

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 is an important regulator of plant growth, development and defense. The jasmonate-ZIM domain (JAZ) proteins are key regulators in jasmonate 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*, and initiated systematic functional characterization of these proteins by reverse genetic approaches. In this report, *Nicotiana attenuata* plants silenced in the expression of *NaJAZd* (irJAZd) by RNA interference 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 wild type plants, both in the glasshouse and the native habitat of *Nicotiana attenuata* in the Great Basin Desert, Utah, USA. Interestingly, irJAZd plants produced fewer seed capsules than did wild type 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 jasmonic acid and jasmonoyl-L-isoleucine, 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 *NECTARIN 1* transcript levels, phenotypes which are copied in irJAZd plants. We propose that the NaJAZd protein is required to counteract flower abscission, possibly by regulating jasmonic acid and jasmonoyl-L-isoleucine levels and/or expression of *NaMYB305* gene in *Nicotiana attenuata* flowers. This novel insight into the function of JAZ proteins in flower and seed development highlights the diversity of functions played by jasmonates and JAZ proteins.

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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 phytohormone 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 transcriptional 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 (CORONATINE INSENSI-

TIVE1), 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 (TOPELESS) proteins, InsP₅ (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 subsets of JA-mediated responses in plants [30,31] however, specific examples of such interactions remain rare. 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 *Nicotiana attenuata* (*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. attenuata* 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*, *NaNECI*) 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 μ L of water; W+W), simulated herbivore attack (wounds treated with 20 μ L 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*, -*b*, -*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. Similar to our previous study with *NaJAZh*-silencing, which increased the expression of several other *JAZ* genes [31], we found a significantly higher expression of *NaJAZb* in both *irJAZd* examined lines (and higher expression of *NaJAZf*, -*j*, and -*m* in at

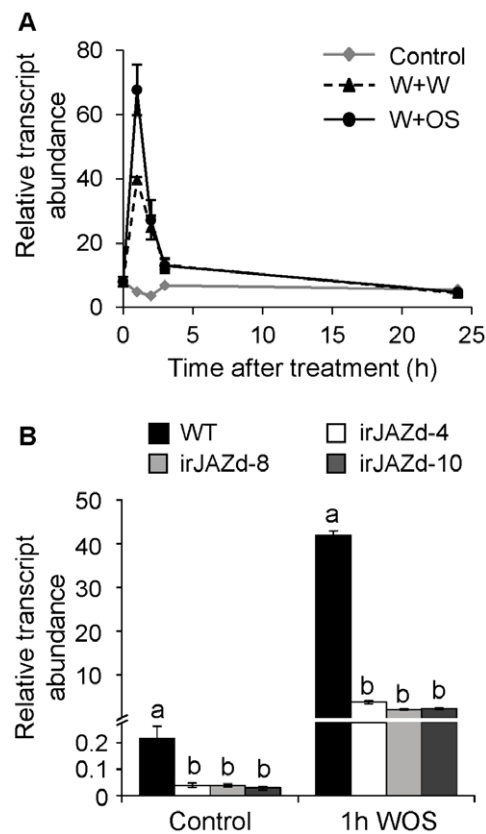


Figure 1. Regulation of *NaJAZd* transcripts and silencing efficiency in *irJAZd* plants. (A) *NaJAZd* transcript abundances \pm SE were determined by quantitative real-time PCR (qPCR, $n=3$) in samples from WT *N. attenuata* leaves treated with wounding and water (W+W), wounding and *M. sexta* oral secretions (W+OS) harvested at 0, 1, 2, 3, and 24 h after treatment (control leaves remained untreated). (B) Transcript abundances \pm 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 housekeeping *EF1 α* transcript abundances determined by qPCR in the same samples. Different letters in B indicate significant differences among the combination of identically treated genotypes (WT vs. independent *NaJAZd* silenced lines, *irJAZd*-4, -8, 10 by one-way-ANOVA ($P\leq 0.05$). doi:10.1371/journal.pone.0057868.g001

least one line), suggesting a possible crosstalk or compensation responses in the JAZ regulatory network. In addition, we found a significant down-regulation of *NaJAZh* transcripts in response to W+OS-elicitation (Figure S2) in both examined lines, consistent with the result of nucleotide alignment of *NaJAZh* gene and *irJAZd*-silencing region which revealed a potential cross-silencing region of 20 nt (Text S1) between these sequences (no other identities ≥ 20 nt were found in any other known *N. attenuata* *JAZ* gene sequences). Recently, we analyzed in detail plants strongly silenced ($>90\%$) in *NaJAZh* expression but the phenotypes found in *irJAZh* plants were not consistent with the *irJAZd* phenotypes described in the following chapters. For example, *irJAZh* plants showed significantly reduced nicotine levels and dramatic increases in TPI activity, as well as they developed spontaneous necrosis during aging [31], none of which could be found in *irJAZd* plants, suggesting that a partial silencing of NaJAZh was not sufficient to induce NaJAZh-associated phenotypes. We therefore proceeded with the search for specific NaJAZd silencing-associated phenotypes.

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. Taking into account the data from three independently transformed irJAZd lines, the levels of JA-Ile (Figure 2B), and of other phytohormones (JA, SA and ABA; Figure S3) in W+OS-treated irJAZd 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 W+OS in the leaves (Figure 1A). The basal levels of SA were slightly lower in all irJAZd lines; however, they rise to WT SA levels after W+OS treatment, suggesting a normal responsiveness and accumulation of SA in irJAZd plants during herbivory stress.

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 constitutive 17-hydroxygeranylinalool-diterpene glycosides (HGL-DTGs) [44,45] was slightly higher in two irJAZd lines but not significantly different after W+OS treatment at 24–72 h compared to WT (Figure S4A). Trypsin protease inhibitors (TPIs) activity [46–48] in W+OS-treated irJAZd-4, -8, and -10 plants were not different from WT levels (Figure S4B). 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 season, we planted empty vector-transformed (EV) and irJAZd-8 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.

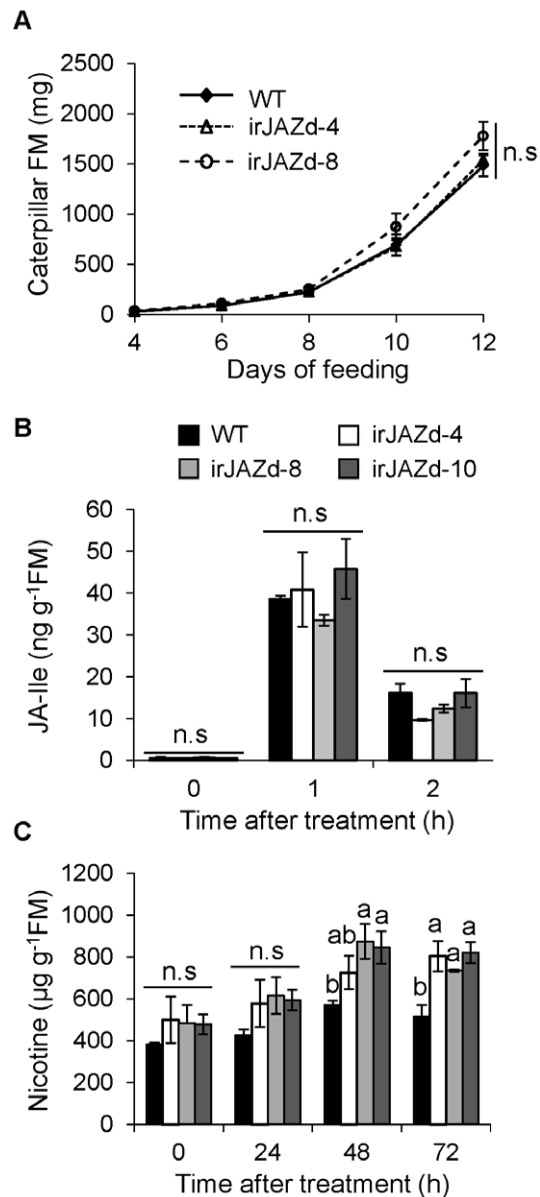


Figure 2. Defense responses against specialized herbivore *M. sexta* are mostly unaltered in irJAZd plants. (A) Herbivory performance of *M. sexta* on rosette leaves of WT and two independent irJAZd lines (irJAZd-4 and -8) was determined by measuring larval mass at 4, 6, 8, 10 and 12 d after placement of freshly hatched neonates on the plants. Mean fresh masses \pm SE of irJAZd-4 and -8-fed caterpillars ($n=20$) were not significantly different from WT-fed caterpillars. (B) Mean \pm 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 \pm 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 indicate significant differences among the different genotypes (WT vs. independent NaJAZd silenced lines, irJAZd-4, -8, 10) at the same time points; n.s., not significantly different; FM, fresh mass. doi:10.1371/journal.pone.0057868.g002

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

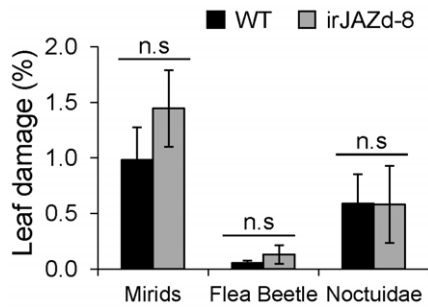
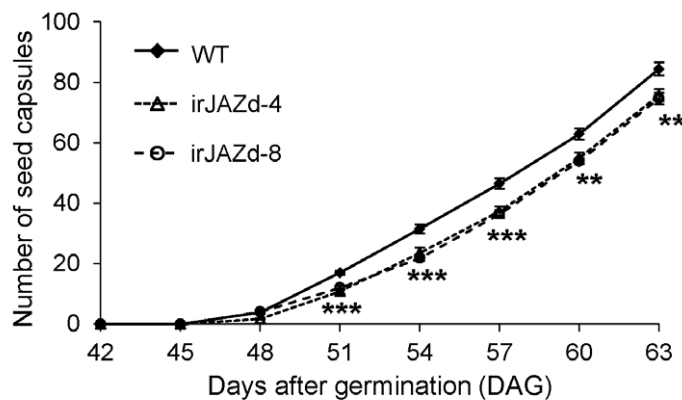


Figure 3. Plant damage caused by herbivores in *N. attenuata*'s native habitat. EV and irJAZd-8 plants were planted in a size-matched paired-design in their native habitat, Great Basin Desert, Utah, USA and damage by native herbivores was scored throughout the 2010 field season. Herbivore damage was 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). No significant differences (n.s) between the two genotypes determined by Student t-test were observed.
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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 11–35% fewer capsules compared to WT between 51–63 d after germination (Figure 4).



	42 DAG	45 DAG	48 DAG	51 DAG	54 DAG	57 DAG	60 DAG	63 DAG
WT	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.4	17.0 ± 0.7 ^a	31.5 ± 1.4 ^a	46.5 ± 1.8 ^a	62.9 ± 1.8 ^a	84.4 ± 2.2 ^a
irJAZd-4	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.2	10.9 ± 0.9 ^b	23.5 ± 1.8 ^b	37.3 ± 1.6 ^b	55.0 ± 1.7 ^b	75.7 ± 2.0 ^b
irJAZd-8	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 0.4	12.0 ± 0.6 ^b	21.8 ± 0.8 ^b	36.4 ± 1.4 ^b	53.9 ± 1.0 ^b	74.7 ± 1.9 ^b

Figure 4. NaJAZd-silencing negatively affects seed capsule production. WT and two independent 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≤0.01, ***P≤0.001). There were no significant differences in number of seed capsules between two independent irJAZd lines (irJAZd-4 and -8). The exact numbers of capsules are displayed in table under the graph and different letters in the table indicate significant differences among the different genotypes (WT vs. independent NaJAZd silenced lines, irJAZd-4, -8) at the same time points. Differences at 42–48 d were not significant.
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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 and spreading it 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 plants to WT levels. These results suggested that irJAZd flowers have normal anthesis and otherwise completely normal morphology (Figure S6) but experience 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 30×52 cm plastic tray which captured all abscised flowers from a single plant. 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

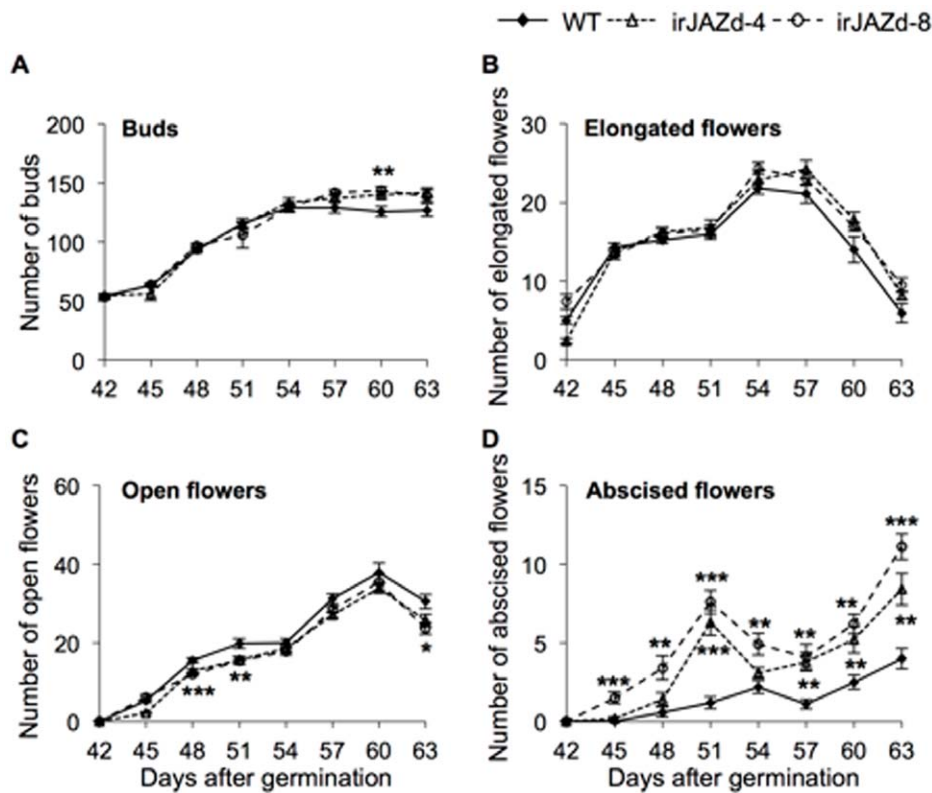


Figure 5. NaJAZd counteracts flower abscission in *N. attenuata*. Each individual plant was placed in an individual plastic tray (30×52 cm) in the glasshouse to avoid mixing of abscised flowers, and number of buds (A), elongated flowers (B), open flowers (C), and abscised flowers (D) in each plant from 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 (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). doi:10.1371/journal.pone.0057868.g005

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-8 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-8 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 levels were strongly reduced in irJAZd-8 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-8 plants. Ethylene emissions increased in a stage dependent manner; however, we found no significant

differences between WT and irJAZd-8 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-8 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-8 flowers had significantly reduced levels of JA and JA-Ile compared to WT flowers (Figure 6B). It suggested that NaJAZd might 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: their flowers failed to enter anthesis and eventually, the plants did not produce any seed capsules. 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

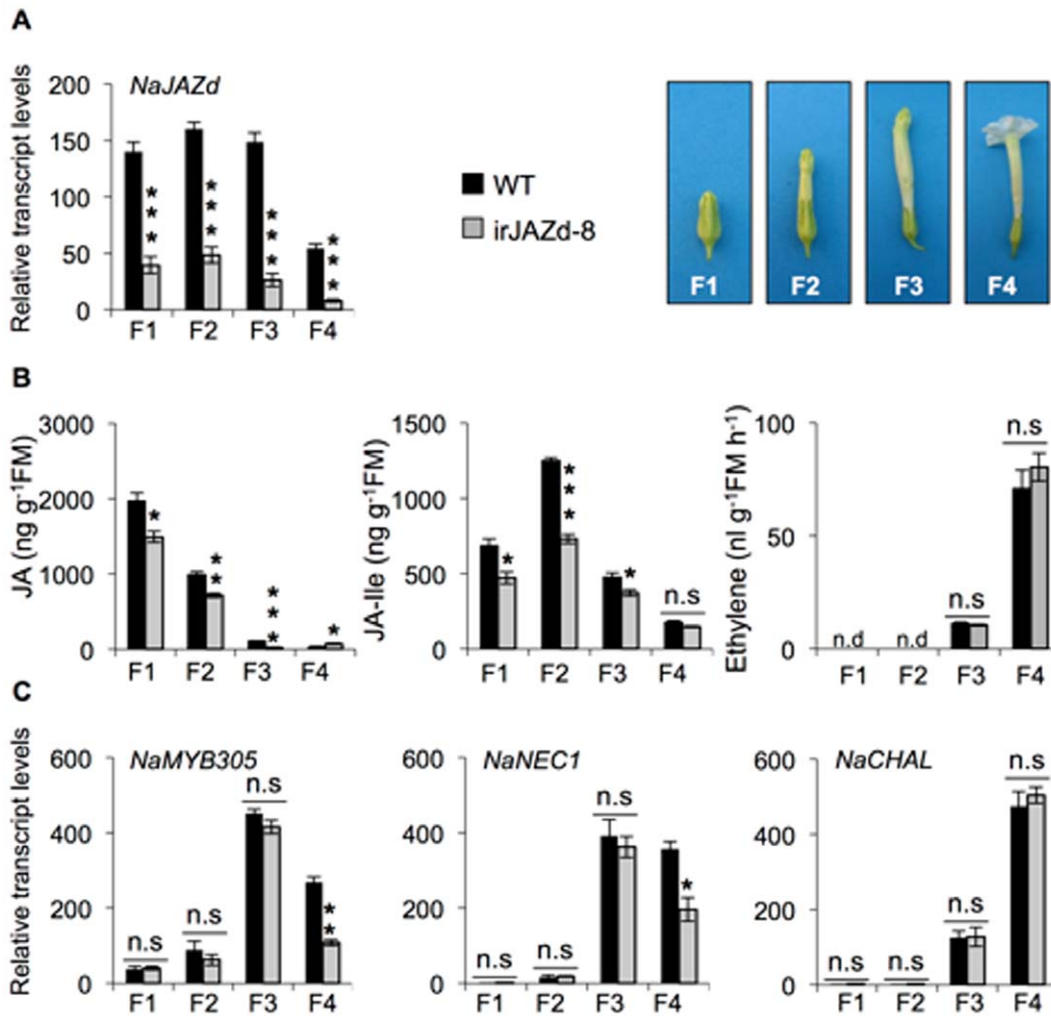


Figure 6. NaJAZd regulates phytohormone levels and flower development-related genes. WT and *irJAZd-8* plants (*irJAZd-8*) were grown in the glasshouse and four different developmental stages of flowers (F1, F2, F3, and F4) were collected 57 d after germination. (A) Transcript abundances of *NaJAZd* determined by qPCR in *irJAZd-8* flowers were significantly lower compared to WT. (B) Mean JA and JA-Ile levels \pm SE determined by LC-ESI-MS/MS using individual stage flowers and mean ethylene levels \pm SE measured by photoacoustic spectrometer using a mixture of five flowers of each stage. (C) Transcripts abundances \pm SE 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 remained unaltered in *irJAZd-8* compared to WT flowers. Bars \pm SE in (C) show *EF1 α* -normalized relative transcript abundances. Statistical differences in phytohormones, JA, JA-Ile, ethylene ($n=4$) and transcript abundances ($n=4$) were determined by Student t-test. Asterisks represent significant differences between WT and *irJAZd* in same stage of flowers (* $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.001$); n.s., not significantly different; FM, fresh mass. doi:10.1371/journal.pone.0057868.g006

relationship between *NaMYB305* and *NaJAZd*, we analyzed *NaMYB305* expression at four different stages of WT and *irJAZd-8* flowers (Figure 6C). In both WT and *irJAZd-8* flowers, *NaMYB305* expression gradually increased from F1 to F3, corroborating previous studies [52]. However, the *irJAZd-8* 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 *irMYB305* 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 regulat-

ed in F4 stage flowers in *irJAZd-8* plants compared to WT flowers. However, *NaCHAL* expression was not significantly different between WT and *irJAZd-8* flowers (Figure 6C), showing an expression pattern which closely tracked flower ethylene emissions (Figure 6B). In an independent experiment, we also analyzed in detail the hormone contents and gene transcript levels in *irJAZd-4* and WT flowers (Figure S7). Identical changes in JA, JA-Ile contents and *NaMYB305* transcript accumulation were observed but the changes were less pronounced compared to the independently transformed line *irJAZd-8*, which was consistent with the lower number of abscised flowers found in *irJAZd-4* line (Fig. 5D). In *irJAZd-4* line, the levels of *NaNEC1* transcripts were not yet reduced, showing a time delay between accumulation of the *MYB305* regulator and the expression of downstream-regulated gene, *NEC1*.

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 number 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 (20)- and down (99)-regulated genes in *irJAZd* compared to WT plants was annotated and categorized according to established GO categories (Tables 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 might regulate sink-source relationship by regulating expression and/or accumulation of 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 NtJAZ1 and 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 suppress 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 the follow up experiments, we therefore decided to use 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 native herbivores of *N. attenuata* in native habitat of this plant (Figure 3).

Our initial data thus suggested that NaJAZd might 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 *N. attenuata* inflorescences, a finding not surprising 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 (Figures 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 unaltered in *irJAZd* flowers compared to WT (Figure 6B). Plants blocked in ethylene perception (*etr1* mutants) show a typical flower corolla-retention phenotype where 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 abscised after separation of pedicels from inflorescences, which was 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. *irJAZd* 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* genotypes, 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 a 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 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 link 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 *NEC5* proteins and high levels of antimicrobial H_2O_2 (up to 4 mM) are thought to protect the gynoceum 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 31st inbred 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; Figure 1B) 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 RevertAidTM 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 1α) 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 two independent *irJAZd* line plants (*irJAZd*-4 and -8). The larval fresh mass was measured on 4th, 6th, 8th, 10th, 12th 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 [²H₂]JA, and 40 ng each of JA-[¹³C₆]Ile, [²H₄]SA and [²H₆]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 to clean microcentrifuge tubes and dried in vacuum concentrator (Eppendorf) at 30°C. The residues were re-suspended 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 five flowers were collected from each stage of flowers to measure ethylene in 250 mL flasks. Flowers were incubated for 5 h to accumulate ethylene in the flasks and accumulated ethylene in the headspace was flushed with a 130 to 150 mL/min flow of purified air into spectrometer where it was 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 NH₄OH, 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 the Animal and Plant Health Inspection Service (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 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 for approximately 2 weeks, after which the plants were grown without watering. 15 pairs of EV and irJAZd-8 plants were planted and grown in the field plot and monitored for damage from native herbivores. Damage of plants by native herbivores was determined by estimating the percentages of total leaf area of plants damaged by each herbivore: Noctuidae larvae, *Spodoptera* spp.; flea beetles, *Epitrix* species; and mirids, *Tupiocoris notatus*. A result of representative measurement conducted on 15th, May, 2010 is shown in the Figure 3.

Seed Capsule and Flower Counts

The number of seed capsules at 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 (30×52 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 a 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, log₂ 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) ≤2.09% and greater than 3-fold signal changes (irJAZd vs. WT) were used. QC table showing the technical variability of 30 selected control genes spotted in 10 different locations of microarray chip is shown in Table S4. All data were deposited to public GEO (Gene Expression Omnibus) repository under accession number GSE43395. 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.

Supporting Information

Figure S1 Generation of stable *NaJAZd*-silenced *N. attenuata* plants. (A) A 303 bp 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, -9, and -10) lines and WT. The genomic DNA was digested with *Xba*I enzyme and hybridized with a ³²P-labeled probe coding for the hygromycin resistance gene located between the 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. (TIF)

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 1 h 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-8, 10) and treatments determined by one-way-ANOVA (*P*≤0.05); n.s., not significantly different. (TIF)

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); n.s., not significantly different. FM, fresh mass. (TIF)

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 (irJAZd-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 TPI activities 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*≤0.05); n.s., not significantly different. FM, fresh mass. (TIF)

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 d 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 ($P \leq 0.05$). (TIF)

Figure S6 Flowers and buds of irJAZd transgenic and WT plants at F1–F4 stages of development. Flower buds and flowers were detached from 57-d-old plants and photographed to capture the highly similar morphology of flowers in two transgenic irJAZd-4 and -8 lines and WT. (TIF)

Figure S7 NaJAZd regulates phytohormone levels and flower development-related genes. WT and irJAZd-4 plants were grown in glasshouse and four 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-4 flowers were significantly lower compared to WT. (B) Mean JA and JA-Ile levels \pm SE determined by LC-ESI-MS/MS using four individual stage flowers and mean ethylene levels \pm SE measured by photoacoustic spectrometer using a mixture of five flowers of each stage. (C) Transcripts abundances of flower development-related genes, *NaMYB305*, *NaNEC1*, and *NaCHAL* determined by qPCR: irJAZd-4 plants were impaired in expression of *NaMYB305* gene in last stage of flower development (F4) while *NaNEC1* and *NaCHAL* transcripts were unaltered in irJAZd-4 compared to WT flowers. Bars \pm SE in (C) show *EF1 α* -normalized relative transcript abundances. Statistical differences in phytohormones, JA, JA-Ile, ethylene ($n = 4$), and transcript abundances ($n = 4$) were determined by Student t-test. Asterisks represent significant differences between WT and irJAZd in same

stage of flowers (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$); n.s., not significantly different; FM, fresh mass. (TIFF)

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

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

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

Table S4 QC table showing technical variability of 30 selected control genes spotted in 10 different locations on microarray chips. (PDF)

Text S1 Nucleotide sequence alignment of *NaJAZ* genes and inverted repeat (ir) construct used for *NaJAZd* silencing in irJAZh plants. (PDF)

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Author Contributions

Conceived and designed the experiments: IG YO ITB. Performed the experiments: YO IG ITB. Analyzed the data: YO IG ITB. Contributed reagents/materials/analysis tools: ITB. Wrote the paper: YO IG ITB.

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