Jasmonoyl-L-isoleucine hydrolase 1 (JIH1) contributes to a termination of jasmonate signaling in *Nicotiana attenuata*

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The jasmonate signaling pathway is essential for plant development, reproduction, and defense against herbivores and pathogens. When attacked by herbivores, plants elicit defense responses through the rapid accumulation of jasmonates. Although the transduction of the jasmonate burst into downstream responses has been largely resolved in the past decade, how the jasmonate burst is switched off remained unknown. Recently, two mechanisms that involve cytochrome p450-mediated hydroxylation/carboxylation and NaJIH1-mediated hydrolysis of JA-Ile were identified as major termination mechanisms of JA signaling. Due to a lack of hydrolysis, *Nicotiana attenuata* plants silenced in the expression of the *JIH1* gene accumulated significantly more JA-Ile than did wild type plants and became more resistant to herbivore attack. Although less likely, additional functions of JIH1, such as contributing to the pool of free Ile and thereby increasing JA-Ile accumulation, remained untested. Here we show that increased isoleucine availability does not explain the observed phenotype in JIH1-deficient *N. attenuata* plants.

In the world of complex ecological interactions, optimal performance requires maintenance of homeostasis. For instance, in response to herbivore attack, plants reconfigure their metabolism and produce potent defensive secondary metabolites¹⁻³; a process mediated by the highly conserved jasmonate signaling pathway.⁴⁻⁷ The jasmonate signaling cascade is induced in response to herbivore attack and leads to the rapid accumulation of jasmonic acid (JA) and its bioactive form - jasmonoyl-L-Ile (JA-Ile) - which, through a series of molecular interactions, results in the transcriptomic and metabolic reconfigurations and plant defense responses.⁸⁻¹¹ However, due to the potential metabolic cost of jasmonate-induced defenses,¹²⁻¹⁴ plants tightly regulate the biosynthesis and catabolism of jasmonates.

Generally, plants maintain the homeostasis of active hormone by either conjugating them with small molecules such as sugars or amino acids, or inactivating them through a series of chemical modifications.¹⁵⁻¹⁷ Recently, two *Arabidopsis thaliana* cytochrome p450 enzymes (CYP94B3 and CYP94C1) were demonstrated to attenuate jasmonate responses by catabolizing JA-Ile to its inactive forms, 12-OH-JA-Ile and 12-COOH-JA-Ile. *A. thaliana* plants that ectopically expressed CYP94B3 were male sterile and insensitive to jasmonate-mediated root growth inhibition (and chlorosis); phenotypes reminiscent of jasmonate

deficiency. Conversely, a significant increase was observed in the accumulation of JA-Ile when knockout *cyp94b3* and *cyp94c1* *A. thaliana* plants were wounded, confirming the negative role of these enzymes in the regulation of the herbivore-induced jasmonate burst. However, at later time points, the level of the wound-induced JA-Ile in *Atcyp94b3* plants waned to the basal level, indicating the existence of alternative mechanisms to attenuate the JA-Ile burst.¹⁸⁻²⁰

In our group, we identified a novel hydrolase in *Nicotiana attenuata* and demonstrated its hydrolytic function on jasmonoyl-L-isoleucine in vitro and in vivo (hence named as NaJIH1).²¹ After simulated herbivory, silenced *N. attenuata* plants (irJIH1) accumulated significantly more JA-Ile, and consequently, significantly more defense metabolites (nicotine, 17-hydroxygeranyllinalool diterpene glycosides and proteinase inhibitors) than did wild type (WT) plants. The increased accumulation of defense metabolites correlated with the reduced performance of the specialist (*Manduca sexta*) and the generalist (*Spodoptera littoralis*) herbivores reared on irJIH1 plants. In the field (Great Basin Desert, Utah, USA), we attached *Manduca sexta* eggs to the underside leaves of WT and irJIH1 plants and compared the percentage of eggs predated upon by egg predators (*Geocoris* spp.). We found that irJIH1 plants experienced

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Figure 1. Endogenous level of isoleucine and leucine in WT and irJIH1 plants after simulated herbivory. Fully elongated leaves of Wt and irJIH1 plants were treated with simulated herbivory and the total level of isoleucine and leucine was measured. No significant differences were observed in the accumulation among wild type and transgenic irJIH1 plants.

significantly more predation than did WT plants, results consistent with the higher production of volatile organic compounds by these plants that function as indirect defenses by attracting predators.

Consistent with function of the putative *N. attenuata* AtCYP94b3 homolog, three hours after the simulated herbivory, we observed a significantly higher accumulation of

12-OH-JA-Ile and 12-COOH-JA-Ile in irJIH1 plants compared with WT plants.²¹ Our findings demonstrated the importance of *NaJIH1*, both in maintaining jasmonate homeostasis and affecting the ecological interactions of *N. attenuata* plants. Similar to the results obtained from knockout *cyp94b3* and *cyp94c1* *A. thaliana* plants, the wound/herbivory-induced JA-Ile burst in irJIH1 plants waned to basal levels after 2h, suggesting the highly congruent function of the CYP94B3/CYP94C1- and JIH1-mediated attenuation mechanisms.

In addition to the catabolic processes described above, the accumulation and dynamics of hormones is also affected by the rates of biosynthesis. For example, the amplitude of the herbivore-induced JA-Ile burst is determined by the availability of JAR enzyme and its substrates, jasmonic acid (JA) and isoleucine.²²⁻²⁴ *N. attenuata* plants that were deficient in the activity of the threonine deaminase gene (involved in the biosynthesis of isoleucine) showed a significant reduction in the accumulation of herbivore induced JA-Ile. The accumulations of the herbivore-induced JA-Ile, and the defense secondary metabolites that it elicits, were completely restored by the exogenous application of isoleucine (Ile), indicating the importance of isoleucine availability in mediating JA-Ile burst/responses.²⁴ Hence, the significantly higher accumulation of JA-Ile in irJIH1 plants could be associated with an increased availability of isoleucine in irJIH1 plants compared with WT plants. Physiological processes that may or may not require the hydrolytic function of the JIH1 protein could affect the biosynthesis, transport or

Figure 2. Isoleucine availability does not explain the JA-Ile phenotype in irJIH1 plants. Fully elongated leaves of wild type and irJIH1 plants were wounded with pattern wheel and 0.625 μ mol isoleucine was applied on the wounds. Samples ($n = 4$) were collected 2h, 4h and 6h after treatment and the accumulations of wound-induced jasmonates were determined. IrJIH1 plants still accumulated significantly more (Independent *t* test; $P < 0.05$) JA-Ile (B), OH-JA-Ile (C) and COOH-JA-Ile (D) than wild type plants, while no difference was observed on the level of JA (A). Different letters indicate statistically significant differences.

Figure 3. Comparable amounts of endogenous and ¹³C₆-labeled jasmonates are produced after JA and ¹³C₆-Ile feeding. Fully-expanded leaves (n = 4) of WT and irJRH1 plants were wounded with pattern wheel and treated with 0.625 μmol JA and 0.625 μmol ¹³C₆-Ile (wound + JA + ¹³C₆-Ile). The accumulation of the endogenous (A, B and C) and ¹³C₆-labeled (D, E and F) jasmonates were determined. IrJRH1 plants accumulated significantly more (independent t test; *P* < 0.05) ¹³C₆-labeled and endogenous jasmonates than WT plants, though the levels of the endogenous and labeled jasmonates were similar. Significant differences are indicated by different letters.

partitioning of isoleucine in irJRH1 plants, and consequently, in the higher JA-Ile phenotype in these plants. On the other hand, the higher JA-Ile phenotype could be explained by a complex regulation that involves altered substrate and JIH1 protein localization. Consistent with this prediction, the JIH1 protein was shown to have an HDEL motif that localizes the protein to the endoplasmic reticulum.

Taking these reports into consideration, we asked if the increased availability of Ile in irJRH1 plants, rather than NaJIH1-mediated hydrolysis of JA-Ile could explain the higher accumulation of JA-Ile in these plants. When we measured the level of endogenous isoleucine and leucine in WT and irJRH1

plants treated with simulated herbivory, we found no significant difference (Fig. 1; ANOVA, $F_{2,15} = 1.796$, *P* = 0.200). We hypothesized that, though there was no significant difference in the total endogenous level of isoleucine between WT and irJRH1 plants, there could be differences in the amount of immediately available isoleucine to be conjugated with JA during herbivory. And to test this possibility, we wounded fully-expanded leaves of WT and irJRH1 *N. attenuata* plants with a pattern wheel and treated the wounds with diluted (5X in distilled water) *M. sexta* oral secretions supplemented with 0.625 μmol Ile. Samples were collected 2h, 4h and 6h post-treatment and the levels of JA, JA-Ile, OH-JA-Ile and COOH-JA-Ile were compared in WT and

irJ1H1 plants. We hypothesized that under “unlimited” Ile supply, the differences in JA-Ile burst between WT and irJ1H1 plants should be minimized, if not eliminated.

No significant differences were observed in the JA content between WT and irJ1H1 plants at all time-points. In contrast, despite the exogenous application of Ile both to irJ1H1 and wild type plants, irJ1H1 plants still accumulated significantly more JA-Ile and JA-Ile metabolites than WT plants. Specifically, the content of **JA-Ile** (independent *t* test; 2 h, *t*(5) = 2.716, *P* = 0.04; 4 h, *t*(5) = 3.892, *P* = 0.01; 6 h, *t*(6) = 2.976, *P* = 0.02), **OH-JA-Ile** (independent *t* test; 2 h, *t*(5) = 2.852, *P* = 0.03; 4 h, *t*(5) = 8.713, *P* < 0.001; 6 h, *t*(6) = 7.878, *P* < 0.001) and **COOH-JA-Ile** (independent *t* test; 4 h, *t*(5) = 10.955, *P* < 0.001; 6 h, *t*(6) = 6.023, *P* = 0.01) were significantly higher in irJ1H1 plants than in wild type plants (Fig. 2). These results suggest that the higher JA-Ile accumulation in irJ1H1 plants could not be explained by altered availability of isoleucine in these plants.²⁴

To further test the effect of isoleucine availability on the jasmonate burst and follow the jasmonate flux, we wounded elongated leaves of WT and irJ1H1 plants with a pattern wheel, treated the wounds with a 20 µL mixture of JA and ¹³C₆-isoleucine (0.625 µmol each) and compared the accumulations of the endogenous and ¹³C₆-labeled jasmonates 1 h or 2 h after induction. We reasoned that if increase in Ile (or JA) availability was the reason for the increased JA-Ile accumulation in irJ1H1 plants, WT and irJ1H1 plants would again produce comparable amounts of ¹³C₆-labeled and endogenous JA-Ile (and metabolize them similarly) when we added an additional competitive pool of ¹³C₆-isoleucine and JA.

We observed that unstressed wild type and irJ1H1 plants accumulated very low levels of endogenous and ¹³C₆-labeled jasmonates, but after the treatment, their levels gradually increased. Interestingly, despite the availability of an additional pool of isoleucine and JA, WT and irJ1H1 plants produced different amounts of jasmonates. Similar to our previous report,²¹ irJ1H1 plants accumulated significantly higher levels of jasmonates (JA-Ile, 1 h, *t*(6) = 2.769, *P* = 0.03; 2 h, *t*(6) = 2.577, *P*

= 0.04; 12-OH-JA-Ile, 1 h, *t*(6) = 3.930, *P* = 0.008; 2 h, *t*(6) = 6.472, *P* = 0.001 and 12-COOH-JA-Ile, 2 h, *t*(6) = 2.795, *P* = 0.03) than WT plants. At early time points, the accumulation of JA-(¹³C₆)-Ile in irJ1H1 plants was also significantly higher (1 h, *t*(6) = 2.399, *P* = 0.053; 2 h, *t*(6) = 0.889, *P* = 0.408) than in WT plants. Similarly, irJ1H1 plants accumulated significantly more metabolites of JA-(¹³C₆)-Ile than WT plants (12-OH-JA-(¹³C₆)-Ile, 1 h, *t*(6) = 2.910, *P* = 0.02; 2 h, *t*(6) = 5.665, *P* = 0.001 and 12-COOH-JA-(¹³C₆)-Ile, 1 h, *t*(6) = 3.434, *P* = 0.01; 2 h, *t*(6) = 2.756, *P* = 0.03) (Fig. 3). These results clearly demonstrate that the higher JA-Ile content in irJ1H1 plants is not associated with altered Ile or JA content in irJ1H1 plants. They also reconfirmed our previous report that the hydrolytic function of NaJ1H1 against JA-Ile was a major contributor to the waning of JA-Ile burst.

Recently, two close homologs of NaJ1H1 were identified in *A. thaliana* (AtIAR3 and AtILL6) and were demonstrated to function in the attenuation of the wound-induced JA-Ile. Interestingly, these hydrolases also hydrolyzed 12-OH-JA-Ile, though less efficiently.^{25,26} Further experiments are required to directly test the hydrolytic activity of NaJ1H1 against conjugates/metabolites of JA-Ile and study the ecological relevance of this mechanism. Such comparative studies of the functional conservation of attenuation mechanisms among different families of plants raise interesting questions: are the cytochrome p450-mediated and J1H1/ILL6-mediated attenuation mechanisms the only mechanisms of attenuating the jasmonate burst? Are there other JA-derived molecules used in attenuating the jasmonate signaling? How conserved are these mechanisms in other families of plants?

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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