- -----LVAAI DKS
SlJAZ12  136 - ---------- --------AR ---------- ---------- ---------- ---
SlJAZ2    84 - ---------- --------IA ---------- ---------- ---------- ---
SlJAZ3    70 - ---------- ---------- PMNLFPQE-- ---------- V-----ISTA KSE
SlJAZ4    52 - ---------- ---------- PHSILPSSGS V--------- -------AGI TEP
SlJAZ5    79 - ---------- --------RK SMNLFPQ--- ---------- -------TEA KSE
SlJAZ6   113 - ---------- --------AA TMQLFPPR-- ---------- -------AAA ADG
SlJAZ7   101 - ---------- --------RR AAAAAAAS-- ---------- -------ATK SN-
SlJAZ8    66 - ---------- --------SE -------PPP P--------- -------STA PVP
SlJAZ9    50 - ---------- --------QD ---------- ---------- ---------- ---
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**ZIM motif**

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AtJAZ1    97 ------- --------AP AAPLTIFYGG NATQA----- ---------- AVSPVLTR
AtJAZ10   90 ------- ---------- KRQLTIFYGG SVAVFD-VS- ---------- HDKAEAIM
AtJAZ11   70 PE----- ---------- RRQLTIFYGG SMVKFDNVP- ---------- REKIRYAC
AtJAZ12  107 RD----- ---------- ---------- ---------- ---------- --------
AtJAZ2   124 AQ----- ---------- ---------- ---------- ---------- --------
AtJAZ3   127 GSTVGVY GGPRDLQNPK VTQMTIFYDG RMVVFEDFP- ---------- ADKAAEVM
AtJAZ4   110 ------- --------PE KAQMTIFYGG KVLVFDDFP- ---------- AEKAKDLM
AtJAZ5    70 ------- ---------- SQQLTIFYNG KVLVFNDFP- ---------- ADKAKGLM
AtJAZ6   102 GDGVDKN ---------- ---------- ---------- ---------- --------
AtJAZ7   137 GSLPSAP TTVARMPEAH VFPPAKVTVP ---------- ---------- --------
AtJAZ8    92 SWKPD-- -------QPE KAQMTIFYGG LVNVFDNIP- ---------- VEKAQELM
AtJAZ9   111 AP----- ------KEPK AAQLTMFYDG QVIVFNDFP- ---------- ADKAKEIM
NaJAZa    95 IPH---- ------KEQK LAQLSIFYGG KFV-ASHVT- ---------- QLQAKAII
NaJAZb   141 ETEEKTN KSL------- ---------- ---------- ---------- --------
NaJAZc   123 LKFRDAV SSEKQLFVET LSGAQPLPRR EVSPTKPMML QKP------- ------QL
NaJAZd   113 KNYD--- -----NLSPN ESTLTIFYMG QVIVFDDFP- ---------- ADKANEIM
NaJAZe   112 RGTP--- -----RPSPG PAQLTMFYAG KVIVFDDFP- ---------- ADKARAVM
NaJAZf    52 ------- ---------- SQQLTIFYHG KVVVFDDFP- ---------- AEKARAVM
NaJAZg   108 APPVTPN ATSTLSPVQA PIPKSSAIDS ---------- ---------- --------
NaJAZh    97 GCAPPNV VQPRFQLQAS ASKPAAADGV NSMLGFSEK- ---------- --------
NaJAZj    96 DVLKMTQ TTRSVKPESQ TAPLTIFYAG EVHIFPGIS- ---------- PEKAELII
NaJAZk   129 RSST--- -----ELVSG TVPMTIFYNG SVCVYDNIS- ---------- PEKAQAIM
NaJAZl    80 ------- -------PAL NRALSFSTVA KFV-VSDAT- ---------- ELQAKAII
NaJAZm    71 VSS---- -------PAL NRAPSFSSTS FVVNQCRN-- ---------- ------TT
NtJAZ1   130 DVQMIKE TTKSVKPESQ SAPLTIFYGG CVNQTPNML- ---------- ------PA
NtJAZ2    84 RSSS--- -----KPIGS PAQLTIFYAG QVIVFNDFS- ---------- AEKAKEVI
NtJAZ3   144 RSSS--- -----KPL-- PPQLTIFYAG SVSVFQ-VS- ---------- RNKAGEIM
OsJAZ1    77 QP----- ---------G SSQLTIFFGG DLPIARRRSL QRFLEKRRDR STKPDGSM
OsJAZ10   96 APE---- --------SG NSQLTIFFGG NVASP----- ---------- AAQP----
OsJAZ11  105 ------- ---------- SQILTIFYNG RVMVFDDFS- ---------- AEKAKEVI
OsJAZ12   58 ------- ---------- SQRITIFYNG SVCVYDDIS- ---------- PEKAKAIM
OsJAZ2   137 VPRV--- -----ASSGS SPQLTIFYGG SVLVYQDIA- ---------- PEKAQAIM
OsJAZ3    66 ------- --------SE KAQMTIFYGG KVLVYNEFP- ---------- VDKAKEIM
OsJAZ4    98 KNSD--- -----HLRKP ESTLTIFYMG KVMVFNEFP- ---------- EDKAKEIM
OsJAZ5    79 WFNS--- -----KGSAA PAQLTIFYGG HMCVSSDLT- ---------- HLEANAIL
OsJAZ6    56 ------- --------PE KAQMTIFYGG KMCFSSDVT- ---------- HLQARSII
OsJAZ7    90 RGAP--- -----KTPPG PAQLTIFYGG TISVFNDIS- ---------- PDKAQAIM
OsJAZ8    71 ------- ---------- QQQLTIFYNG QVIVFNDFP- ---------- ADKAKEIM
OsJAZ9    99 RGAP--- -----KTSAG PAQLTIFYAG EVHIFQDIT- ---------- PEKAELIM
SlJAZ1    96 AP----- ------KEPK AAQLTMFYDG TVNVFDDIS- ---------- PEKAQAIM
SlJAZ10   74 ------- ---------- PQQLTIFYNG QVIVFDDFP- ---------- AAKANEIM
SlJAZ11   92 APSRTIG -----SVDTS AGQMTIFYSG SVCVYDNVS- ---------- PEKAQAIM
SlJAZ12  138 ------- ---------- SEQLTIFYAG KVV-VSDAT- ---------- ELQAKAII
SlJAZ2    86 ------- ---------- -KHITIFYNA SVSVYDNIS- ---------- PEKAQAIM
SlJAZ3    86 ------- --------PE KAQMTIFYGG KVIVFDDFP- ---------- ADKARAVM
SlJAZ4    69 WFNS--- -----KGSAA PAQLTIFYGG KLV-VSDAT- ---------- ELQAKAII
SlJAZ5    94 ------- --------PE KAQMTIFYGG KVNVYDDVP- ---------- ADKAQTIM
SlJAZ6   129 VATPS-- --------AG TAPLTIFYDG IVHVYDNLP- ---------- VEKAQSIM
SlJAZ7   116 ------- -------AAI AVKPPVMPAA QAG-ECDIS- ---------- EVQALAIL
SlJAZ8    78 EEMP--- -----GAAAA AAPMTLFYNG QVIVFDDFP- ---------- ADKANEIM
SlJAZ9    52 ------- ---------T TEQLTIFYSG TVNVFDDIS- ---------- PEKAQAIM
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AtJAZ1   122 SL SLQSTSVATG ---------- ---------- ---------- ---------- --
AtJAZ10  116 RM ATEATK---- ---------- ----AKG--- ---------- ---------- --
AtJAZ11   99 RL RRLYSSLQRS ---------- ---------- CVNQTPNML- ---------- --
AtJAZ12  109 -- ---------- ---------- ---------- ---------- -SPISESSSP IS
AtJAZ2   126 -- ---------- ---------- ---------- ---------- ---------- --
AtJAZ3   171 RM ASSG------ ---------- ---------- ---------- ---------- --
AtJAZ4   139 QM ASKSSSTAQN CVLLPSSA-- ---------- ---------- ---------- --
AtJAZ5    97 QL ASKGSPVAPQ NAAAP--A-- ---------- ---------- ---------- --
AtJAZ6   109 -- ---------- ---------- ---------- AN--ISV--- ---ETPKIVT SL
AtJAZ7   164 -- ---------- ---------- ---------- -ATQIQKTAE SASD------ --
AtJAZ8   127 LL ASRASIPSPP S-AARKSDSP ISAAAKLTVP ---------- ---------- --
AtJAZ9   144 LM ASTSKGN-NP AKPLESAA-- ---------- ---------- ---------- --
NaJAZa   128 YL ASREMEEKTN K--------- ---------- ---------- ---------- --
NaJAZb   151 -- ---------- ---------- ---------- ---------- ---------- LQ
NaJAZc   165 VS SPVPAISKPI SVVSQATSLP RSASSSNVDS ---------- ------STAA AA
NaJAZd   149 KL ANKQNPT--- -----NNF-- ---------- EALPARQIVV QKP------- --
NaJAZe   148 LL ASKGCPQSSF GTFQAINIDK INTCS--PAP ---------- ---------- --
NaJAZf    79 LL ASKGISNNSC AIFQTP---- ---------- ---------- ---------- --
NaJAZg   135 -- ---------- ---------- ---------- AAVTDNTKAP MAVPAPVSSL PT
NaJAZh   133 -- ---------G KERVCGGSKN AKEISLLTIC ---------- --------TG TA
NaJAZj   140 DL VSKSTT---- ---------- -LHMDEILEK ---------- ---------- --
NaJAZk   165 LL AGNTP---IS TT-------- -IRNSPSLDH ASLTSNRTDS VAP------- -Q
NaJAZl   109 YL ASRGMEMKTN K--------- ---------- ---------- ---------- --
NaJAZm    97 PT LASPISITSH GGAQAARVST TTNGVTI-IK ---------- ---LSEPSS- --
NtJAZ1   168 SG LSSPMSVSSH PIGQSDGSSG NKDDMKMSKT ---------- -SPISEPSS- --
NtJAZ2   120 NL ASKGTANSLA KNQTDIRS-- ---------- VMNKEKYE-- ---------- --
NtJAZ3   177 KV ANEAAS---- ---------- ----KKDESS HH-------- ---------- --
OsJAZ1   118 IL PSQLTIIFGG SFSVFDGIPA EKVQEI---- ---------- ---------- --
OsJAZ10  120 -F P--------- ---------- ---------- TQTNG----- ---------- --
OsJAZ11  132 DL ANKGSAKSFT CFTAEVNN-- ---------- SIGVLPS-PS LKAEPSKVTS SV
OsJAZ12   85 LL AGNGSSMPQV FSPPQTHQQV VHHTRASVDS ---------- ---------- --
OsJAZ2   173 LL AGNGPHAKPV SQP--KPQKL VHHSLPTTDP METDLSVI-- ---------- --
OsJAZ3    95 EV AKQAKPVTEI NIQTPI---- ---------- ---------- ---------- --
OsJAZ4   134 EV AKEANHVAVD SKNS------ ---------- ---------- ---------- --
OsJAZ5   115 SL ASRDVEEKSL SL-------- ---------- ---------- ---------- --
OsJAZ6    85 SI ASREMKTKS- ---------- ---------- SAMPPSFMP- ---------- --
OsJAZ7   126 LC AGNGLKG--- ---------- ----ETGDSK PTMPPSFLP- ---------- --
OsJAZ8    98 LM ASCAKGN-NN S--------- ---------- ---------- ---------- --
OsJAZ9   135 DL ASKSTN---- ---------- -LHMTEILEK ---------- ---------- --
SlJAZ1   129 FL AGNGCVPPNV VQPRFQVQAS TPKLAAVDGT ---------- ---------- --
SlJAZ10  101 KL ASKKNNNNNK QNLATNIF-- ---------- ---------- ---------- --
SlJAZ11  131 LL AGNAPPVTPS ATSTLSPVQA PIPKSSSVDS PVREAERMY- ---------- --
SlJAZ12  164 YL ASRETEENTK TS-------- ---------- ---------- ---------- --
SlJAZ2   112 LL AGNAQPAGIP STTSTASPVQ RIPKSSSVDA -ANKEKYE-- ---------- --
SlJAZ3   115 LL ASKGCPQSSF GTFHTTTIDK INTSATAAAT CVNQTSNIL- ---------- --
SlJAZ4   104 YL ASREMEKKIK IR-------- ---------- ---------- ---------- --
SlJAZ5   123 RV ASSSLCVPSE TP-------- ---------- FVVNQSHN-- ---------- --
SlJAZ6   163 DF ARESSLFSGS TN-------- ---------- ---------- ---------- --
SlJAZ7   145 WH A-RQVKYNN- ---------- ---------- FVGNKCHRT- ---------- --
SlJAZ8   114 KL ATKKTNN--K QNLASNIF-- ---------- ASLTCNKTNQ LKPSTVSIAP PQ
SlJAZ9    80 FL AGNGCVPPNV VQPRFQVQAS TPKLAAVDGT ---------- ---------- --
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AtJAZ1   134 -------- ---------- ---------- ---------- ---------- -SPISES
AtJAZ10  127 -------- ---------- ---------- --------SY SIGVLTNSPS NKTEPSK
AtJAZ11  120 ----PASG HSSPMSVSSH PIGQSAGNSG NKDDMKISKT SKTDQLKPVS TSSASQK
AtJAZ12  120 EPSSPLLQ SP----ASDI SMKRSLQRFL QKRK---NRI TSTAPYQICN KNAASAK
AtJAZ2   126 -------- ----LNATVA AQHSTCCLQV ANTKLRPDSD ISASPYDT-S KQSPECS
AtJAZ3   177 -------- ---------- ---------- ---------- ---------- --VKFPP
AtJAZ4   159 -------- ---------- ---------- LARGNAIVGN ---------- -------
AtJAZ5   115 -------- ---------- ---------- ---------- PMVNNQNSAE SVTTNFS
AtJAZ6   123 GPVGATTI MPA---AVPQ ARKASLARFL EKRK---ERV QATSPYHR-- -------
AtJAZ7   177 -----LVP QPIISGDLPI ARRASLTRFL EKRK---DRL MVLLPTIQTE AVENPSS
AtJAZ8   158 -------- ---------- ---------- ---------- ---------- -------
AtJAZ9   163 -------- ---------- ---------- ---------T ---------- ----QPQ
NaJAZa   141 -------- ---------- ---------- ---------P ---------- -------
NaJAZb   153 TQDTSMFP SSSSLHIQTK RRGYSVIRLL REMLMVCSST MNAAPYGL-S KKSGECS
NaJAZc   203 VSDAGAAA GQPCLPDMPI ARKVSLQRFL EKRK---NRI TAKAPYQLSN TNKQ-AA
NaJAZd   174 ----EASV PLVSGVSNPI TIVSQAVTLP KSFSSSNDSA ---------- ------G
NaJAZe   178 -------- ---------- ---------- --------DL ---------- -------
NaJAZf    97 -------- ---------- ---------- ---------- ATVADNTKVS -AVPAPA
NaJAZg   157 AQADAQKP ARANASDMPI ARKASLHRFL EKRK---DRL RTKPMLVHSD SIGAQRT
NaJAZh   158 ATQSLPRP AHNSLPDLPI ARRNSLHRFL EKRK---GRM VVAEPLPESE KKEAESS
NaJAZj   157 -------- ---------- ---------- --------TY NVTKSSGPLV VPPTSLP
NaJAZk   199 QQHLQIKP DSCSAAPQQH KHNSPP---- ----LHVCSS GPKSGGLPLA VTPLSQA
NaJAZl   122 -------- ---------- ---------- --------TT VVPSFGKTSI QENQ---
NaJAZm   134 ----PLLQ P--------Q TVKKSLQRFL QKRK---NRI NAKTPYQASP SDATPVK
NtJAZ1   208 ----PLLQ P--------Q TVKKSLQRFL QKRK---SRT NANAPYQANC TAAP--S
NtJAZ2   148 -------- ---------- ----ENKSDP S--NASTNYA YSEMVASFYV TSDKAHD
NtJAZ3   193 -------- -------HHH HNNNNNNNNN STNETTI-IR PMIKNQKTAD QSGVSFG
OsJAZ1   146 -------- ---------- ---------- ---------- TKTDQLKLGS VSSAPLV
OsJAZ10  127 -------- --SNNFDLPI ARRSSLYRFL EKRK---DRD QITSPYHH-- -------
OsJAZ11  173 GS-FPASL VPS---AVPQ ARKASLARFL EKRK---ERV QTTSPYHH-- -------
OsJAZ12  117 -------- ---------- ---------- --------N- AN--ISVTPH VKLDTSK
OsJAZ2   211 -------- ---------- --------LP TTLRPKLFGQ KG-------- -------
OsJAZ3   113 -------- ---------- ---------- ------LHIA SIGVLKS--- --HELSK
OsJAZ4   150 -------- ---------- ---------- ---------- ---------- ---MSEP
OsJAZ5   129 -------- ---------- ---------- --------NH ISASPYPLNS KQSPECS
OsJAZ6   105 ----TISY LSPEAGSS-- --TNGLGATK ATRGLTSTYH MNLAPYGL-S KKSPECS
OsJAZ7   150 ----SISY IVSETRSSGS NGVTGLGPTK TKASLASTRN -------IAT IANQVPH
OsJAZ8   110 -------- ---------- ---------- --------ND NL-------- -------
OsJAZ9   152 -------- ---------- ---------- --------QS AAAKATETIN LTSINPA
SlJAZ1   161 -------- ---------- ---------- ---------- ---------- ---IQP-
SlJAZ10  121 -------- ---------- ---------- ---------- SAYSQKEIAS SPNPVCS
SlJAZ11  172 ----GKQI HNTAATSSSS --------AT HTDNFSRCRD NN-------- --QANGS
SlJAZ12  178 -------- ---------- ---------- ---------- NQ-------- --TAAFS
SlJAZ2   151 -------- ---------- ----ENKSEP STPNASTNYA ENNNNKSSMV LPDLNEP
SlJAZ3   156 ----PASG HSSPMSVSSH PIGQSAGNSG NKDDMKISKT HMNLDKSNVV IPDLNEP
SlJAZ4   118 -------- ---------- ---------- --------SY ---------- --RSSDG
SlJAZ5   145 ----TTPT LPSPISITSH CGSQSAGVSS NTNGVTI-IK ---------- ---SSNG
SlJAZ6   177 -------- ---------- ---------- ---------- TP--VAAT-- ---NAMS
SlJAZ7   164 ----TSPS FSSPIPITTH GASQSIGVSN NTNQITMSIR -TTQIQKTAE SALD---
SlJAZ8   154 QKQQQIHV SYSKSDQLKP GYNSATPQVL QQQLVHVSST KG-------- -------
SlJAZ9   112 -------- ---------- ---------- ---------- AN--ISV--- ---ETPK
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                                                         Jas motif
AtJAZ1   140 SS- ------PLLQ T-----QTGL SMKKSLQRFL QKRK---NRI IAKAPYQLSN T
AtJAZ10  146 VVR SQES-HPPSH TLS---AVPQ ARKASLARFL EKRK---ERI ITARPYQY-G E
AtJAZ11  171 QQE QHQQTQSQTP GTSSS-ELPI ARRSSLHRFL EKRK---DRA MNAAPYGL-S K
AtJAZ12  168 NE- -ENKAWLGLG GKFVPVKTEQ FF-------- ---------- ---------- -
AtJAZ2   168 TLG YGSRSFAKYS LGSCPPQVIN LVKET----- ---------- ---------- -
AtJAZ3   182 KEA EPNQKSQVPF ACKFQAELPI ARRKSLKRFF EKRH---NRI QETSPYHH-- -
AtJAZ4   169 --- ---------- --------GL SLRKSLQRFL QKRRE--RRI LSASPYDN-S K
AtJAZ5   132 QEL RTRT-HVPIS QSSVA-DLPI ARRNSLTRFL EKRK---DRI TARAPYQVVH N
AtJAZ6       --- ---------- ---------- ---------- ---------- ---------- -
AtJAZ7   224 RKA SVQRYLEKRK DR-------- ---------- ---------- ---------- -
AtJAZ8   158 -AC SPPQPAHAAA ----LPEMPI ARKASLQRFL QKRK---HRI ISKHPYASPV I
AtJAZ9   167 VAA DPSS------ ICKLQADLPI ARRHSLQRFL EKRR---DRL QAANPY---- -
NaJAZa   142 --- ---------- ----FAKEPL TRTKSLQRFL SKRK---ERL TSTAPYQICN K
NaJAZb   207 TPE SIGVGFSATS SVGTSPLIAG KET------- ---------- ---------- -
NaJAZc   254 VS- -ENKVWLGLG AQFP-VKAEQ F--------- ---------- ---------- -
NaJAZd   209 GGG APEAPP---- ---ALVDMPI ARKASLKRFL AKRK---ATP TTTS---EPY K
NaJAZe   180 -MA AAPAQREGAA ----LADMPI MRKASLQRFF AKRK---DRL VSKAPYPTKS S
NaJAZf   113 SAL PVAQANAPKP VRPNAADLPQ ARKASLHRFL EKRK---DRL TSLGPYQVGG P
NaJAZg   209 GTP PSRRRIHARG KSRSCQEMSH CW-------- ---------- ---------- -
NaJAZh   210 KRA KK-------D DGGASWLQVN PT-LSL---- ---------- ---------- -
NaJAZj   176 PPA QPETLATTTA AAIMPRAVPQ ARKASLARFL EKRK---ERV ASARSSYVVR A
NaJAZk   246 SPS QPIPVATTNA SAIMPRAVPQ ARKASLARFL EKRK---ERV AATTPYARPS P
NaJAZl   138 --- -------MPN QPIVS-DLPI ARRASLTRFL EKRK---DRL QAKAPYQGSP S
NaJAZm   174 KEP -ESQP----W LGLGPNAVVK PIERGQ---- ---------- ---------- -
NtJAZ1   246 KQA NGDKS----W LGFGQEMTIK QEI------- ---------- ---------- -
NtJAZ2   179 ILK FAETSRVRND FSPNFGDLSL TRSISLPRFL EKPKTRLDYI TTVAPYPLAK S
NtJAZ3   232 NKL IQELPKLSMP QPSVA-DLPI ARRNSLTRFL EKRK---DRV SSVAPYPSSK S
OsJAZ1   163 EQE QHKQIQSQAA EISSSSELPI ARRSSLHRFL EKRK---DRA TAKVPYHREE A
OsJAZ10      --- ---------- ---------- ---------- ---------- ---------- -
OsJAZ11      --- ---------- ---------- ---------- ---------- ---------- -
OsJAZ12  133 IVT SLGPVGATTI MTAGMASVPQ ARKASLARFL EKRK---ERV MPMTFFYEGN T
OsJAZ2   225 --- ---------- ------ALAM ARRATLARFL EKRK---HRL TSIAPYQISN N
OsJAZ3   129 IVT SQESRQPPNH NLS---AVPQ ARKASLARFL ERRK---ERV TVRAPYQVVR N
OsJAZ4   154 SS- ------PLLQ P--------Q TVKKSLQGFL QKRK---KRV TARAPYQMH- -
OsJAZ5   148 TPE LGSRSLSMNS SGSCPPHIIS LVK------- ---------- ---------- -
OsJAZ6   151 TPE SNGVGFSATS ----TPLLAG KET------- ---------- ---------- -
OsJAZ7   194 PRK TTTQEPIQSS PTPLT-ELPI ARRASLHRFL EKRK---DRV IKARPYLY-G E
OsJAZ8   114 --- ---------- ----EGDLPI ARRKSLQRFL EKRK---ERL VSASPYGN-G K
OsJAZ9   171 LKR AISFSNASTV ACVSTADVPI ARRRSLQRFF EKRR---HRF QATSPYHK-- -
SlJAZ1   164 --- -ISF------ -CRSTADLPI ARRHSLQRFL EKRR---DRL ---------- -
SlJAZ10  138 PAK TAAQEPIQPN PASLACELPI ARRASLHRFL EKRK---DRI ---------- -
SlJAZ11  205 NIN CPVPVSCSTN VMAPTVALPL ARKASLARFL EKRK---ERV TSKAPYQLCD P
SlJAZ12  185 --- ---------- -MAPTVGLPQ TRKASLARFL EKRK---ERV VSTSPYYPTS A
SlJAZ2   184 TDN NHLTKEQQQQ QEQNQIVERI ARRASLHRFF AKRK---DRA VHTKPYSATT S
SlJAZ3   207 TSS G--NNEDQET GQQHQVVERI ARRASLHRFF AKRK---DRA VNKNPYPTSD F
SlJAZ4   125 SDP PTIPNNSTRF HY-----QKA SMKRSLHSFL QKRS---LRI TSKAPYQIDG S
SlJAZ5   182 SDP ---PNKSTSF HHNQLPNPKA SMKKSLQSFL QKRK---IRI TSVSPYCLDK K
SlJAZ6   187 MIE SFN-AAPRNM IPS----VPQ ARKASLARFL EKRK---ERL INVSPYYVDN K
SlJAZ7   211 --- -------LVP QPIISGDLPI ARRASLTRFL EKRK---DRL VARAPYQVNQ N
SlJAZ8   194 --- ---------- ------ALAM ARRATLARFL EKRK---HRL VARAPYQVNQ H
SlJAZ9   121 IMT SLGPVGASTI MSA---AVPQ ARKASLARFL EKRK---ERV QATSPYHRYR -
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AtJAZ1   179 NKQ-AAVS- -ENKAWLGLG AQFP-VKAEQ F--------- ---------- ------
AtJAZ10  192 KTPKFPFEM HQ-EEETASS SVHWES---- ---------- ---------- KIEEGQ
AtJAZ11  220 KSGECSTPE SNGVGFSATS SVGTSPLIAG KET------- ---------- --KEGQ
AtJAZ12      --------- ---------- ---------- ---------- ---------- ------
AtJAZ2       --------- ---------- ---------- ---------- ---------- ------
AtJAZ3   230 --------- ---------- ---------- ---------- ---------- ------
AtJAZ4   199 QSSQYSTPG SSSWSFFVNS SGSSTVLPAT I--------- ---------- ------
AtJAZ5   181 NP-LPSSSN NNGESSSKDC EDQLDLNFKL ---------- ---------- ------
AtJAZ6       --------- ---------- ---------- ---------- ---------- ------
AtJAZ7       --------- ---------- ---------- ---------- ---------- ------
AtJAZ8   204 TQHEDECND QSGNYSLKEK NS-------- ---------- ---------- ------
AtJAZ9       --------- ---------- ---------- ---------- ---------- ------
NaJAZa   176 KIADSKNE- -ENKAWLGLG AKFVPVKTEQ FF-------- ---------- ------
NaJAZb       --------- ---------- ---------- ---------- ---------- ------
NaJAZc       --------- ---------- ---------- ---------- ---------- ------
NaJAZd   250 KAAVASPAP EK-------S FAVAPVKDEP ATWLGL---- ---------- ------
NaJAZe   226 EGMEASGME VTAEGKAQ-- ---------- ---------- ---------- ------
NaJAZf   164 AAVGATTST TTKSFLAKEE EHTAS----- ---------- ---------- ------
NaJAZg       --------- ---------- ---------- ---------- ---------- ------
NaJAZh       --------- ---------- ---------- ---------- ---------- ------
NaJAZj   227 AAAEEEQPP AK-------- -KAKAAVERR EDWLALGSLG HMHSR----- ------
NaJAZk   297 AETKASEPE EKKTPTSWLD LAASASAAAR RDSLTIAL-- ---------- ------
NaJAZl   178 DASPVKKEL QESQP----W LGLGPQVAAP DLSLRQESSQ ---------- ------
NaJAZm       --------- ---------- ---------- ---------- ---------- ------
NtJAZ1       --------- ---------- ---------- ---------- ---------- ------
NtJAZ2   233 PLESSDTMG SAND-NKSSC TDIALSSN-R DESLSLGQPR TISFCE-ESP ------
NtJAZ3   282 PLESSDTIG SPSTPSKSSC TDITPSTNNC EDSLCLGQPR NISFSSQEPP ------
OsJAZ1   214 AAP-KKEE- -HKAPWLGLG GQFA-VKTEQ Y--------- ---------- ------
OsJAZ10      --------- ---------- ---------- ---------- ---------- ------
OsJAZ11      --------- ---------- ---------- ---------- ---------- ------
OsJAZ12  184 TTLNVSIDK AVNILAFVER SNFGDACPSG KKFPAEILSG DQHLARRNSV ------
OsJAZ2   257 KKS--KNE- -DNKAWLGLG AQFV--KTEQ YF-------- ---------- STKLQI
OsJAZ3   177 NPLLPSSSN TNGESSSKDS EDQLDLNFKL ---------- ---------- STKLQI
OsJAZ4   188 NP-LQSSSR TRG------- -DHFDLNF-- ---------- ---------- ------
OsJAZ5       --------- ---------- ---------- ---------- ---------- ------
OsJAZ6       --------- ---------- ---------- ---------- ---------- ------
OsJAZ7   243 NLSKFPFDI QQQEEETASS SVHWEN---- ---------- ---------- LGFSEK
OsJAZ8   147 QSSQHMMN- -----FTINS SGSSTSLPAA N--------- ---------- ------
OsJAZ9       --------- ---------- ---------- ---------- ---------- ------
SlJAZ1       --------- ---------- ---------- ---------- ---------- ------
SlJAZ10      --------- ---------- ---------- ---------- ---------- ------
SlJAZ11  256 AKASSNPQT TGNMSWLGLA AEI------- ---------- ---------- ------
SlJAZ12  222 --------- ---------- ---------- ---------- ---------- ------
SlJAZ2   235 EADKNETSP IVT------- ---------- ---------- ---------- ------
SlJAZ3   256 KKTDVPTGN VSIKEEFPTA ---------- ---------- ---------- ------
SlJAZ4   171 AEASSKP-- -TNPAWLSSR ---------- ---------- ---------- ------
SlJAZ5   230 SSTDCRRSM SECISSSLSS AT-------- ---------- ---------- ------
SlJAZ6   233 SSIDCRTLM SECVSCPPAH HLH------- ---------- ---------- ------
SlJAZ7   252 AGHHRYPPK PEIVTGQPLE AGQSSQRPPD NAIGQTMAHI KSDGDKDDIM ------
SlJAZ8   226 GSH--LPPK PEMVAPS-IK SGQSSQHIAT PPKPKAHNHM PMEVDK---- ------
SlJAZ9       --------- ---------- ---------- ---------- ---------- ------
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AtJAZ1   205 ---- ---------- ---------- ---------- ---------- ----------
AtJAZ10  222 SSKD LDLRL----- ---------- ---------- ---------- ----------
AtJAZ11  256 SSKN LELKL----- ---------- ---------- ---------- ----------
AtJAZ12      ---- ---------- ---------- ---------- ---------- ----------
AtJAZ2       ---- ---------- ---------- ---------- ---------- ----------
AtJAZ3   230 ---- ---------- ---------- ---------- ---------- ----------
AtJAZ4       ---- ---------- ---------- ---------- ---------- ----------
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NaJAZd       ---- ---------- ---------- ---------- ---------- ----------
NaJAZe       ---- ---------- ---------- ---------- ---------- ----------
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NaJAZg       ---- ---------- ---------- ---------- ---------- ----------
NaJAZh       ---- ---------- ---------- ---------- ---------- ----------
NaJAZj       ---- ---------- ---------- ---------- ---------- ----------
NaJAZk       ---- ---------- ---------- ---------- ---------- ----------
NaJAZl       ---- ---------- ---------- ---------- ---------- ----------
NaJAZm       ---- ---------- ---------- ---------- ---------- ----------
NtJAZ1       ---- ---------- ---------- ---------- ---------- ----------
NtJAZ2       ---- ---------- ---------- ---------- ---------- ----------
NtJAZ3       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ1       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ10      ---- ---------- ---------- ---------- ---------- ----------
OsJAZ11      ---- ---------- ---------- ---------- ---------- ----------
OsJAZ12      ---- ---------- ---------- ---------- ---------- ----------
OsJAZ2   288 ---- ---------- ---------- ---------- ---------- ----------
OsJAZ3   212 ---- ---------- ---------- ---------- ---------- ----------
OsJAZ4       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ5       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ6       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ7   274 QKDR LCDGSKNGLR TALLTICYWG MVGNFNVSND EANEILKFAK RSITNDVFSS
OsJAZ8       ---- ---------- ---------- ---------- ---------- ----------
OsJAZ9       ---- ---------- ---------- ---------- ---------- ----------
SlJAZ1       ---- ---------- ---------- ---------- ---------- ----------
SlJAZ10      ---- ---------- ---------- ---------- ---------- ----------
SlJAZ11      ---- ---------- ---------- ---------- ---------- ----------
SlJAZ12  222 ---- ---------- ---------- ---------- ---------- ----------
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SlJAZ6       ---- ---------- ---------- ---------- ---------- ----------
SlJAZ7   301 ---- ---------- ---------- ---------- ---------- ----------
SlJAZ8       ---- ---------- ---------- ---------- ---------- ----------
SlJAZ9       ---- ---------- ---------- ---------- ---------- ----------
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AtJAZ1   205 ---------- ---------- ---------- ---------- ---------- -----
AtJAZ10      ---------- ---------- ---------- ---------- ---------- -----
AtJAZ11      ---------- ---------- ---------- ---------- ---------- -----
AtJAZ12      ---------- ---------- ---------- ---------- ---------- -----
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NaJAZa       ---------- ---------- ---------- ---------- ---------- -----
NaJAZb       ---------- ---------- ---------- ---------- ---------- -----
NaJAZc       ---------- ---------- ---------- ---------- ---------- -----
NaJAZd       ---------- ---------- ---------- ---------- ---------- -----
NaJAZe       ---------- ---------- ---------- ---------- ---------- -----
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NaJAZg       ---------- ---------- ---------- ---------- ---------- -----
NaJAZh       ---------- ---------- ---------- ---------- ---------- -----
NaJAZj       ---------- ---------- ---------- ---------- ---------- -----
NaJAZk       ---------- ---------- ---------- ---------- ---------- -----
NaJAZl       ---------- ---------- ---------- ---------- ---------- -----
NaJAZm       ---------- ---------- ---------- ---------- ---------- -----
NtJAZ1       ---------- ---------- ---------- ---------- ---------- -----
NtJAZ2       ---------- ---------- ---------- ---------- ---------- -----
NtJAZ3       ---------- ---------- ---------- ---------- ---------- -----
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OsJAZ11      ---------- ---------- ---------- ---------- ---------- -----
OsJAZ12      ---------- ---------- ---------- ---------- ---------- -----
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OsJAZ9       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ1       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ10      ---------- ---------- ---------- ---------- ---------- -----
SlJAZ11      ---------- ---------- ---------- ---------- ---------- -----
SlJAZ12  222 NSGDLSLPKI NSSLRFSEKP QIRLGCESEN GVKVEPLTIF YDGKIVVYDV SIEKA
SlJAZ2       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ3       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ4       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ5       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ6       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ7   301 ---------- ---------- ---------- ---------- ---------- -----
SlJAZ8       ---------- ---------- ---------- ---------- ---------- -----
SlJAZ9       ---------- ---------- ---------- ---------- ---------- -----
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AtJAZ1   205 ----- ---------- ---------- ---------- ---------- ----------
AtJAZ10      ----- ---------- ---------- ---------- ---------- ----------
AtJAZ11      ----- ---------- ---------- ---------- ---------- ----------
AtJAZ12      ----- ---------- ---------- ---------- ---------- ----------
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AtJAZ3   230 ----- ---------- ---------- ---------- ---------- ----------
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AtJAZ9       ----- ---------- ---------- ---------- ---------- ----------
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NaJAZb       ----- ---------- ---------- ---------- ---------- ----------
NaJAZc       ----- ---------- ---------- ---------- ---------- ----------
NaJAZd       ----- ---------- ---------- ---------- ---------- ----------
NaJAZe       ----- ---------- ---------- ---------- ---------- ----------
NaJAZf       ----- ---------- ---------- ---------- ---------- ----------
NaJAZg       ----- ---------- ---------- ---------- ---------- ----------
NaJAZh       ----- ---------- ---------- ---------- ---------- ----------
NaJAZj       ----- ---------- ---------- ---------- ---------- ----------
NaJAZk       ----- ---------- ---------- ---------- ---------- ----------
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NtJAZ1       ----- ---------- ---------- ---------- ---------- ----------
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OsJAZ8       ----- ---------- ---------- ---------- ---------- ----------
OsJAZ9       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ1       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ10      ----- ---------- ---------- ---------- ---------- ----------
SlJAZ11      ----- ---------- ---------- ---------- ---------- ----------
SlJAZ12  277 TNILK ---------- ---------- ---------- ---------- ----------
SlJAZ2       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ3       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ4       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ5       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ6       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ7   301 ----- FVERDAISSK KEFSMESLSG DLHLAKRNFM HGVLENRNER FCPPGFEDIK
SlJAZ8       ----- ---------- ---------- ---------- ---------- ----------
SlJAZ9       ----- ---------- ---------- ---------- ---------- ----------
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AtJAZ1   205  ---------- KLAIFDVRSY KVDEILKFAE SSKQQTLSQD VLTTRKSSVR FLEK
AtJAZ10       ---------- ---------- ---------- ---------- ---------- ----
AtJAZ11       ---------- ---------- ---------- ---------- ---------- ----
AtJAZ12       ---------- ------
AtJAZ2
AtJAZ3   230  ---------- APENKK
AtJAZ4        ---------- ---------- ---------- ---------- ---------- ----
AtJAZ5        ---------- ---------- ---------- ---------- ---------- ----
AtJAZ6        ---------- ------
AtJAZ7
AtJAZ8        ---------- ------
AtJAZ9        ---------- ---------- ---------- ---------- ---------- ----
NaJAZa        ---------- ---------- ---------- ---------- ---------- ----
NaJAZb        ---------- ------
NaJAZc        ----------
NaJAZd        ---------- ---------- ---------- ---------- ---------- ----
NaJAZe        ---------- ---------- ---------- ---------- ---------- ----
NaJAZf        ---------- ---------- ---------- ---------- ---------- ----
NaJAZg        ---------- ------
NaJAZh        ---------- ------
NaJAZj        ---------- ---------- ---------- ---------- ---------- ----
NaJAZk        ---------- ---------- ---------- ---------- ---------- ----
NaJAZl        ---------- ---------- ---------- ---------- ---------- ----
NaJAZm        ---------- ------
NtJAZ1        ---------- ------
NtJAZ2        ---------- ---------- ---------- ---------- ---------- ----
NtJAZ3        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ1        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ10       ---------- ------
OsJAZ11       ---------- ------
OsJAZ12       ---------- ---------- ---------- ---------- ---------- ----
OsJAZ2        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ3        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ4        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ5        ---------- ------
OsJAZ6        ---------- ------
OsJAZ7        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ8        ---------- ---------- ---------- ---------- ---------- ----
OsJAZ9        ---------- ---------- ---------- ---------- ---------- ----
SlJAZ1        ---------- ------
SlJAZ10       ---------- ------
SlJAZ11       ---------- ---------- ---------- ---------- ---------- ----
SlJAZ12       ---------- ---------- ---------- ---------- ---------- ----
SlJAZ2        ---------- ---------- ---------- ---------- ---------- ----
SlJAZ3        ---------- ---------- ---------- ---------- ---------- ----
SlJAZ4        ---------- ------
SlJAZ5        ---------- ------
SlJAZ6        ---------- ---------- ---------- ---------- ---------- ----
SlJAZ7   351  IAPLSIIYNG ---------- ---------- ---------- ---------- ----
SlJAZ8        ---------- ---------- ---------- ---------- ---------- ----
SlJAZ9        ---------- ---------- ---------- ---------- ---------- ----
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AtJAZ1   249 RQERMT MVSPYGFPHA
AtJAZ10      ------ ----------
AtJAZ11      ------ ----------
AtJAZ12
AtJAZ2
AtJAZ3
AtJAZ4       ------ ----------
AtJAZ5       ------ ----------
AtJAZ6
AtJAZ7
AtJAZ8
AtJAZ9       ------ ----------
NaJAZa       ------ ----------
NaJAZb
NaJAZc
NaJAZd       ------ ----------
NaJAZe       ------ ----------
NaJAZf       ------ ----------
NaJAZg
NaJAZh
NaJAZj       ------ ----------
NaJAZk       ------ ----------
NaJAZl       ------ ----------
NaJAZm
NtJAZ1
NtJAZ2       ------ ----------
NtJAZ3       ------ ----------
OsJAZ1       ------ ----------
OsJAZ10
OsJAZ11
OsJAZ12      ------ ----------
OsJAZ2       ------ ----------
OsJAZ3       ------ ----------
OsJAZ4       ------ ----------
OsJAZ5
OsJAZ6
OsJAZ7       ------ ----------
OsJAZ8       ------ ----------
OsJAZ9       ------ ----------
SlJAZ1
SlJAZ10
SlJAZ11      ------ ----------
SlJAZ12      ------ ----------
SlJAZ2       ------ ----------
SlJAZ3       ------ ----------
SlJAZ4
SlJAZ5
SlJAZ6       ------ ----------
SlJAZ7       ------ ----------
SlJAZ8       ------ ----------
SlJAZ9       ------ ----------
```

**Supplemental Text S2.** Nucleotide sequence alignment of *NaJAZ* genes that are regulated by silencing of *NaJAZh* against sequence of inverted repeat (ir) construct used in irJAZh plants.

**A.** Sequence alignment of NaJAZe and ir-construct of NaJAZh

```
NaJAZe            1 TTATGGGTTTGACTCATCATGTGAAGCAAGAAGTAATTGAAGAACATATAGATCCAGCAC   60

ir construct        ------------------------------------------------------------

NaJAZe           61 CTCTGAGAAGTTCAGCAATGCAGTGGTCATTCTCGAACAACATCTCGACTCATCCTCAAT  120

ir construct        ------------------------------------------------------------

NaJAZe          121 ACCTCTCTTTCAAGGGTGCTCAAGAGGATAGGCTGAAAACTGGTTTTGATTCACTTGCAT  180

ir construct        ------------------------------------------------------------

NaJAZe          181 CAACTGGATTGGTGACTATAACCACAACTGAAGCTGTCGACTCAAGTCATCGACCGTACT  240

ir construct        ------------------------------------------------------------

NaJAZe          241 CTGGTGTCACACAGAATAATATGATGCTTGAAAAGCAAGGTGGAACGCACTACACGTCGA  300

ir construct        ------------------------------------------------------------

NaJAZe          301 CAACTTTCTCTCCTCATCACTATGATGCTCACTCCGTGCATCGATCTCATGGAGTCAGAG  360

ir construct        ------------------------------------------------------------

NaJAZe          361 TGCTCCCAGTTTCCAACCTAGCAAATCAGATTTCTGTATCTATGACTATGCCTGGTCATA  420

ir construct        ------------------------------------------------------------

NaJAZe          421 AGTCCTTTGTTTCTCCTCTTGGACAGAATCCAGTTGCTAGCCCCATTTCAGCTGTTCCAA  480

ir construct        ------------------------------------------------------------

NaJAZe          481 CTAACAGCGCTGTCGTGGGCACAACTGATTTAAGGGGTGCTCCGAAAACTCCCCCAGGTC  540

ir construct        ------------------------------------------------------------

NaJAZe          541 CTGCTCAGTTGACCATCTTTTATGGTGGTTCCGTCTGTGTTTATGATAATGTTTCGCCAG  600

ir construct        ------------------------------------------------------------
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NaJAZe          601 AGAAGGCTCAAGCTATTATGTTGCTTGCTGGAAATGCACCACCTGTTACACCAAGTGCAA  660

ir construct        ------------------------------------------------------------

NaJAZe          661 CATCTACTCTATCTCCAGTTC--AGGC-GCCTATACCCAAGTCCTCTTC-TGTTGACTCT  716
                      TCTA T  A C CCA   C  A GC GC    A C AA      TTC TGTT   TC
ir construct      1 --TCTATTGCACCACCACAACAAAAGCAGC----AGCAAA------TTCATGTT---TC-   44

NaJAZe          717 TTTGTTGTAAATCAGTCCCACAACACAA--CA-CCTACTCTTC--CCA-GCC-CCATTTC  769
                     TT T GTAAA  AGT  C CAAC CAA  CA   TA   TTC  C A GCC C A T C
ir construct     45 -TTATAGTAAA--AGTGAC-CAACTCAAGCCAGGGTATAATTCTGCTACGCCGCAAGTAC  100

NaJAZe          770 TATAACATCTCATTGTGGATCTCAATCTGCTGGAGTGTCTAGTAATACAAATGGAG--TA  827
                    T  A CA C   T GT  AT T     T CT  AGT  CTAGTAA AC  AT  AG  TA
ir construct    101 TGCAGCAGCAGCTAGTCCATGT-----TTCT--AGTA-CTAGTAAAACTGAT-CAGCTTA  151

NaJAZe          828 A-CTATTATCAAATCAACTGGGGTCCTACCATCTCCTTCT-AATAA-AGCAGGACTTTCC  884
                    A C A T     ATCAACT    TC T    TCT C TC  AA AA AGCAGGA     C
ir construct    152 AGCCAGT-----ATCAACT----TC-T----TCTGCGTCGCAAAAACAGCAGGA----GC  193

NaJAZe          885 AA-ATTTTCCAGTTCCATAGGATCTGTTCCTGCCACCTTTGTTCCATCAGCTGTACCGCA  943
                    AA AT    CAG   CA A G  C GT  C G CACCT  G  C A CAGCT   C G A
ir construct    194 AACAT----CAG---CAAACG--CAGTCACAGACACCTG-GAACTAGCAGCT---CTG-A  239

NaJAZe          944 GGCACGCAAGGCATCATTGGCTCGGTTCTTGGAGAAGCGCAAAGAAAGGGTAATAAGTGC 1003
                    G
ir construct    240 G-----------------------------------------------------------  240

NaJAZe         1004 ATCACCTTACGACACCAGCAAGCAATCCCCAGAATGTAGCACTCTTGGATATGGAAGCAG 1063

ir construct        ------------------------------------------------------------

NaJAZe         1064 AAGTTTCGCTAAATATTCTTTAGGCTCTTGTCCTCCCCAAGTAATCAATTTGGTCAAGGA 1123

ir construct        ------------------------------------------------------------

NaJAZe         1124 GACGTGAAGTGCCAACGGTGGCAAAATAATGTAGACACAAGATGAAGACTGTACCAGATT 1183

ir construct        ------------------------------------------------------------

NaJAZe         1184 AGATTATTAAAGCTAAATTGGTGTTCATTTTGACTTCAATACTTCAGTTCTCTCTTAGAT 1243

ir construct        ------------------------------------------------------------

NaJAZe         1244 GCGATATATTATTCTAGGTTGTTTTCCTTGTAATTGTGATCAGAGCCTTTT 1294

ir construct        ---------------------------------------------------
```

**B.** Sequence alignment of NaJAZm and ir-construct of NaJAZh

```
NaJAZm          1 ATGAAGCACAGAATTGGCCTTCAACTTTTCCCTCTATTGTCGTCTTCTTCTTCTTCAGAG  60

ir construct      ------------------------------------------------------------

NaJAZm         61 TTCCACAGTAGTGATCACTCTGTGGCCTGCTCGTCTACAAGAAAGCATATCACAATTTTC 120

ir construct      ------------------------------------------------------------

NaJAZm        121 TACAACGCACAAGCTGGAGAATGTGATATATCTGAAGTGCAGGCCTTAGCCATCTTATGG 180

ir construct      ------------------------------------------------------------

NaJAZm        181 CATGCAAGACAAGTAAAATATAATAATGGGCTGTCCCTGAGAAAATCCCTACAGAGGT-- 238

ir construct    1 ----------------------------------------------------------TC   2

NaJAZm        239 TTCTGCA-AAAGAGAAGAGAAAGGAGGATTCAAGCAGCTAATCCAT-------ACTAGCT 290
                  T  TGCA  A  A AA A AAAG       C AGCAGC AAT CAT       A TAG T
ir construct    3 TATTGCACCACCACAACA-AAAG-------C-AGCAGCAAATTCATGTTTCTTA-TAG-T  51

NaJAZm        291 AAAAG-----AA--------ATGATATGATGTTGGTGATA-GGCGAATTCAGAATTG--- 333
                  AAAAG     AA         A G TAT A  TT  TG TA G CG A   AG ATG
ir construct   52 AAAAGTGACCAACTCAAGCCAGGGTATAA--TTC-TGCTACGCCGCA---AGTACTGCAG 105

NaJAZm        334 -AGAATTTAGT-----TATCTAGAATGTGTATGATTTTGTGATATGCGGATAGGTCACTA 387
                   AG A  TAGT    T TCTAG A  GTA  A     TGAT  GC   TA G CA TA
ir construct  106 CAGCAGCTAGTCCATGTTTCTAGTACTAGTAAAA----CTGATCAGC--TTAAGCCAGTA 159

NaJAZm        388 --ATTTTTTTCTGCATATGTATCTGCATTTCCAACGGGAACAGTGATTTCAGC------- 438
                    A  TT TTCTGC   GT  C GCA    CA C GGA CA   A  TCAGC
ir construct  160 TCAACTTCTTCTGC----GT--C-GCAAAAACAGCAGGAGCA---ACATCAGCAAACGCA 209

NaJAZm        439 -TGA-ATTTACC--GAACTAGCTATATATGTGTTGATTCCTAATTATCGAAATTTTGATT 494
                   T A A  ACC  GAACTAGC A  T TG G
ir construct  210 GTCACAGACACCTGGAACTAGC-AGCTCTGAG---------------------------- 240

NaJAZm        495 CTCTCGTCCTAACT 508

ir construct      --------------
```

**C.** Sequence alignment of NaJAZf and ir-construct of NaJAZh

```
NaJAZf          1 ATGAGAAGAAACTGTAACTTGGAACTAAGGCTTGTCCCTCCTTGTGTTTCTGTTTCTCCT  60

ir construct      ------------------------------------------------------------

NaJAZf         61 AAAGATTGCACTACGACCCCTTACTTCTCCATGAGGGATAACCAGGGCACAGAAGAGAAG 120
                                                           C A  GCAC  A  A AA
ir construct    1 ----------------------------------------TCTATTGCACC-ACCACAA-  18

NaJAZf        121 CAACAGCAGCAGCTAACAATATTCTACAATGGAAAAGTTGTGGTTTCTGATGCTACA--- 177
                  CAA AGCAGCAG    CAA ATTC    AT            GTTTCT AT  TA A
ir construct   19 CAAAAGCAGCAG----CAA-ATTC----AT------------GTTTCTTATAGTAAAAGT  57

NaJAZf        178 GAGCT--TCAGGCGAAAGCAATAATATAT-CT----CGCAAGTAGAGAAACGGAGGAG-- 228
                  GA C   TCA GC  A G  ATAAT T T CT    CGCAAGTA  G A  G AG AG
ir construct   58 GACCAACTCAAGC-CAGGGTATAAT-TCTGCTACGCCGCAAGTACTGCA--GCAGCAGCT 113

NaJAZf        229 AATACAAAGACTTC-ATCACCAATTTCAGAATCATCATCACCATTGTTA---CAAACTCA 284
                  A T CA  G  TTC A  AC A T   A AA C T ATCA C    TTA   CA   TCA
ir construct  114 AGTCCA-TGT-TTCTAGTACTAGT---AAAA-C-TGATCAGC----TTAAGCCAGTATCA 162

NaJAZf        285 AACTGGTCTT-TCCAT-GAAGAAATCTCTGCAAAGATTTCTGCAA-AAGAGAAAAAATAG 341
                   ACT  TCTT T C T G A AAA   C GC A GA     GCAA A  AG AAA    G
ir construct  163 -ACT--TCTTCTGCGTCGCAAAAA---CAGC-AGGA-----GCAACATCAGCAAA---CG 207

NaJAZf        342 AATTCA-AGAAACTTCTCCA--TATCATCACT-AGCTAGTTGATACTATTTGTACTGATT 397
                   A TCA AGA AC  CT  A  TA CA C CT AG
ir construct  208 CAGTCACAGACAC--CTGGAACTAGCAGCTCTGAG------------------------- 240

NaJAZf        398 TCTGATAACCTTTTATTAATTTGTTTTTTCATTCTTTTTGCCCTTCTTTATCTGGTGTGG 457

ir construct      ------------------------------------------------------------

NaJAZf        458 TCTTGGGGGTATAAAGAAGGAGAACCTTTTCGTGTCATTACTGATGACATGCTCTCAGAT 517

ir construct      ------------------------------------------------------------

NaJAZf        518 ATGTAAATTAGTACTATCATATAT 541

ir construct      ------------------------
```

# *EOBII* Controls Flower Opening by Functioning as a General Transcriptomic Switch[1[C][W]]

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R2R3-MYB transcription factors (TFs) are involved in diverse aspects of plant biology. Recently an R2R3-MYB was identified in *Petunia x hybrida* line P720 to have a role in the transcriptional regulation of floral volatile production. We propose a more foundational role for the R2R3-MYB TF *EMISSION OF BENZENOIDS II* (*EOBII*). The homolog of *EOBII* was isolated and characterized from *P. x hybrida* 'Mitchell Diploid' (MD) and *Nicotiana attenuata*. For both MD and *N. attenuata*, *EOBII* transcript accumulates to high levels in floral tissue with maximum accumulation at flower opening. When *EOBII* transcript levels are severely reduced using a stable RNAi (*ir*) approach in MD and *N. attenuata*, *ir-EOBII* flowers fail to enter anthesis and prematurely senesce. Transcript accumulation analysis demonstrated core phenylpropanoid pathway transcripts and cell wall modifier transcript levels are altered in *ir-EOBII* flowers. These flowers can be partially complemented by feeding with a sucrose, *t*-cinnamic acid, and gibberellic acid solution; presumably restoring cellular aspects sufficient for flower opening. Additionally, if ethylene sensitivity is blocked in either MD or *N. attenuata*, *ir-EOBII* flowers enter anthesis. These experiments demonstrate one R2R3-MYB TF can control a highly dynamic process fundamental to sexual reproduction in angiosperms: the opening of flowers.

Regulation of gene transcription is essential for numerous aspects of biology. Transcription factors (TFs) are one way to regulate transcription. To be considered a TF, a protein must bind DNA in a sequence-specific manner (Latchman, 1997). A TF can function to activate or repress the transcription of specific genes (Lee and Young, 2000). Because TFs are essential for the regulation of transcription, TFs are found in all living organisms. The number of TFs per genome follows the power law; the larger the genome the greater the number of TFs (van Nimwegen, 2003).

In plants, an important and relatively large family of TFs is the R2R3-MYB TF family, which in Arabidopsis (*Arabidopsis thaliana*) consists of 126 R2R3-MYB genes (Stracke et al., 2001). R2R3-MYBs have been associated with a large number of divergent plant processes such as primary and secondary metabolism, cell fate and identity, and biotic and abiotic stress response (for review, see Dubos et al., 2010). Recently, a *Petunia x hybrida* line P720 (P720) R2R3-MYB transcript sequence, *EMISSION OF BENZENOID II* (*PhEOBII*), was reported to be involved in floral volatile production during open flower stages of development (Spitzer-Rimon et al., 2010). The authors demonstrate that a PhEOBII:GFP fusion protein localizes to the nucleus in leaf mesophyll cells, transcript accumulation of *PhEOBII* is flower specific and coincides with floral volatile emission from P720, and ectopically expressed PhEOBII can activate phenylpropanoid-related gene promoters. Also, upon a transient reduction of *PhEOBII* transcript levels, reduced levels of emitted and internal volatile benzenoid/phenylpropanoid compounds are detected from the P720 flower. PhEOBII shares high amino acid similarity to the seemingly redundant Arabidopsis MYB21 and MYB24 (AtMYB21/24), which belong to R2R3-MYB subgroup 19 (Stracke et al., 2001).

AtMYB21/24 function is associated with the regulation of jasmonate signaling during stamen and pollen development in an Arabidopsis flower (Mandaokar et al., 2006). Both *AtMYB21/24* transcripts accumu-

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[C] Some figures in this article are displayed in color online but in black and white in the print edition.

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

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late to high levels in floral tissue of Arabidopsis, and AtMYB21/24 are involved in the activation of phenylpropanoid-related genes (Shin et al., 2002; Yang et al., 2007). Reduction in *AtMYB21/24* transcript (T-DNA insertion lines) results in floral developmental defects like failure of anther dehiscence and flower opening; the latter was an observation and not the focus of experimentation (Mandaokar et al., 2006). Another putative homolog of *EOBII* in ornamental tobacco (*Nicotiana langsdorffii x Nicotiana sanderae*; *NlxNsMYB305*) has been characterized through stable RNAi silencing, and the authors reported on floral developmental defects (Liu et al., 2009). Among which were nectary formation and a failure of flower petals to expand at anthesis, which again, the latter was an observation and not the focus of experimentation. Lastly, EOBII homologs from *Antirrhinum majus* (*AmMYB305* and *AmMYB340*) and *Pisum sativum* (*PsMYB26*) have been reported as flower specific and involved in phenylpropanoid pathway regulation (Sablowski et al., 1994; Uimari and Strommer, 1997).

In a *P. x hybrida* 'Mitchell Diploid' (MD) flower, an R2R3-MYB TF termed *ODORANT1* (*PhODO1*; 27.5% amino acid identity with PhEOBII) is involved with regulating genes in primary and secondary metabolism beginning at anthesis, resulting in the indirect regulation of floral volatile benzenoid/phenylpropanoid (FVBP) biosynthesis throughout open flower stages (Verdonk et al., 2005). Since FVBPs can function as pollinator attractants (Hoballah et al., 2005), it is logical that FVBP production in petunia (*P. x hybrida*) is turned on (Verdonk et al., 2003; Colquhoun et al., 2010b) when the sexual organs are receptive to pollination, which occurs at anthesis (Hoekstra and Weges, 1986; Weiss et al., 1992; Wang and Kumar, 2007).

During flower development in petunia, the biosynthesis of phenylpropanoids is a tightly controlled process. Pigment biosynthesis, anthocyanins and flavonoids, occurs during the first stages of flower de-

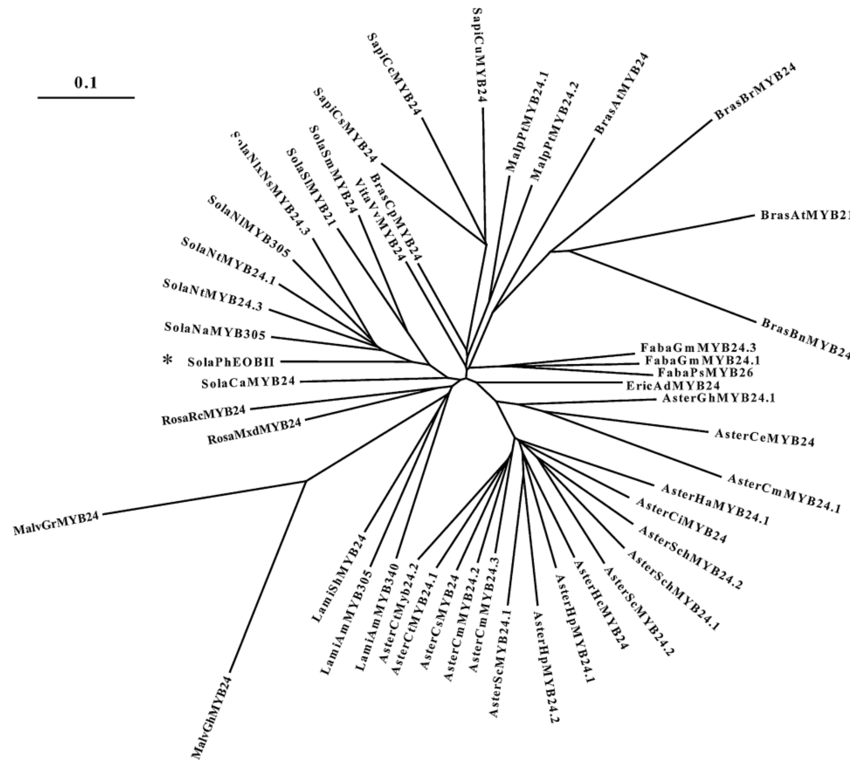


**Figure 1.** An unrooted neighbor-joining tree of PhEOBII-like amino acid sequences. TREEVIEW (Win32) software version 1.6.6, nearest-joining method, was used to create the resulting phylogenetic tree. Scale bar represents distance as the number of substitutions per site (i.e. 0.1 amino acid substitutions per site). An asterisk denotes the PhEOBII sequence.

velopment (Weiss et al., 1992). Petunia corolla growth starts with a slow growth phase during which cell division occurs followed by a phase of rapid growth that mostly results from cell expansion (Ben-Nissan and Weiss, 1996). The phytohormone, gibberellin (GA) is important during the first stages of petunia flower development. GA is necessary to induce anthocyanin biosynthesis and the initiation of rapid cell elongation, but it is not necessary for their maintenance (Weiss et al., 1992; Weiss, 2000). GA is likely synthesized in the anthers, which is then transported to the corolla (Weiss et al., 1993). At the later stages of petal growth, after the transition to rapid elongation, the corolla is no longer dependent on the anthers or GA for growth and pigmentation. In fact, transcription of the genes associated with anthocyanin production is down-regulated at the onset of anthesis by an unknown mechanism, and the application of GA is insufficient to prevent the decline in transcripts because the floral tissue has become insensitive to GA (Weiss, 2000). These results suggest that, in petunia, GA is not involved in the process of anthesis itself.

The transition phase of the flower, anthesis, has not been well defined from a molecular and genetic prospective. In MD, flower opening occurs after several days of bud elongation when the flower bud is approximately 65 mm from the base of the receptacle to the tip of the corolla (Colquhoun et al., 2010b). In petunia, flower opening is irreversible; the flower stays open until senescence, when the corolla wilts and abscises from the receptacle (Negre et al., 2003; Underwood et al., 2005; Colquhoun et al., 2010b). It is assumed that the opening of the flower occurs due to cell elongation and expansion, and is controlled by phytohormones such as auxin, GAs, cytokines, ethylene, and/or brassinosteroids (for review, see van Doorn and Van Meeteren, 2003).

Here, we have examined *EOBII* function in MD flowers. We propose that *PhEOBII* functionality is dynamic in the MD flower, and ultimately controls flower opening (anthesis). In MD, *PhEOBII* transcript accumulation peaks at anthesis, and a severe reduction of *PhEOBII* transcript using a stable, reverse-genetics approach results in a failure to enter anthesis. Additionally, the transgenic phenotype can be complemented by hormone sensitivity manipulation without complimenting FVBP emission. EOBII amino acid sequence is highly conserved throughout multiple orders of angiosperm species, and the MD *PhEOBII* functional characterizations can be replicated in *Nicotiana* species, which provides support for a conservation of function among angiosperms.

## RESULTS

### Identification of *PhEOBII*

Our initial aim was to isolate transcriptional regulators associated with the FVBP gene network in *P. x hybrida* MD. Colquhoun et al. (2011) assembled MD R2R3MYB-like transcript sequences, and one of these Contig 4, since named *PhEOBII*, was chosen for further analysis based on the similarity to Arabidopsis *MYB21* and *MYB24*. The predicted protein sequence of PhEOBII is 197 amino acids in length with a predicted nuclear localization (WoLF PSORT). When aligned with putative homologs, orthologs, and paralogs (Supplemental Table S1) a conserved, N-terminal R2R3-MYB domain (Stracke et al., 2001) and a C-terminal transactivation (W/Y-MDDIW) domain (Li et al., 2006) are obvious (Supplemental Fig. S1). The transactivation domain appears highly conserved through 12 orders and 38 species of angiosperms. Three amino acids in particular (Trp-183, Asp-187, and Trp-189) are conserved in every sequence analyzed (one mismatch

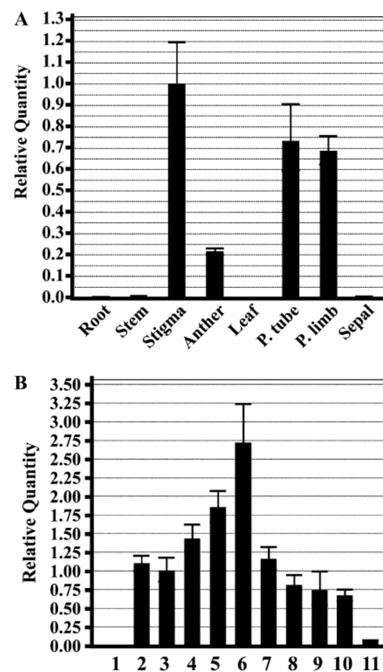


**Figure 2.** qRT-PCR transcript accumulation analysis of *PhEOBII* from MD plants. A, Spatial analysis used total RNA from root, stem, stigma, anther, leaf, petal (P.) tube, petal (P.) limb, and sepal tissues collected at 4 PM (mean ± SE; $n$ = 3). B, Floral developmental analysis used total RNA from staged flower tissues collected at 4 PM of the same day (mean ± SE; $n$ = 3). Stage 1 represents a 1-cm floral bud from the base of the receptacle to the tip of the emerging corolla tissue. Each stage is developmentally separated by approximately 1 d, except for stages 10 and 11, where 3 d separates the stages (adapted from Colquhoun et al., 2010b). All histograms are representative of multiple experiments with multiple biological replicates.

for Asp-187). After placing the extensive list of sequences in an unrooted neighbor-joining tree, the family and genus members group together with the compactness of the tree easily identifiable (Fig. 1).

#### *PhEOBII* Transcript Accumulation Analysis and Promoter Sequence Features

*PhEOBII* transcript accumulation has been examined from whole plant, a flower developmental series, and over a time course in flowers of P720 (Spitzer-Rimon et al., 2010). Since *PhODO1* transcript accumulates differentially between petunia lines (Verdonk et al., 2005), we chose to examine *PhEOBII* transcript accumulation in MD utilizing quantitative reverse-transcriptase PCR (qRT-PCR). From a spatial context, high levels of *PhEOBII* transcripts were detected in the stigma, petal tube, and petal limb tissues with a relatively low detection of transcript in anthers (Fig. 2A). To investigate the transcriptional profile of *PhEOBII* in MD further, staged flowers from a 1-cm bud to the end of the floral life cycle (detailed in Colquhoun et al., 2010b) were used to examine *PhEOBII* transcript accumulation over the course of floral development. The results resembled a bell curve with a maximum at stage six, which is anthesis (Fig. 2B). *PhEOBII* transcript was not detected in stage 1 MD flower buds, and accumulation was reduced to comparatively very low levels in senescing flower tissue (Fig. 2B).

The specificity of *PhEOBII* transcript accumulation in MD flowers led us to clone approximately 1.25 kb of DNA sequence upstream of the *PhEOBII* translational start codon in the MD genome, via GenomeWalker. Utilizing the plant cis-acting regulatory DNA elements database (Higo et al., 1999), putative cis-acting regulatory elements were identified including the abundant etiolation induced, light regulated, Dof binding, enhancers, basic helix-loop-helix, and WRKY binding motifs. Less-abundant elements include: ethylene responsive, GA induced, jasmonic acid (JA) responsive, a plastid responsive, and a prolamine box element (Wu et al., 2000).

#### Functional Characterization of *PhEOBII*

A 3′ segment of the *PhEOBII* transcript (Supplemental Fig. S2) was used to create approximately 50 independent T0 *ir-PhEOBII* (stable RNAi, inverted repeat) plants. These transgenic plants were generated in the MD genetic background by leaf-disc transformation (Jorgensen et al., 1996; Underwood et al., 2005; Dexter et al., 2007; Colquhoun et al., 2010a). A striking phenotype was visually observable when the *ir-PhEOBII* plants were grown to a mature, reproductive stage; flowers from multiple independent transgenic events failed to enter anthesis (Fig. 3) and prematurely senesced as closed buds. *PhEOBII* transcripts are not detected in a stage 1 MD flower (Fig. 2B), so a physiological study was conducted by tagging stage 1 flower buds and measuring the floral growth rate from T0 plants and MD (Fig. 3A). Flowers from MD and *ir-PhEOBII-1* (a transgenic plant with a wild-type [Fig. 3B] flower opening) developed at an equivalent rate, entering anthesis between the sixth and seventh stage with an almost exponential growth curve between stage 4 and 7. In contrast, floral bud growth from the *ir-PhEOBII-5* and *ir-PhEOBII-12* plants (complete nonopening floral phenotype; Fig. 3D) diverged from that of MD between the fourth and fifth stage, and resembled more of a linear function between stage 4 and 7 (Fig. 3A). A representative of an intermediate phenotype, *ir-PhEOBII-3* floral growth rate bisected the difference between MD and the complete nonopening lines (Fig. 3A). The *ir-PhEOBII-3* flowers opened but they failed to completely expand the petal limb tissue (Fig. 3C).

Twelve T0 plants showing the nonopening phenotype and a reduction of *PhEOBII* transcript during an initial semiquantitative (sq)RT-PCR screen (Supplemental Fig. S3) were chosen for further analysis and

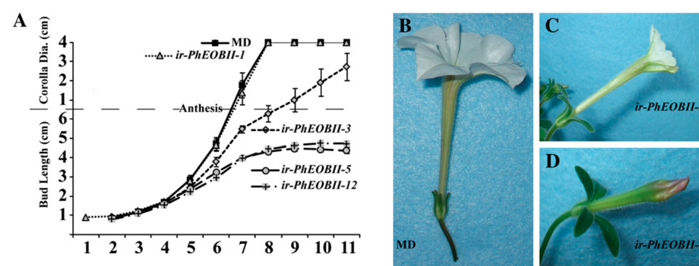


**Figure 3.** Phenotypic growth comparison of MD and T0 *ir-PhEOBII* flowers. Shown are representatives from transgenic plants showing a wild type (*ir-PhEOBII-1*), an intermediate (*ir-PhEOBII-3*), and two strong floral phenotypes (*ir-PhEOBII-5* and *12*). A, All flower buds were tagged at a 1-cm bud and followed through development (mean ± SE; $n = 12$). B to D, Pictures of the *ir-PhEOBII* phenotype. B, An entire open MD flower. C, A semiopening flower from an intermediate line, *ir-PhEOBII-3*. D, A nonopening flower from a strong phenotype line, *ir-PhEOBII-5*. [See online article for color version of this figure.]

**Table I.** *Phenotypic characterization of the vegetative growth of T1 ir-PhEOBII RNAi lines*

Measurements of three independent plants were made; averages and SE were then calculated. Significant differences from MD ($P < 0.01$) were not found except for the field marked with an asterisk.

| Plant Aspects | MD | *ir-3* | *ir-5* | *ir-12* |
|---|---|---|---|---|
| No. of branches | 12.7 ± 0.82 | 11.3 ± 0.41 | 12.0 ± 0.71 | 13.0 ± 0.71 |
| No. of subbranches | 27.0 ± 3.74 | 11.3 ± 8.44 | 24.0 ± 8.86 | 21.7 ± 1.78 |
| No. of nodes per branch | 16.2 ± 0.32 | 16.4 ± 0.84 | 16.1 ± 0.98 | 18.6 ± 1.08 |
| No. of flowers or buds | 116.3 ± 14.57 | 61.0 ± 22.26 | 148.7 ± 6.1 | 107.3 ± 19.8 |
| Aerial fresh weight (g) | 220.3 ± 18 | 248.3 ± 96.65 | 325.9 ± 28.53 | 353.6 ± 15.27* |
| Total branch length (cm) | 381.0 ± 54.55 | 507.7 ± 71.37 | 715.7 ± 63.25* | 835.7 ± 87.37* |
| Average branch length (cm) | 60.6 ± 2.7 | 45.1 ± 7.51 | 59.5 ± 2.19 | 64.3 ± 5.74 |

self pollinated by cutting the corolla open of an approximately 3-cm flower bud, allowing the anthers to dehisce, and pollinating an adjacent mechanically opened flower bud. Segregating T1 populations were used for gross physiological and more intricate floral measurements such as: the total number of branches per plant, the total number of flowers per plant, and the average branch length per plant (Table I); or petiole length, sepal length, and stigma length (Table II). The only per plant measurement that demonstrated a significant difference ($P < 0.01$) between MD and both nonopening transgenic lines was total branch length, where *ir-PhEOBII-5* and *ir-PhEOBII-12* plants demonstrated a longer total branch length than MD (Table I). Comparative floral measurements between MD and the two nonopening lines illustrated significant differences in petiole length, stigma length, and anther filament length (Table II). Note, no difference was measured for sepal length between MD and transgenic flowers. All T1 populations segregating in a Mendelian fashion were self pollinated as previously described, and a T2 homozygous *ir-PhEOBII* line (*ir-5*, name originated from T0 number) was produced and used for further analysis (Fig. 4B). When flowers from MD and *ir-5* were tagged, developmentally staged, and visualized photographically, the nonopening phenotype was evident (Fig. 4).

The development of an *ir-5* flower is disconnected from that of a MD flower (Figs. 3 and 4). To normalize a targeted transcript accumulation analysis, we compared *ir-5* flowers to MD flowers staged over time (Fig. 4). One-centimeter flower buds were tagged and allowed to progress through what would be stage 7 in MD flowers. Again, qRT-PCR was utilized, but a two-step reaction was preferred to eliminate any *PhEOBII* RNA expressed from the transgene (Colquhoun et al., 2011). *PhEOBII* transcripts were severely reduced (approximately 82% reduction at floral developmental stage 5) in *ir-5* flowers compared to MD flowers (Fig. 5A). A petunia transcript very similar to a rose (*Rosa hybrida*) *AQUAPORIN* necessary for flower petal expansion in rose, *RhPIP2;1* (Ma et al., 2008), was unaltered between MD and *ir-5* flowers (Fig. 5B). AtMYB21, NlxNsMYB305, and PhEOBII can positively regulate a *PHENYLALANINE AMMONIA-LYASE* (*PAL*) promoter in the respective species (Shin et al., 2002; Liu et al., 2009; Spitzer-Rimon et al., 2010). In MD, two *PhPAL* transcripts accumulate to high levels around anthesis, but no increase in transcript accumulation was observed for the two *PhPALs* in *ir-5* flowers (Fig. 5C). As expected from the nonopening phenotype (Fig. 4) and the lack in up-regulation of the *PhPALs* (Fig. 5C), *ir-5* flowers do not emit FVBPs (Supplemental Fig. S4). Petunia sequences very similar to xyloglucan endotransglycosylases/hydrolases (cell wall modifiers) *PhXTR6* and *PhXTR7* (Yokoyama and Nishitani, 2007) were also altered in *ir-5* flowers compared to MD (Fig. 5D). Whether *PhEOBII* directly or indirectly regulates the *PhPALs* and the *PhXTRs* has yet to be determined, however, we conclude when levels of *PhEOBII* are reduced in MD, these transcripts (associated with divergent biological processes) are altered and may collectively contribute to the nonopening phenotype.

#### Complementation of *ir-PhEOBII* Phenotype

*PhEOBII* has a wide-ranging effect on the development of a petunia flower, e.g. slowed corolla growth to

**Table II.** *Phenotypic characterization of the floral organs of T1 ir-PhEOBII RNAi lines*

Measurements of five independent flowers at stage 8 were made; averages and SE were then calculated. Significant differences from MD ($P < 0.01$) are marked with an asterisk. In reference to anthers, d1 denotes tube segment attachment.

| Flower Aspects | MD | *ir-3* | *ir-5* | *ir-12* |
|---|---|---|---|---|
| Petiole length (cm) | 4.47 ± 0.26 | 3.63 ± 0.20 | 3.28 ± 0.13* | 3.26 ± 0.14* |
| Sepal length (cm) | 1.12 ± 0.03 | 1.10 ± 0.02 | 1.13 ± 0.03 | 1.13 ± 0.02 |
| Ovary length (cm) | 0.47 ± 0.02 | 0.48 ± 0.02 | 0.48 ± 0.02 | 0.52 ± 0.02 |
| Stigma length (cm) | 4.10 ± 0.04 | 3.98 ± 0.06 | 2.73 ± 0.08* | 2.80 ± 0.11* |
| Anther filament length (cm) | 4.35 ± 0.06 | 4.31 ± 0.06 | 3.02 ± 0.08* | 3.22 ± 0.01* |
| Anther attached [d1] (cm) | 1.77 ± 0.07 | 1.80 ± 0.03 | 1.47 ± 0.07* | 1.50 ± 0.04* |

the failure to enter anthesis (Fig. 3, A and D), or phenylpropanoid metabolism to cell wall modification (Fig. 5, C and D). We hypothesized that the supplementation of downstream compounds from the effected molecular steps would rescue the transgenic nonopening phenotype (Fig. 4). After investigating numerous chemicals and combinations, petiole feeding with a Suc, *t*-cinnamic acid, and GA (STG) solution resulted in an increased bud elongation of the *ir-5* flowers compared to flowers in water (Fig. 6). Additionally, the *ir-5* flowers in the STG solution enter anthesis; however, complete limb expansion was not observed (Fig. 6, inset picture). JA feeding resulted in a hypersensitive response (bud growth impairment was exacerbated) from *ir-5* flowers, which was abolished in the presence of the STG solution (Fig. 6).

The nonopening, transgenic flowers senesce prematurely (Fig. 4). Therefore, we hypothesized the phytohormone ethylene was responsible for the premature senescence of the *ir-5* flowers. The ethylene-insensitive *P. x hybrida* MD line 44568 (Wilkinson et al., 1997), which displays an increased floral longevity phenotype (Wilkinson et al., 1997; Colquhoun et al., 2010b), was utilized to cross pollinate with the *ir-5* flowers. The 44568 × *ir-5* flowers did not senesce early compared to MD. The F1 generation 44568 × *ir-5* flowers displayed a partial complementation by entering anthesis on the same day the *ir-5* flowers displayed signs of senescence (Fig. 7). Again, anthesis was not complete, but the petal limb did expand further than the mechanical complementation (Fig. 6). The premature senescence of the closed *ir-5* flower bud is most likely attributed to the ethylene senescence program. Also, if the ethylene signal is not perceived, anthesis is triggered by an unknown mechanism.

### Conservation of *PhEOBII* Function

To test the functional conservation suggested by amino acid conservation through many angiosperm

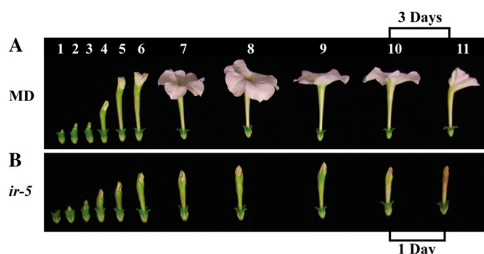


**Figure 4.** Relation of MD floral development and a T2 *ir-PhEOBII* homozygous line. A, Developmentally staged MD flowers from a small bud, stage 1; 1 cm, to a senescing flower, stage 11 (adapted from Colquhoun et al., 2010b). B, Developmentally staged flowers from the *ir-PhEOBII-5* (*ir-5*) homozygous line. Approximately 1 d separates each stage except for stages 10 and 11 from MD flowers, where these stages are separated by 3 d. [See online article for color version of this figure.]

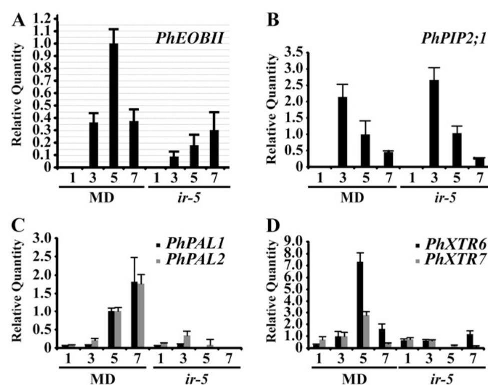


**Figure 5.** Comparative transcript accumulation analysis using MD and *ir-5* flowers. A to D, Developmentally staged flowers (an abbreviated staging since *ir-5* flowers initially differs from MD between stage 4 and 5) were collected on one day at 4 PM. qPCR was carried out with 10 times diluted cDNA samples and run with the ΔΔCt method using *PhFBP1* as an internal reference. Histograms are representative of two biological replicates (mean ± SE; *n* = 3).

species, the *PhEOBII* homolog from the ecological model system *Nicotiana attenuata* (Baldwin, 1998) was isolated (*NaMYB305*) and cloned. *NaMYB305* transcript accumulation in a developing *N. attenuata* flower is very similar to *PhEOBII* in MD (Figs. 2 and 8). Much like in petunia, a stable RNAi approach was used to reduce the *NaMYB305* transcript accumulation in *N. attenuata*, which resulted in 14 independent T0 plants with flowers that failed to enter anthesis, senesced prematurely, and were reduced in *NaMYB305* transcript levels compared to wild-type *N. attenuata* (Fig. 9A). The *ir-NaMYB305* plants displaying the nonopening phenotype were extremely difficult to sexually propagate because the closed flower buds would drop if mechanically manipulated as was done for the MD *ir-PhEOBII* flowers.

Of interest was the phenotypic complementation by ethylene insensitivity in petunia (Fig. 7). When the *ir-NaMYB305* plants were treated with 1-methylcyclopropene (MCP; an ethylene receptor blocker), a partial complementation was observed (Fig. 9B). However, the frequency (flowers/plant) of complementation was low. Regardless, the results obtained from experimentation with *N. attenuata* resemble that of MD, and further support a conservation of function for EOBII between these two genuses.

## DISCUSSION

Since we were interested in FVBP biosynthesis regulation, we initially isolated the Contig 4 nucleotide (nt) sequence (Colquhoun et al., 2011) from MD be-

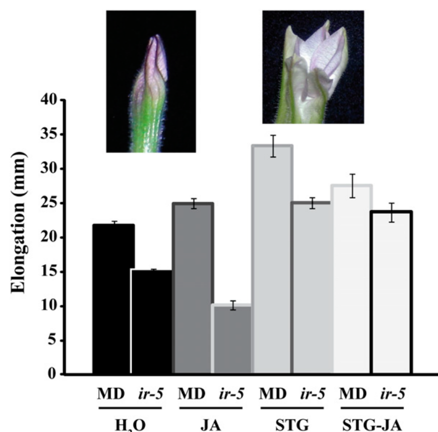


**Figure 6.** Feeding experiments with excised MD and *ir-5* flowers. All flowers were excised at 3 cm and placed in solutions indicated: water, 50 $\mu$M JA, and STG. Growth was measured every 24 h in millimeters (mean ± SE; $n = 15$). Inset pictures are representative of the *ir-5* flowers used in this experiment after four days of feeding. [See online article for color version of this figure.]

cause it was most similar to *AtMYB21* and *AtMYB24*, of which AtMYB21 can positively regulate the *AtPAL1* promoter (Shin et al., 2002). During our time of experimentation with *P. x hybrida* MD, another group published quality results communicating a floral volatile function for *PhEOBII* in an open flower of a commercially available *P. x hybrida* line P720 (Spitzer-Rimon et al., 2010). This group used a virus-induced gene silencing-based system to illustrate that upon reduction of *EOBII* transcript a concomitant reduction of emitted and internal levels of FVBP compounds were measured from open flowers. A nonopening phenotype was not reported (Spitzer-Rimon et al., 2010). A double T-DNA insertion line for Arabidopsis *EOBII* homologs (*AtMYB21* and *AtMYB24*) was generated and a nonopening flower phenotype was observed, but no further analysis was conducted (Mandaokar et al., 2006). Additionally, a stable RNAi-based approach was used to reduce the transcript of another *EOBII* homolog in ornamental tobacco *N. langsdorffii x N. sanderae*, *NlxNsMYB305*, and the authors reported on a "failure of the flower petals to expand at anthesis," with no further analysis conducted (Liu et al., 2009, p. 2682). We have demonstrated through a stable RNAi approach that a drastic reduction of *EOBII* transcript in MD and *N. attenuata* plants results in a nonopening floral phenotype (Figs. 3, 4, and 9), and that *EOBII* function is highly dynamic (Figs. 3, 5–7, and 9).

A floral volatile function for EOBII in an open flower is acknowledged (Spitzer-Rimon et al., 2010). Indirectly our experimentation supports for a similar conclusion. In the MD genetic background, the nonopening phenotype of *ir-5* was partially complimented by ethylene insensitivity (Fig. 7), but the lack in emission of FVBPs was not complemented (Supplemental Fig. S4). This result implies the petal limb tissue can transition into and partially through the physical processes of opening, but induction of the FVBP pathway is not necessary for that complementation. In contrast, EOBII may be necessary for the induction of the FVBP pathway.

The *EOBII* gene is an R2R3-MYB TF that is expressed in flowers at high levels in genus like *Petunia*, *Arabidopsis*, *Antirrhinum*, *Nicotiana*, and *Pisum* (Figs. 2A and 8; Sablowski et al., 1994; Uimari and Strommer, 1997; Shin et al., 2002; Schmid et al., 2005; Liu et al., 2009; Spitzer-Rimon et al., 2010). Interestingly, *EOBII* transcripts accumulate to relatively high levels in the petal tube tissue of MD (Fig. 2A), but in P720 and *Antirrhinum* very little transcript was detected in petal tube tissue (Sablowski et al., 1994; Spitzer-Rimon et al., 2010). Also noteworthy, *PhEOBII* transcript was not detected in sepal tissue of MD (Fig. 2A) and sepal development was not affected in the *ir-PhEOBII* plants by our observation (Table II), which suggests the functionality of this transcript is regulated after organ identity (Coen and Meyerowitz, 1991) has been established.

The primary amino acid sequence of EOBII is well conserved at the R2R3 domain and the transactivation domain through 12 orders of eudicots (Fig. 1; Supplemental Fig. S1). Work carried out in the aforementioned plant species indicates the EOBII protein can activate promoter regions of genes like *PAL1*, *PAL2*, *ISOEUGENOL SYNTHASE1*, *ODO1*, *NECTARIN1*, and *NEC5*. Therefore, EOBII has been established to have multiple target genes, and with evidence like protein sequence and functional conservation, appears conserved in many angiosperms. The *N. attenuata* experimentation was an initial attempt to test the conservation of function in an angiosperm system that

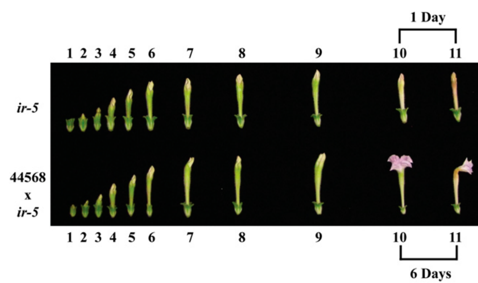


**Figure 7.** Complementation of the *ir-PhEOBII* phenotype by ethylene insensitivity. Shown are developmentally staged *ir-5*, and 44568 × *ir-5* flowers, from a small bud (stage 1, 1 cm) to a senescing flower (stage 11). Approximately 1 d separates each stage except for stages 10 and 11 in the 44568 × *ir-5* line, where 10 and 11 are separated by approximately 6 d. [See online article for color version of this figure.]

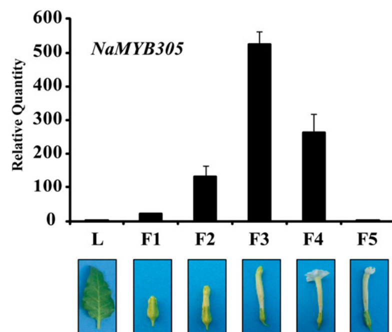


**Figure 8.** Transcript accumulation of the *PhEOBII* homolog in *N. attenuata*, *NaMYB305*. qPCR analysis of *NaMYB305* transcript accumulation through the development of a *N. attenuata* flower (mean ± se; *n* = 3). The level of *NaMYB305* transcript in floral tissue is set relative to the level in leaf tissue. [See online article for color version of this figure.]

was accessible (Figs. 8 and 9), which resulted in a conservation of function down to the ethylene insensitivity complementation (Fig. 9).

Our results indicate that a more foundational role for *PhEOBII* exists, which is the control of flower opening. The concept of TFs affecting different numbers of promoters at differing protein concentrations is not novel, e.g. the morphogen gradient model from *Drosophila melanogaster* or haploinsufficiency in *Homo sapiens* (Deutschbauer et al., 2005; Ashe and Briscoe, 2006). However, various other explanations exist such as: necessary coregulators produced at differing developmental stages effecting different target pathways, or cascades of factors with different developmental timing. Intriguing though, is that one gene (*PhEOBII*), through a developmental stimulus of some sort (floral developmental stage 2; Fig. 2B), controls divergent aspects of flower biology (phenylpropanoid metabolism to cell wall modifications) to set forth a different cellular paradigm (FVBP biosynthesis). This model resembles that of a trans-epigenetic state (Bonasio et al., 2010).

## CONCLUSION

In petunia, *Nicotiana*, and Arabidopsis EOBII function is required for sexual reproduction. That is not to say in all cases of severe *EOBII* deficiency the sexual organs are defective. The tissue that encases developing sexual organs and later may attract potential pollinators (the corolla in general) is most likely affected in secondary metabolite production, cell wall modifications, turgor pressure manipulation, and phytohormone response. Together, these biological limitations result in a failure to display sexual organs to the environment by undergoing anthesis. Whether EOBII has a direct role in all these systems, or EOBII is a central member of a cascade of molecular events, all culminating in the dynamic process of flower opening, is not clear. However, if the molecular mechanism of anthesis is conserved among most angiosperms, exploitation of *EOBII* expression in genetically modified angiosperm crops may aid in reducing the risk of outcrossing for particular applications.

## MATERIALS AND METHODS

### Plant Material

Inbred *Petunia x hybrida* MD plants were used as a wild-type control in all experiments (Mitchell et al., 1980). The homozygous ethylene-insensitive *cauliflower mosaic virus 35S:etr1-1* line, 44568, generated in the MD genetic background (Wilkinson et al., 1997), was used as a negative control for ethylene sensitivity (Underwood et al., 2005; Dexter et al., 2007, 2008; Colquhoun et al., 2010a, 2010b, 2011) and in cross pollinations to the MD *ir-PhEOBII* lines. All petunia (*P. x hybrida*) plants were grown in glass greenhouses as previously described (Dexter et al., 2007). *Nicotiana attenuata* (30th inbred generation) seeds, originally collected from a native population from a field site located in Utah, were used for all described experiments. Wild-type and *ir-NaMYB305* RNAi plants were grown in glass greenhouses as previously described (Kaur et al., 2010).

### Identification of *EOBII*

Sequences with similarity to Arabidopsis (*Arabidopsis thaliana*) R2R3-MYB TFs (*AtMYBs*) were gathered using the National Center for Biotechnology Infor-

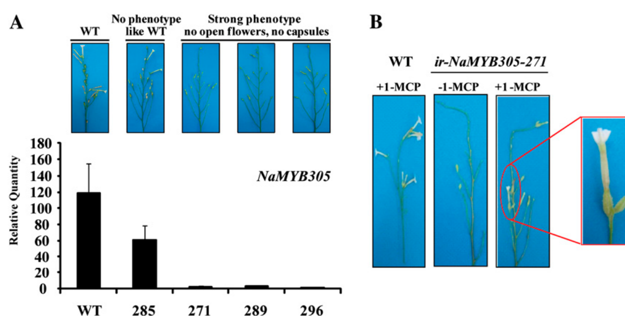


**Figure 9.** A reduction of *NaMYB305* transcript levels in *N. attenuata* by RNAi. A, Transcript accumulation of *NaMYB305* levels in representative T0 *ir-NaMYB305* lines compared to wild-type *N. attenuata* (mean ± se; *n* = 3) with phenotypic pictures and descriptions included. B, Complementation study using MCP to block ethylene sensitivity in *N. attenuata* and *ir-NaMYB305* with a magnified inset picture of an *ir-NaMYB305* that has entered anthesis. [See online article for color version of this figure.]

mation, Sol Genomics Network, and the 454 petunia databases (www.ncbi.nlm.nih.gov, http://solgenomics.net, and http://140.164.45.140/454petuniadb). The resulting sequences were used to construct a petunia nt alignment (ContigExpress module, Vector NTI Advance 11, Invitrogen Corp., http://invitrogen.com) representing the MD *PhEOBII*, and sequence-specific primers (*PhEOBII* forward primer 5′-TCCCTATCCCATCTCTTTCTCTCTCCCTCT-3′; *PhEOBII* reverse primer 5′-CACTCATGAGATGGTTCAATCTAGGG-3′, ordered through Integrated DNA Technologies) were designed approximately 80 to 100 nt 5′ and 3′ of the deduced 594-nt coding region (GenBank accession number: *P. x hydrida* MD *NON-OPENING 1* [*PhNON1*], EU374207). Replicates of the expected, approximate 700-nt product were amplified using Advantage 2 polymerase mix (Clontech Laboratories Inc., http://www.clontech.com) from gene transcript pools of MD flower tissue, and purified using QIAquick spin columns (Qiagen, http://qiagen.com). Amplicons were ligated into pGEM-T-easy vector (Promega Corp., http://promega.com), transformed into One Shot Mach1-T1$^R$ chemically competent *Escherichia coli* (Invitrogen Corp., http://invitrogen.com), multiple clones were isolated and sequenced (Big Dye V1-2; University of Florida Sequencing Core Facility, http://www.biotech.ufl.edu) to at least a 4× coverage to check for errors.

nt sequence of *NaMYB305* was assembled from 109 individual reads (size 400–450 bp) obtained from 454 GS FLX titanium series sequencing of *N. attenuata* cDNAs (vertis Biotechnologie AG; http://www.vertis-biotech.com/). Individual 454 reads were assembled by the PAVE program for assembling and viewing ESTs (Soderlund et al., 2009). Gene-specific primers were designed to amplify *NaMYB305* gene fragments that were ligated into pGEM-T-easy vector (Promega Corp., http://promega.com) and sequenced using a standard sequencing method. The sequence of *NaMYB305* was deposited in GenBank under accession number: *N. attenuata MYB305* (*NaMYB305*), Na_454_02248.

## Promoter Cloning and Motif Scan

Approximately 1.25 kb of the MD *EOBII* promoter was cloned with a gene-specific primer 5′-ACGTTTGAGACCAGCAGATTTAGCTAAGG-3′ and a nested specific primer 5′-TTCTTCCATAGTCCAAGGTCCTTTCC-3′ using the GenomeWalker kit and protocol according to the manufacturer's instructions (Clontech Laboratories Inc., http://www.clontech.com). Plant cis-acting regulatory DNA elements, Signal Scan Search, an online database of nt sequence motifs found in plant cis-acting regulatory DNA elements was used to screen the *PhEOBII* promoter sequence (www.dna.affrc.go.jp).

## Generating Transgenic *PhEOBII* RNAi Plants

To directly test the gene function of MD *PhEOBII*, stable RNAi-based gene silencing was utilized. A 283-nt sequence at the 3′ end of the *PhEOBII* transcript was developed as the RNAi-inducing fragment (*PhEOBII* forward primers: 5′-GCTCTAGAGCACATTAAGCAAGCAGA-3′, 5′-CGGGATCCGCACATTAAGCAAGCAGA-3′; and reverse primers: 5′-GGAATTCAGATGGTTCAATCTCAGG-3′, 5′-GGAATTCATAGGCACCTCCATGCAT-3′). The corresponding nt region in the most similar petunia sequence is 65.6% identical. Also, when the 283-nt *PhEOBII* RNAi trigger is fractionated into 22- to 25-nt fragments, then used as queries for Blast analysis on the petunia EST databases, the only identical sequences that return belong to *EOBII* petunia sequences. Additionally, the corresponding nt region in *AtMYB24* is 60.9% and 46.1% identical to the two closest Arabidopsis sequences (*AtMYB21* and *AtMYB57*, respectively). In planta expression of this fragment is driven by a constitutive promoter, pFMV. Fifty independent *PhEOBII* RNAi (*ir-PhEOBII*, inverted repeat) plants were generated in the MD background by leaf disc transformation (Jorgensen et al., 1996). Further details of the technical cloning have been previously described (Underwood et al., 2005; Dexter et al., 2007).

At least 12 T0 plants showing a transcriptional (sqRT-PCR) and physiological phenotype were self pollinated by cutting the corolla open of an approximate 3-cm flower bud, allowing the anthers to dehisce, and pollinating an adjacent, mechanically opened flower bud. The T1 generation was analyzed for a 3:1 segregation based on the presence of the transgene and the observable phenotype. Segregating T1 lines were self pollinated as before, and T2 generation was examined for nonsegregating lines with the phenotype. In this manner, a T2 homozygous *ir-PhEOBII* line (*ir-5*) was produced.

An RNAi approach was utilized to reduce the *NaMYB305* transcript accumulation, resulting in 30 independently transformed *ir-NaMYB305* plants. An RNAi-inducing 161-bp long fragment of *NaMYB305* coding sequence was introduced into *N. attenuata* plants using *Agrobacterium tumefaciens*-mediated plant transformation method as previously described (Krügel et al., 2002). PCR-amplified inverted repeat of *NaMYB305* gene (forward primers: 5′-CTGCAGCAGAAAACATGAATGGACAA-3′, 5′-GAGCTCGCAGAAAACATGAATGGACAA-3′; and reverse primers: AAGCTTGTTTCAGTGAGAAAAGGTC, CTCGAGTTTCAGTGAGAAAAGGTC) was cloned into *Hin*dIII/*Pst*I and *Sac*I/*Xho*I restriction sites of pSOL8 (*S12*) as inverted repeat to generate transformation vector pSOL8MYB3.

## RNA Isolation, Tissue Collection, and Treatments

In all cases, total RNA was extracted as previously described (Verdonk et al., 2003) and subjected to TURBO DNase treatment (Ambion Inc.) followed by total RNA purification with RNeasy mini protocol for RNA cleanup (Qiagen). Total RNA was then quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific) and 50 ng/$\mu$L dilutions were prepared and stored at −20°C. Generation of cDNA samples used 2 $\mu$g of total RNA with SuperScript reverse transcriptase II (Invitrogen Corp., http://invitrogen.com) and was conducted more than three times for technical replications.

The physiological study was performed by tagging newly emerging flower buds of *ir-PhEOBII* and MD plants at stage 1: a bud 1 cm from the base of receptacle to corolla tip. Buds were measured in length of the corolla limb for 11 d. *ir-PhEOBII* were self pollinated by slicing open approximate 3-cm flower bud corolla, allowing the anthers to dehisce, and pollinating an adjacent, mechanically opened, emasculated flower bud.

The feeding experiments were performed using the compounds: Glc, Fru, Suc, sorbitol, auxin, JA, GA, salicylic acid, abscisic acid, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid, Phe, *t*-cinnamic acid, and benzoic acid. Excised *ir-PhEOBII* flowers were harvested at 3 cm and exogenously fed designated solutions and water as a control. Elongation was measured every 24 h in millimeters from the original 3-cm flower bud.

All petunia tissue collections were done as previously described (Colquhoun et al., 2010b). The spatial transcript accumulation analysis consisted of total RNA isolated from petunia root, stem, stigma, anther, leaf, petal tube (stage 8), petal limb (stage 8), and sepal tissues from multiple greenhouse-grown MD plants harvested at 4 PM. The developmental transcript accumulation analysis used total RNA isolated from whole (from the base of the receptacle up) petunia flowers (MD, 44568, *ir-5*, and 44568 × *ir-5*) collected at 11 consecutive stages beginning at a small bud to floral senescence from multiple greenhouse-grown plants at 4 PM. For all tissue collections, individual samples consisted of three flowers. All samples were frozen in liquid $N_2$ and stored at −80°C. Total RNA was then isolated from all samples, with multiple biological replicates included.

RNA extractions, cDNA synthesis, and qRT-PCR analyses of transcript abundances of *NaMYB305* gene were performed essentially as described in Kaur et al. (2010). The developmental transcript accumulation analysis used whole *N. attenuata* flowers collected at five consecutive stages beginning at a small bud to floral senescence from multiple greenhouse-grown plants at 1 PM. Rosette leaf samples were collected and analyzed to compare expression levels of *NaMYB305* in reproductive and vegetative tissues. Silencing efficiency of *NaMYB305* gene relative to wild-type levels was determined in four independently transformed *ir-NaMYB305* lines and wild type using floral buds at F2 stage of development.

To inhibit ethylene perception, we grew isolated branches of *ir-NaMYB305* plants with F1-F2 stage flower buds in 0.1× Murashige and Skoog salt solution and exposed them to ethylene receptor antagonist 1-MCP vapors: 10 mg of SmartFresh (3.3% 1-MCP; AgroFresh; Rohm and Haas) was dissolved in 1 mL of alkaline solution (0.75% KOH + NaOH in a 1:1 ratio) to release the active volatile substance, 1-MCP. Alkaline solution without 1-MCP was used in control experiment. The branches with flower buds were maintained in closed containers during whole treatment (6 d); once a day the containers were fully opened and new vial with freshly activated 1-MCP solution or alkaline solution alone were inserted before closing.

## Transcript Accumulation Analysis

All transcript accumulation analyses were conducted multiple times with multiple biological replicates and equivalent results were observed. sqRT-PCR was performed using a Qiagen one-step RT-PCR kit (Qiagen Co.) with 50 ng total RNA. To visualize RNA-loading concentrations, samples were amplified with *Ph18S* primers (forward primer 5′-TTAGCAGGCTGAGGTCTCGT-3′; reverse primer 5′-AGCGGATGTTGCTTTTAGGA-3′) and analyzed on an agarose gel. The following primers were designed and utilized for the

visualization of the mRNA levels corresponding to *PhEOBII* (forward primer 5′-TCCCCCATATATGTGAGTTAAGTG-3′; reverse primer 5′-CCATAGG-CACCTCCATGCAT-3′) ΔΔCt. qRT-PCR was performed and analyzed using a StepOnePlus real-time PCR system (Applied Biosystems). Power SYBR green RNA-to-Ct 1 and 2-step kits (Applied Biosystems) were used to amplify and detect the products according to the manufacturer's protocol. The following qRT-PCR primers were constructed in Primer Express software v2.0 (Applied Biosystems): *PhEOBII* forward primer, 5′-CATTTGCCTGGAA-GAACAGA-3′; *PhEOBII* reverse primer, 5′-TGCTTGTCCATTCATGGTTT-3′; *PhFBP1*, forward primer, 5′-TGCGCCAACTTGAGATAGCA-3′; *PhFBP1* reverse primer, 5′-TGCTGAAACACTTCGCCAATT-3′; *PhPIP2;1*, forward primer, 5′-TGCTGGTCTCAAACGTACCG-3′; *PhPIP2;1* reverse primer 5′-GGACATCAGGCCGGAGATAA-3′; *SGN-U207589* (*PhXTR6*), forward primer, 5′-GCATCTTCTACTTCTGCCAGAA-3′; *SGN-U207589* (*PhXTR6*), reverse primer 5′-TCTGCACCCATTTCATCCTT-3′; *SGN-U207700* (*PhXTR7*), forward primer, 5′-GCAAATCCCAATTCTCAGATG-3′; *SGN-U207700* (*PhXTR7*), reverse primer 5′-GGAAAACCCTGTGGAAACCT-3′: *PhCM1*, forward primer, 5′-CCCTGATGAGCACCCATTC-3′; *PhCM1*, reverse primer 5′-ACTGCATGGGTGGCAACAC-3′; *PhPAL1*, forward primer, 5′-GCTAGG-CGGTGAGACGCTAA-3′; *PhPAL1* reverse primer 5′-CTCGGACAGCTGC-ACTGTCA-3′; *PhPAL2*, forward primer, 5′-ACTGGCAGGCCTAATTCCAA-3′; *PhPAL2* reverse primer 5′-GCGAAACGCTTCTTCAGCAT-3′. Optimization of primers was conducted and demonstrated gene specificity during melt curve analysis.

A qPCR core kit for SYBR green I (Eurogentec; http://www.eurogentec.com) was used following the manufacturer's instructions on a Stratagene Mx3005P real-time PCR system (http://www.stratagene.com) to determine relative expression of *NaMYB305* gene. Relative expression was calculated using the elongation factor-1 housekeeping gene (GenBank accession number: *Nicotiana tabacum*, D63396) as an endogenous reference. *NaMYB305* gene-specific primers were used: forward primer 5′-ATGCTAAGTGGGGAAA-CAG-3′ and reverse primer 5′-GCAATTGCATGGACCAGA-3′.

### Volatile Emission

For all volatile emission experiments, emitted floral volatiles from excised flowers were collected at 6 PM and quantified as previously described (Underwood et al., 2005; Dexter et al., 2007). All samples consisted of three flowers per sample with at least three biological replicates.

### GenBank Accession Numbers

*PhODO1*, AY705977; *PhEOBII* (P720), EU360893; *PhEOBII* (MD), EU374207; *promPhEOBII* (MD), HQ450382; *NlxNsMYB305*, EU111679; *RhPIP2;1*, EU572717; *AtMYB21*, AT3G27810; *AtMYB24*, AT5G40350; *PhPAL1*, AY705976; *PhPAL2*, CO805160; *AtXTR6*, AT4G25810; *AtXTR7*, AT4G14130; and *NaMYB305*, Na_454_02248.

### Supplemental Data

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

**Supplemental Figure S1.** Predicted amino acid sequence alignment of putative, homologous EOBII proteins.

**Supplemental Figure S2.** Schematic representation of the *PhEOBII* gene model.

**Supplemental Figure S3.** sqRT-PCR transcript accumulation analysis in floral tissues of independent T0 *ir-PhEOBII* lines and MD plants.

**Supplemental Figure S4.** Floral volatile emission analysis of representative plants from MD, *ir-5*, and 44568 × *ir-5*.

**Supplemental Table S1.** Accession numbers and respective identifiers of sequences used for the phylogenetic comparisons.

## ACKNOWLEDGMENTS

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**S1**

Figure S1. Predicted amino acid sequence alignment of putative, homologous EOBII proteins from various species. Sequences were aligned using the AlignX program of the Vector NTI Advance™ 11 software. Residues highlighted in: blue represent consensus residues derived from a block of similar residues at a given position, green represent consensus residues derived from the occurrence of greater than 50 % of a single residue at a given position, and yellow represent consensus residues derived from a completely conserved residue at a given position. A red bar underlines the C-terminal trans-activation domain of interest. * denotes the PhEOBII sequence.

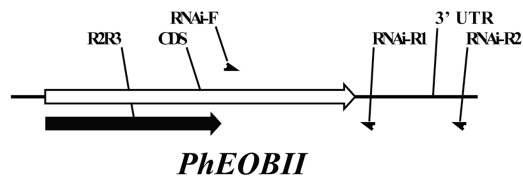


Figure S2. Schematic representation of the *PhEOBII* gene model as shown in Vector NTI Advance™ 11. Depicted are the highly conserved R2R3-MYB domain, the coding sequence, and the location for primers used in constructing the RNAi plasmid.

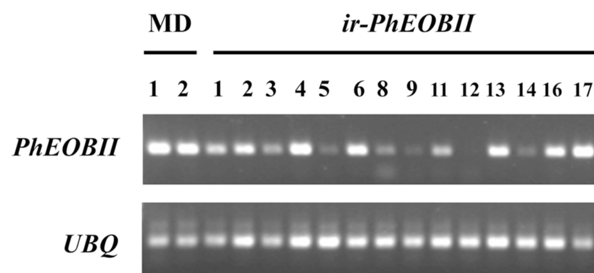


Figure S3. sqRT-PCR transcript accumulation analysis in floral tissues of independent $T_0$ *ir-PhEOBII* lines and MD plants. Gene specific primers for *PhEOBII* were used with 26 cycles of amplification. *PhUBIQUITIN* primers were used as a loading control with 18 cycles used, and 50 ng of total RNA was used in each reaction.

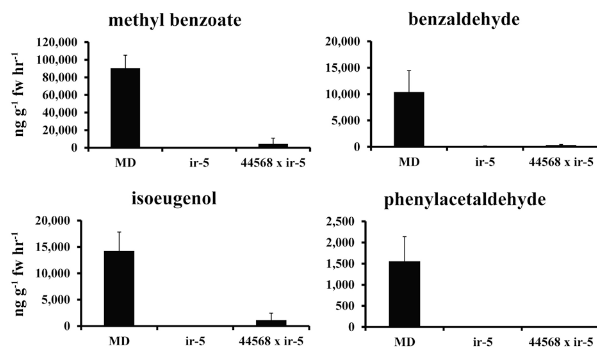


Figure S4. Floral volatile emission analysis of representative plants from MD, *ir-5*, and 44568x*ir-5*. Developmentally staged flowers (what would be stage 8 in MD) were used to collect FVBP emission at 18:00 h (mean ± se; n = 3). Shown are four major FVBP compounds with all measurements ng/g fresh weight/hour.

**SUPPORTING DATA**

**TABLES**

Table S1. Accession numbers and respective identifiers of sequences used for the phylogenetic comparisons. Sequences identifiers given generally follow established terms. Unnamed sequences were given default names according to Arabidopsis nomenclature. Additionally, a family abbreviation was added to the beginning of each name.

| PhEOBII Homolog | NCBI Accession |
|---|---|
| AsterCeMYB24 | EL359998.1 |
| AsterCiMYB24 | EH701388.1 |
| AsterCmMYB24.1 | EH741019.1 |
| AsterCmMYB24.2 | EH749972.1 |
| AsterCmMYB24.3 | EH743134.1 |
| AsterCsMYB24 | EH789579.1 |
| AsterCtMYB24.1 | EL402561.1 |
| AsterCtMyb24.2 | EL400467.1 |
| AsterGhMYB24.1 | AJ751525.1 |
| AsterHaMYB24.1 | GE522594.1 |
| AsterHcMYB24 | EL414154.1 |
| AsterHpMYB24.1 | EL491532.1 |
| AsterHpMYB24.2 | EL490232.1 |
| AsterSchMYB24.1 | CO553429.2 |
| AsterSchMYB24.2 | DY659178.1 |
| AsterScMYB24.1 | DY661292.1 |
| AsterScMYB24.2 | DY662068.1 |
| BrasAtMYB21 | NM_113696.2 |
| BrasAtMYB24 | NM_123399.4 |
| BrasBnMYB24 | GR440457.1 |
| BrasBrMYB24 | GR724986.1 |
| BrasCpMYB24 | EX292075.1 |
| EricAdMYB24 | FG428833.1 |
| FabaGmMYB24.1 | EV266129.1 |
| FabaGmMYB24.3 | FG989549.1 |
| FabaPsMYB26 | Y11105.1 |
| LamiAmMYB305 | P81391.1 |
| LamiAmMYB340 | P81396.1 |

| | |
|---|---|
| LamiShMYB24 | FS446813.1 |
| MalpPtMYB24.1 | XM_002298498.1 |
| MalpPtMYB24.2 | XM_002323767.1 |
| MalvGhMYB24 | ES817664.1 |
| MalvGrMYB24 | CO113188.1 |
| RosaMxdMYB24 | GO506355.1 |
| RosaRcMYB24 | BI978095.1 |
| SapiCcMYB24 | CX290217.1 |
| SapiCsMYB24 | CV885038.1 |
| SapiCuMYB24 | DC899681.1 |
| SolaCaMYB24 | GD094622.1 |
| SolaNaMYB305 | EU111679.1 |
| SolaNlMYB305 | EU111679.1 |
| SolaNlxNsMYB24.3 | EB694062.1 |
| SolaNtMYB24.1 | EB427455.1 |
| SolaNtMYB24.3 | FG630317.1 |
| SolaPhEOBII | EU360893.1 |
| SolaSlMYB21 | AI486576 |
| SolaSmMYB24 | FS083560.1 |
| VitaVvMYB24 | EE107959.1 |

**SUPPORTING FIGURE LEGENDS**

Figure S1. Predicted amino acid sequence alignment of putative, homologous EOBII proteins from various species. Sequences were aligned using the AlignX program of the Vector NTI Advance™ 11 software. Residues highlighted in: blue represent consensus residues derived from a block of similar residues at a given position, green represent consensus residues derived from the occurrence of greater than 50 % of a single residue at a given position, and yellow represent consensus residues derived from a completely conserved residue at a given position. A red bar underlines the C-terminal trans-activation domain of interest. * denotes the PhEOBII sequence.

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**A jasmonate ZIM-domain protein NaJAZd regulates floral jasmonic acid levels and counteracts flower abscission in *Nicotiana attenuata* plants**

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**Abstract**

Jasmonic acid (JA) is an important regulator of plant growth, development and defense. The Jasmonate-ZIM domain (JAZ) proteins are key regulators in JA signaling ubiquitously present in flowering plants but their functional annotation remains largely incomplete. Recently, we identified 12 putative JAZ proteins in native tobacco, *Nicotiana attenuata* (*N. attenuata*), and initiated systematic functional characterization of these proteins by reverse genetic approaches. In this report, *N. attenuata* plants silenced in the expression of *NaJAZd* (irJAZd) by RNAi-mediated gene silencing were used to characterize NaJAZd function. Although *NaJAZd* transcripts were strongly and transiently up-regulated in the rosette leaves by simulated herbivory treatment, we did not observe strong defense-related phenotypes, such as altered herbivore performance or the constitutive accumulation of defense-related secondary metabolites in irJAZd plants compared to WT plants, both in the glasshouse and the native habitat of *N. attenuata* in the Great Basin Desert, Utah, USA. Interestingly, irJAZd plants produced fewer seed capsules than did WT plants as a result of increased flower abscission in later stages of flower development. The early- and mid-developmental stages of irJAZd flowers had reduced levels of JA and JA-Ile, while fully open flowers had normal levels, but these were impaired in *NaMYB305* transcript accumulations. Previously, *NaMYB305*-silenced plants were shown to have strong flower abscission phenotypes and contained lower *NEC1* transcript levels, phenotypes which are copied in irJAZd plants. We show that the NaJAZd protein is required to counteract flower abscission, possibly by regulating JA, JA-Ile levels and/or expression of *NaMYB305* gene in *N. attenuata* flowers. This novel insight into the

function of JAZ proteins in flower and seed development highlights the diversity of functions played by JA and JAZ proteins.

**Introduction**

Plants are frequently exposed to various abiotic and biotic stresses such as high light, water deficit, salinity stress, variable temperature, lack of nutrients, and attack from pathogens and herbivores. Survival of plants in nature thus strongly depends on a balance between growth and defense related processes, which is regulated by a complex phytohormonal network [1-6]. In this network, jasmonic acid (JA) controls both growth and defense responses in plants (reviewed in [7]). JA is synthesized from membrane-derived fatty acids (18:3) via the octadecanoid pathway [8] and is known to activate transcription factors (TFs) that trigger a large-scale transcription reprogramming of growth and development, such as root growth and adventitious root formation, trichome initiation, fruit ripening, anthocyanin accumulation, senescence, pollen and flower development, tuber formation and tendril coiling, and defense against wounding, herbivore attack and pathogen infection [9-13].

Recently, the mode of action and role of several core components in JA signaling, COI1 (CORNATINE INSENSITIVE1), JAZ (Jasmonate ZIM-domain), and (+)-*7-iso*-JA-L-Ile (JA-Ile) were identified [14-18]. In the presence of the active hormone, JA-Ile, JAZ proteins are degraded by the action of $SCF^{COI1}$-E3 ubiquitin ligase complex associated with 26S proteasome that releases the positive regulators of JA signaling, MYC2/3/4 transcription factors and triggers the expression of JA-dependent genes in *Arabidopsis* (reviewed in [19]). In addition, the function of several co-regulators of the core complex of JA signaling, such as NINJA (Novel Interactor of JAZ) and TPL (TOPLESS) proteins, $InsP_5$ (inositol pentakisphosphate), EIN3/EIL1 (ethylene-stabilized transcription factors), R2R3-MYB transcription factors MYB21 and MYB24, WD-repeat/bHLH (GL3, EGL3, TT8)/MYB75 complexes and DELLA proteins were elucidated [20-25].

JAZ proteins that are generally classified as negative regulators of JA signaling contain two functionally conserved domains, ZIM with TIF[F/Y]XG motif (or its variant) and Jas with S-L-X(2)-F-X(2)-K-R-X(2)-R motifs, both of which are essential for JA signal transduction [26-29]. ZIM domains mediate the homo- and heteromeric interactions among the JAZ proteins as well as their interaction with the co-repressor NINJA-TPL complex; the

Jas domain is required for binding several core- (COI1, MYC2/3/4) and co- (EIN3/EIL1, MYB21/24, TT8/GL3/EGL3 and DELLA) regulatory proteins that transduce downstream signaling (reviewed in [19]). It was proposed that different combinations and interactions between JAZ proteins and co-regulators can control specific subset of JA-mediated response in plants [30,31] however, specific examples of such interactions remain rare. Nevertheless, emerging JAZ interactome and the discovery of JAZ proteins in many plant species are continuously expanding our knowledge of jasmonate signaling (reviewed in [19]). Functional studies with genetically modified plants have provided evidence of the direct involvement of JA and JAZ proteins in developmental processes such as secondary growth (interfascicular cambium initiation) [32], phytochrome A-mediated shade responses [33], anthocyanin accumulation and trichome initiation [24], stamen development [23], flower induction [34], and defense responses against biotic [31,35-37] and abiotic [38-41] stresses. However, additional experiments are required to better understand the complex networking among JA, JAZ, and downstream responses in plants.

Previously, we cloned 12 novel *JAZ* genes from the native tobacco plant *N. attenuata* and reported unique roles for NaJAZh in defense and development [31]. Here, we examine the function of NaJAZd, both in development and defense against herbivores. The *NaJAZd*-silenced plants had normal levels of defense-related phytohormones and only slightly altered defense metabolic profiles in the leaves. In development, irJAZd plants had significantly impaired seed production which is one of the most important fitness parameters in *N. attenuat*a plants. We show that NaJAZd is involved in the regulation of flower abscission which in turn is associated with reduced jasmonate levels and impaired expression of genes (*NaMYB305*, *NaNEC1*) known to be important for flower development.

## Results

### *NaJAZd* transcript accumulation is strongly induced by wounding and herbivory

Previously, we reported 12 *JAZ* genes in *N. attenuata* [31], including the *NaJAZd* gene characterized in this study. First, we examined *NaJAZd* expression in the rosette leaves of *N. attenuata* plants after wound and water treatment (puncturing leaves with a fabric pattern wheel and supplying with 20 µL of water; W+W), simulated herbivore attack (wounds treated with 20 µL of 1:10 diluted oral secretions isolated from specialist

herbivore *Manduca sexta* (*M. sexta*) larvae; W+OS), and in untreated leaves by quantitative real-time PCR (qPCR). While both treatments strongly increased *NaJAZd* transcript accumulations compared to the levels in untreated leaves, W+OS-treatment dramatically amplified these increases (Figure 1A). The gene transcripts rapidly returned to basal levels within 3 h after treatment. To further explore the function of NaJAZd, we generated the inverted-repeat (ir) RNAi-mediated *NaJAZd*-silenced plants (irJAZd; Figure S1A and S1B) and selected the three best silenced lines (irJAZd-4, -8, and -10; Figure 1B) for functional analysis. A single copy T-DNA insertion status of each line was confirmed by Southern blot analysis (Figure S1C).

While measuring the silencing efficiency of *NaJAZd* by RNAi, we also examined the expression of other *NaJAZ* genes (*NaJAZa*, *c*, *e*, *f*, *h*, *i*, *j*, *k*, *l*, and *m*) in irJAZd plants to identify possible cross-silencing effects and/or crosstalk among *JAZ* genes. In contrast to our previous study with *NaJAZh*-silenced plants [31], we did not find any strong evidence of *NaJAZd*-mediated changes in expression of other *JAZ* genes in *N. attenuata* except for a slight but significant down-regulation of *NaJAZh* transcript accumulation in response to W+OS-elicitation (Figure S2). This suggests that *NaJAZd* may be required for a full up-regulation of *NaJAZh* expression after herbivore attack.

**_NaJAZd_-silencing weakly affects JA-dependent defenses**

To determine the role of NaJAZd in defense, we carried out performance assays with the specialist herbivore, *M. sexta,* with rosette stage WT and irJAZd plants. We placed a freshly hatched *M. sexta* neonate on the leaves of each 20 replicates of WT and irJAZd-4 and -8 plants and determined the mass of caterpillars after 4, 6, 8, 10, and 12 d of feeding on the plants (Figure 2A). *NaJAZd*-silencing did not affect the performance of *M. sexta* caterpillars as on both irJAZd genotypes and WT plants the larvae had similar growth rates.

To further test the hypothesis that NaJAZd is not a major player in defense against herbivores, we examined the levels of herbivore-induced phytohormones, JA-Ile (Figure 2B), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA; Figure S3) in rosette stage WT and irJAZd-4, -8, and -10 plants at 0, 1, and 2 h after W+OS treatment. The levels of JA-Ile (Figure 2B), and of other phytohormones (JA, SA and ABA; Figure S3) in irJAZd transgenic plants were similar to those in WT at all examined time points, confirming that *NaJAZd*-silencing alone does not significantly alter the leaf levels of

defense-related phytohormones, even though the transcript levels of *NaJAZd* were strongly elicited by wounding and herbivory in the leaves (Figure 1A).

To gain additional insight in potential targets of NaJAZd, we analyzed several defense-related secondary metabolites after W+OS treatment. Nicotine is a well-known defense-related secondary metabolite in *Nicotiana* species [42,43]. In contrast to the unaltered phytohormone levels, irJAZd leaves contained significantly more nicotine at 48 and 72 h after W+OS (Figure 2C), suggesting that NaJAZd may negatively contribute to biosynthesis of nicotine and/or suppress its transport from the roots. The accumulation of 17-hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs) [44,45] and trypsin protease inhibitors (TPIs) activity [46-48] in W+OS-treated irJAZd-4, -8, and -10 plants were not different from WT levels (Figure S4). Apparently, higher amounts of nicotine in irJAZd compared to WT plants alone were not sufficient to alter the performance of a specialist herbivore (Figure 2A).

**NaJAZd-silencing does not alter the preferences of native herbivores in nature**

In natural environments, plants are exposed to substantially more stresses compared to their relatively safe containment in the glasshouse. We therefore examined if *NaJAZd*-silenced plants could perform differently in high stress conditions characterized by high UV irradiance, high and variable temperatures, low humidity and communities of voracious native herbivores. In the 2010 field seasons, we planted empty vector-transformed (EV) and irJAZd plants in a pairwise design in the native habitat of *N. attenuata* (Great Basin Desert, Utah, USA) and compared herbivore damage to these plants (Figure 3). Field-grown irJAZd plants showed similar levels of damage from native herbivores, mirids (*Tupiocoris notatus*), flea beetles (*Epitrix* spp.), and noctuidae larvae (*Spodoptera* spp.) compared to WT plants, providing additional evidence that NaJAZd has only a minor role in defense against biotic and abiotic stresses. This prompted our intensive search for alternative functions of this protein.

**NaJAZd-deficiency causes increased flower abscission**

*NaJAZd*-silencing only slightly affected defense responses. Considering the extensively described role of JA in growth and development (reviewed in [9]), we decided to carefully examine the growth and development of irJAZd plants. The irJAZd plants

showed no obvious vegetative growth deficiencies: they had similar size of rosettes, leaf shape, and stalk length (data not shown). However, in contrast to vegetative growth, their reproductive fitness was significantly compromised. During the harvesting of seeds, we noticed that irJAZd plants produced significantly less seed mass compared to WT plants. When we carefully counted the number of mature seed capsules during entire reproduction of WT and irJAZd-4 and -8 plants, both irJAZd lines had about 20% fewer capsules compared to WT (Figure 4).

We hypothesized that NaJAZd was involved in flower initiation, which would ultimately affect the lifetime seed capsule production in irJAZd plants. However, the numbers of flower buds in irJAZd plants seemed comparable in WT and irJAZd plants and flower parameters such as degree of flower opening, pollen maturation or length of pistils were not visibly altered in irJAZd plants. In addition, we examined if self-pollination ability was impaired in irJAZd flowers by hand pollination experiments using ripe pollen from the same flowers by spreading pollen on stigma with fine brush (Figure S5). The hand pollination, assuring that each stigma received sufficient amount of pollen in a timely coordinated fashion, failed to recover the formation of seed capsules in irJAZd to WT levels. These results suggested that irJAZd flowers have normal anthesis but another problem in flower development. We therefore conducted another more detailed experiment in which we quantified flower production distinguishing 4 categories: buds, elongated flowers, fully opened flowers and abscised flowers, which were counted every 3 d starting 42 d after germination when the first buds and a few elongated flowers but no open flowers were present on the plants (Figure 5). To prevent mixing of abscised flowers from different plants, we placed each single plant in individual 30 x 52 cm plastic tray which captured all abscised flowers. While irJAZd plants had similar or even higher number of buds and elongated flowers, they produced significantly fewer open flowers on 48, 51, and 63 d-old plants and correspondingly higher numbers of abscised flowers at these and additional time points. Notably, the abscised flowers were all fully open flowers; abscission of younger stages or flower buds was not occurring. These data suggested that the function of NaJAZd is to prevent flower abscission in the later stages of flower development that directly affects lifetime production of seed capsules and fitness of *N. attenuata*. Whether this was mediated by direct function of NaJAZd in flowers was examined next.

**Phytohormones and gene expression in *N. attenuata* flowers**

To elucidate the molecular mechanisms involved in NaJAZd-regulated flower abscission, we analyzed the levels of phytohormones and flower-related gene expression at four different developmental stages of flowers in WT and irJAZd plants: buds (F1), early elongated flowers (~ 10 mm length, F2), fully elongated flowers (still green and completely closed corollas, F3) and open flowers (completely opened white corollas, F4). First, we determined the expression of *NaJAZd* in WT and irJAZd flowers to examine if (1) NaJAZd is expressed in stage-specific manner, and (2) to evaluate the efficiency of gene silencing in irJAZd flowers by RNAi. In WT plants, the gene showed comparably high transcript levels during F1-F3 stages but its expression declined in the F4 stage. *NaJAZd* transcript level was strongly reduced in irJAZd flowers compared to WT levels (Figure 6A).

Ethylene is known to be one of the important signals controlling flower abscission in plants (reviewed in [49]). The analysis of nearly 300 plant species showed that flower abscission in plants is highly sensitive to ethylene [50]. We therefore investigated the levels of ethylene and its possible role in enhanced flower abscission in irJAZd plants. Ethylene emissions increased in a stage dependent manner; however, we found no significant differences between WT and irJAZd flowers at all examined developmental stages (Figure 6B). These data suggest that enhanced flower abscission in irJAZd plants is independent of ethylene concentrations in irJAZd flowers.

Because JA is also known to regulate flower development in plants, we analyzed JA and JA-Ile levels using entire homogenized flowers. Interestingly, at three developmental stages (F1, F2, and F3) irJAZd flowers had significantly reduced levels of JA and JA-Ile compared to WT flowers (Figure 6B). It suggested that NaJAZd may be regulating flower abscission process via the regulation of JA and JA-Ile levels and/or JA-Ile-mediated signaling process. How a putative negative regulator NaJAZd contributes to the accumulation of JA remains to be elucidated.

Finally, we examined the expression of several flower development-related genes. The R2R3-MYB transcription factors are known to regulate stamen maturation, flower opening and nectar production (reviewed in [51]). Recently, the function of *MYB305* gene in controlling flower opening and floral nectar production in petunia, *N. tabacum* and *N. attenuata* was reported [52,53]. The *N. attenuata* plants strongly silenced in the expression of *MYB305* showed premature flower abscission in early flower developmental stages

because their flowers failed to enter anthesis and eventually, these plants could not produce any seed capsules due to a lack of self-fertilization. Although irJAZd flowers did not show anthesis-related phenotypes as described in the previous section, premature flower abscission phenotype strongly resembled those of irMYB305 plants but the abscission was shifted to later stages in flower development. To examine a possible relationship between NaMYB305 and NaJAZd, we analyzed *NaMYB305* expression at four different stages of WT and irJAZd flowers (Figure 6C). In both WT and irJAZd flowers, *NaMYB305* expression gradually increased from F1 to F3, corroborating previous studies [52]. However, the irJAZd flowers contained significantly fewer *NaMYB305* transcripts than did F4 stage WT flowers, suggesting that NaJAZd might be required for maintaining the appropriate levels of *NaMYB305* in open stage flowers. Because fully silenced irNaMYB305 plants lost all their flowers, it is likely that moderate reductions in *NaMYB305* levels observed in this study could be responsible for the abscission of a certain portion of flowers in irJAZd plants.

To further examine the NaMYB305 deficiency, we analyzed the expression of *NaNEC1* (nectarine 1) and *NaCHAL* (chalcone synthase) genes (Figure 6C) which are located downstream of MYB305 regulator in petunia and tobacco [52,54,55]. Consistent with *NaMYB305* expression, *NaNEC1* was similarly down-regulated in F4 stage flowers in irJAZd plants compared to WT flowers. However, *NaCHAL* expression was not significantly different between WT and irJAZd flowers (Figure 6C), showing an expression pattern which tracked flower ethylene emissions (Figure 6B).

**Global transcriptional changes associated with *NaJAZd*-silencing in *N. attenuata* leaves**

Because *NaJAZd* gene was strongly induced by W+OS treatment in the leaves, we conducted an additional microarray experiment focused on global leaf gene expression 2 h after W+OS-treatment. *NaJAZd*-silencing down-regulated a large numbers of genes (10321 of 43504 microarray probes) but it up-regulated only a relatively small number (38) of genes. The list of more than 3 times up- and down-regulated genes in irJAZd plant compared to WT plants was annotated and categorized according to established GO categories (Table S1 and S2). Interestingly, several primary metabolic genes, such as sugar

transporter SWEET3 (4.93-fold), unknown glycosyltransferase (4.17-fold), fructokinase (3.76-fold), putative beta-1, 3-glucan synthase (3.76-fold) and 6-phosphofructokinase 4 (3.7-fold) were strongly down-regulated in irJAZd leaves compared to WT leaves. These results suggest that, apart from direct changes in flowers caused by *NaJAZd*-silencing, the enhanced flower abscission phenotype in irJAZd plants could be due to a reduced nutrient availability in the flowers as leaves are providers of all essential nutrients required for successful flower development. Previous studies suggested that JA signaling may regulate sink-source relationship by regulating expression and accumulating vegetative storage proteins (VSPs) in soybean plants [56,57]. Further experiments are required to elucidate the emerging pleiotropic roles of NaJAZd in plant metabolism, development and defense.

## Discussion

### NaJAZd is a minor defense regulator in *N. attenuata*

Previously, a dominant negative truncated forms of NtJAZ1and NtJAZ3 proteins from *N. tabacum*, a close homologues of *N. attenuata* NaJAZd and NaJAZa, respectively, repressed the MeJA-induced nicotine and related alkaloid accumulations in cultivated tobacco cells [35]. However, truncation of JAZ proteins affects the overall JAZ-mediated signaling so the plants become completely “deaf” to JA signaling. In other words, experiments with truncated JAZs can only tell us that certain metabolites, such as nicotine, are indeed JAZ-regulated but cannot pinpoint the causative JAZ protein(s) involved. In contrast, targeted gene silencing is more useful but such analyses are frequently confounded by redundancy of gene function, and/or the lack of sophisticated, ecologically-realistic phenotypic screens. Despite predicted and/or observed redundancy in the function of JAZ proteins [16,17,58], we reported that NaJAZh alone is able to suppressed the accumulation of two herbivore-induced defense metabolites, HGL-DTGs and TPIs in *N. attenuata*. In addition, silencing of NaJAZh by RNAi strongly reduced the performance of *M. sexta* larvae on these plants [31]. In these follow up experiments, we therefore used gene silencing to examine the function of NaJAZd.

Overall, our data suggest that NaJAZd protein is another negative JAZ regulator involved in defense, particularly in nicotine accumulation. *NaJAZd*-silencing allowed higher accumulation of nicotine in simulated herbivory-treated plants at 48 and 72 h (Figure 2C). Regulation of nicotine levels by NaJAZd was specific to this alkaloid as other

defensive secondary metabolites such as HGL-DTGs or TPIs, previously shown to be controlled by NaJAZh [31], were not altered. However, the control of NaJAZd over nicotine levels was marginal as irJAZd plants did not contain constitutively increased nicotine levels as would be expected if NaJAZd was a master repressor of nicotine biosynthesis. Previously, silencing of NaJAZh, a master repressor of HGL-DTGs and TPIs caused significant increase in basal levels of these otherwise inducible metabolites in irJAZh plants [31]. Eventually, the changes in nicotine levels in *NaJAZd*-silenced plants were not sufficient to affect growth of a specialist herbivore *M. sexta* feeding on irJAZd plants in glasshouse (Figure 2A) and several natural herbivores of *N. attenuata* in native habitat of this plant (Figure 3).

Our initial data suggested that NaJAZd may not be a major player in defense. We therefore searched for alternative functions of this protein, finding an independent, fitness-related role of NaJAZd. The expression of *NaJAZd* was required for WT-level retention of flowers in the *N. attenuata* inflorescences, a finding not unsurprising as JA is known to control various aspects of flower development. Furthermore, functional specialization of JAZ proteins in both defense and growth has already been proposed by other authors [10,30,31].

**NaJAZd affects JA signaling in flowers and counteracts flower abscission**

The irJAZd plants were not different from WT in their vegetative growth; however, more irJAZd flowers abscised compared to WT, which significantly reduced the number of mature seed capsules (Figure 4 and 5). Ethylene is known to be a critical regulator of flower abscission (reviewed in [49]), but in follow up analyses, ethylene emissions were found not to be altered in irJAZd flowers compared to WT (Figure 6B). Plants blocked in ethylene perception (*etr1* mutants) show a typical flower corolla retention phenotype and corollas remain attached to even ripe capsules [59-61], demonstrating that perception of the post-pollination ethylene burst triggers corolla abscission after successful pollination [61-63]. However, in irJAZd plants, whole flowers are abscised after separation of pedicels from inflorescences, a distinctly different process from that of the ethylene-mediated corolla abscission.

In contrast to ethylene, the patterns of JA and JA-Ile accumulation are altered in irJAZd flowers compared to WT (Figure 6B), which re-connects NaJAZd to its expected

function as an endogenous regulator of JA signaling, albeit in flowers. It has been widely reported that JA affects flower development [23,64,65], but JA function has been typically associated with male sterility. For example, defects in pollen maturation and pistil elongation disabled efficient self-pollination in COI1 mutant plants [14,66,67]). Here, our data provide a novel insight into JA function in flower development. This phenotype is likely a combined effect of reduced JA and JA-Ile levels and/or impaired JA signaling due to silencing of NaJAZd repressor in the flowers. At present, no other JA-deficient *N. attenuata* plants, including irAOC (strongly silenced in expression of allene oxide cyclase) and irCOI1 (silenced in expression of coronatine insensitive 1) plants have been reported to show similar flower abscission phenotype. Whether the effect of NaJAZd is on the enzymes that degrade JA or promotes JA biosynthesis in the flowers by suppressing a putative negative regulator of biosynthetic genes, remains to be determined. From our data and the expression of the key flower regulator NaMYB305, we propose that the function of NaJAZd is to maintain optimal levels of JA throughout flower development, which in turn, provides sufficient expression and function of MYB305 transcriptional regulator. Previously, plants silenced in expression of *NaMYB305* gene were completely sterile due to a complete abscission of buds and early elongated flowers [52]. The silencing of *NaMYB305* in *N. attenuata* was partially counteracted by inhibiting ethylene perception with 1-MCP treatments, and it is therefore possible that the lack of NaJAZd and dysfunction of MYB305 may be caused by an exaggerated sensitivity to otherwise normal levels of ethylene in irJAZd flowers.

The homologues of NaMYB305 in petunia and *N. tabacum* regulate flower-specific flavonoid biosynthetic genes (phenylalanine ammonia-lyase; *PAL*, chalcone isomerase; *CHI*, and chalcone synthase; *CHS*) and two nectarines (nectarin1; *NEC1*, nectrain5; *NEC5*). Nectarines, in particular, are known to be involved in direct flower defense which may reconnect NaJAZd function back to defense. Previously, NEC1 has been shown to control the production of hydrogen peroxide ($H_2O_2$) in nectar together with NEC3 and NEC 5 proteins and high levels of antimicrobial $H_2O_2$ (up to 4 mM) are thought to protect the gynoecium and developing ovules from invading microorganisms [55,68,69]. Recently, MYB305 has been also shown to mediate additional functions in the maturation of the tobacco nectary by controlling the expression of several starch metabolic genes [53].

Although we found altered JA levels and direct changes in flower gene expression, it should not be forgotten that NaJAZd is strongly regulated by herbivory stress in *N. attenuata* leaves. The regulatory role of NaJAZd over several primary metabolic genes in leaves during simulated herbivory, as revealed by microarray analysis of the leaves, offers an alternative mode of action for NaJAZd via control and/or redistribution of nutrients, which then might indirectly affect flower and capsule development in *N. attenuata*.

**Conclusions**

Increased flower abscission in *NaJAZd*-silenced plants points to a novel function of JAZ proteins in plants. The absence of NaJAZd negatively affected the fitness of plants as the production of seed capsules (and seeds) in irJAZd plants were reduced by around 20 percent. Our data suggest that NaJAZd is required for a proper accumulation and/or maintenance of *NaMYB305* transcript levels in developing flowers, revealing a new function and requirement of *NaMYB305* in flower retention during later stages of flowering that can optimize fitness and seed production in plants.

**Materials and Methods**

**Plant material and growth conditions**

All experiments were conducted with 31$^{st}$ inbreed generation of *N. attenuata*. Seeds were germinated and grown in the glasshouse as previously described in Krügel *et al.* [70]. Plants were maintained under 16 h daylight supplemented by Philips Master Sun-T PIA Agro 400 W or 600 W sodium lights at 23-25 °C and 8 h dark at 19-23 °C, 45 to 55 % relative humidity.

To generate inverted repeat (ir) JAZd plants, we cloned a 303 bp fragment of *NaJAZd* gene (Figure S1A) as an inverted repeat into pSOL8 transformation vector [71] containing hygromycin (*hptII*) resistance gene as plant selection maker (Figure S1B). *Agrobacterium tumefaciens*-mediated plant transformation was conducted essentially as described in Krügel *et al.* [70]. The best *NaJAZd*-silenced, single T-DNA insertion transgenic lines (irJAZd-4, -8, and -10) were selected on hygromycin and subjected to Southern blot (Figure S1C) and quantitative real-time PCR (qPCR) analyses as described in Oh *et al.* [31].

Experiments were conducted with transition leaves (i.e., leaves undergoing the source-sink transition at node -1) using approximately 30-d-old rosette-stage *N. attenuata* plants. Four different developmental stages of flowers were collected from approximately 57-d-old flowering *N. attenuata* plants.

**Quantitative real-time PCR**

Total RNA was extracted from approximately 100 mg of frozen leaves or flower tissues ground in liquid nitrogen using Trizol reagent as recommended by manufacturer (Invitrogen). Total RNA was treated with RQ1 RNase-Free DNase (Promega), phenol extracted and precipitated by addition of 3M sodium acetate (pH 5.2) and 100% ethanol. First strand cDNA was synthesized from 1 μg of RNA using oligo-dT primer (Fermentas) and RevertAid™ H Minus reverse transcriptase (Fermentas) following manufacturer's protocol. Quantitative real-time PCR (qPCR) was conducted with the core reagent kit for SYBR Green I (Eurogentec) and gene-specific primer pairs (Table S3) using Mx3005P PCR cycler (Stratagene). Relative transcript abundances were calculated from dilution series of standard curves and normalized by *NtEF1α* gene (*N. tabacum* elongation factor 1*α*) expression.

**Herbivore performance in the glasshouse**

To determine herbivore performance, freshly hatched specialist herbivore *M. sexta* neonates were placed on selected rosette leaves of 20 each WT and 2 independent irJAZd line plants (irJAZd-4 and -8). The larval fresh mass was measured on 4$^{th}$, 6$^{th}$, 8$^{th}$, 10$^{th}$, 12$^{th}$ d after initial feeding.

**Phytohormone analyses**

To determine JA, JA-Ile, SA and ABA levels in WT and irJAZd plants, phytohormones were extracted from approximately 100 mg frozen leaves or flowers. Plant tissues were homogenized with 1 mL of internal standard (200 ng of [$^{2}H_2$]JA, and 40 ng each of JA-[$^{13}C_6$]Ile, [$^{2}H_4$]SA and [$^{2}H_6$]ABA)-spiked ethyl acetate and 2 steel balls in a Genogrider 2000 (SPEX Certi Prep) at 1000 strokes per minute for 10 min. The extracts were centrifuged at 16,100 *g* at 4 °C for 15 min, and the upper organic phases were transferred and dried in vacuum concentrator (Eppendorf) at 30 °C. The residues were

resuspended in 500 µL (for leaf) or 200 µL (for flowers) of 70 % (v/v) methanol: water and centrifuged. 10 µL of particle free supernatant was analyzed in Varian 1200 LC-ESI-MS/MS system (Varian) as described in Oh *et al.* [31].

Ethylene emissions were measured with a photoacoustic spectrometer (INVIVO; https://www.invivo-gmbh.de) as described in von Dahl *et al.* [72]. irJAZd and WT plants were grown in the glasshouse until flowering stage and 5 flowers were collected from each stage of flowers and used for ethylene measurements in 250 mL flasks. Flowers were incubated for 5 h to accumulate ethylene in the flasks and accumulated ethylene in the head space was flushed with a 130 to 150 mL/min flow of purified air into spectrometer and measured against known amount of ethylene standard. The results were normalized by fresh mass of flowers used in each measurement.

**Analysis of secondary metabolites by HPLC**

Plants materials (~100 mg) were homogenized with 1 mL of acetate buffer (60 % buffer A; 2.3 mL/L of acetic acid, 3.41 g/L ammonium acetate adjusted to pH 4.8 with 1 M $NH_4OH$, and 40 % (v ⁄v) methanol) and analyzed by HPLC (Agilent-HPLC 1100 series) coupled with PDA (Photo Diode Array, Agilent) and ELS (Evaporative Light Scattering, Varian) detectors as described in *Oh et al.* [31].

**Field bioassays**

The field experiments were performed in the native habitat of *N. attenuata*, the Lytle Ranch Preserve, Utah, Santa Clara, USA. The release of transgenic plants was carried under APHIS notification 06-242-3r-a3 and the seeds were imported to USA under permit number 07-341-101n. The seeds of EV and irJAZd-8 plants were germinated on the seeds on Gamborg's B5 medium as described in earlier section (Plant material and growth conditions). About 15 d-old seedlings were transferred to pre-hydrated 50 mm peat pellets (Jiffy 703, http://www.jiffypot.com) and seedlings were gradually adapted to the high light and low relative humidity of the habitat over a 2-week-period. Finally, pre-adapted rosette-stage plants were transplanted on the field plot and watered daily until roots have established, approximately 2 weeks, then plants were grown without watering. 15 pairs EV and irJAZd-8 plants were planted and grown in the field plot and monitored for damage from native herbivores. Damage of plants by natural herbivores was determined by

estimating the percentages of total leaf area of plants, which was damaged by each herbivore, Noctuidae larvae, *Spodoptera* spp.; flea beetles, *Epitrix* species; mirids, *Tupiocoris notatus*. A result of representative measurement conducted on 15$^{th}$, May, 2010 is shown in the Figure 3.

**Seed capsule and flower counts**

The number of seed capsules and four different developmental stages of flowers (buds, elongated-, open-, and abscised flowers) were counted in 3 d intervals from 42 d until 63 d after germination that covered complete reproductive stage of *N. attenuata* plants. Seed capsules were counted after complete maturation of plants. For counting abscised flowers, the plants were placed in separate plastic trays (30 X 52 cm) and kept apart to avoid mixing of abscised flowers. Every 3 d, buds, elongated- and open flowers on the plants, and newly abscised flowers on each tray were counted.

**Hand-pollination experiments**

Plants were kept in the glasshouse until flowering stage (approximately 55 d after germination) and all fully elongated but still green flowers of each WT, irJAZd-4 and irJAZd-8 plants were labeled previous evening and half of the flowers were hand-pollinated when the flowers opened next morning. Control half-set of the plants remained intact and was allowed to self-pollinate only. Hand-pollinations were repeated 4-times with ripe pollen from the same flowers by spreading pollen on stigma with fine brush and the percentage of mature capsules resulting from hand- and self-pollinated flowers were counted after 10 d period.

**Microarray experiment**

Untreated and W+OS-treated leaves of 30-d old WT and irJAZd-8 plants were used for microarrays. Total RNA was extracted as described in Kistner and Matamoros, [73] and cDNA preparation and hybridizations were performed as described in Kallenbach *et al.* [74]. Raw microarray data were normalized by 75 percentile and log 2 transformed and processed by SAM software version 3.11 (Significance Analysis of Microarrays; Stanford University, USA; [75]). For selection and annotation of genes, false discovery rates (FDR) $\leq$ 2.09 % and greater than 3-fold signal changes (irJAZd vs. WT) were used. The genes

were annotated after processing each entry by Blast-X program (E-value < 1-5e) and classified into groups based on GO classification from TAIR (http://www.arabidopsis.org).

**Statistical analyses**

Data were analyzed with StatView 5.0 software (SAS institute) using appropriate methods such as Student t-test for pair comparisons and ANOVA Fisher's PLSD for multiple samples.

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**Figure legends**

**Figure 1. Regulation of *NaJAZd* transcripts and silencing efficiency in irJAZd plants.** (A) *NaJAZd* transcript abundances ± SE were determined by quantitative real-time PCR (qPCR, *n=3*) in samples from WT *N. attenuata* leaves treated with wounding and water (W+W), wounding and *M. sexta* oral secretions (W+OS) harvested at 0, 1, 2, 3, and 24 h after elicitation (control leaves remained untreated). (B) Transcript abundances ± SE of *NaJAZd* determined in untreated (control) and 1 h W+OS-treated leaves of three independent inverted repeat (ir)-*NaJAZd*-silenced genotypes (irJAZd-4, -8, and -10) by qPCR (*n=3*). Signals in A and B were normalized by house-keeping *EF1* □transcript abundances determined by qPCR in the same samples. Different letters in B indicate significant differences among the combination of genotypes (WT vs. independent *NaJAZd* silenced lines, irJAZd-4, -8, 10) and treatments determined by one-way-ANOVA ($P \leq 0.05$).

**Figure 2**. **Defense responses against specialized herbivore *M. sexta* are mostly unaltered in irJAZd plants.** (A) Herbivory performance of *M. sexta* on rosette leaves of WT and two independent irJAZd lines (irJAZd-4 and -8) was determined by measuring larval mass at 4, 6, 8, 10 and 12 d after placement of a freshly hatched neonate on the plants. Mean fresh masses ± SE of irJAZd-4 and -8-fed caterpillars (*n=20*) were not significantly different from WT-fed caterpillars. (B) Mean ± SE levels of JA-Ile (*n=3*) determined by LC- ESI-MS/MS showed no significant differences in irJAZd compared to WT leaves. (C) Mean ± SE levels of nicotine (*n=3*) determined by HPLC coupled to PDA (Photo Diode Array) detector were significantly higher at 48 and 72 h after W+OS treatment of irJAZd plants compared to WT. Statistical differences in (A)-(C) were determined by one-way-ANOVA ($P \leq 0.05$). Different letters in C indicate significant differences among the different genotypes (WT vs. independent NaJAZd silenced lines, irJAZd-4, -8, 10) at the same time points. FM, fresh mass.

**Figure 3. Plant damage caused by herbivores in *N. attenuata*'s native habitat**. EV and irJAZd-8 plants were planted in a size-matched paired-design in their native habitat, Great Basin Desert, Utah, USA and natural herbivore damage was scored throughout the 2010 field season. Herbivore damage was determined as the % of leaf canopy damaged by (1)

cell-damaging feeding of *Tupiocoris notatus* mirid bugs (mirids), (2) the small feeding holes that characterize flea beetle feeding, and (3) leaf chewing Lepidopteran larvae (Noctuidae).

**Figure 4**. ***NaJAZd*-silencing negatively affects seed capsule production.** WT and two independent of irJAZd lines (irJAZd-4 and -8) were grown in the glasshouse and their capsules were counted at specified time points. irJAZd plants produced significantly fewer seed capsules at 51, 54, 57, 60 and 63 d after germination compared to WT plants; significant differences between genotypes were determined at each time point by one-way-ANOVA (**$P \leq 0.01$, ***$P \leq 0.001$). There were no significant differences in number of seed capsules between two independent irJAZd lines (irJAZd-4 and -8).

**Figure 5**. **NaJAZd counteracts flower abscission in *N. attenuata*.** Each individual plant was placed in an individual plastic tray (30 x 52 cm) in the glasshouse to avoid mixing of abscised flowers, and number of buds (A), elongated flowers (B), open flowers (C), and abscised flowers (D) in each plant from 42 d to 63 d after germination was determined in 3 d intervals. Both irJAZd-4 and -8 plants had similar number of buds and elongated flowers as WT plants but displayed significantly reduced numbers of open flowers (significant at 48, 51, 63 d) and higher numbers of abscised flowers (significant at most time points) compared to WT plants. Significant differences between genotypes were determined separately for each time point by one-way-ANOVA (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

**Figure 6. NaJAZd regulates phytohormone levels and flower development-related genes.** WT and irJAZd plants (irJAZd-4 and -8) were grown in glasshouse and 4 different developmental stages of flowers (F1, F2, F3, and F4) were collected 57 d after germination. (A) Transcript abundances of *NaJAZd* determined by qPCR in irJAZd flowers were significantly lower compared to WT. (B) Mean JA and JA-Ile levels ± SE determined by LC-ESI-MS/MS and mean ethylene levels ± SE measured by photoacoustic spectrometer using 5 flowers of each stage. (C) Transcripts abundances of flower development-related genes, *NaMYB305*, *NaNEC1*, and *NaCHAL* determined by qPCR: irJAZd-8 plants were impaired in expression of *NaMYB305* and *NaNEC1*genes in last stage of flower development (F4) while *NaCHAL* transcripts remain unaltered in irJAZd-8 compared to

WT flowers. Bars in (C) show *EF1α*-normalized relative transcript abundances ± SE (*n=4*). Statistical differences in phytohormones, JA, JA-Ile, (*n=4*) and transcript abundances were determined by Student t-test, and differences in ethylene was determined by one-way-ANOVA. Asterisks represent significant differences between WT and irJAZd in same stage of flowers (*P≤0.05, **P≤ 0.01, ***P≤ 0.001). FM, fresh mass.

**Supporting information**

**Figure S1. Generation of stable *NaJAZd*-silenced *N. attenuata* plants.** (A) A 303bp region in *NaJAZd* gene used for gene silencing is shown in red letters. (B) The pSOL8JAZd vector containing inverted repeat of *NaJAZd* gene used for *Agrobacterium tumefaciens*-mediated transformation and generation of stably silenced *N. attenuata* irJAZd plants. (C) Southern blot analysis of 6 independently transformed irJAZd (irJAZd-1, -2, -4, -8, and -10) lines and WT. The genomic DNA was digested with XbaI enzyme and hybridized with a $^{32}$P-labeled probe coding for the hygromycin resistance gene located between right and left T-DNA borders of the transformation vector pSOL8JAZd. The black boxes indicate single T-DNA insertion lines selected for further experiments: irJAZd-4, -8 and -10.

**Figure S2. Transcript abundances of other *NaJAZ* genes in irJAZd plants.** Transcript abundances of other *NaJAZ* genes were determined by qPCR in the leaves of irJAZd and WT plants before and 1h after W+OS elicitation; bars indicate *EF1α*-normalized relative transcript abundances ± SE (*n=3*) and different letters indicate significant differences among the combination of genotypes (WT vs. independent *NaJAZd*-silenced lines, irJAZd-4, -8, 10) and treatments determined by one-way-ANOVA (P ≤ 0.05).

**Figure S3. *NaJAZd*-silencing does not significantly alter basal or herbivory-induced phytohormones levels.** Rosette stage plants of WT and irJAZd (irJAZd-4, -8 and -10) were treated with W+OS and harvested before, 1 and 2 h after treatment. Mean ± SE levels of JA, ABA and SA (*n=3*) were determined by LC- ESI-MS/MS using internal deuterium-labeled phytohormone standards. Different letters indicate significant differences among the different genotypes (WT vs. independent *NaJAZd* silenced lines, irJAZd-4, -8, 10) at the same time points by ANOVA (P ≤ 0.05). FM, fresh mass.

**Figure S4.** ***NaJAZd*****-deficiency does not affect levels of defense-related secondary metabolites, HGL-DTGs and TPIs, in irJAZd plants.** Rosette stage WT and irJAZd (irJAZh-4, -8 and -10) plants were treated with W+OS and harvested before and 24, 48, and 72 h after treatment for determination of total HGL-DTGs levels and trypsin protease inhibitors (TPIs) activity. (A) Mean ± SE levels of total HGL-DTGs measured by HPLC coupled to ELS (Evaporative Light Scattering) detector (*n=3*). (B) Mean ± SE levels of TPIs determined by radial diffusion assay (*n=3*). Different letters in A and B indicate significant differences among the different genotypes (WT vs. independent *NaJAZd* silenced lines, irJAZd-4, -8, 10) at the same time point determined by one-way-ANOVA ($P \leq 0.05$). FM, fresh mass.

**Figure S5. Hand-pollination does not rescue seed capsule formation in irJAZd plants.** Plants were kept in the glasshouse until flowering stage (approximately 55 d after germination) and, in the previous evening, all fully elongated flowers ready to open next morning were labeled with color strings. In half of the plants, hand- pollination was conducted while second half remained exclusively self-pollinated. Approximately 10 days later, mature seed capsules resulting from labeled flowers in each group were counted and percentage of capsules originating from self- and hand-pollination groups of WT and irJAZd plants were determined (*n=24*). Different letters indicate significant differences among the different genotypes (WT vs. independent *NaJAZd*-silenced lines, irJAZd-4, -8) at the same condition determined by one-way-ANOVA ($P \leq 0.05$).

**Table S1.** Up-regulated genes in irJAZd plants compared to WT plants determined by microarrays

**Table S2.** Down-regulated genes in irJAZd plants compared to WT plants determined by microarrays

**Table S3.** Primer sequences used in quantitative real time PCR (qPCR)

**Figure 1.**

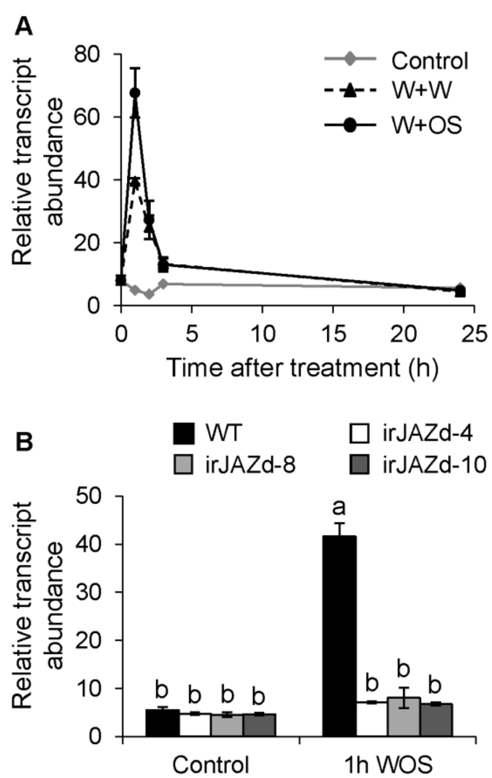


**Figure 2.**

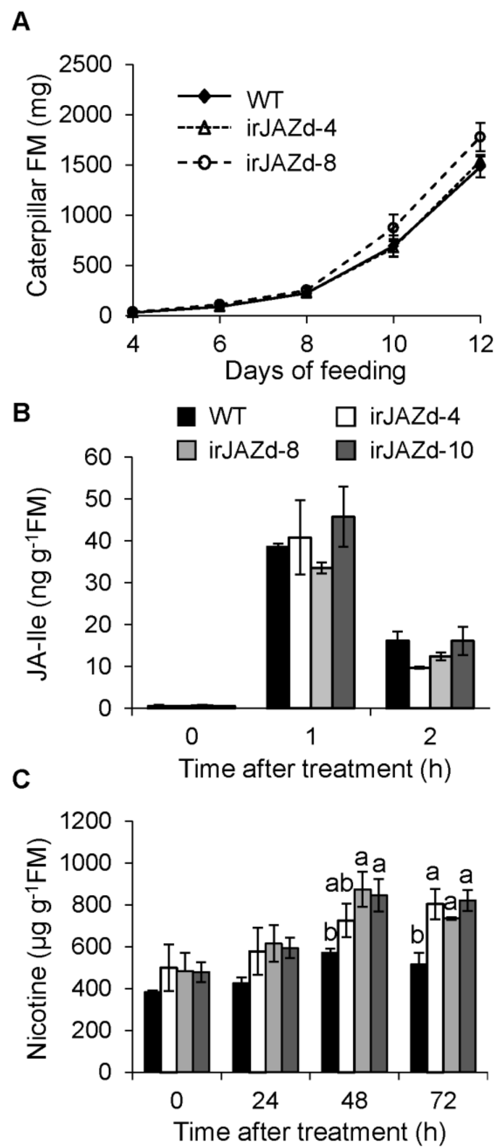


**Figure 3.**

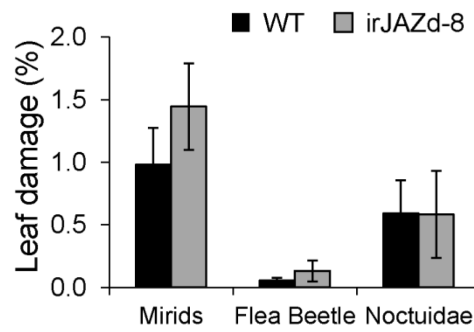
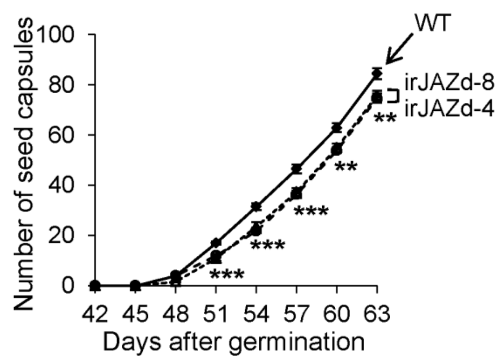


**Figure 4.**



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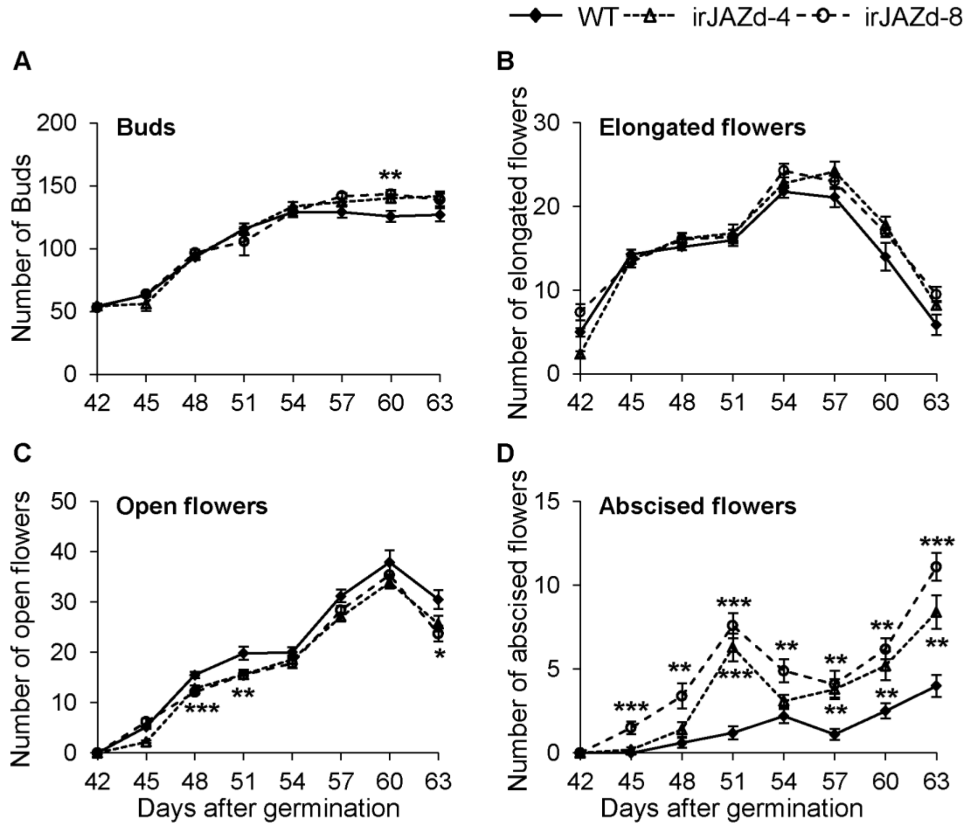

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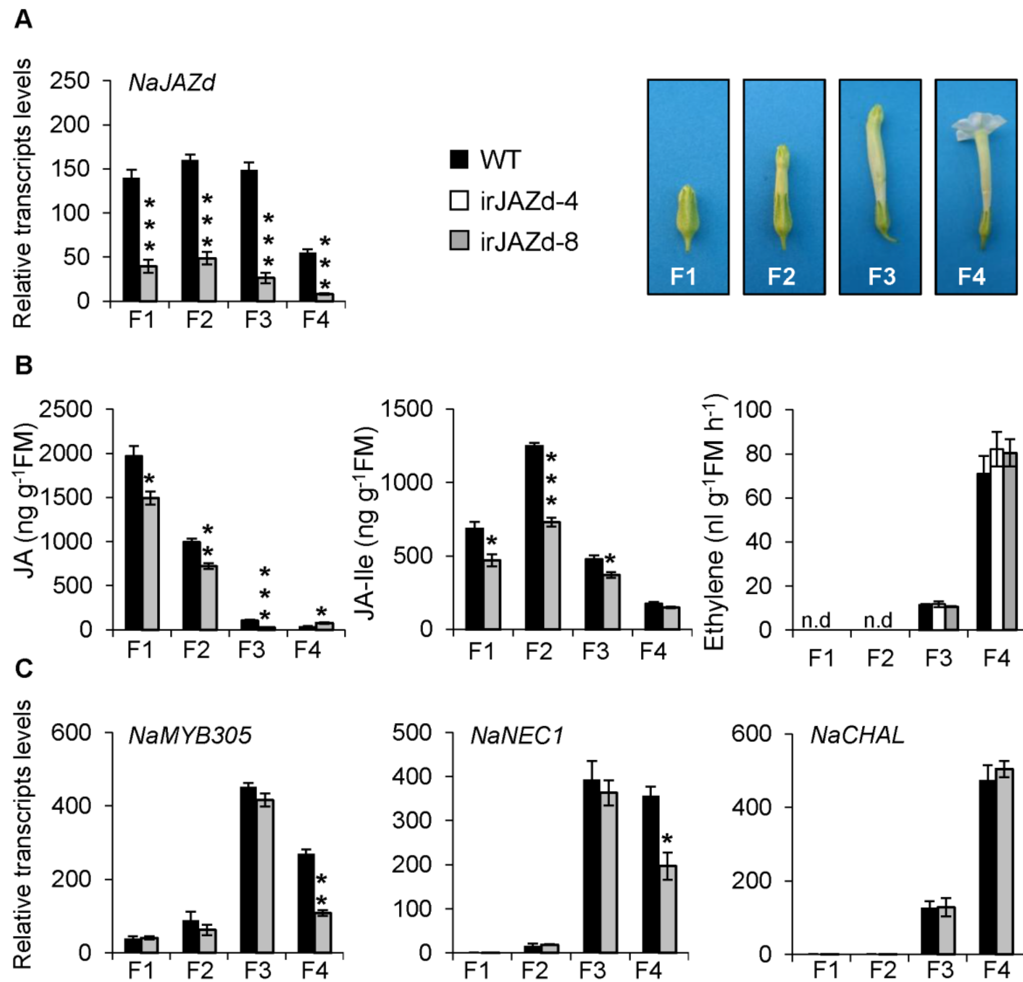

**Figure S1.**

**A**

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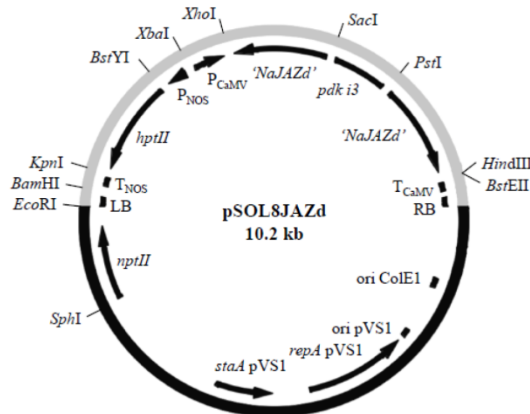
**B**




**C**

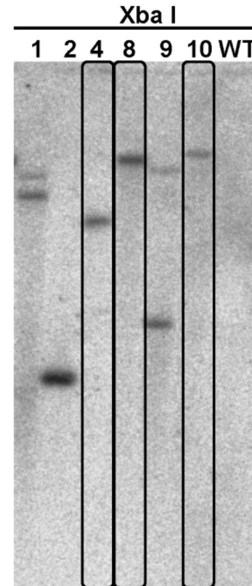


**Figure S2.**

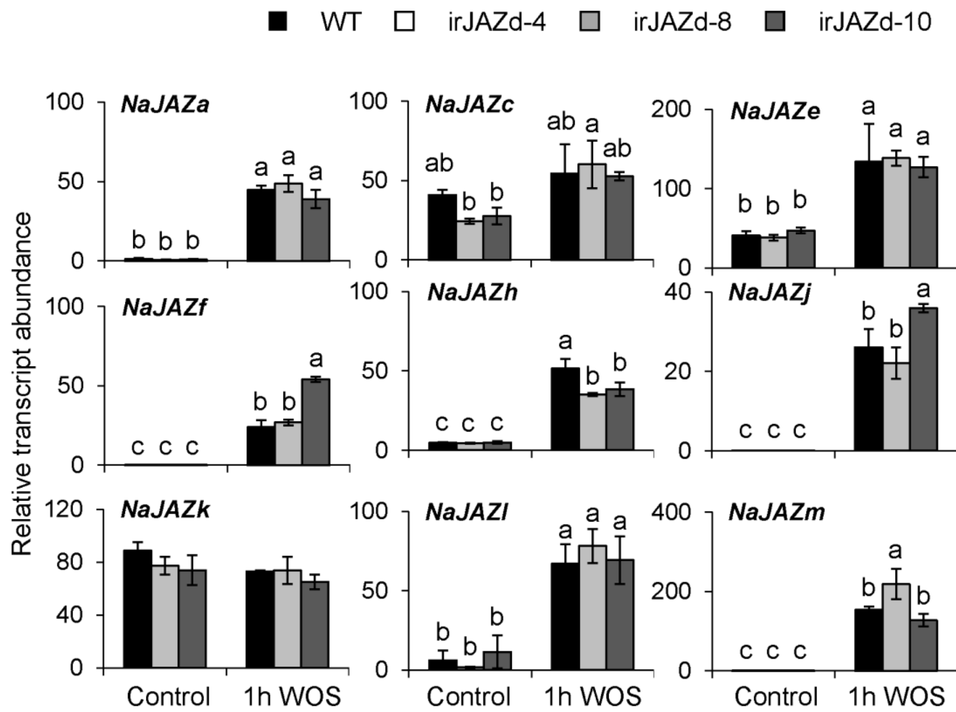
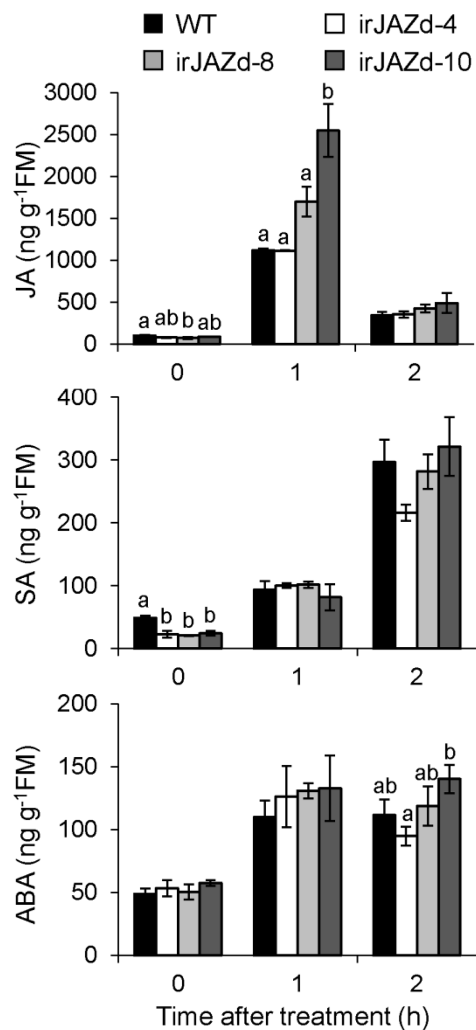


**Figure S3.**



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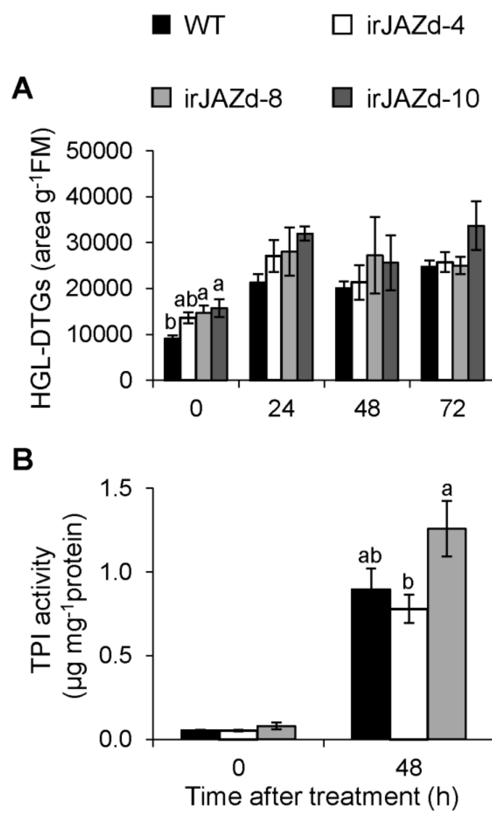

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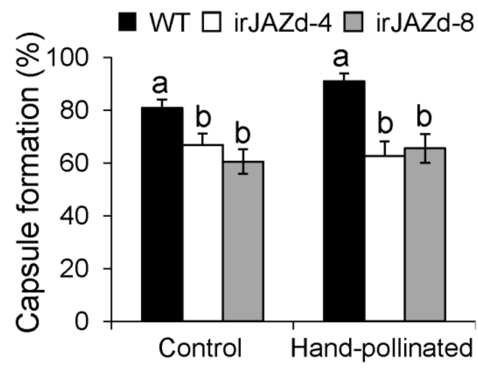

# Table S1.

**Table S1.** Up-regulated genes in irJAZd plants compared to WT plants determined by microarrays

| Classification | Fold Change | Bincode (TAIR) | Annotation | Gene ID | ACC number |
|---|---|---|---|---|---|
| **TCA / org. transformation.** | 4.30 | 8.3 | Carbonate dehydratase | Na_454_18630 | P27141 |
| **Miscellaneous enzyme families** | 4.39 | 26.12 | Peroxidase | Na_454_32590 | Q9XIV8 |
| **RNA.regulation of transcription** | 4.11 | 27.3.5 | ARR17 (ARABIDOPSIS RESPONSE REGULATOR 17) | Na_454_26424 | NP_567037 |
| | 3.49 | 27.3.99 | Leucine zipper factor-related | Na_454_15857 | NP_563798 |
| **DNA.synthesis/chromatin structure** | 3.72 | 28.1.3 | Histone H1 | Na_454_08010 | AAC41651 |
| **Protein.targeting.nucleus** | 3.02 | 29.3.1 | IMPORTIN ALPHA ISOFORM 2 | Na_454_15992 | ABM05488 |
| **Protein.postranslational modification** | 3.05 | 29.4 | Protein kinase | Na_454_34744 | CAA50374 |
| **Signalling.receptor kinases** | 3.50 | 30.2.26 | CCR1 (ARABIDOPSIS THALIANA CRINKLY4 RELATED 1) | Na_454_31246 | NP_187589 |

**Table S1.** (continued)

| Classification | Fold Change | Bincode (TAIR) | Annotation | Gene ID | ACC number |
|---|---|---|---|---|---|
| Not assigned | 26.28 | 35.1 | Carbon-nitrogen hydrolase family protein | Na_454_20897 | NP_196765 |
| | 4.85 | 35.1 | Carbon-nitrogen hydrolase family protein | Na_454_17587 | NP_196765 |
| | 3.00 | 35.1 | Glycosyl transferase family 1 protein | Na_454_37011 | NP_173105 |

* Microarray data is processed by SAM after 75% percentile normalization (FDR=2.09).
* Up-regulated genes were determined by a greater than 3-fold induction of normalized signals in their expression ratio (irJAZd / WT) of 2h-WOS treated leaves. The values are the average ratio of 3 biological replicates of the microarrays.
* All changes in gene expression were statistically significant by t-test ($P < 0.05$)
* Gene annotation is processed by Blast X (E-value < 1e-5)
* Classification of genes is based on GO classification from TAIR (http://www.arabidopsis.org).

# Table S2.

**Table S2.** Down-regulated genes in irJAZd plants compared to WT plants determined by microarrays

| Classification | Fold Change | Bincode (TAIR) | Annotation | Gene ID | ACC number |
|---|---|---|---|---|---|
| **Major CHO metabolism** | 3.86 | 2.2.1.1 | Fructokinase | Na_454_06965 | NP_001234206 |
| **Minor CHO metabolism** | 3.76 | 3.60 | Putative beta-1,3-glucan synthase | Na_454_25961 | AAK49452 |
| **Glycolysis** | 3.70 | 4.2.5 | 6-phosphofructokinase 4 | Na_454_38726 | NP_001032120 |
| **Cell wall** | 3.79 | 10.1.9 | UDP-glucose 4-epimerase | Na_454_18676 | AAP42567 |
| | 3.28 | 10.5.2 | Proline-rich protein | Na_454_40169 | AAF28387 |
| **Amino acid metabolism** | 5.02 | 13.1.3.1.1 | Asparagine synthetase 3 | Na_454_21256 | NP_001031864 |
| | 3.19 | 13.2.3.4 | S-adenosyl-L-homocysteine hydrolase | Na_454_21170 | AAV31754 |
| **Secondary metabolism** | 4.17 | 16.8.3 | Glycosyltransferase | Na_454_07441 | ADI33725 |
| | 2.99 | 16.4.1 | Iron ion binding / oxidoreductase/ oxidoreductase protein | Na_454_22715 | NP_190233 |
| **Hormone metabolism** | 3.07 | 17.7.1.2 | Lipoxygenase (NaLOX1b) | Na_454_37768 | AAP83135 |
| **Stress.abiotic** | 3.67 | 20.2.1 | DnaJ-like protein isoform | Na_454_21360 | ABI34703 |

**Table S2.** (continued)

| Classification | Fold Change | Bincode (TAIR) | Annotation | Gene ID | ACC number |
|---|---|---|---|---|---|
| **Biodegradation of Xenobiotics** | 3.37 | 24.10 | Glyoxalase II 3 | Na_454_20653 | NP_564636 |
| **Miscellaneous enzyme families** | 3.18 | 26.10 | Cytochrome P450, family 78, subfamily A, polypeptide 10 | Na_454_31815 | NP_177551 |
| **RNA.regulation of transcription** | 8.13 | 27.3.22 | Class 2 knotted1-like protein | Na_454_34641 | BAF95776 |
| **RNA.RNA binding** | 3.64 | 27.40 | RNA recognition motif-containing protein | Na_454_41880 | NP_973888 |
| **Protein.synthesis** | 3.31 | 29.2.3 | Eukaryotic translation initiation factor SUI1-like protein | Na_454_11378 | NP_177291 |
| **Protein.postranslational modification** | 3.55 | 29.40 | Protein kinase family protein-like | Na_454_17022 | BAD61275 |
| | 3.48 | 29.40 | O-methyltransferase | Na_454_25230 | AAA34088 |
| | 9.47 | 29.4.1 | Putative serine/threonine protein kinase | Na_454_15886 | AAC69450 |
| | 3.28 | 29.4.1 | MAP kinase, putative | Na_454_21759 | AAM20643 |
| **Protein.degradation** | 3.39 | 29.5.11.4.2 | RING/U-box domain-containing protein | Na_454_30344 | NP_175132 |
| | 3.20 | 29.5.11.4.2 | E3 ubiquitin-protein ligase ATL41 | Na_454_21141 | NP_181765 |
| | 3.71 | 29.5.5 | Serine carboxypeptidase-like 16 | Na_454_04811 | NP_566414 |
| **Signalling** | 3.95 | 30.1.1 | PAR-1c (photoassimilate-responsive 1c) | Na_454_25707 | CAA58732 |
| | 3.30 | 30.11 | Phytochrome F | Na_454_38145 | AAC49301 |
| | 2.96 | 30.2.99 | Leucine-rich repeat-containing protein | Na_454_03038 | NP_188563 |
| | 4.25 | 30.30 | Calcium-binding EF-hand domain-containing protein | Na_454_06154 | NP_564623 |

**Table S2.** (continued)

| Classification | Fold Change | Bincode (TAIR) | Annotation | Gene ID | ACC number |
|---|---|---|---|---|---|
| **Development** | 5.36 | 33.99 | Nodulin MtN3 family protein | Na_454_41228 | NP_200131 |
| | 3.44 | 33.99 | NAC domain protein NAC2 | Na_454_11638 | ABK96797 |
| | 3.06 | 33.30 | Squamosa promoter binding-like protein | Na_454_11778 | ABH07904 |
| **Transporter.sugars** | 4.93 | 34.20 | Bidirectional sugar transporter SWEET3 | Na_454_16634 | XP_002267886 |
| **Not assigned** | 11.02 | 35.20 | Protein LURP-one-related 5 | Na_454_11287 | NP_178129 |
| | 9.35 | 35.20 | Jasmonate ZIM-domain protein 1 | Na_454_02978 | BAG68655 |
| | 6.10 | 35.20 | Putative non-LTR retrolelement reverse transcriptase | Na_454_31619 | AAD26953 |
| | 4.65 | 35.20 | UDP-glycosyltransferase family protein | Na_454_43115 | NP_001190226 |
| | 3.09 | 35.20 | Ninja-family protein AFP2 | Na_454_29306 | NP_563933 |
| | 3.72 | 35.10 | Rho GTPase activation protein (RhoGAP) with PH domain | Na_454_36722 | NP_196776 |
| | 3.64 | 35.1.5 | Pentatricopeptide repeat-containing protein | Na_454_25757 | XP_002273247 |

* Microarray data is processed by SAM after 75% percentile normalization (FDR=2.09).
* Down-regulated genes were determined by a greater than 3-fold repression of normalized signals in their expression ratio (irJAZd / WT) of 2h-WOS treated leaves. The values are the average ratio of 3 biological replicates of the microarrays.
* All changes in gene expression were statistically significant by t-test ($P < 0.05$)
* Gene annotation is processed by Blast X (E-value < 1e-5)
* Classification of genes is based on GO classification from TAIR (http://www.arabidopsis.org).

**Table S3.**

**Table S3.** Primer sequences used in quantitative real time PCR (qPCR)

| Gene name | Primer sequences |
|---|---|
| NaJAZa FP | CAGTGAAAGCTGAGCAATTCTAGTACTC |
| NaJAZa RP | AGCCTTAGACGAATTGAATACCTACAC |
| NaJAZc FP | GGAAAGGGTGATGAACGCTGCA |
| NaJAZc RP | TATGGCAATAGGCTGCCTTCAGAC |
| NaJAZd FP | GCCTTGGCGTAACTGATGAAGTTG |
| NaJAZd RP | GGCAGCCCAGTGCACTAAGC |
| NaJAZe FP | CAATTTGGTCAAGGAGACGTGA |
| NaJAZe RP | GGCTCTGATCACAATTACAAGG |
| NaJAZf FP | CAAGTAGAGAAATGGAGGAG |
| NaJAZf RP | GCTAGTGATGATATGGAGAAG |
| NaJAZh FP | TTCTGCTACGCCGCAAGTACTG |
| NaJAZh RP | GGTATGGCGCTCTAGCCGTTG |
| NaJAZj FP | CATCATCACCAATTTCAGAGCCTTC |
| NaJAZj RP | TCCAATTTTCCAATTTCTCCCAGCA |
| NaJAZk FP | TCTATGGTGATGTGCCTGCTGAC |
| NaJAZk RP | AACGGATATCCAAGCTAGCTGTTG |
| NaJAZl FP | TTGCCAGAAGGAAATCCCTGAAGAG |
| NaJAZl RP | TCCATCAAAAGCTAGCCCTACTTAGC |
| NaJAZm FP | AGTGCGTCAAATTTGAGAGCACCA |
| NaJAZm RP | GCTGCTTGAATCCTCCTTTCTCTTC |
| NaMYB305 FP | ATGCTAAGTGGGGAAACAG |
| NaMYB305 RP | GCAATTGCATGGACCAGA |
| NaNEC1 FP | TGCTGTTTTTGCCGCTCCTT |
| NaNEC1 RP | ACCACATCGTGGCACAGAGAGT |
| NaCHAL FP | TTCACGTTTCAAGGCCCAA |
| NaCHALRP | TGCTCCATCAGCGAAAAGG |
| NtEF1a FP | CCACACTTCCCACATTGCTGTCA |
| NtEF1a RP | CGCATGTCCCTCACAGCAAAAC |

# Discussion

Despite the long history of plant-herbivore interactions (about 450 million years), the first peer-reviewed scientific articles reporting that plants defense metabolites are toxic to herbivores and affect herbivore performance and herbivore selection of host plants according to their metabolites were published in early 1960s (Barnett, 1959; Farkas and Kiraaly, 1962; Pringle and Scheffer, 1964). The observation of such interactions in the field led to the hypothesis of the co-evolution of plants and herbivores, first proposed by Ehrlich and Raven (Ehrlich and Raven, 1964). Our knowledge of plant-herbivore interactions has greatly increased since those first papers, now including the effects of complex abiotic and biotic factors on various plants and herbivore species (Whittaker and Feeny, 1971; McKey, 1974; Feeny, 1977; Berenbaum, 1983; Coley, 1988; Thompson, 1999; Cunningham et al., 2001; Leimu et al., 2012). The accumulated observations of changes in morphology and chemical composition of plants following stress or damage from herbivores led to the proposal of optimal defense theory. Defense responses help plants to survive and propagate in nature, but production of defense metabolites is costly (production, storage, translocation, breakdown of functional compounds), while plants have only limited resources to use. Plants allocate their resources to both chemical and mechanical (structural) defense, depend on the optimized cost-benefit ratio for maximization of fitness, considering the risk of attack (how often, what kind), how valuable plant parts are which are being attacked, and what is the cost of defense production (McKey, 1974; Coley et al., 1985; Karban and Myers, 1989; Zangerl and Rutledge, 1996; Ohnmeiss and Baldwin, 2000). Because plant tissues (e.g. young and old leaves, roots, flowers, etc.) have different value depending on ontogeny, plants tend to allocate more resources to more valuable tissues according to optimal defense. In other words, plants use different strategies of defense to protect differently valuable tissues: (1) constitutive levels of defensive secondary metabolites are typically much higher in reproductive, younger and developing tissues than old tissues, (2) young tissues are more responsive to stress (herbivory) and show greater and faster increases of induced metabolites compared to older tissues and (3) reproductive organs such as flowers and developing seeds have the strongest defense responses, with much higher constitutive levels of secondary metabolites compared than other organs. Reproductive organs are obviously the most valuable tissues for annual plants because they

bear the genetic information which will be passed on to the next generation, and attract pollinators and seed dispersers to do so. The values of specific plant tissues can also change over ontogeny: different developmental stages of plants respond differently to the same type and strength of stress. Previous studies showed that younger stages of plants respond more to stress compared to flowering stages, and the reduced responsiveness can be recovered by removing flowers (Diezel et al., 2011). Optimal defense theory states that tissue value and resource allocation are positively correlated in plants: it allows us to understand herbivore behavior on the plants, their feeding patterns, and preferences for particular stages of plants and tissue types (reviewed in (Meldau et al., 2012)).

***Jasmonate signaling cascade in plants***

Jasmonates are well known phytohormones that regulate defense against abiotic and biotic stress, as well as growth and development of plants, as I described in my general introduction. In response to jasmonates, plants accumulate a large variety of metabolites that reflect the extreme chemical diversity of terrestrial plants. However, in contrast to the well-characterized jasmonate biosynthesis pathway, many regulatory mechanisms of jasmonate signaling still remain unclear. Although the jasmonate co-receptor, $SCF^{COI1}$ complex; crucial negative regulators, JAZ proteins; and active hormone, JA-Ile, have been discovered; precise functions of the individual JAZ proteins as repressors of jasmonate signaling rarely have been reported (reviewed in (Kazan and Manners, 2012)). In this dissertation, I identified a novel JAZ family (12 JAZ proteins) in the native tobacco *Nicotiana attenuata* (*N. attenuata*), and examined the function of two individual JAZ proteins, JAZh and JAZd, in both plant development and defense responses. In addition, I identified a potential regulatory partner of NaJAZd, the NaMYB305 protein that is known as a master regulator of flower development in plants.

***Identification of a novel JAZ family in* Nicotiana attenuata**

In 2007, 12 novel jasmonate-inducible ZIM domain-containing proteins, Jasmonate ZIM domain (JAZ) proteins, were identified by microarray analysis of methyl jasmonate (MeJA)-treated plants (7 JAZs) and sequence homology search (5 additional JAZs) in *Arabidopsis* (Chini et al., 2007; Thines et al., 2007). Following this report, JAZ proteins were identified in many other plant species including *N. attenuata*. **In chapter 3**, we

identified 12 putative JAZ proteins in *N. attenuata* that complemented three already known JAZ1, JAZ2 and JAZ3 proteins from *N. tabacum* (Shoji et al., 2008). Similar to the JAZ family discovered in *Arabidopsis*, all NaJAZ proteins contain two conserved domains, ZIM and Jas, and are highly responsive to simulated herbivory. Interestingly, *NaJAZ* genes showed spatially and temporally different transcript accumulation patterns in local and systemic tissues induced by different stimuli, such as mechanical wounding, simulated herbivory, and even in untreated tissues. These data are consistent with previous studies which showed that wounding and herbivore feeding induced local and systemic *JAZ* gene expression in *Arabidopsis* (Chung et al., 2008; Koo et al., 2009). In contrast to previous studies focused on expression and systemic signaling in the leaves, I examined, apart from the leaves, the expression and spread of systemic signals into roots. These data suggest that a subset of JAZ proteins may actually play an important role in roots when herbivores attack the leaves. Nicotine is a well-known defensive secondary metabolite in *Nicotiana* spp. which is synthesized in roots after herbivore attack, then transported to locally damaged leaves (Baldwin, 1989; Baldwin et al., 1997; Shoji et al., 2000; Steppuhn et al., 2004). Accumulation of specific *NaJAZ* transcripts in the roots further supports the hypothesis that JAZ proteins may have specific functions in herbivory-induced nicotine biosynthesis and/or transport to the shoot in *Nicotiana attenuata*.

NaJAZ proteins can be categorized into different sub-groups according to their structural features as described in **chapter 3**. It is already known that JAZ proteins have quite diverse sequences except for the two highly conserved motifs, TIFY and Jas. Previous studies showed that some of the *JAZ* genes may have alternative spliceoforms which could play important roles in JAZ-SCF$^{COI1}$ complex interactions and jasmonate signaling in *Arabidopsis*: two spliceoforms of the AtJAZ10 protein showed differential binding affinities to the SCF$^{COI1}$ complex (Thines et al., 2007; Chung and Howe, 2009; Chung et al., 2010). I also found two cases of alternative splicing in *N. attenuata*: *NaJAZc* and *NaJAZk* showed different spliceoforms (cDNA): while NaJAZc proteins differed in the presence of an internal 32 amino acid sequence (NaJAZc.1 and NaJAZc.2), NaJAZk occurred in two forms, one that completely lacked the Jas motif (NaJAZk.2) and another which contained an incomplete Jas sequence (NaJAZk.1). Further analysis is required to understand the exact functions of these different modifications in regulating JAZ-mediated jasmonate signaling.

Recently, Sheard et al. (2010) showed that JAZ proteins have a typical degradation sequence (degron; LPIARR) in their Jas motif that is required for interaction with the COI1protein (Sheard et al., 2010) and is therefore conserved in most AtJAZ proteins (Chung et al., 2009). However, several AtJAZ proteins (AtJAZ 4, 5, 6, 7 and 8) have an $X_3$SMK sequence in the corresponding region of the degron sequence in Jas, and these JAZ proteins are therefore unable to directly interact with $SCF^{COI1}$ complex (Pauwels and Goossens, 2011; Shyu et al., 2012). *N. attenuata* JAZ proteins show a similar pattern: while NaJAZa, -d, -h and -l contain typical degron sequence, NaJAZf and -j contain the $X_3$SMK sequence in the corresponding region of degron (**Text 1**, unpublished data). Additional experimental evidence is required to demonstrate whether variation in the degron sequence in NaJAZ proteins also plays an important role in interaction of NaJAZ-$SCF^{COI1}$ complex. The expectation would be that degron sequence-containing NaJAZ proteins are able to interact with NaCOI1, while this should not occur in $X_3$SMK sequence-containing NaJAZ proteins. Several questions arise from this assumption, such as the presence of alternative COI proteins capable of interacting with non-degron-containing JAZ proteins, or the intrinsic role and targets of non-degron JAZ proteins in jasmonate signaling.

The data in both *Arabidopsis* and *N. attenuata* suggest that a subgroup of JAZ proteins require one or more co-factors to interact with $SCF^{COI1}$ complex, or interact with other adaptor protein(s), such as NINJA, to regulate distinct JAZ-mediated jasmonate signaling. However, another subgroup of AtJAZ proteins contains LxLxL-type EAR (ERF-associated amphiphilic repression) or DNLxxP-type EAR-like motifs in the N-terminus region of the protein (Shyu et al., 2012). While most JAZ proteins recruit a NINJA-TPL complex as a co-repressor of jasmonate signaling (Pauwels et al., 2010), these AtJAZ (AtJAZ5, -6, -7 and-8 ) can directly recruit TPL protein and repress downstream jasmonate-responsive transcriptional events without the NINJA adaptor protein and its EAR motif. Shyu et al. (2012) demonstrated that the EAR-motif is required for the AtJAZ8-mediated response of jasmonate-dependent root growth inhibition, but not for regulation of reproductive processes such as pollen development. In my work, I found that NaJAZf, -j, -m and -l also contain their own LxLxL-type EAR motif, while NaJAZb contained a DLNxxP-type EAR-like motif in its N-terminus (**Text 1**, unpublished data). This suggests that these NaJAZ proteins are likely to recruit TPL protein and repress target gene transcription without assistance from NINJA, but further experiments are required to

confirm this hypothesis.

Alternative splicing, sequence variation, and distinct domain architectures in NaJAZ proteins support the general theory proposed in several studies that different subgroups of JAZ proteins have different regulatory functions with different co-regulator(s) and target transcription factors, in order to fine-tune numerous jasmonate-controlled responses in growth, development, and defense.

**Text 1.** The degradation sequence (LPIARR) and EAR (LxLxL- or DNLxxP-type) motif in *N. attenuata* JAZ proteins

```
                          DLNxxP-type EAR motif

NaJAZe       MG---LTHHVKQEVIEEH-IDPAPLR-SSAMQWSFSNNISTHPQYLSFKGAQEDRLKTGF
NaJAZg       MERDFMGLAVKQEIPEEQPTDPAMARISAILQRSFSN--KALPQYLSFKNAQGNTPKTGF
NaJAZc       -----MGLNSKDSVVLVK-EEPVETCKDSGFRWPLSS-KVGIPHFMSLNSAQDEKP----
NaJAZf       ------------------------------------------------------------
NaJAZj       ------------------------------------------------------------
NaJAZa       ------------MASS-EIVDSGKFSAGGGQKSHFSQTCNLLSQYLKEKKGSFGDLS---
NaJAZd       ------------MGLS-EIVDSGKVT---GQKSQFSQTCNLLSQFLK-KKGSFGDLNN--
NaJAZh       ------------MSNSQNSFDGGRRAGKAPERSNFVQTCNLLSQFIK-GKATIRDLN---
NaJAZm       ------------------------------------------------------------
NaJAZl       ------------------------MYCSSKVANNFLKIEKFNKNFDYQKQINESNLKG—
NaJAZb       MDSSIIEIDFMDLNSRPQSEMAKQQTKASGMKWPFSSMANLATQAESRFFQNYNSSP---
NaJAZk       -------MPPEESVSKSLLDKPLQQLTEDDISQLTREDCRRYLKLKGMRRPSWNKSQAIQ


NaJAZe       DSLASTGLVTITTT-EAVDSSHRPYSGVTQNNMMLEKQGGTHYTSTTFSPHHYDAHSVHR
NaJAZg       DSLASAGLVTITTSHEAVDSNYRPYTAVTQKNLMLEKQGITNYTMTTYPPHKIGTNSVQQ
NaJAZc       --------FKAQSAADGVDSCLKRQSGEIQN-----------------------VHAMHL
NaJAZf       -------------------------------------------------------MRRNC
NaJAZj       --------------------------------------------------------RKNC
NaJAZa       --------LGIHR----AG------TTTMDLFPMIEK-----------------SGESN-
NaJAZd       --------LGIYRSFEPTGNQ--TTTTTMNLLPMIEK-----------------SGDSAE
NaJAZh       --------LGIAGKSEISGKSDVTEAATMDLLTIMEN-----------------PSIETK
NaJAZm       ---------------------------------------------------------MKH
NaJAZl       --------IGNNG-----------------------------------------SHRRMS
NaJAZb       -----------------------------------------------------IVSSNSK
NaJAZk       QVISLKALLETTPDSDAGTRKKLYIPRSDTKLHHVQRGK--------------NTDEEFI


             LxLxL-type EAR motif

NaJAZe       SHGVRVLPVSNLANQISVSMT-MPGHKSFVSPLGQN-------------PVASPISAVPT
NaJAZg       SHEVRVLPVANQTHQISVSTRNMHGRQPLISPAGQNLISIINQNPARGAQISSSISILPN
NaJAZc       LHDVMMLPFNRSNPSYKTHFG-GTGQISAAATMKQQLLG--------GIPVTAPHSILPS
NaJAZf       NLELRLVPPCVSVSPKDCTTT---------------------------------------
NaJAZj       NLELRLMPPSFSFSPKSCTT----------------------------------------
NaJAZa       --PQKPMNLFPQ---TEAKS----------------------------------------
NaJAZd       KKSQKPMNLFPQEVISTTKS----------------------------------------
NaJAZh       EQEQKSIDPVRQSAVTESSRD---------------------------------------
NaJAZm       RIGLQLFPLLSSSSSSEFHSS---------------------------------------
NaJAZl       ALELAILPGIKHDTQLPVLQR---------------------------------------
NaJAZb       SSPLKSYKLTTKDSRKNSDHL---------------------------------------
NaJAZk       ELAEETVPDGRTLQDKADLSG---------------------------------DATPNL
```

**Text 1. (continued)**

```
NaJAZe          NSAVVGTTDLRGAPKTPPGPAQLTIFYGGSVCVYDNVSPEKAQAIMLLAGNAPPV-TPSA
NaJAZg          RNGVVGTTELRGAPKTSAGPAQLTIFYAGSVSVYDNISPEKAQAIMLLAGNAQPAGIPST
NaJAZc          SGSVAGITEPWFNSKGSAAPAQLTIFYGGTVNVFDDISPEKAQAIMFLAGNGCVP--PNV
NaJAZf          --PYFSMR--DNQGTEEKQQQQLTIFYNGKVVVSD-ATELQAKAIIYLAS----------
NaJAZj          --PYFSMNREDKENTEEKEPQQLTIFYNGKLVVSD-ATELQAKAIIYLAS----------
NaJAZa          ----------------ESEKAQMTIFYGGQVIVFNDFPADKAKEIMLMASC---------
NaJAZd          ----------------EPEKAQMTIFYGGQVIVFDDFPAAKANEIMKLASK---------
NaJAZh          MEVAVNEPSTSKEAPKEPKAAQLTMFYDGKVIVFDDFPADKARAVMLLASKGCPQSSFGT
NaJAZm          ----------DHSVACSSTRKHITIFYNAQAGECD-ISEVQALAILWHAR----------
NaJAZl          ---------------EKSESEQLTIFYAGIVHVYDNLPVEKAQSIMDFARE---------
NaJAZb          ----------------RKPESTLTIFYMGEVHIFQDITPEKAELIMDLASKSTN------
NaJAZk          VAAIDKSAPSRTIGSVDTSAGQMTIFYSGKVNVYDDVPADKAQTIMRVASSS--------

NaJAZe          TSTLSPVQAPIPKSSSVDSFVVNQSHNTT--PTLPSPISITSHCGSQSAGVSSNT-----
NaJAZg          TSTASPVQR-IPKSSSVDAFVGNKCHRTTS-PSFSSPIPITTHGASQSIGVSNNT-----
NaJAZc          VQPRFQVQASTPKLAAVDGTCVNQTSNILPASGHSSPMSVSSHPIGQSAGNSGNK-----
NaJAZf          -------------------------RETEENTKTSSPISESSS-----------------
NaJAZj          -------------------------REMEKKIKIRSPISESSSPISEPSS----------
NaJAZa          ---------------------AKGNNN-----------STTQIQ----------------
NaJAZd          ---------------------KNNNNNKQNLATNIFSYPMVNNQ----------------
NaJAZh          FHTTTIDKINTSATAAATASLTCNKTNQLKPSTVSIAPPQQKQQQIHVSYSKSDQLKPGY
NaJAZm          ------------------------------------QVKYNN------------------
NaJAZl          ------------------SSLFSGSTNVKFPPKEAEPNQKSQVP----------------
NaJAZb          ---------------LHMTEILEKANKEKYEENKSEPSTPNASTN---------------
NaJAZk          ---------------------LCVPSETPLNATVAAQHSTCCLQVAN-------------
                                                                     -------
                                                                      xxxSMK
                                                                   or LPIARR
NaJAZe          -------------NGVTI-IKSTGVLP-SPSNKAGLSKFSSSIGSVPATFVPSAVPQARK
NaJAZg          -------------NQITMSIRSIGVLTNSPSNKTEPSKVVRSQESHPPSHTLSAVPQARK
NaJAZc          -------------DDMKI-SKTANISVETPKIMTSLGPVGAS------TIMSAAVPQARK
NaJAZf          -----------------------------------------------PLLQT-QTGLSMK
NaJAZj          -----------------------------------------------PLLQSPASDISMK
NaJAZa          --------------KTAESALDLVP-----------QPIIS-----------GDLPIARR
NaJAZd          --------------NSAESVTTNLT-----------QELRSRTHVPISQSSVADLPIARR
NaJAZh          NSATPQVLQQQLVHVSSTSKTDQLKPVSTSSASQKQQEQHQQTQSQTPGTSSSELPIARR
NaJAZm          -------------------------------------------------------GLSLR
NaJAZl          ----------------------------------------------FACKFQAELPIARR
NaJAZb          -------------------------------------------------YAKGALAMARR
NaJAZk          ---------------------------------TKLRPDSDMVLLPTIQTEAVENPSSRK

                      Jas motif
                ---------------------
NaJAZe          ASLARFLEKRKER-VISASPYDTSKQSPECSTLGYGSRSFAKYSLGSCPPQVINLVKET
NaJAZg          ASLARFLEKRKER-ILSASPYDNSKQSSQYSTPGSSSWSFFVNSSGSSTVLPATI----
NaJAZc          ASLARFLEKRKER-VMNAAPYGLSKKSGECSTPESNGVGFSATSSVGTSPLIAGKET--
NaJAZf          KSLQRFLQKRKNR-IQETSPYHH------------------------------------
NaJAZj          RSLQRFLQKRKNR-IQATSPYHR------------------------------------
NaJAZa          ASLTRFLEKRKDR-LIAKAPYQLSNTNKQAAVSE-NKAWLGLGAQF-PVKAEQF-----
NaJAZd          NSLTRFLEKRKDR-ITSTAPYQICNKNAASAKNEENKAWLGLGGKFVPVKTEQFF----
NaJAZh          SSLHRFLEKRKDR-ATARAPYQVVHNNPLPSSSNNNGESSSKDCEDQLDLNFKL-----
NaJAZm          KSLQRFLQKRRERRIQAANPY--------------------------------------
NaJAZl          KSLKRFFEKRHNR-IISKHPYASPVITQHEDECNDQSGNYSLKEKNS------------
NaJAZb          ATLARFLEKRKHR-LITARPYQYGEKTPKFPFEMHQEEETASSSVHWES----------
NaJAZk          ASVQRYLEKRKDR----------------------------------------------
```

***Crosstalk in JAZ proteins family***

I used targeted gene silencing by inverted repeat (ir)-mediated RNA interference (RNAi) to study the function of individual JAZ proteins in *N. attenuata*. Targeted gene silencing is very useful in studies of individual gene function, but it also carries a considerable risk compared to insertion mutagenesis of non-specific cross-silencing for genes that belong to large gene families with high sequence homology. This is especially true in plant species with unknown or incomplete genome sequences. To be sure that the phenotype of both *NaJAZh*- and *NaJAZd*-silenced plants described in **chapter 3** and **chapter 5** was only caused by single gene silencing, I measured the accumulation of all 12 *NaJAZ* gene transcripts in WT vs. *NaJAZh*- and *NaJAZd*-silenced plants. Neither *NaJAZh*- nor *NaJAZd*-silenced plants showed cross-silencing of the other non-target *JAZ* genes. However, I observed an unexpected up-regulation of several *JAZ* genes in *NaJAZh*-silenced plants: NaJAZb, -j, and -f transcripts were significantly higher after simulated herbivory in *NaJAZh*-silenced plants compared to WT plants (**Chapter 3**), which suggested that *NaJAZh* silencing can directly or indirectly affect the expression of other *JAZ* genes. This data were further supported by the nicotine phenotype of *JAZh*-silenced plants. Unexpectedly, *NaJAZh* silencing repressed nicotine accumulation in the leaves after simulated herbivory, in sharp contrast to the resulting increase in other defensive secondary metabolites, HGL-DTGs and TPI activity (**Chapter 3**). *NaJAZh*-silencing-mediated increases in *NaJAZ* expression may affect nicotine biosynthesis or transport to leaves if these *JAZ* genes control the accumulation of this metabolite.  Recently, *JAZ* expression (*AtJAZ1*, *AtJAZ2* and *AtJAZ9*) in roots was also reported in *Arabidopsis* after local wounding (Hasegawa et al., 2011; Sogabe et al., 2011), supporting the hypothesis that a subset of JAZ proteins may have specific functions in the roots together with recently reported root-expressed MYC2-like genes and/or AP2/ERF transcription factors reported as positive regulators of nicotine biosynthesis in tobacco (Shoji et al., 2010; Shoji and Hashimoto, 2011; Zhang et al., 2012). Based on my results, an active cross-talk among JAZ proteins may exist and play important role in JAZ-mediated jasmonate-dependent plant responses. In addition, although very weak relative to *NaJAZh* silencing by RNAi, I observed a slight but significant down-regulation of *NaJAZh* transcripts after simulated herbivory in *NaJAZd*-silenced plants (**Chapter 5**), suggesting that *NaJAZd* may be required for a full up-regulation of *NaJAZh* expression after herbivore attack, and presenting another example of JAZ crosstalk.

Although further studies are required to demonstrate the precise function of crosstalk among the JAZ proteins in JAZ-mediated jasmonate signaling, we provided the first insight into this phenomenon.

***Functions of JAZ proteins in plant defense against herbivory***

Plants accumulate a huge variety of defense metabolites to defend themselves against herbivory such as glucosinolates (amino acid derivatives) in *Arabidopsis* (Rask et al., 2000; Mewis et al., 2006; Shroff et al., 2008), nicotinic alkaloids and 17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) in tobacco (Baldwin et al., 1997; Shoji et al., 2000; Steppuhn et al., 2004; Heiling et al., 2010), and protease inhibitors (PIs) in most plants (Koiwa et al., 1997; Zavala et al., 2004; Habib and Fazil, 2007; Hartl et al., 2010). These defense metabolites can reduce damage from herbivores by acting as toxins or anti-digestive compounds. Since the discovery of JAZ proteins as negative regulators of jasmonate signaling, much effort has been focusing on functional studies of these proteins in plants. Shoji et al. (2008) showed that a dominant negative form of NtJAZ1 and NtJAZ3 proteins repressed MeJA-induced nicotine accumulation and related alkaloids in tobacco hairy roots and cell cultures. Demianski et al. (2012) showed that JAZ10-silenced *Arabidopsis* is more susceptible to *Pseudomonas syringae* DC3000 infection, suggesting that AtJAZ10 is a negative regulator of disease symptom development during *Pseudomonas syringae* DC3000 infection. Although jasmonates are known to be important hormones in defense against herbivory (Browse, 2009), and JAZ proteins are central switches in jasmonate signaling, the defense-related phenotypes associated with silencing of individual JAZ have been rarely reported. The lack of phenotypes was mainly considered to be functional redundancy of JAZ proteins in plants.

Nevertheless, in **chapter 3** and **chapter 5**, I was able to demonstrate the individual function of two *N. attenuata* JAZ proteins, NaJAZh and NaJAZd. I used inverted-repeat (ir) RNAi-mediated *NaJAZd*- or *NaJAZh*-silenced plants to conduct functional studies of these genes in jasmonate signaling and defense.

*Direct (HGL-DTGs and TPIs) and indirect (VOCs) defenses against herbivory*

*NaJAZh* silencing strongly reduced the performance of the specialist herbivore *M. sexta* on *NaJAZh*-silenced plants compared with WT plants in the glasshouse. Correlated to

herbivore performance, *NaJAZh* silencing strongly upregulated both constitutive and inducible levels of secondary metabolites, HGL-DTGs and TPIs that act as direct defense (see general introduction) as well as volatile organic compounds (VOCs such as GLVs, sesquiterpenes) emissions serving as indirect defense (see general introduction) in *N. attenuata*. In contrast to other defense metabolites described above, nicotine was significantly reduced in *NaJAZh*-silenced plants compared to WT plants. This data suggested that nicotine is most likely regulated by a distinct set of regulators (possibly NaJAZf), while production of HGL-DTGs, TPIs and volatile organic compounds in *N. attenuata* is connected to a communal regulatory defense node. It is also supported by crosstalk among individual JAZ proteins described in the earlier section.

To examine the ecological relevance of NaJAZh function, I planted EV (control) and *NaJAZh*-silenced plants in a paired design in the native habitat of *N. attenuata*, the Great Basin Desert, Utah, USA. However, in two field seasons, I did not observe any differences in damage from native herbivores between WT and *NaJAZh*-silenced plants, leading to two hypothesises: (1) the Utah ecotype of *N. attenuata* used in this study may already have the most efficient defense system, so additional defensive metabolites are not advantageous to these plants, and (2) because nicotine is one of the most effective metabolites against native herbivores in natural habitat of *N. attenuata*, lower nicotine levels in *NaJAZh*-silenced plants counteracted the advantage gained from high levels of TPIs and HGL-DTGs. Further experiments are required to understand the equally good performance of EV and *NaJAZh*-silenced plants in nature. **In chapter 3**, I demonstrated that NaJAZh is an important negative master regulator of a specific subset of JAZ-mediated jasmonate-dependent defense responses, including both constitutive and inducible direct (TPIs, HGL-DTGs) and indirect (VOCs) defense in *N. attenuata*. It remains to be determined if this is a general case in plants or specific evolutionary trait acquired by *N. attenuata* during its long and exceptionally intense interactions with herbivores in nature.

*Nicotine biosynthesis and/or transport*

In a follow-up study (**Chapter 5**), I used another transgenic ir-line silenced in the expression of *NaJAZd* by RNAi to examine the function of NaJAZd in *N. attenuata*. *NaJAZd*-silenced plants displayed greater accumulation of nicotine after simulated herbivory, but other defense metabolites, HGL-DTGs and TPIs that are known as typical

defense related secondary metabolites in *Nicotiana* spp. remained unchanged. In addition, increased nicotine levels in *NaJAZd*-silenced plants were not sufficient to affect the performance of the specialist herbivore *M. sexta* in glasshouse as well as several native herbivores of *N. attenuata* monitored in the native habitat of *N. attenuata*, the Great Basin Desert, Utah, USA. Overall, the data suggest that NaJAZd is only a minor negative regulator involved in defense against herbivory, particularly in nicotine biosynthesis and/or transport to the leaves.

Despite predicted or observed redundancy in the function of JAZ proteins in *Arabidopsis* (Chini et al., 2007; Thines et al., 2007; Chung and Howe, 2009), I was able to show that silencing of a single *JAZ* gene can affect various jasmonate-dependent defense responses, suggesting a branched regulation of jasmonate-mediated defense metabolites in tobacco and their control by separate JAZ proteins (**Chapter 3 and Chapter 5**).

***Functions of JAZ proteins in plant development***

*NaJAZ*-silenced *N. attenuata* plants showed not only defense-related phenotypes but also non-defense related phenotypes such as alteration of developmental processes. This finding is not surprising as jasmonates are already known to regulate various aspects of plant development apart from defense responses (reviewed in (Wasternack, 2007)). To date, the lack of JAZ proteins in plants resulted in desensitization of jasmonate-induced root inhibition, defects in pollen development, anthocyanin accumulation, and trichome initiation in *Arabidopsis* (reviewed in (Browse and Wager, 2012)).

*Control of ROS accumulation in leaves*

In *N. attenuata*, *NaJAZh* silencing did not cause a strong growth phenotype until the transition stage between vegetative growth and flowering, when the plants displayed visible necrotic lesions on the leaves. These symptoms remained restricted to the leaves and did not affect flowers and seed capsules (**Chapter 3**). The necrotic lesion symptoms first spontaneously appeared as small spots on cotyledonary leaves, spreading to other leaves in ontogenic order. Necrotic lesions resembled programed cell death (PCD) that occurs in response to pathogen infection, wounding, ozone and UV exposure, cold and high light stress (Pennell and Lamb, 1997; Rao et al., 2000a; Rao et al., 2000b; Beers and McDowell, 2001; Pasqualini et al., 2003; Hatsugai et al., 2004; Van den Burg et al., 2008). Apart from

stress responses, PCD also occurs in plant growth and development during pollen development, senescence and vascular tissue differentiation (Wang et al., 1996; Calderon-Urrea and Dellaporta, 1999; Buckner et al., 2000; Wu and Cheung, 2000; Lee and Chen, 2002). However, the *NaJAZh*-silencing-induced necrotic lesion symptoms not related to stress because they occurred spontaneously in *NaJAZh*-silenced plants. PCD is also closely associated with accumulation of ROS (reactive oxygen species; e.g. hydrogen peroxide, superoxide anion, hydroxyl radicals, singlet oxygen and nitric oxide) that leads to oxidative damage and/or apoptotic death of plant cells (reviewed in (Quan et al., 2008)). **In chapter 3**, *NaJAZh*-silenced plants showed higher accumulation of hydrogen peroxide ($H_2O_2$) as well as transcripts of PCD marker genes (*Hin1*, *Hsr203*, and *NaVPE361*). From these data I formulated two hypothesises: (1) the first hypothesis is that NaJAZh works as a suppressor of ROS in *N. attenuata*. Previously, it has been shown that MeJA treatment induces ROS production in plants (Orozco-Cárdenas et al., 2001; Hung and Kao, 2007; Reinbothe et al., 2009), and it was shown that *Atrboh* (respiratory burst oxidase homolog) D and F genes were required for COI1-dependent $H_2O_2$ production in *Arabidopsis* leaves treated with MeJA (Maruta et al., 2011). In particular, $H_2O_2$ accumulation was essential for the induction of jasmonate-dependent genes such as *VSP1*, *ANAC019* and *ANAC055*, suggesting that jasmonate-controlled ROS is playing an active role as secondary messenger in various physiological and defense-related processes in plants. (2) NaJAZh plays a role in control of senescence process occurring in mature tobacco plants, possibly by controlling ROS levels in the oldest leaves. A number of previous studies suggested that jasmonate signaling is involved in senescence in plants (Weidhase et al., 1987; Parthier, 1990; He et al., 2002; Shan et al., 2011). However, the role of jasmonates in senescence process remains unclear because (1) jasmonate-induced senescence generally differs from natural age-related senescence process and (2) no jasmonate signaling and biosynthetic mutant plants showed obvious senescence-related phenotypes (Schommer et al., 2008; Seltmann et al., 2010). Although further research is required to establish the role of jasmonates in ROS accumulation in plants, my work addressed a novel function of JAZ proteins: NaJAZh plays an important role in accumulation of $H_2O_2$ and transcripts of PCD marker genes in *N. attenuata*.

*Flower abscission and seed capsule production*

By connecting two originally independent parts of my project, I demonstrated that JAZd counteracts flower abscission in *N. attenuata* plants via control of a master regulator of flower development, NaMYB305 (**Chapter 5**). Similar to *NaJAZh*-silenced plants, *NaJAZd*-silenced plants did not show any strong growth phenotypes until flowering and seed production when they showed significantly fewer seed capsules (reduced by ~20% compared to WT level) caused by increased premature flower abscission in later flower developmental stages. I examined the function of NaJAZd in four different developmental stages of flowers using WT and *NaJAZd*-silenced plants. Interestingly, *NaJAZd*-silenced flowers showed no difference in ethylene emissions compared to WT flowers, although ethylene is well known to regulate flower abscission (reviewed in (Klee and Clark, 2010)). In addition, *NaJAZd*-silencing-mediated flower abscission occurred in pedicels of inflorescences that is different from ethylene-mediated corolla abscission and separation of sepals in plants (Patterson and Bleecker, 2004; Cho et al., 2008). My data therefore suggested that NaJAZd-mediated flower abscission is independent of ethylene concentrations.

In contrast to ethylene, *NaJAZd*-silencing significantly repressed JA and JA-Ile in early- and middle developmental stages of flowers which re-linked NaJAZd to its expected function in jasmonate signaling. Altered JA and/or JA-Ile levels, or jasmonate signaling, caused by *NaJAZd* silencing likely affects flower abscission rates in late flower developmental stages in *N. attenuata*. However, other jasmonate-deficient *N. attenuata* plants such as irAOS (silenced in the expression of allene oxide synthase) and irCOI1 (silenced in the expression of coronatine insensitive 1) plants do not show similar flower abscission phenotypes. Therefore, the question of how NaJAZd-regulated jasmonate signaling actually controls flower abscission remains to be determined. Nevertheless, the phenotype caused by *NaJAZd* silencing and described in **chapter 5** provides another novel insight into jasmonates' function in plants and flower development. Until now, known jasmonate-mediated regulation of flower development was limited to male sterility caused by defects in pollen development, such as short pistil length (Feys et al., 1994; McConn and Browse, 1996; Xie, 1998; Mandaokar et al., 2006; Paschold et al., 2008; Song et al., 2011). Interestingly, the premature flower abscission phenotype in *NaJAZd*-silenced plants closely resembled *NaMYB305*-silenced plants that I described in **chapter 4**. MYB transcription

factors are key components in regulatory networks controlling development, metabolism and responses to abiotic and biotic stresses in all eukaryotes (reviewed in (Dubos et al., 2010)). R2-R3-type MYB transcription factors regulate several flower developmental processes including flower opening, floral nectar production, and nectary maturation ((Liu et al., 2009; Liu and Thornburg, 2012) and **chapter 4**). The plants silenced in expression of the *NaMYB305* gene showed premature flower abscission in the early flower developmental stage: they fail to enter anthesis and later did not produce any seed capsules or seeds required for propagation. In the case of *NaMYB305*-silenced plants, the flower abscission phenotype was partially recovered by treatment with the ethylene inhibitor, 1-MCP, which produced some open flowers but at a very low success rate. To study the relationship between NaJAZd and NaMYB305 in flower abscission, I examined transcript abundances of *NaMYB305* in four different developmental stages of WT and *NaJAZd*-silenced flowers (**Chapter 5**). Both WT and *NaJAZd*-silenced flowers showed gradually increasing levels of *NaMYB305* expression through the early- and middle developmental stages, but the last stage (open flowers) of *NaJAZd*-silenced flowers showed significantly lower transcript accumulation compared to WT flowers, suggesting that NaMYB305 may play a role in regulation of flower abscission. I proposed that NaJAZd is required to maintain optimal levels of JA and/or JA-Ile throughout flower development, which in turn provides for the sufficient expression and function of the MYB305 transcriptional regulator.

In addition, I examined global transcriptional changes in WT and *NaJAZd*-silenced plants after simulated herbivory (**Chapter 5**). Remarkably, *NaJAZd* silencing repressed several primary metabolic genes (e.g. sugar transporter, unknown glycosyltransferase, fructokinase, putative beta-1, 3-glucan synthase, 6-phosphofructokinase 4 and several other genes) in the leaves. It proposed an alternative hypothesis for *NaJAZd*-silencing-mediated phenotype in flowering plants: NaJAZd-mediated control and redistribution of nutrients that indirectly affects flower abscission and seed capsule development in *N. attenuata*. Although potentially interesting, this hypothesis remains to be tested.

***Conclusions and future prospects in research of JAZ proteins***

Many complex plant behaviors such as plant growth, development, and defense are regulated by phytohormonal networks. JAZ proteins play important roles not only in jasmonate signaling but also in hormone crosstalk. In this dissertation, I provided experimental evidence of individual functions of *N. attenuata* JAZ proteins, NaJAZh (**chapter 3**) and NaJAZd (**chapter 5**) together with NaMYB305 (**chapter4**). My data strongly support the hypothesis that JAZ proteins are functionally specialized in JAZ-mediated jasmonate signaling in plants. Furthermore, I provided novel insights into JAZ-mediated jasmonate signaling in plant developmental processes such as ROS accumulation, PCD and flower abscission. It is already known that functions of jasmonate-mediated defense responses are attenuated in the flowering stage of plants (Diezel et al., 2011), suggesting a possible transition of jasmonate function from defense to reproduction according to optimal defense theory. Each subgroup of JAZ proteins is likely to be involved in different parts of jasmonate signaling in different developmental stages of plants to fine-tune growth, development, and defense responses, leading to better survival in nature.

Our knowledge of mechanistic understanding in JAZ-mediated jasmonate signaling is constantly expanding as many new signaling components and target transcription factors are constantly reported; however, large unknown areas still remain to be clarified. (1) Research needs to identify the remaining co-regulators and target proteins that moderate pleiotropic downstream responses regulated by JAZ proteins. A growing number of functional studies is conducted using genetically modified plants and increasing numbers of plants species; JAZ proteins have been implicated in an increasing number of plant developmental processes such as secondary growth (interfascicular cambium initiation) (Sehr et al., 2010), phytochrome A-mediated shade responses (Robson et al., 2010), flower induction (Kim et al., 2011), and defense responses against biotic (Shoji et al., 2008), (Sun et al., 2011; Demianski et al., 2012; Oh et al., 2012) and abiotic (Ye et al., 2009; Seo et al., 2011; Ismail et al., 2012; Zhu et al., 2012) stresses in plants; however, detailed mechanisms and interactions involved in these regulatory processes remain unclear. (2) More functional studies using different species of plants are clearly required. Although JAZ proteins are ubiquitous in flowering plants, most current studies are exclusively performed in *Arabidopsis*. However, plants have different ways to balance their growth, development and defense according to their specific environments, so it is of high priority to investigate

JAZ-mediated processes in alternative plant species with interesting physiologies and ecologies. (3) The precise roles of individual JAZ proteins in JAZ protein family are still largely unknown. Early studies of JAZ proteins proposed that JAZ proteins repress the MYC2 transcription factor, leading to the repression of jasmonate-responsive genes, and thus that JAZ protein function is highly redundant. However, more recent research has demonstrated the existence and roles of several co-regulators and several target transcription factors other than MYC2 in regulating downstream responses by different stimuli (reviewed in (Browse and Wager, 2012; Kazan and Manners, 2012)). According to these trends, we can expect to discover specificity and functional diversity of individual JAZ proteins in various aspects of plants growth, development, and defense responses.

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# Summary


Plants and herbivores formed one of the earliest terrestrial communities and co-evolved in time, adapting to each other, both trying to maximize their own fitness. In the early 1960s, the first studies demonstrated that plant defense metabolites produced after herbivore attack can negatively affect herbivore performance via toxic or anti-digestive mechanisms. These effects resulted in specialization of herbivores and selection of their host plants according to species-specific spectra of defensive metabolites. To survive and maximize fitness in nature, plants learned to cope with multitudes of harmful factors, finding a proper balance between growth and defense that involves complex phytohormonal regulatory networks. One group of hormones, jasmonic acid (JA) and its derivatives, is known to control both growth and defense responses in plants. In the past decade, several core components of jasmonate signaling: the receptor $SCF^{COI1}$ complex, key negative regulators, JAZ (Jasmonate ZIM-domain) proteins, and the active jasmonate hormone (+)-7-iso-JA-L-Ile (JA-Ile) were discovered and contributed to our current understanding of regulatory mechanisms involved in jasmonate signaling. Nevertheless, it remained to decipher specific roles of these multiple players. In particular, the functional characterization of JAZ proteins remained unresolved, and I addressed this in my work.

In my dissertation, I identified 12 novel JAZ proteins in the native tobacco *Nicotiana attenuata*, a summer annual plant native to southwestern North America. The *NaJAZ* genes showed spatially and temporally resolved expression patterns characterized by highly induced local and systemic accumulation of transcripts after stimulated herbivory. To identify the function of individual JAZ proteins by reverse genetics, I used two sets of transgenic plants that were silenced in the expression of either *NaJAZd* (irJAZd) or *NaJAZh* (irJAZh), using inverted repeat (ir) RNAi constructs to stably silence the specific *JAZ* genes. The silencing of either *NaJAZd* or *NaJAZh* strongly affected both plant defense responses and development.

*NaJAZh* silencing altered the performance of the specialist herbivore *Manduca sexta* by inducing both constitutive and induced levels of direct defenses (trypsin protease inhibitors, TPIs and 17-hydroxygeranyllinalool diterpene glycosides, HGL-DTGs) and indirect defenses (volatile organic compounds, VOCs) in *N. attenuata*. In contrast, *NaJAZh* silencing repressed nicotine accumulation in leaves, which could be explained by a

crosstalk of *JAZ* genes: *NaJAZh* silencing also up-regulated several other *JAZ* genes such as *NaJAZf*, *NaJAZj*, and *NaJAZb*. In addition, I found that NaJAZh is required to repress programmed cell death in the leaves by regulating ROS levels, especially levels of hydrogen peroxide.

I found that in contrast to NaJAZh, the NaJAZd protein plays only a minor role in defense, specifically in nicotine biosynthesis and/or transport. These changes, however, were not sufficient to alter herbivore performance on these plants. In contrast, *NaJAZd* silencing strongly affected the lifetime production of seed capsules by causing enhanced flower abscission in late flower developmental stages. I found that *NaJAZd* silencing significantly repressed JA and JA-Ile levels, but not ethylene, in flowers. Interestingly, the *NaJAZd* silencing-mediated flower abscission phenotype resembled the phenotype of *NaMYB30*5 (homologue to *PhEOBII*)-silenced plants that I recently characterized as part of my dissertation. NaMYB305 plays a major role in flower opening and its silencing caused premature flower abscission. Notably, *NaJAZd*-silenced flowers had altered transcript abundance of this master flower regulator at the last open stage of flowering. I propose that NaJAZd may be required to maintain sufficient levels of *MYB305* transcripts and prevent premature flower abscission: NaJAZd thus counteracts flower abscission by regulating JA, JA-Ile levels and/or expression of *NaMYB305* gene in *N. attenuata*, permitting seed production in plants.

Current identification of core components of jasmonate signaling, JAZ, $SCF^{COI}$ complex, and JA-Ile, significantly contributed to the understanding of jasmonate signaling, and identification of the roles of several co-regulatory components and target transcription factors. However, one of the major gaps in our knowledge – the role of individual JAZ proteins – remained. In my dissertation, I showed for the first time that a single JAZ protein can control specific jasmonate-dependent defense and developmental processes, which lends more support to the hypothesis that JAZ proteins are functionally specialized both in defense and development, proposed by several previous studies. Apart from defense, my work provided several novel insights into the function of jasmonates and JAZ regulators in plant development, including spontaneous necrosis, ROS accumulation, flowering and seed development.

# Zusammenfassung

Pflanzen und Pflanzenfresser bilden eine der frühesten terrestrischen Gemeinschaften. Sie entwickelten sich gemeinsam und versuchen durch gegenseitiges aneinander Anpassen ihre eigene Fitness zu maximieren. Erste Studien in den frühen 1960er Jahren zeigten, dass pflanzliche Abwehrstoffe nach Fraßattacken produziert werden und in Form von Gift oder als verdauungshemmende Stoffe die Leistung von Pflanzenfressern negative beeinflussen können. Diese Effekte führten zu einer Spezialisierung von Pflanzenfressern und der Auswahl ihrer Wirtspflanzen nach artspezifischen Aspekten der Abwehrstoffe. Um zu überleben und die darwinsche Fitness zu maximieren, haben die Pflanzen gelernt, mit einer Vielzahl von schädlichen Faktoren umzugehen und ein ausgewogenes Gleichgewicht zwischen Wachstum und Verteidigung mit Hilfe eines komplexen, regulatorischen Pflanzenhormonnetzwerkes zu finden. Eine spezielle Gruppe der Pflanzenhormone, die Jasmonsäure (JA) und ihren Derivaten, sind dafür bekannt, dass sie sowohl das Wachstum als auch die Abwehrreaktionen in Pflanzen regulieren. Im letzten Jahrzehnt wurden mehrere Kernkomponenten der Jasmonat-Signalkette gefunden: der Jasmonat-rezeptor $SCF^{COI1}$ Komplex, negative Regulatoren der Jasmonat-Signalkaskade (JAZ, Jasmonate ZIM-Domain) und das aktive Jasmonat-Derivat (+)-7-iso-JA-L-Ile (JA-Ile), die dazu beigetragen haben, unser gegenwärtiges Verständnis der Regulationsmechanismen in der Jasmonat-Signalkette zu verstehen. Dennoch müssen die spezifischen Rollen dieser Komponenten erst noch entschlüsselt werden. In meiner Arbeit befasste ich mich speziell mit der Rolle der JAZ Proteine und deren funktioneller Charakterisierung, da diese Fragen bisher ungelöst blieben.

In meiner Dissertation identifizierte ich 12 neue JAZ Proteine im wilden Tabak (*Nicotiana attenuata*), einer einjährigen Pflanze, die im südwestlichen Nordamerika heimisch ist. Die *NaJAZ* Gene waren durch ein räumlich und zeitlich aufgelöstes Expressionsmuster durch stark induzierte lokale und systemische Akkumulation von Transkripten nach Fraß Attacke gekennzeichnet. Um die Funktion der einzelnen JAZ Proteine mittels reverser Genetik zu identifizieren, verwendete ich zwei Sätze von stabilen transgenen Pflanzen, die, mittels selbstkompatibler, sogenannter *inverted repeat* (ir) RNAi s*ilencing*-Konstrukte, entweder in der Expression von *NaJAZd* oder *NaJAZh* herunter-

reguliert wurden. Das Silencing beider Gene, *NaJAZd* oder *NaJAZh,* zeigte starke Auswirkungen auf die Abwehrreaktion und Entwicklung der betroffenen Pflanze.

In *N. attenuata* veränderte *NaJAZh* silencing das Wachstum der Raupen des Tabakschwärmers *Manduca sexta* durch Erhöhung von sowohl konstitutiven und induzierbaren direkten Abwehrstoffen wie Trypsin-Protease-Inhibitoren (TPIs) und 17-hydroxygeranyllinalool Diterpenglycoside (HGL-DTGS) als auch von indirekt zu Verteidigung beitragenden Duftstoffen (volatile organic compounds, VOCs). Im Gegensatz dazu zeigten ir-*NaJAZh* Pflanzen eine verringerte Akkumulation von Nikotin in den Blättern. Diese Beobachtung könnte durch eine Vernetzung der JAZ-Signalkaskade erklärt werden, denn *NaJAZh* silencing führte zu einer Hochregulierung mehreren anderen JAZ Gene wie *NaJAZf*, *NaJAZj* und *NaJAZb*. Darüber hinaus konnte ich zeigen, dass *NaJAZh* auch dafür erforderlich ist, den programmierten Zelltod nach Verwundung in den Blättern durch Regulierung der Bildung von reaktiven Sauerstoffverbindungen (ROS) insbesondere Wasserstoffperoxid zu unterdrücken.

In meiner Arbeit konnte ich außerdem zeigen, dass *NaJAZd* im Gegensatz zu *NaJAZh* nur eine untergeordnete Rolle in der Verteidigung spielt, nämlich bei der Nikotinbiosynthese und/oder dem Nikotintransport. Diese Änderungen in ir-*NaJAZd* Pflanzen reichten jedoch nicht aus, um die Raupen des Tabakschwärmers zu beeinflussen. Nichtsdestotrotz führte *NaJAZd* silencing zu einer starken Beeinflussung der Dauer der Samenkapselproduktion durch vermehrtes Abwerfen von Blüten in den späten Entwicklungsstadien. Ich fand heraus, dass *NaJAZd* silencing die Akkumulation von JA und JA-Ile in den Blüten deutlich unterdrückt, nicht aber die Menge von Ethylen. Interessanterweise ähnelte dieser ir-*NaJAZd* Blütenabwurf dem Phänotyp von *NaMYB305* (*master flower regulator*, homolog *PhEOBII*) gesilencten Pflanzen, die ich als Teil meiner Arbeit charakterisierte. *NaMYB305* spielt eine wichtige Rolle beim Öffnen der Blüten und dessen Herunterregulierung verursacht ein vorzeitiges Abwerfen der Blüten. Bemerkenswerterweise zeigten ir-*NaJAZd* Pflanzen auch veränderte Transkriptlevel von MYB305 in offenen Blüten. Dies induziert, dass *NaJAZd* möglicherweise in der Regulierung von MYB305 eine Rolle spielt und verhindert, dass Blüten vorzeitig abgeworfen werden: *NaJAZd* wirkt durch die Regulierung von JA und JA-Ile und/oder der Expression von NaMYB305 dem Blütenabwurf vor und ermöglicht die Samenproduktion von *N. attenuata*.

Die Identifikation von Kernkomponenten der Jasmonat -Signalkette (JAZ, $SCF^{COI}$ Komplex und JA-Ile) als auch die Identifikation von mehreren Co-regulatorische Komponenten und Transkriptionsfaktoren trug im Wesentlichen zum Verständnis der Jasmonat -Signalkette bei. Eine Frage blieb jedoch unbeantwortet - die funktionale Rolle der einzelnen JAZ Proteine. In meiner Dissertation habe ich zum ersten Mal gezeigt, dass ein einzelnes JAZ Protein spezifische Jasmonat-abhängige Verteidigungs- und Entwicklungsprozesse kontrolliert, was die Hypothese anderer Studien unterstützt, dass JAZ Proteine sowohl für Verteidigungs- als auch Entwicklungsprozesse funktionell spezialisiert sind. Neben pflanzlichen Verteidigungsprozessen gibt meine Arbeit neue Einblicke in die Funktion von Jasmonaten und JAZ-Regulatoren in der Pflanzen-entwicklung, einschließlich spontaner Nekrose, ROS Akkumulation und Blüten- sowie Samenbildung.

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# Selbständigkeitserklärung

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

______________________

Youngjoo Oh

Jena, 06. 11. 2012

# Curriculum vitae

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## Oral Presentation

- Youngjoo Oh, Ian T. Baldwin, and Ivan Galis. JAZh regulates jasmonic acid (JA) signaling which controls defense responses against herbivore attack in *Nicotiana attenuata*. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, 2011

# Poster Presentation

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- Youngjoo Oh, Ian T. Baldwin, and Ivan Galis. JAZh regulates JA-related direct and indirect plant defense responses against herbivory in *Nicotiana attenuata*. ICE Symposium, MPI for Chemical Ecology, Jena, Germany, 2011
- Youngjoo Oh, Ian T. Baldwin, and Ivan Galis. JAZ repressors of jasmonate signaling in *Nicotiana attenuata*. 9th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, 2010
- Young Joo Oh, Young Jun Cho, Young Na Lee, In-yong Hwang, and Woong June Park. Studies on the activity regulation of aldehyde oxidases (AO) in maize (*Zea mays*). Plant winter conference, Pohang, Korea, 2008
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