The *HERBIVORE ELICITOR-REGULATED1* Gene Enhances Abscisic Acid Levels and Defenses against Herbivores in *Nicotiana attenuata* Plants[1][C][W][OPEN]

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Nicotiana attenuata plants can distinguish the damage caused by herbivore feeding from other types of damage by perceiving herbivore-associated elicitors, such as the fatty acid-amino acid conjugates (FACs) in oral secretions (OS) of *Manduca sexta* larvae, which are introduced into wounds during feeding. However, the transduction of FAC signals into downstream plant defense responses is still not well established. We identified a novel FAC-regulated protein in *N. attenuata* (NaHER1; for herbivore elicitor regulated) and show that it is an indispensable part of the OS signal transduction pathway. *N. attenuata* plants silenced in the expression of *NaHER1* by RNA interference (irHER1) were unable to amplify their defenses beyond basal, wound-induced levels in response to OS elicitation. *M. sexta* larvae performed 2-fold better when reared on irHER1 plants, which released less volatile organic compounds (indirect defense) and had strongly reduced levels of several direct defense metabolites, including trypsin proteinase inhibitors, 17-hydroxygeranyllinallool diterpene glycosides, and caffeoylputrescine, after real and/or simulated herbivore attack. In parallel to impaired jasmonate signaling and metabolism, irHER1 plants were more drought sensitive and showed reduced levels of abscisic acid (ABA) in the leaves, suggesting that silencing of *NaHER1* interfered with ABA metabolism. Because treatment of irHER1 plants with ABA results in both the accumulation of significantly more ABA catabolites and the complete restoration of normal wild-type levels of OS-induced defense metabolites, we conclude that NaHER1 acts as a natural suppressor of ABA catabolism after herbivore attack, which, in turn, activates the full defense profile and resistance against herbivores.

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

The identification of herbivore-associated elicitors and their apparent structural diversity is consistent with the expectations of strong (co)evolutionary interactions between herbivores and their host plants (Ehrlich and Raven, 1964; Voelckel and Baldwin, 2004; Agrawal et al., 2012; Ali and Agrawal, 2012). FACsfound in oral secretions (OS) of most lepidopteran larvae are introduced into wounds during feeding on the plants. They are biosynthesized from fatty acids, such as linolenic and linoleic acids, of plant origin that are conjugated with Gln or Glu in the insect guts. Because FACs are essential for the larval digestion and nitrogen utilization (Yoshinaga et al., 2008) and do not occur in intact plants, FACs provide plants with an accurate and reliable signal of herbivore attack. FAC perception has been demonstrated in both monocots (maize [*Zea mays*]) and dicot (eggplant [*Solanum melongena*] and *Nicotiana*

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attenuata) plants, suggesting an ancient origin or convergent evolution of FAC perception (Halitschke et al., 2001; Truitt et al., 2004; Schmelz et al., 2009). As FACs in OS are rapidly metabolized on wounded leaf surfaces, it remains unclear if FACs function directly, or as FAC metabolic products, to activate downstream defenses against herbivores in *N. attenuata* (Halitschke et al., 2001; VanDoorn et al., 2010; Bonaventure, 2012). In contrast to the well-established activity of FACs as insect elicitors, the signal transduction of FAC perception into defense responses is less understood (Bonaventure, 2012).

When synthetic FACs are applied to mechanical wounds, *N. attenuata* plants accumulate dramatically larger amounts of jasmonic acid (JA) and its bioactive form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) and, consequently, activate both quantitatively and qualitatively adapted (tuned) defense responses compared with standardized mechanical wounding (Halitschke et al., 2001, 2003; Giri et al., 2006; von Dahl et al., 2007; Gaquerel et al., 2009). One of the first committed steps in JA biosynthesis is the oxygenation of α-linolenic acid by 13-lipoxygenase (LOX), followed by the activity of several other chloroplast- and peroxisome-localized enzymes (Vick and Zimmerman, 1984; Halitschke and Baldwin, 2003). JA is then conjugated with Ile to form JA-Ile, known to be the major endogenous bioactive jasmonate regulating downstream defense responses in plants (Kang et al., 2006; Fonseca et al., 2009). Consistent with the proposed role of FACs upstream of JA biosynthesis, FAC-elicited herbivore resistance in *N. attenuata* was dramatically attenuated when JA or JA-Ile biosynthetic genes were silenced by RNA interference or an antisense approach (Halitschke and Baldwin, 2003; Kessler et al., 2004; Heiling et al., 2010; Kallenbach et al., 2012).

In the presence of JA-Ile, JASMONATE ZIM DOMAIN (JAZ) repressors that physically interact with the positive transcription factors of JA signaling, such as MYC2 proteins, are recruited to the SCF^{COI1} E3 ubiquitin ligase complex, ubiquitinated, and consequently degraded by 26S proteasome. The removal of JAZ repressors releases MYC2, and other related transcription factors, from inhibition that triggers downstream JA-dependent defense responses (Lorenzo et al., 2004; Chini et al., 2007; Dombrecht et al., 2007; Thines et al., 2007; Kazan and Manners, 2008, 2012, 2013; Sheard et al., 2010; Fernández-Calvo et al., 2011; Hiruma et al., 2011; Oh et al., 2012). In *N. attenuata*, such defense responses include the accumulation of trypsin proteinase inhibitors (TPIs) and enzymes involved in the biosynthesis of direct defense secondary metabolites such as 17-hydroxygeranyllinalool (HGL)-diterpene glycosides (DTGs), caffeoylputrescine (CP), dicaffeoylspermidine (DCS), and nicotine. In addition, plants use indirect defenses, such as the biosynthesis and release of volatile organic compounds (VOCs) from the leaves or the production of extrafloral nectar that attract and/or increase the presence of natural enemies of herbivores (Kessler and Baldwin, 2001; van Dam et al., 2001; Halitschke and Baldwin, 2003; Steppuhn et al., 2004; Kost and Heil, 2008; Radhika et al., 2008; Skibbe et al., 2008; Heiling et al., 2010; Kaur et al., 2010; Kessler et al., 2012; Schuman et al., 2012; Heinrich et al., 2013).

In Arabidopsis (*Arabidopsis thaliana*), several plant hormones have been shown to have synergistic or antagonistic effects on JA signaling. For example, interaction between salicylic acid (SA) and JA in defense (Takahashi et al., 2004; Beckers and Spoel, 2006) suggests that the relative concentration of each hormone, JA and SA, can strongly affect the final outcome of the responses (Mur et al., 2006). The NONEXPRESSER OF PR GENES1 (NPR1) protein is known to regulate cross talk between SA and JA (Spoel et al., 2003), and ethylene and methyl jasmonate (MeJA) synergistically induce several members of the pathogenesis-related genes in groups 1 and 5 (Xu et al., 1994). Abscisic acid (ABA) antagonizes JA-ethylene signaling in response to biotic and abiotic stresses (Anderson et al., 2004); however, ABA also elicits JA responses via an interaction with MYC2 transcription factors (Dombrecht et al., 2007). In fact, MYC2, which is the central transcription factor in JA signaling in Arabidopsis, was first reported as an ABA-regulated gene (Abe et al., 2003). Conversely, JA has positive effects on ABA signaling; for example, it modulates the expression of the ABA receptor PYRABACTIN RESISTANCE1-LIKE4 (PYL4) in leaves (Lackman et al., 2011). Cross talk between JA and SA and other signaling pathways, such as ethylene, auxin, gibberellins (GAs), and cytokinins (Pieterse et al., 2012), and the signaling networks with nitric oxide and reactive oxygen species in response to pathogen attack remain topics of intensive investigations (Sato et al., 2010).

In *N. attenuata*, synergistic or antagonistic interactions of plant hormones such as SA, ethylene, brassinosteroids, auxin, and GAs with JA signaling in defense and development have been reported. Most of these interactions are modulated by insect-derived FACs (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; von Dahl et al., 2007; Onkokesung et al., 2010; Gilardoni et al., 2011; Yang et al., 2011; Heinrich et al., 2013). Therefore, phytohormone cross talk and FACs have important roles in the organization and execution of optimal defense responses in natural ecosystems in this plant.

In addition to phytohormone signaling, some molecular mechanisms involved in FAC signaling have already been described in *N. attenuata* (typically by using one of the most abundant FACs in *Manduca sexta* OS, *N*-linolenoyl-L-glutamate [C18:3-Glu]). FACs activate mitogen-activated protein kinases, WRKY transcription factors, and more than 500 other herbivory-responsive genes. This large transcriptional response is associated with significant changes in resource allocation and the accumulation of secondary metabolites such as nitrogen-containing compounds (nicotine, TPIs), phenolic compounds (CP), terpenoids (HGL-DTGs, terpenoid volatiles), and fatty acid derivatives (volatile oxylipins; Halitschke et al., 2001, 2003; Giri et al., 2006; Schwachtje et al., 2006; Paschold et al., 2007; von Dahl et al., 2007; Wu et al., 2007;

Gaquerel et al., 2009; Meldau et al., 2009; Kaur et al., 2010; VanDoorn et al., 2010). The analysis of FAC-responsive genes by Super SAGE (Gilardoni et al., 2010) identified a novel LECTIN RECEPTOR KINASE1 (NaLecRK1) that is rapidly induced by FACs and *M. sexta* OS. At a functional level, NaLecRK1 modulates and/or integrates SA and JA signal transduction pathways during herbivore attack in a FAC-dependent manner (Gilardoni et al., 2010, 2011).

Here, we identified another specific FAC (*N*-linolenoyl-L-glutamate)-regulated protein in *N. attenuata* plants, NaHER1 (for herbivore elicitor regulated), which functions downstream of herbivory perception and upstream of JA-regulated defenses and metabolite accumulation. The negative impact of *NaHER1* silencing on free ABA levels and the experimentally demonstrated positive effect of ABA signaling on the accumulation of several antiherbivore defense metabolites in *N. attenuata* highlight the role of ABA in FAC/OS-mediated JA signaling and the essential role played by ABA in plant innate defense against herbivores.

RESULTS

NaHER1 Is Specifically Induced by OS and FACs But Not JA

While searching for herbivory-elicited genes and their regulators, we identified several genes strongly responding to WOS treatment (a standardized mechanical wounding treatment in which the puncture wounds are immediately treated with insect OS) but less to wounds treated with water (WW). In particular, the transcripts corresponding to the *N. attenuata* complementary DNA (cDNA) fragment Na_454_06813 (named *NaHER1*) accumulated strongly and specifically 1 h after WOS but remained at low levels after WW treatment (Fig. 1A). As suggested from its weak response to WW, which in turn is associated with a significant burst of JA in *N. attenuata*, *NaHER1* induction is independent of JA signaling: *NaHER1* was normally induced in *N. attenuata* plants silenced in JA biosynthesis (irLOX3; Kallenbach et al., 2010) and perception (irCOI1; Paschold et al., 2007; Fig. 1C). In addition, *NaHER1* was not induced by the direct application of MeJA to leaves in a lanolin paste (Fig. 1B), even when combined with WW (MeJA can be rapidly deesterified to JA in *N. attenuata* leaves [Wu et al., 2008b], and it is routinely used as functional probe of JA signaling). In contrast, *NaHER1* strongly responded to the synthetic FAC, C18:3-Glu, found in the OS of *M. sexta* larvae (Fig. 1B).

The full-length protein sequence of NaHER1 was used for homologous protein search using BLAST (blast.ncbi.nlm.nih.gov/). Proteins similar to HER1 were found in a large variety of plant species, including tomato (*Solanum lycopersicum*; 68% identity), soybean (*Glycine max*; 44%), poplar (*Populus* spp.; 42%), and Arabidopsis (40%). However, they were mostly classified as predicted, hypothetical, or uncharacterized proteins without specific function (Supplemental Table S1). Only six of 56 hits (E value cutoff of 1e-20) were annotated as (1) calmodulin-binding protein (*Arabidopsis lyrata*) or (2) yeast pheromone receptor protein AR781 (*Medicago truncatula*, *Quercus robur*, and Arabidopsis). However, no direct experimental evidence exists to support a proposed calmodulin-binding function. The pheromone receptor-like protein was up-regulated in *Q. robur* during the premycorrhizal infection phase by the *Piloderma croceum* ectomycorrhizal fungus (Kruger et al., 2004). Although functional mechanisms remain unknown, these responses suggested a possible general function of HER1-like proteins in sensing and regulating biotic stimuli.

Using previously published microarray data (Kim et al., 2011), we extracted additional *NaHER1* expression profiles. Apart from its activation in OS-elicited

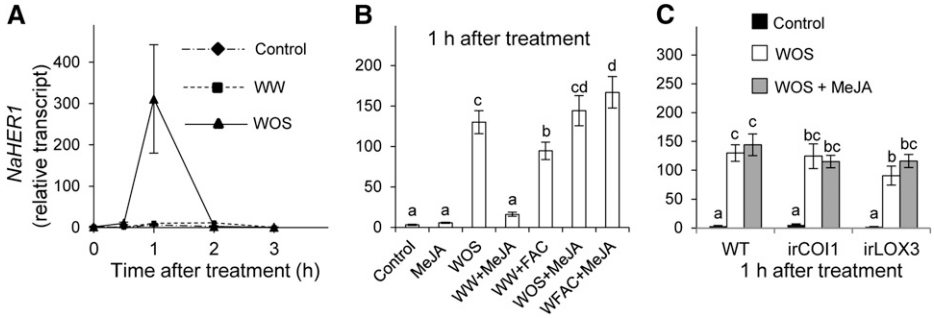

Figure 1. *NaHER1* is regulated by OS and FAC independently of JA. A, *NaHER1* transcript abundances (±SE; $n \geq 3$) in wild-type leaves wounded and immediately treated with 20 μL of water (WW) or 1:5 water-diluted OS (WOS) from *M. sexta* larvae. Samples were collected at the designated time points and analyzed by qPCR. B, *NaHER1* transcript abundances in wild-type plants wounded and treated with OS (WOS) or with C18:3-Glu (0.26 mM) suspended in water (WW+FAC) and complemented with 150 μg (0.625 μmol) of MeJA in 20 μL of lanolin paste as described; 20 μL of pure lanolin was used as a control. Samples were collected after 1 h of treatment. C, *NaHER1* transcript abundances in JA biosynthesis-deficient (irLOX3) and perception-deficient (irCOI1) plants after WOS and WOS + MeJA treatments; samples were collected after 1 h of treatment. Different letters show significant differences determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P \leq 0.05$). WT, Wild type.

leaves, *NaHER1* was strongly but transiently induced in systemic untreated leaves and moderately elevated in the roots of *N. attenuata* after WOS treatment of rosette leaves (Supplemental Fig. S1). The root transcript abundances detected by microarrays were significantly lower compared with the strong microarray signals found in the leaves (Supplemental Fig. S1). To further explore the function of the *NaHER1* gene in plant defense, we used RNA interference-mediated gene silencing to knock down the expression of *NaHER1* in *N. attenuata*. Three well silenced, single-insert-containing, and independently transformed lines, irHER1-6/4, irHER1-8/6, and irHER1-9/6, showing approximately 6- to 10-fold reduced levels of *NaHER1* expression 1 h after WOS treatment, were selected for further analysis (for more details, see "Materials and Methods" and Supplemental Fig. S2).

irHER1 Plants Are Impaired in Indirect Defense

In the next experiment, we asked if NaHER1 directly connects OS perception to OS-specific plant defenses in *N. attenuata*. *N. attenuata* plants emit a blend of VOCs after herbivore attack that functions as an indirect defense by attracting natural enemies of herbivores (Pare and Tumlinson, 1999; Kost and Heil, 2006; Schuman et al., 2009). Some VOCs are instantly released from the mechanically disrupted tissues after herbivore attack (Pare and Tumlinson, 1999; Kessler and Baldwin, 2001; Huang et al., 2012; Schuman et al., 2012) and frequently modified by the constituents in OS other than FACs (Allmann and Baldwin, 2010). Additional VOCs (e.g. terpenoids) are released from the entire plant, usually within one light cycle of the start of the attack (Loughrin et al., 1994).

Because WW treatment does not induce volatiles such as the strongly FAC- and OS-elicited α-bergamotene in *N. attenuata* (Wu et al., 2008a), examination of indirect defenses in irHER1 plants was used to parse OS (FAC)-specific responses by contrasting WW and WOS treatments. In the systemic leaf (i.e. eighth leaf above the WOS-treated leaf), four groups of VOCs were released at reduced levels from irHER1 plants compared with wild-type plants. These included the green leaf volatiles cis-3-hexenyl-3-methylbutyrate, cis-3-hexenyl-2-methylbutyrate, and cis-3-hexenylbutyrate; the monoterpenes α-terpineol, D-limonene, and β-pinene; the diterpene trans-α-bergamotene; and the benzenoid/phenylpropanoid derivatives methylsalicylate, methylbenzoate, and benzylalcohol. Interestingly, the levels of these volatiles in irHER1 plants equaled the amounts released from WW-treated wild-type plants (Fig. 2), suggesting that the putative OS signal was blocked in irHER1 plants. The local VOC emissions were similarly affected in irHER1 (Supplemental Fig. S3), consistent with the hypothesis that NaHER1 mediates *N. attenuata* responses to OS and/or FACs. When the typically low constitutive/basal levels of volatiles were determined by a newly established sensitive solid phase microextraction protocol, we found no significant differences between irHER1 and wild-type plants in all analyzed compounds (data not shown).

irHER1 Plants Are Less Defended against Attack from *M. sexta* Larvae

Because *NaHER1* was specifically induced by FACs (Fig. 1) and irHER1 plants were compromised in indirect defenses (Fig. 2), we next examined direct defense responses in irHER1 plants, such as the accumulation of secondary metabolites and the performance of *M. sexta* on these plants. We first quantified the levels of the defense metabolites HGL-DTGs (Heiling et al., 2010), CP, DCS (Kaur et al., 2010), and TPIs (Steppuhn and Baldwin, 2007). In addition, the transcripts of two regulatory genes, *NaMYB8* (Kaur et al., 2010) and *NaMYC2* (cDNA fragment Na_454_00400; Supplemental Fig. S4), which is the homolog of the Arabidopsis *MYC2* gene (AB000875; Abe et al., 2003), were determined. Silencing *NaHER1* in *N. attenuata* reduced the expression of *NaMYB8* and *NaMYC2* (Fig. 3B), which translated into significantly reduced levels of CP, DCS, HGL-DTGs, and TPIs in these plants (Fig. 3C). The reduced levels of defense metabolites resulted in the better performance of *M. sexta* larvae fed on the irHER1 plants, which grew up to two times larger on irHER1 plants compared with those reared on wild-type plants (Fig. 3A).

irHER1 Plants Show Symptoms of ABA Deficiency

While studying defense responses of irHER1 plants in the glasshouse, we noticed that irHER1 plants wilted faster than wild-type plants when, accidentally, they were not watered for a few days. This observation suggested an ABA signaling-related function of NaHER1 and a possible connection between ABA and JA in defense against herbivores. To examine this hypothesis, we checked leaf transpiration rates in the glasshouse and found that, indeed, both irHER1 lines had significantly higher transpiration rates compared with wild-type plants (Fig. 4B; ANOVA, Fisher's protected LSD [PLSD] test, $P < 0.05$). We then conducted a water competition experiment by pairing initially size-matched wild-type and irHER1-6/4 plants in one pot to provide equal conditions for both genotypes and observed the growth parameters of the plants under increasing drought stress. The drought symptoms started to develop after 4 d without watering in both wild-type and irHER1 plants; however, at this time point, the irHER1-6/4 plants almost stopped growing while wild-type plants continued to elongate. After 12 d without water, wild-type plants were twice as tall and flowered, while none of the irHER1 plants entered the flowering stage (Fig. 4A). These results suggested that irHER1 plants either had lower ABA levels or were affected in ABA signaling, since ABA sensitivity is well known to regulate water loss (Cutler et al., 2010).

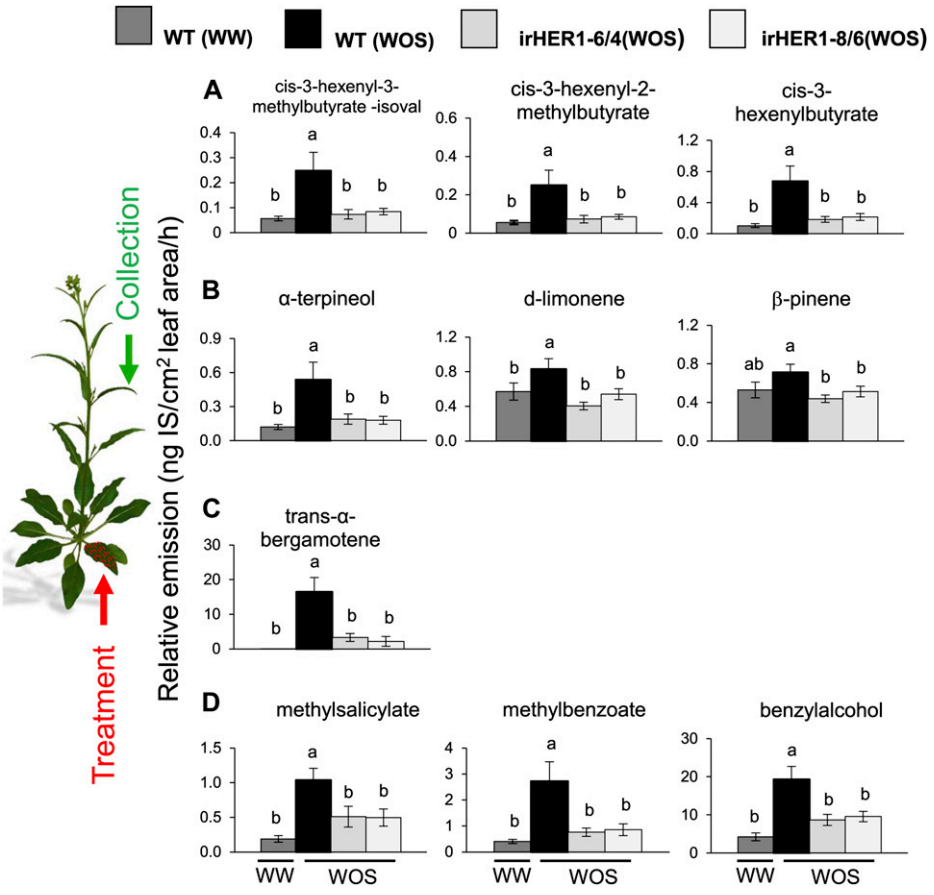

Figure 2. WOS-induced volatile emissions are compromised in irHER1 plants. Relative emission rates (means ± SE; $n \geq 6$) are shown for green leaf volatiles (A), monoterpenes (B), diterpenes (C), and benzenoid/phenylpropanoid derivatives (D) released from the systemic leaves of wild-type (WT) and irHER1 plants treated with WW or WOS. One local rosette leaf in each 44-d-old *N. attenuata* plant was mechanically wounded and treated with 20 μL of water (WW) or diluted OS (WOS) 18 h before the start of volatile collections. Volatiles were collected for 3 h from the head space of a systemic leaf at position +8 relative to the leaf undergoing its transition from source to sink (position 0). Different letters show significant differences determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P \leq 0.05$). IS, Internal standard. [See online article for color version of this figure.]

To examine this possibility, we measured water loss in detached leaves: while the leaves were not different under control conditions, irHER1 leaves lost more water compared with the wild type after spraying a 300 μM ABA solution on the leaves (Fig. 4C; Mann-Whitney *U* test). These data suggested that irHER1 plants are not compromised in ABA biosynthesis but, rather, deficient in ABA perception and/or were able to catabolize the exogenously applied ABA more rapidly, resulting in higher leaf water loss.

irHER1 Plants Accumulate Less JA and ABA

To assess the possible function of NaHER1 in local and systemic signaling, we determined phytohormone and defense metabolite levels in four different parts of the leaves, while only one part was treated with WOS (Fig. 5). A similar system was previously used to study the systemic defense responses in *N. attenuata* leaves (Wu et al., 2007; Stork et al., 2009), providing one local and three systemic samples from each OS-treated leaf. In most zones, the levels of the phytohormones JA and ABA determined 30 min after WOS, and the secondary metabolites chlorogenic acid (CHA), CP, DCS, and HGL-DTGs determined 48 h after WOS, were reduced in both irHER1 lines compared with wild-type plants (Fig. 5). This effect was particularly clear in the case of ABA, which was not limited to the directly wounded sections of the leaf that experienced water loss from open wounds; rather, it affected the entire leaf. The levels of defense metabolites in irHER1 leaf parts (Fig. 5) were consistent with the previous data from the whole-leaf treatments (Fig. 3). Because the basal levels of phytohormones and secondary metabolites did not differ between irHER1 and the wild type (Supplemental Fig. S5), we only focused on and report the induced levels of hormones and metabolites in the following section.

Exogenous JA Application Does Not Restore Defense Metabolite Levels in irHER1

In previous research, we used exogenous applications of MeJA to complement the impaired JA levels of several JA-deficient plant genotypes such as antisense LOX3, irWRKY3, and irWRKY6 (Halitschke and Baldwin, 2003; Skibbe et al., 2008). We also applied MeJA to irHER1 and wild-type plants to test if reduced levels of HGL-DTGs, CP, and DCS and reduced TPI activity are due to the reduced JA and/or JA-Ile levels in irHER1 plants. To our surprise, the local and systemic levels of CP, DCS, and HGL-DTGs in irHER1 were not restored to wild-type levels by a combined WOS and MeJA treatment (Fig. 6). Only in the case of CHA, while still

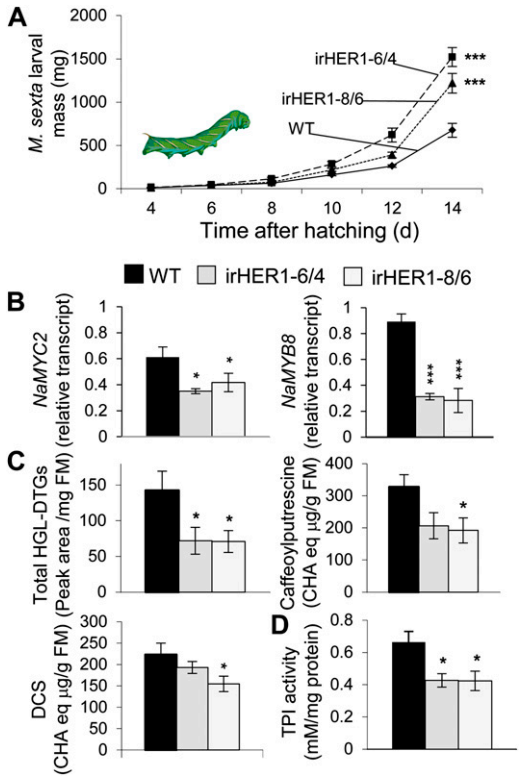

Figure 3. Silencing of *NaHER1* suppressed plant defense metabolite accumulation and increased the performance of the *M. sexta* specialist herbivore. A, Fresh masses (±SE; $n \geq 10$) of *M. sexta* neonates feeding on wild-type (WT) and irHER1 plants recorded at designated time points to estimate the specialist herbivore performance. B, Relative transcript abundances (±SE; $n = 5$) of *NaMYB8* and *NaMYC2* genes determined by qPCR. C, Accumulations (means ± SE; $n = 5$) of secondary metabolites CP, DCS, and total HGL-DTGs in wild-type and irHER1 leaves fed by *M. sexta* neonates for 4 d. D, TPI activity (means ± SE; $n = 5$) determined in local WOS-treated leaves 24 h after elicitation. Asterisks represent significant differences between the wild type and irHER1 lines determined by ANOVA followed by Fisher's PLSD post hoc test (*$P \leq 0.05$, ***$P \leq 0.01$). CHA eq, CHA equivalents; FM, fresh mass. [See online article for color version of this figure.]

lower in irHER1, did the difference between wild-type and irHER1 plants become nonsignificant after MeJA treatment. Together with the previously demonstrated lack of regulation of *NaHER1* by JA (Fig. 1, B and C), these results demonstrated that NaHER1 protein regulates JA responses via a pathway that is independent of JA signaling. Since ABA levels were lower in irHER1 leaves (Fig. 5), this phytohormone became the first suspect to investigate.

Silencing the ABA Receptor Partially Phenocopies irHER1 Plants

ABA has been shown to influence both local and systemic responses of maize plants attacked by root herbivores (Erb et al., 2011, 2012). Therefore, we hypothesized that NaHER1 could modulate ABA signaling in an OS-dependent manner. To examine this hypothesis, we silenced *NaPYL4* (cDNA fragment Na_454_17098; Supplemental Fig. S6), an *N. attenuata* homolog of the PYL4 ABA receptor in tobacco (*Nicotiana tabacum*; Lackman et al., 2011). Interestingly, the PYL4 protein is known to be involved in JA signaling during metabolic reprogramming in Arabidopsis and tobacco plants (Lackman et al., 2011).

We used virus-induced gene silencing (VIGS) to test if silencing of *NaPYL4* can affect OS-elicited defense metabolite accumulation in *N. attenuata* plants. In parallel, we silenced *NaHER1* in wild-type plants with an independent VIGS construct to allow for a direct comparison, as the interpretation of VIGS results can sometimes be confounded by the virus (*Tobacco rattle virus*) presence in the inoculated plants (for silencing efficiency of

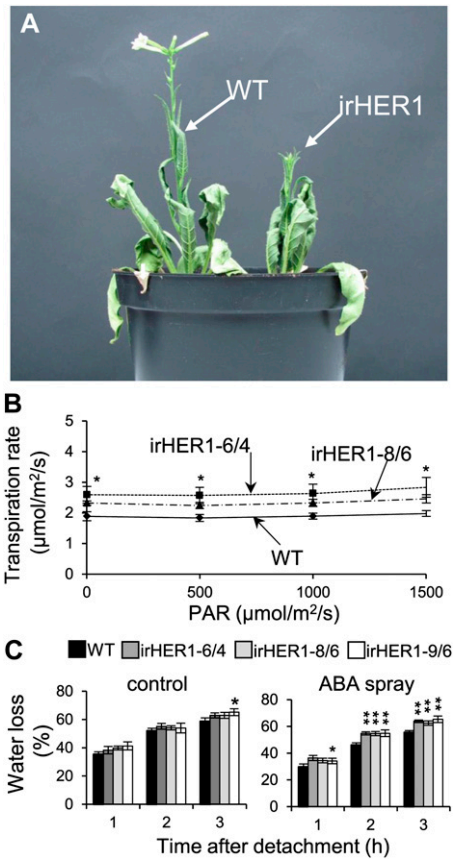

Figure 4. Silencing of *NaHER1* affects transpiration rates and plant growth under drought stress. A, Wild-type (WT; left) and irHER1-6/4 (right) plants after 12 d without watering. B, Transpiration rates (±SE; $n = 4$) determined by an LI-6400 Portable Photosynthesis System in the intact leaves of glasshouse-grown wild-type and irHER1 plants. C, Water loss (±SE; $n = 9$) from detached leaves of wild-type and irHER1 plants determined at designated time points after spraying with 0.5% ethanol (control) or with 0.5% ethanol supplied with 300 µM ABA. Transpiration rates were compared by ANOVA followed by Fisher's PLSD post hoc test; water loss data in C were analyzed by the Mann-Whitney *U* test (*$P \leq 0.05$, **$P \leq 0.01$). [See online article for color version of this figure.]

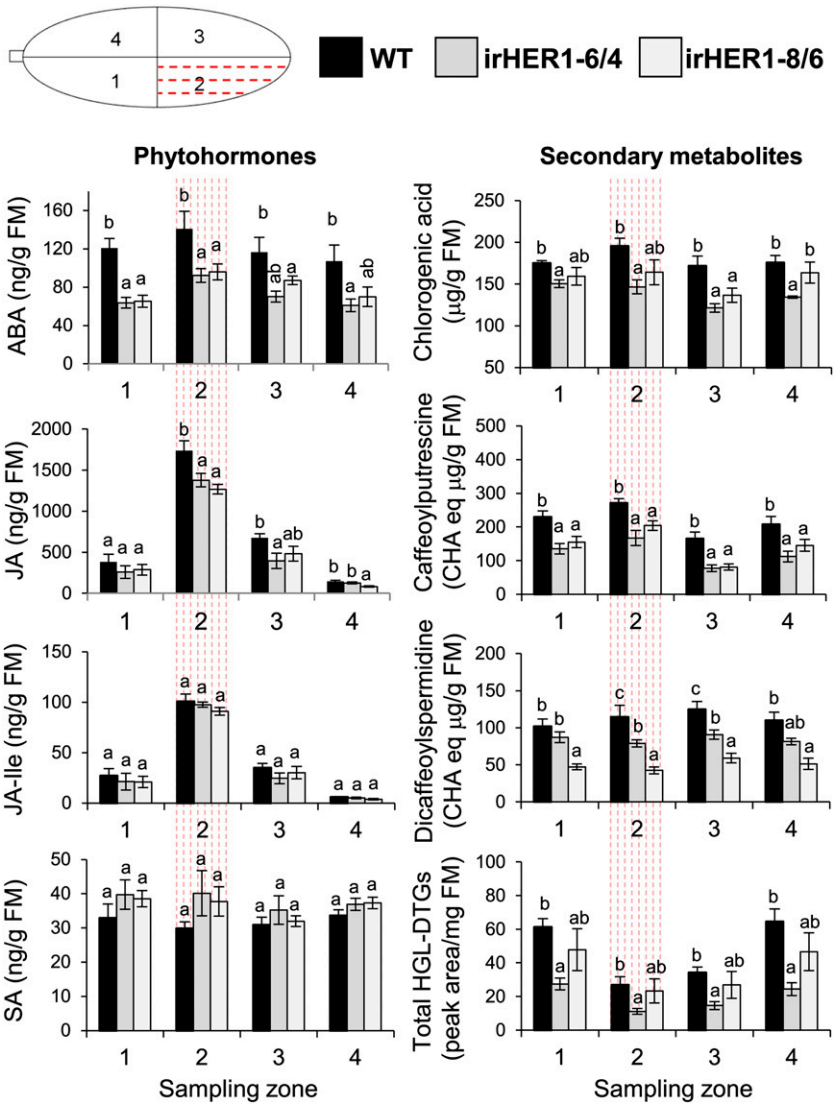

Figure 5. Silencing of *NaHER1* affects ABA, JA, and defense metabolite levels. Rosette-stage *N. attenuata* leaves were treated with WOS in zone 2, and samples were collected after 30 min and 48 h and analyzed by LC-MS/MS (ABA, JA, JA-Ile, SA [±SE; $n = 4$]) and HPLC (CHA, CP, DCS, HGL-DTGs [±SE; $n = 4$]), respectively. Leaves were divided into four equal parts during sampling, and each part was analyzed separately. Different letters show significant differences determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P \leq 0.05$). CHA eq, CHA equivalents; FM, fresh mass; WT, wild type. Red dashed lines show treated areas. [See online article for color version of this figure.]

NaHER1 and *NaPYL4*, see Supplemental Fig. S7). Although reduction in JA and JA-Ile was not statistically significant in *NaHER1*-VIGS plants, possibly due to a lower efficiency or inhomogeneity of silencing compared with stable irHER1 lines, both hormones were significantly reduced 1 h after WOS elicitation in *NaPYL4*-VIGS plants, along with the JA-elicited defense metabolites, CP and DCS, measured 4 d after continuous feeding of *M. sexta* larvae (Fig. 7). These results were consistent with the initial hypothesis that ABA signaling is required for OS (FAC)-triggered defense responses in *N. attenuata*. Interestingly, the levels of HGL-DTGs were not significantly affected in this experiment, prompting a more detailed analysis of these metabolites in the future (see "Discussion").

Exogenous Application of ABA Can Restore Defense Metabolite Levels in irHER1

While treatment with MeJA failed to restore defense metabolites in irHER1 plants, we treated plants with ABA (Pena-Cortes et al., 1989) in an attempt to complement the interrupted NaHER1 function. After ABA treatment, the levels of CHA, CP, DCS, and HGL-DTGs returned to wild-type levels in both local and systemic tissues, and almost no statistically significant differences remained between wild-type and irHER1 leaves sprayed with 100 µM ABA immediately after WOS treatment (Fig. 8). Considering the direct role of ABA in NaHER1 signaling, we examined the transcript levels of *NaHER1* at 0, 1, and 4 h after treatment with 100 µM ABA, but the levels were unchanged compared with mock treatment (Supplemental Fig. S8), consistent with the specific elicitation of *NaHER1* by FACs.

Silencing of *NaHER1* Promotes ABA Catabolism in Leaves

Endogenous ABA levels, like those of most phytohormones, are tightly regulated by a dynamic balance of both biosynthesis and catabolism, which is influenced by development, biotic, and abiotic stresses

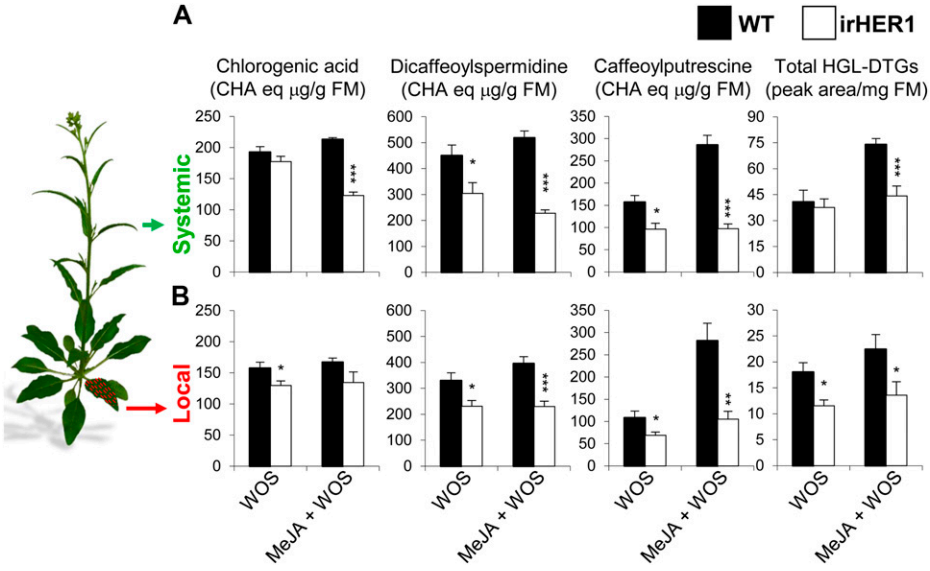

Figure 6. Local and systemic defense metabolite levels in irHER1 cannot be complemented by exogenous MeJA. Local (A) and systemic (B) accumulations (means ± SE; $n \geq 4$) are shown for CHA, DCS, CP, and total HGL-DTGs in 44-d-old *N. attenuata* plants that had their rosette leaves mechanically wounded and treated with 20 μL of diluted OS from *M. sexta* (WOS) in combination with MeJA in lanolin paste as described. After 72 h, local and systemic leaves (+8 position) from each plant were collected and analyzed by HPLC. Asterisks represent significant differences between wild-type (WT) and irHER1-6/4 plants determined by Student's *t* test: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. CHA eq, CHA equivalents; FM, fresh mass. [See online article for color version of this figure.]

(Cutler and Krochko, 1999). In addition, ABA levels are determined by hormone transport between roots and leaves (Seo and Koshiba, 2002; Jiang and Hartung, 2008; Umezawa et al., 2010; Seiler et al., 2011). To address a possible role of *NaHER1* in ABA biosynthesis, we identified and quantified transcript levels of *NaABA1* (cDNA fragment Na_454_00091; Supplemental Fig. S9) encoding a putative *N. attenuata* zeaxanthin epoxidase in ABA biosynthesis using *Nicotiana plumbaginifolia* (X95732) and Arabidopsis (AT5G67030) zeaxanthin epoxidase genes as baits in a BLAST search (Marin et al., 1996; Nambara and Marion-Poll, 2005; Wang

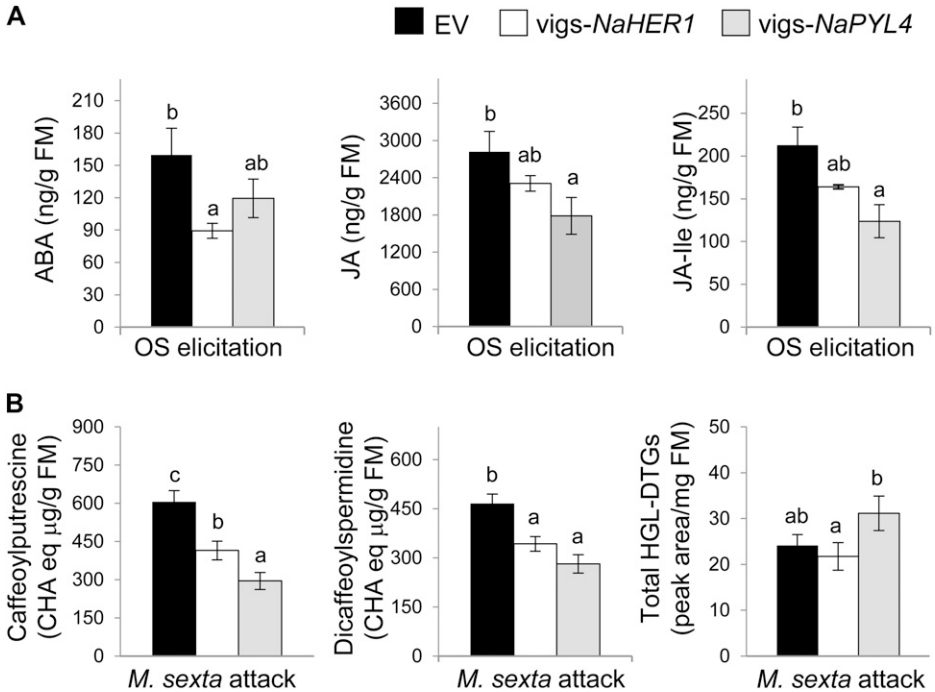

Figure 7. ABA is required for the induction of OS-dependent defense metabolite accumulation. *NaHER1* or *NaPYL4* (ABA receptor) were silenced by VIGS in wild-type *N. attenuata* plants. A, Phytohormone (ABA, JA, JA-Ile) contents (means ± SE; $n = 6$) in mechanically wounded leaves treated with 20 μL of diluted OS from *M. sexta* (WOS), collected after 1 h, and analyzed by LC-MS/MS. B, Secondary metabolites (CP, DCS, HGL-DTGs) in the leaves attacked by *M. sexta* neonates (one per leaf) for 4 d and analyzed by HPLC. Different letters show significant differences between samples determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P \leq 0.05$). EV, Empty vector. CHA eq, CHA equivalents; FM, fresh mass.

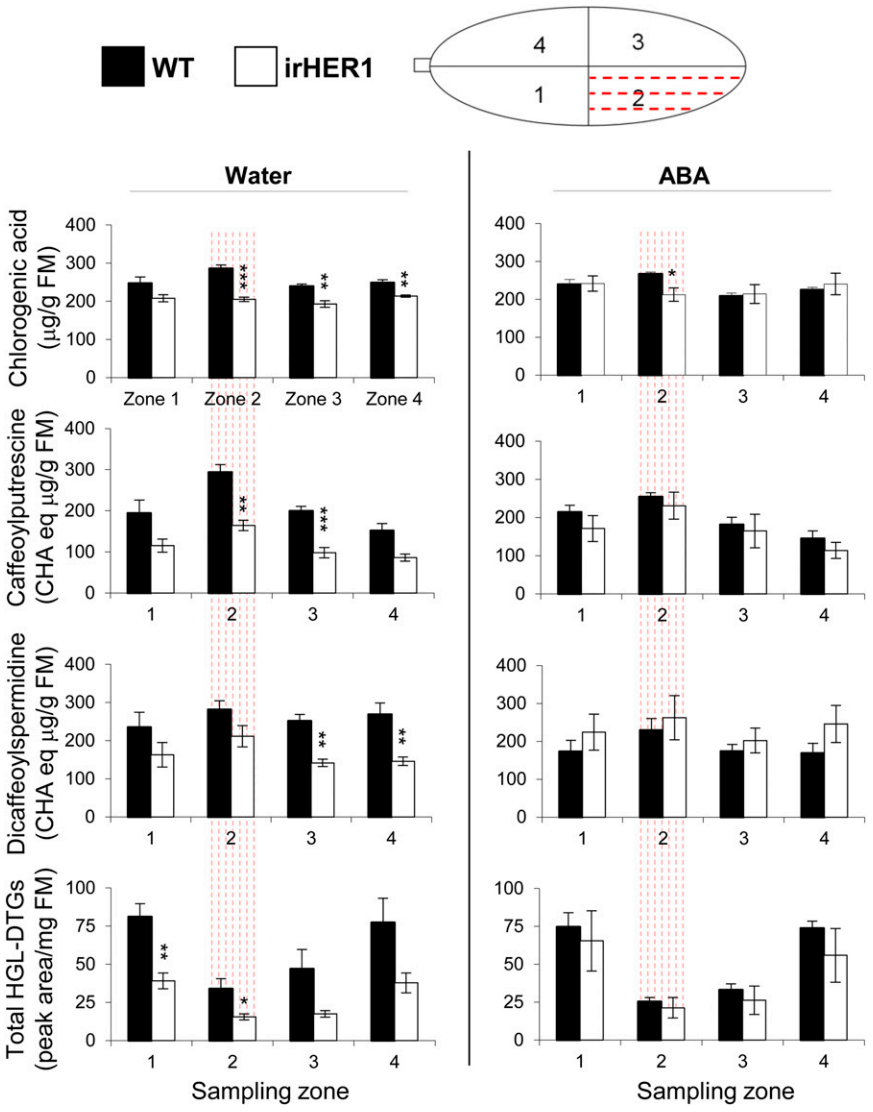

Figure 8. Exogenous application of ABA restores local and systemic defense metabolite levels in irHER1 leaves. Secondary metabolite contents (means ± SE; $n = 4$) are shown for rosette-stage wild-type (WT) and irHER1 leaves wounded with a pattern wheel in zone 2 and treated with 5 μL of diluted OS from *M. sexta*. Immediately after treatment, leaves were sprayed with 0.5% (v/v) ethanol in water (control) or 100 μM ABA diluted in 0.5% ethanol to complement ABA deficiency in irHER1 plants. Samples were collected after 48 h and analyzed by HPLC. Asterisks represent significant differences ($P \leq 0.05$) between wild-type and irHER1-6/4 plants determined by Student's *t* test: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CHA eq, CHA equivalents; FM, fresh mass. [See online article for color version of this figure.]

et al., 2011). In addition, we quantified the transcript levels of *NaPDR12* (cDNA fragment Na_454_00067; Supplemental Fig. S10), which is a putative homolog of the Arabidopsis ATP-binding cassette transporter PLEIOTROPIC DRUG RESISTANCE TRANSPORTER12 (AtPDR12/ABCG40) involved in ABA transport (Kang et al., 2010). However, the transcript levels of *NaABA1* and *NaPDR12* were not consistent with any of the observed differences in ABA levels in wild-type and irHER1 plants (Supplemental Fig. S11), suggesting that NaHER1 may not be directly involved in ABA biosynthesis. However, cloning and expression analysis of several other *N. attenuata* genes involved in ABA biosynthesis, and their enzyme activities, remain to be analyzed to further test this hypothesis. At this point, alternative mechanisms, such as accelerated ABA catabolism, were considered and examined in the following experiment.

To enhance endogenous ABA metabolism, we detached the leaves of irHER1 and wild-type plants and let them desiccate on a filter paper for up to 3 h. When we analyzed ABA metabolites, irHER1 plants accumulated more phaseic acid (PA), dihydrophaseic acid (DPA), and ABA-Glc ester (Supplemental Fig. S12), suggesting an accelerated catabolism of ABA in detached irHER1 leaves. To further enhance the levels of endogenous ABA as well as *NaHER1* expression, we placed the petioles of detached leaves into 2.0 mL of control solution (0.5% mannitol + 0.02% OS from *M. sexta* + 0.5% ethanol) or the same control solution supplemented with 100 μg of ABA for 3 h. ABA was metabolized into four major metabolites, 7-hydroxy-ABA, 9-hydroxy-ABA, neophaseic acid, and PA. Interestingly, all metabolites attained much higher levels in irHER1 leaves compared with those of wild-type leaves. The levels of DPA, although not significantly changed by exogenous ABA feeding in the wild type, were also significantly higher in irHER1 leaves (Fig. 9). These results suggested that *NaHER1* silencing may promote ABA catabolism in *N. attenuata* leaves. In other

words, the function of the FAC-regulated *NaHER1* gene is to inhibit ABA catabolism that positively contributes to, and converges with, JA signaling to amplify the accumulation of defense metabolites (Fig. 10).

NaHER1 Silencing Affects Overall Plant Fitness

Because defense responses such as the accumulation of defense metabolites can be costly for plants (Harvell, 1990; Baldwin, 1998), we hypothesized that irHER1 plants would produce more seed capsules than wild-type plants under very low herbivore pressure in the glasshouse as a result of avoiding the growth costs associated with defense metabolite production (Baldwin, 1998; Zavala et al., 2004). In contrast to our expectations, irHER1 plants were delayed in flowering and produced fewer seed capsules (and flowers at some stages) than the wild type (Supplemental Fig. S13;

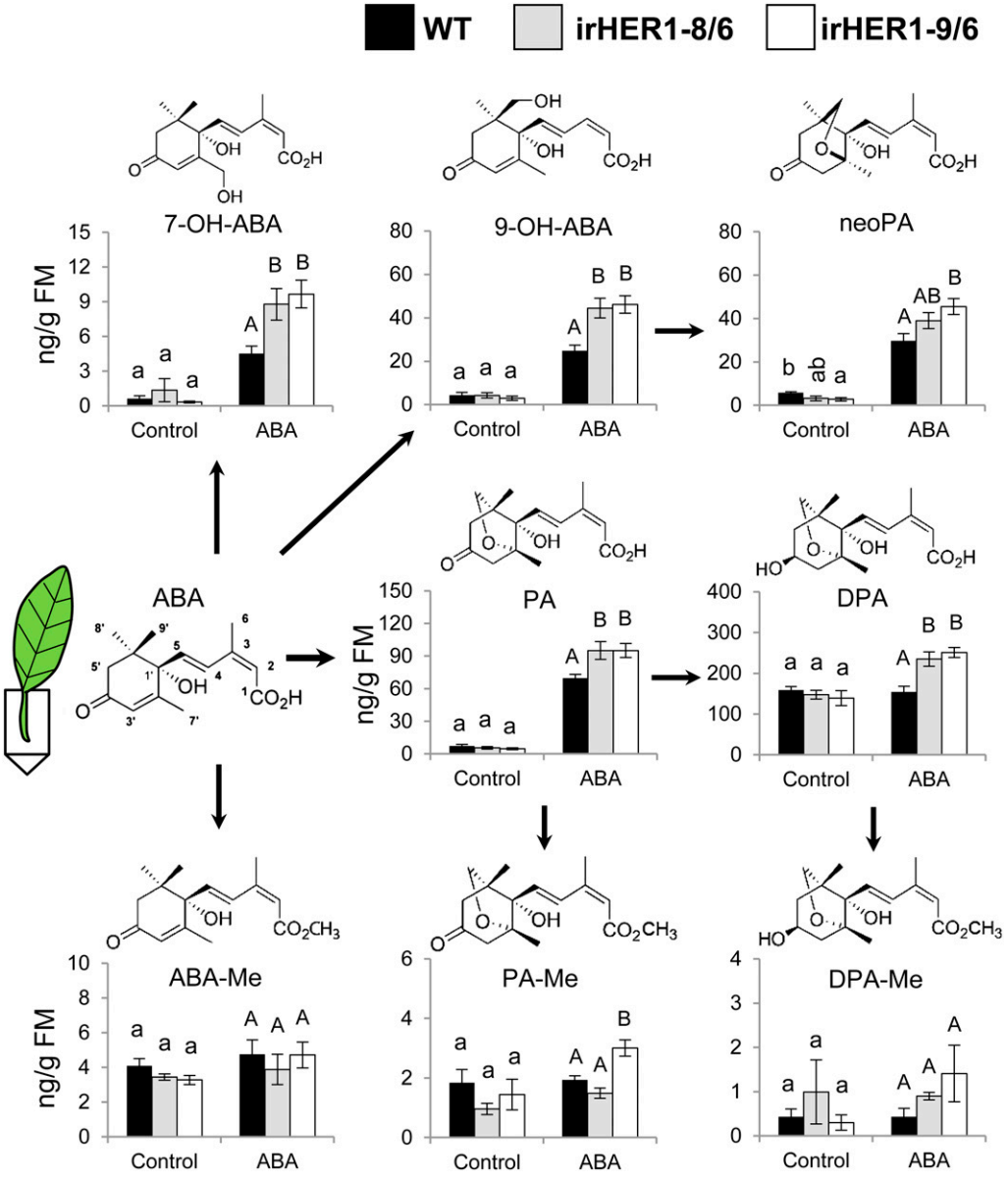

Figure 9. *NaHER1* silencing alters ABA metabolism in *N. attenuata*. Accumulations (means ± SE; $n = 3$) are shown for ABA-derived metabolites in detached leaves of wild-type (WT) and irHER1 plants supplied via petiole with 2 mL of 0.5% (v/v) ethanol in water supplemented with 0.5% (w/v) mannitol and 0.02% (v/v) OS from *M. sexta* larvae (control) or with a control solution supplied with 100 μg of ABA. Samples were collected after 3 h and analyzed by LC-MS/MS for individual ABA metabolites. Different letters show significant differences between samples determined by one-way ANOVA followed by Fisher's PLSD post hoc test ($P \leq 0.05$). 7(9)-OH-ABA, 7(9)-Hydroxy-ABA; FM, fresh mass; neoPA, neophaseic acid; Me, methyl ester. The levels of individual ABA metabolites were determined relative to six atoms of deuterium (D_6)-ABA internal standard. [See online article for color version of this figure.]

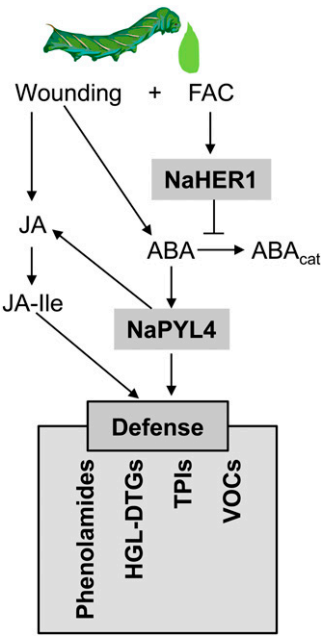

Figure 10. Proposed model of NaHER1 function in *N. attenuata*. The perception and signal transduction of FACs in OS of herbivores induce the expression of the *NaHER1* gene, which, in turn, increases ABA levels by suppressing the catabolism of the hormone (ABA_{cat}). Higher ABA levels promote the accumulation of defense metabolites and JA, which promotes the defense of *N. attenuata* plants and increases resistance against feeding herbivores. [See online article for color version of this figure.]

Mann-Whitney *U* test comparisons). This applied for both control unattacked plants and plants being attacked by *M. sexta* larvae; we assumed that *NaHER1*, despite its relatively low transcript levels detected in noninduced plants (Fig. 1), plays a significant role in a plant's development, possibly due to roles in the regulation of other stresses. In addition, the costs not incurred by irHER1 plants by avoiding the production of defense metabolites when attacked by herbivores were clearly overshadowed by the negative fitness impact of the loss of defense function mediated by NaHER1.

DISCUSSION

During herbivory, a major reconfiguration of plant signaling and metabolism occurs that prevents further mechanical damage and loss of photosynthetic tissues, desiccation from wounds, wound repair and the rebuilding of damaged cell walls, and, in some cases, the bunkering of resources to organs distal and inaccessible to feeding herbivores (Schwachtje et al., 2006; Schwachtje and Baldwin, 2008). Although jasmonate signaling is known to play a key role, it is becoming evident that more than one hormone is required to ensure properly coordinated growth and defense responses in plants (Genoud and Métraux, 1999; Moubayidin et al., 2009; Robert-Seilaniantz et al., 2011; Choudhary et al., 2012). Here, we show, by analysis of the OS/FAC-mediated signaling and the OS/FAC-regulated protein NaHER1, that a typical drought stress hormone, ABA, interacts with JA signaling and enables *N. attenuata* plants to mount a full defense response against chewing herbivores. These data complement the recently recognized role of ABA in regulating JA-mediated defense responses against necrotrophic pathogens (Adie et al., 2007; Fan et al., 2009).

OS/FAC-Mediated Signaling in *N. attenuata*

JA signaling is the core component mediating the elicitation of plant defenses against herbivores. While wound responses in most, if not all, plants are associated with a rapid JA burst, occurring just minutes after mechanical damage, different plants display quite different defense responses when attacked by herbivores. In some plants, such as lima bean (*Phaseolus lunatus*), repeated mechanical damage can closely mimic an herbivory-induced spectrum of VOCs emitted from the plants (Mithöfer et al., 2005), but other plants depend on herbivore-associated molecular patterns to mount herbivore-specific defense responses. For example, mechanically wounded *N. attenuata* plants do not produce substantial amounts of TPIs or VOCs, unless OS containing FAC elicitors are introduced into the wounds (Halitschke et al., 2001; Wu et al., 2007, 2008a), as occurs during caterpillar feeding. Although the accumulation of some other metabolites, such as CP and DCS, also can be increased by wounding, possibly due to their dual role as antimicrobial and antiherbivory substances, these increases are greatly amplified when FACs are introduced into wounds. Interestingly, core forms of the defense metabolites HGL-DTGs undergo a typical conversion into dimalonylated forms, which is triggered by herbivory (Halitschke and Baldwin, 2003; Steppuhn et al., 2004; Kang et al., 2006; Paschold et al., 2007; Steppuhn and Baldwin, 2007; Heiling et al., 2010; Kaur et al., 2010; Woldemariam et al., 2012). Despite a clear role for insect elicitors, such as FACs, in tailoring plant defenses against herbivores, signal perception mechanisms and transduction of the FAC signal are still not well established.

ABA-JA Cross Talk in Herbivory and Pathogen Resistance

Given the complexity of plant defenses, other signaling pathways may strongly interact with OS/FAC signals in plant-insect interactions. SA, ethylene, brassinosteroids, and their signaling pathways have already been shown to act both synergistically and antagonistically with JA during herbivore attack in *N. attenuata* (Voelckel et al., 2001; Rayapuram and Baldwin, 2007; Onkokesung et al., 2010; Yang et al., 2011); a similar level of complex interactions has been shown for ethylene with all other known phytohormones (Chang et al., 2013). The multifaceted role of ABA also suggests

that it may fine-tune biotic stress responses (Ton et al., 2009; Atkinson and Urwin, 2012). For example, plant defense against necrotrophs is known to require both JA and ABA signaling pathways (Adie et al., 2007; Fan et al., 2009). In addition, *Spodoptera littoralis* and *Spodoptera exigua* larvae grew better on Arabidopsis *ABA2-1*-deficient mutants and ABA-deficient tomato plants, respectively (Thaler and Bostock, 2004; Bodenhausen and Reymond, 2007). The application of exogenous ABA and the balance of ABA and SA/JA/ethylene affected rice's defenses against *Hirschmanniella oryzae* (Nahar et al., 2012). Such findings are consistent with a significant role of ABA in regulating plant-herbivore interactions; however, the mechanisms of ABA's role, known as the typical drought-related hormone, in defense against herbivores remain to be understood in detail.

The combination of JA deficiency and impaired ABA accumulation in the WOS-treated leaves of irHER1 plants (Fig. 5) suggested that NaHER1 might regulate two hormone metabolic pathways. Alternatively, NaHER1 could have affected ABA levels, which in turn impaired the accumulation of JA and its associated responses (Fig. 7). Although the changes in hormone levels were relatively modest in NaHER1-silenced plants, we observed dramatic changes in the accumulation of several defense metabolites and resistance to *M. sexta*. This can be explained, for example, by tissue-specific or local accumulation of the hormones, which is likely to be masked by the extraction of entire leaves or their parts during phytohormone analyses. It is also possible that a local change in JA and/or ABA levels is translated into a more distinct change in a yet unknown systemic signal, especially if such changes occur adjacent to or inside the leaf vascular bundles. Indeed, silencing of NaHER1 strongly impaired the emission of volatiles from systemic leaves, and despite that these emissions are known to be JA-Ile dependent, large amounts of JA-Ile are not typically transported between local and systemic leaves in *N. attenuata* (Wang et al., 2008).

Research in potato (*Solanum tuberosum*) and tomato demonstrated that ABA was required for wound-induced JA accumulation (Pena-Cortes et al., 1995), and ABA signaling was also required for normal JA signal transduction (Pena-Cortes et al., 1993, 1995), indicating a close relationship and coordination of these two hormonal pathways. The transfer DNA insertion causing an overexpression of NINE-CIS-EPOXYCAROTENOID DIOXYGENASE5, an important ABA biosynthetic enzyme, not only increases ABA levels but also enhances JA biosynthesis (Fan et al., 2009). When we measured JA and JA-Ile levels 4 d after WOS treatment supplemented with exogenous ABA, JA levels were only marginally higher, but JA-Ile levels were significantly elevated compared with WOS-treated leaves (Supplemental Fig. S14). These results are consistent with a long-term positive effect of ABA on JA-Ile biosynthesis and/or accumulation in *N. attenuata* plants during herbivory, which further enriches the role of ABA in plant defense signaling against herbivore attack.

While higher levels of ABA in the activation-tagged mutant *constitutive disease susceptibility2-1D* compromised Arabidopsis's resistance to various *Pseudomonas syringae* strains, a biotrophic pathogen, these mutants became more resistant to the necrotrophic fungus *Alternaria brassicicola*, consistent with their increased JA levels and the role of ABA in resistance against necrotrophic pathogens (Fan et al., 2009). In addition, ABA regulated the expression of several defense genes, affected JA biosynthesis, and thus contributed to Arabidopsis's defense against another oomycete necrotrophic pathogen, *Pythium irregulare*. While both ABA and JA levels strongly increased upon pathogen infection 6 to 12 h post inoculation, the accumulation of JA was strongly compromised in the ABA-deficient mutant *aba2-12*, thus establishing the role of ABA in defense against necrotrophic pathogen-imposed stress (Adie et al., 2007).

FAC Perception and NaHER1 Are Required for the Contribution of ABA to Defense

The use of the *N. attenuata* ecological model for plant-insect interactions and its strong natural responses to FACs led to the identification of several FAC-responsive genes. Here, the expression of *NaHER1* showed only a weak induction by wounding, but this induction was dramatically amplified (approximately 30-fold) after WOS treatment, which mirrors the response of JA and JA-Ile accumulations to the same treatments but was, surprisingly, independent of JA. In addition, JA and JA-Ile levels were compromised in irHER1 plants, together with the accumulation of several defense metabolites, and plants became more susceptible to attack from the caterpillars of *M. sexta*. Moreover, irHER1 plants showed several ABA-deficient phenotypes.

In tobacco, the overexpression of *NtPYL4*, which is a functional ABA receptor, suggested that cross talk between JA and ABA signaling pathways can affect alkaloid biosynthesis (Lackman et al., 2011). The accumulation of nicotine alkaloids was suppressed in tobacco hairy roots overexpressing *NtPYL4*, suggesting a negative role of ABA in the roots. In leaves, MeJA treatment elicited a transient in *NtPYL4* transcripts with a peak at 30 min. In contrast, in roots, *NtPYL4* expression decreased with a maximum of 5-fold reduction after 2 h of MeJA treatment. This difference in transcriptional responses of the ABA receptor between roots and leaves suggests a different role for ABA in these organs, consistent with the observed positive effect of ABA on leaf defense metabolites (and JA accumulation) found in our study (Fig. 10). While exogenous application of MeJA did not restore wild-type defense metabolites, exogenous ABA treatments recovered wild-type defense metabolite levels in irHER1 plants, indicating that JA signal transduction indeed requires ABA to induce a full defense response. This

was independently confirmed by silencing the ABA receptor *NaPYL4*, which also reduced the accumulation of JA, JA-Ile, and several leaf defense metabolites in these plants (Fig. 7).

At the metabolite level, while CP and HGL-DTGs were strongly induced in the rosette leaves by WOS (compare data in Fig. 5 and Supplemental Fig. S5), the levels of CHA and DCS were rather similar or even lower in 48-h WOS-treated leaves compared with untreated controls. Differences in the accumulation patterns between CP and DCS (or CHA) were already noticed in several other independent studies (Kaur et al., 2010; Onkokesung et al., 2012; Dinh et al., 2013; Woldemariam et al., 2013). This suggests that simulated herbivory attack maintains, in a NaHER1-dependent manner, the higher levels of the "semiconstitutive" DCS (and possibly CHA) metabolites in the naturally aging leaves that can be considered as another part of the *N. attenuata* defense strategy.

A convergence of JA and ABA signaling in Arabidopsis has already been shown at the level of a key transcription factor, MYC2 (Abe et al., 2003; Yadav et al., 2005), pointing to a likely downstream target of JA-ABA cross talk in defense against herbivores. Consistent with this hypothesis, *NaMYC2* transcript levels were reduced in irHER1 plants (Fig. 3), together with another transcription factor, NaMYB8, known to control the biosynthesis of CP and DCS during herbivore attack (Kaur et al., 2010). The cross talk of ABA signaling with signaling pathways such as SA (de Torres Zabala et al., 2009), ethylene (Anderson et al., 2004), cytokinin (Ha et al., 2012), nitric oxide (Hancock et al., 2011), and GAs (Seo et al., 2006) provides additional possibilities for an indirect action of ABA on JA, as the balance of several phytohormones, rather than one hormone alone, often determines the plant responses (Mauch-Mani and Mauch, 2005; Stamm and Kumar, 2010; Peleg and Blumwald, 2011; Chang et al., 2013). For example, the interaction between ABA and SA affects the interaction of rice with *Magnaporthe grisea*, revealing a strong negative impact of ABA on SA-dependent gene expression in rice (Jiang et al., 2010).

While local and systemic CP and DCS levels were consistently reduced in irHER1 plants, total levels of another typical defense metabolite, HGL-DTGs, in *N. attenuata* determined by HLPC-evaporative light-scattering detection (ELSD) showed much less consistent patterns, sometimes being comparable to wild-type levels (Figs. 3 and 5–7). To understand this inconsistency, we conducted additional experiments to analyze the relative amounts of 10 individual HGL-DTGs by highly selective liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in samples showing a comparable level of total HGL-DTGs in irHER1 and wild-type plants. A clear pattern in the accumulation of various forms of HGL-DTGs was observed that provides a plausible explanation for variable HGL-DTG patterns detected by our HPLC-ELSD (Supplemental Fig. S15). In *N. attenuata*, four groups (precursor, core, malonylated, and dimalonylated) of HGL-DTGs exist; while the first three groups showed increased levels in irHER1 plants, only the dimalonylated compounds (nicotianoside II, nicotianoside V, and nicotianoside VII) were reduced. This is consistent with the previously reported conversion of precursor, core, and malonylated HGL-DTGs to dimalonylated HGL-DTGs in response to WOS treatments (Heiling et al., 2010). Because total HPLC-ELSD-detected HGL-DTGs represent a sum of all individual forms, the impaired OS-driven metabolic shift and lack of dimalonylation were likely masked by the increased accumulations of the upstream metabolites.

FAC Perception Regulates ABA Homeostasis via NaHER1

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

In irHER1, the levels of ABA were transiently reduced at 30 min (Fig. 5) to 1 h (Fig. 7) after WOS treatment, which was not reflected in the expression levels of the ABA biosynthetic gene, *NaABA1*, encoding a key biosynthetic enzyme, zeaxanthin oxidase, or the putative *NaPDR12* ABA transporter (Supplemental Fig. S11). In contrast, several ABA-derived catabolites accumulated to higher levels in irHER1 plants, suggesting that a more rapid catabolism of ABA could account for the reduced ABA levels in irHER1. Because this increase was not limited to a single ABA catabolite,

it is likely that NaHER1 simultaneously suppresses the activity of several ABA catabolic enzymes to promote ABA accumulation. This may positively affect JA accumulation and signaling and ultimately promote the accumulation of defense metabolites and defense in herbivore-attacked *N. attenuata* plants (Fig. 10). Further experiments including the identification of ABA catabolic genes, such as microarray experiments with irHER1 and wild-type plants, will help in the future to fully understand the function of NaHER1 and possibly allow for the discovery of novel ABA metabolic genes or their regulators in *N. attenuata*.

NaHER1's Role in Plant Development

ABA metabolism and function are two critical factors involved in plant growth and development. Plants impaired in ABA biosynthesis or sensitivity commonly exhibit severe growth retardation, dwarfing, and wilting phenotypes (Cheng et al., 2002; González-Guzmán et al., 2002; Nambara and Marion-Poll, 2005; Arend et al., 2009). In addition, ABA plays a crucial role in the regulation of gas exchange via the control of stomatal aperture, and it regulates transpirational water loss and desiccation during drought, therefore affecting CO_2 uptake and photosynthesis (Farquhar and Sharkey, 1982; Pei et al., 1998; Schroeder et al., 2001; Gonzalez-Guzman et al., 2012; Kusumi et al., 2012). Although the *NaHER1* gene was induced in a FAC-dependent manner, *NaHER1* silencing apparently caused several other pleiotropic changes in the growth and development of plants in the glasshouse. Higher transpiration rates, accelerated wilting, a delay in flowering time, and reduced seed capsule numbers compared with the wild type (Fig. 4; Supplemental Fig. S13) suggested that *NaHER1* gene may have, first, acquired a role in plant growth and development, which, only later, became associated with OS/FAC signaling and defense responses against herbivores. Interestingly, the OS isolated from *Pieris brassicae* and *S. littoralis* larvae suppressed wound-induced water loss in Arabidopsis (Consales et al., 2012). Because *S. littoralis* OS contains FACs (Maffei et al., 2004), the Arabidopsis homolog of NaHER1 may have promoted ABA levels and reduced water loss from the OS-treated wounds. However, the role of the AtHER1 homolog remains to be investigated.

Because JA signaling is known to affect plant development, especially flowering, seed maturation, and anther dehiscence in Arabidopsis, tomato, and *N. attenuata* (Feys et al., 1994; Li et al., 2004; Paschold et al., 2007), an impaired JA signaling pathway also could have directly contributed to irHER1 reproductive phenotypes, such as delayed flowering and reduced number of seed capsules in the irHER1 plant (Supplemental Fig. S13). Overall, a simple model including the regulation of stomata by NaHER1 and ABA is not sufficient to explain all of the irHER1 phenotypes. For example, wide open stomata with higher transpiration rates should allow for equal or higher emissions of VOCs. In contrast, VOC emissions from irHER1 plants were strongly reduced (Fig. 2), providing strong evidence for NaHER1 and ABA function beyond the control of guard cells, consistent with a direct contribution of endogenous ABA to JA signaling and plant defense.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Plant Treatments

The seeds of *Nicotiana attenuata* wild-type and transgenic plants were sterilized and germinated on Gamborg's B5 medium as described (Krugel et al., 2002). Previously characterized *N. attenuata* transgenic lines silenced in JA biosynthesis (irLOX3) and JA-Ile perception (irCOI1) were used for analysis (Paschold et al., 2007; Heiling et al., 2010; Kallenbach et al., 2010). The irHER1 *N. attenuata* plants silenced in the expression of a novel herbivore elicitor-regulated protein (*NaHER1*) were obtained by transforming the same inbred line of *N. attenuata* used in the previous transformations with an inverted repeat construct containing a 297-bp-long sequence of *NaHER1*, shown in Supplemental Figure S2, A and B. Three homozygous single insertion lines in the T2 generation (irHER1-6/4, irHER1-8/6, and irHER1-9/6) were selected after verification by Southern-blot hybridization (Supplemental Fig. S2C) and growth on hygromycin-containing medium as recommended (Gase et al., 2011). Plants were maintained under standard glasshouse conditions: 16 h of light at 24°C to 28°C, and 8 h of dark at 20°C to 24°C. To simulate herbivory in WOS treatments, leaf lamina were punctured by rolling a serrated fabric pattern wheel three times on each side of the midvein, and the fresh puncture wounds were immediately treated with 20 μL of *Manduca sexta* OS diluted 1:5 in distilled water. Otherwise, wounds were treated with water (WW) or leaves remained untreated as controls (Halitschke et al., 2001).

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

In FAC treatments, C18:3-Glu at a concentration of 0.26 mM, similar to that found in 5-fold-diluted *M. sexta* OS, was used (Halitschke et al., 2001).

In MeJA treatments, 7.5 mg of MeJA was dissolved in 1 mL of melted lanolin, and 20 μL of this lanolin paste, containing an equivalent of 150 μg of MeJA, was applied to the leaf. An aliquot of 20 μL of pure lanolin was used in control treatments (Baldwin et al., 1996; Halitschke et al., 2000).

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

Phytohormone Analyses

Phytohormone extractions were performed as described (Dinh et al., 2013), and individual phytohormones were analyzed by LC-MS/MS on a 1200 Triple-Quadrupole-LC-MS system (Varian). The mobile phases consisted of solvent A (0.05% [v/v] formic acid in water) and solvent B (methanol). The elution profile was as follows: 0:00 to 1:30 min, 15% B in A; 1:30 to 4:30 min, 15% to 98% B in A; 4:30 to 17:00 min, 98% B in A; 17:00 to 18:00 min, 98% to 15% B in A; and 18:00 to 20:00 min, 15% B in A. The flow was as follows: 0:00 to 1:00 min, 0.4 mL; 1:00 to 1:30 min, 0.4 to 0.2 mL; 1:30 to 15:00 min, 0.2 mL; 15:00 to 15:30 min, 0.2 to 0.4 mL; and 15:30 to 20:00 min, 0.4 mL. Multiple-reaction monitoring mode was used to monitor analyte parent ion-to-product ion transitions as described previously (Wang et al., 2007; Owen et al., 2009; Turecková et al., 2009); for detailed information about the collision-induced dissociation energy and precursor (mass-to-charge ratio) and product (mass-to-charge ratio) ions, see Supplemental Table S2. Two atoms of deuterium (D_2)-JA, JA-$^{13}C_6$-Ile, D_6-ABA, and D_4-SA were used to quantify JA, JA-Ile, ABA, and SA accordingly. The relative levels of individual ABA metabolites were quantified by using D_6-ABA internal standard.

Analysis of Secondary Metabolites and Volatiles

Extraction of secondary metabolites was performed as described previously (Dinh et al., 2013). Leaf extracts were analyzed on an HPLC instrument equipped with a PDA detector, essentially as described (Oh et al., 2012). An external calibration curve using an authentic standard with six data points (serial dilutions) was used to quantify CHA contents. CP and DCS contents were quantified using CHA calibration curves and are expressed as CHA equivalents in all graphs. Peak areas were used to estimate total HGL-DTGs contents in HPLC scans detected by ELSD after HPLC separation used for secondary metabolites. VOCs were collected from the head space of selected, treated or systemic, leaves and analyzed as described (Oh et al., 2012) by gas chromatography-mass spectrometry.

Analysis of Gene Expression by Real-Time Quantitative PCR

Total RNA was extracted by TRIzol reagent (Invitrogen) as recommended by the manufacturer and treated with RNase-free DNase-I (Fermentas) to remove all DNA contaminations following the manufacturer's protocol. cDNA synthesis, real-time quantitative PCR (qPCR), and calculation of relative transcript abundances of genes of interest were performed as described (Pfaffl et al., 2002; Dinh et al., 2013). The primer sequences used for qPCR (SYBR Green assays) are listed in Supplemental Figure S16.

VIGS

The cDNA fragments of *NaHER1* and *NaPYL4* (Supplemental Figs. S2A and S6) were inserted into pTV00 vector to create constructs used for the silencing of *NaHER1* and *NaPYL4* expression in *N. attenuata*, respectively. The transformed pTV-PYL4 and pTV-HER1 plasmids were then transferred to *Agrobacterium tumefaciens* for plant transformation. pTV00 plasmid without a gene insert was used as an empty vector control, and pTV00 plasmid carrying a 206-bp fragment of the phytoene desaturase gene from *Nicotiana benthamiana* was used as a visual indicator for silencing efficiency (bleaching leaves).

The *A. tumefaciens* strains containing pTV plasmids were inoculated in YEB medium (10 g L^{-1} yeast extract, 10 g L^{-1} peptone, and 5 g L^{-1} sodium chloride) plus 50 mg L^{-1} kanamycin and cultivated at 200 rpm overnight at 28°C until reaching the optical density at 600 nm of 0.4 to 0.6. Cells were harvested by centrifugation at 4,000 rpm for 12 min at 20°C, and pellets were resuspended in a 10-mL solution of 5 mM $MgCl_2$ and 5 mM MES. Before infiltration, each solution containing the *A. tumefaciens* cells carrying the construct of interest was combined with an equal volume of similarly prepared *A. tumefaciens* culture carrying pBINTRA6 plasmid encoding an intron-disrupted RNA1 of *Tobacco rattle virus* that is essential for virus multiplication.

Approximately 5 d after transfer from small Teku pots to larger 1-L pots with soil (approximately 25 d after germination), *N. attenuata* seedlings were pressure infiltrated into three young expanding leaves with a syringe containing the *A. tumefaciens* solution. After 2 d in dark and high-humidity conditions, the plantlets were grown in the culture room at 20°C to 22°C with a 16-h/8-h light/dark regime until bleaching in pTV-PDS plants had developed and plants were ready for treatment (Galis et al., 2013).

Measurement of TPI Activity

TPI activity was measured by a radial diffusion assay as described (Jongsma et al., 1994). Briefly, plant tissues were ground in liquid nitrogen, and 100 mg of fine powder from each sample in the microcentrifuge tube was suspended in 300 μL of extraction buffer containing 0.1 M Tris-HCl, pH 7.6, 5% polyvinylpolypyrrolidone, 0.2% phenylthiourea, 0.5% diethyldithiocarbamate, and 0.05 M Na_2EDTA (Jongsma et al., 1994). After thorough vortexing and homogenization, samples were centrifuged at 12,000*g* for 20 min at 4°C, and supernatants (100–150 μL) were transferred to a new tube. Cleared supernatants were kept on ice until further analysis. A serial dilution of five bovine serum albumin concentrations, ranging from 0.03 to 0.50 mg mL^{-1}, were used as calibration curves to quantify total protein content in extracts by the method of Bradford (1976). TPI activities were determined by radial diffusion assays using bovine trypsin protease (Sigma) dissolved in agar (50 μg per 25 mL of agar). A series of soybean (*Glycine max*) trypsin protease inhibitors (Sigma) on the same plate, with concentrations ranging from 0.29 to 4.59 μM, were used to generate external calibration curves for TPI quantification (Jongsma et al., 1994).

Statistical Analyses

Data were analyzed with SPSS Statistic 17.0 (SPSS) and INFOSTAT version 2011e (Universidad Nacional de Córdoba). Student's *t* test and ANOVA followed by post hoc Fisher's PLSD were used. Comparisons of means were calculated at a minimal 0.05 level of significance. Data in the water-loss experiment (Fig. 4C) and differences in seed capsules and flower numbers (Supplemental Fig. S13) were analyzed by the Mann-Whitney *U* test.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NaHER1 (full-length CDS, KF321733).

Supplemental Data

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

Supplemental Figure S1. *NaHER1* is systemically regulated after WOS treatment.

Supplemental Figure S2. Sequences, silencing construct, and transgenic plant information.

Supplemental Figure S3. *NaHER1* silencing reduces emissions of several isoprenoid and phenylpropanoid/benzenoid volatiles from local WOS-treated *N. attenuata* leaves.

Supplemental Figure S4. Sequence of *NaMYC2*.

Supplemental Figure S5. Silencing of *NaHER1* does not affect the constitutive levels of phytohormones (ABA, JA, JA-Ile, and SA) and defense metabolites (CHA, CP, DCS, and total HGL-DTGs).

Supplemental Figure S6. cDNA fragment of the *NaPYL4* gene.

Supplemental Figure S7. *NaHER1* and *NaPYL4* silencing efficiency by VIGS.

Supplemental Figure S8. ABA does not induce *NaHER1* expression.

Supplemental Figure S9. Sequence of the *NaABA1* gene.

Supplemental Figure S10. Sequence of *NaPDR12*.

Supplemental Figure S11. Silencing of the *NaHER1* gene does not affect the expression of the ABA biosynthetic gene *NaABA1* or the expression of the ABA transporter *NaPDR12*.

Supplemental Figure S12. *NaHER1* silencing alters ABA metabolism and JA and JA-Ile accumulation in detached leaf.

Supplemental Figure S13. *NaHER1* silencing does not affect rosette diameter but delays flowering time and reduces seed capsule numbers in *N. attenuata*.

Supplemental Figure S14. ABA affects the JA accumulation of OS-elicited plants.

Supplemental Figure S15. Accumulation of individual HGL-DTGs in wild-type and irHER1 plants.

Supplemental Figure S16. Specific primer sequences used in qPCR (SYBR Green) analyses.

Supplemental Table S1. BLAST results of *N. attenuata* HER1 protein against the National Center for Biotechnology Information protein databases.

Supplemental Table S2. Multiple reaction monitoring transitions used for LC-MS/MS quantification of phytohormone levels.

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