

# The rapidly evolving associations among herbivore associated elicitor-induced phytohormones in *Nicotiana*

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**Keywords:** ABA, herbivore associated elicitors, JA, JA-Ile, SA, JA-SA correlation, JA - JA-Ile correlation, *Nicotiana*, phytohormone

**Abbreviations:** herbivore associated elicitors, HAE; jasmonic acid, JA; jasmonic acid isoleucine, JA-Ile; salicylic acid, SA; abscisic acid, ABA.

In response to herbivore attack, plants perceive herbivore associated elicitors (HAE) and rapidly accumulate jasmonic acid (JA) and other phytohormones, which interact in complex ways, such as the crosstalk between JA and salicylic acid (SA). Although recent studies have shown that HAE-induced individual phytohormones can be highly specific among closely related species, it remains unclear how conserved and specific the relationships among HAE-induced phytohormones are. Here we analyzed the correlations among 4 different phytohormones, JA, JA-isoleucine (JA-Ile), SA, and abscisic acid (ABA) in 6 closely related *Nicotiana* species that were induced by 3 different HAEs. Our results showed that while no clear association between ABA and other phytohormones were found, the positive association between JA and JA-Ile is mostly conserved among closely related *Nicotiana* species. Interestingly, the association between JA and SA are highly variable and can be regulated by different HAEs.

In response to herbivore attack, plants induce rapid phytohormonal changes through the perception of chemical cues (herbivore-associated elicitors: HAE) in insect oral secretion (OS).<sup>1</sup> The phytohormonal responses include rapid accumulations of jasmonic acid (JA) and its derivatives, JA-Ile, which are the key plant hormones responsible for the activation of defense responses against most insect herbivores in plants.<sup>2-4</sup> At same time, HAE can also induce accumulations of other phytohormones, such as SA, ABA and ethylene (ET). These different phytohormones can play either synergistic and/or antagonistic roles in defensive reactions.<sup>4</sup> For example, the accumulation of SA, which plays a key role in defending against attack from piercing-sucking insects and biotrophic pathogens,<sup>3-6</sup> can antagonize the accumulation of JA and JA induced anti-herbivore defensive functions.<sup>7,8</sup> In addition to the antagonistic interaction of JA-SA, ABA was shown to have synergistic effects with JA on anti-herbivore defenses in *Nicotiana attenuata*.<sup>9</sup> Therefore, the specificity of HAE-induced defense responses are thought to be depend on both induced individual phytohormones and their crosstalk.<sup>4,10,11</sup> While HAE-induced individual phytohormonal responses were known to be highly variable among plant species and herbivore-dependent,<sup>10-12</sup> how conserved and specific the relationships are among induced phytohormones remains unclear. Here, by analyzing our recently published phytohormone data that were collected from the leaves of 6 closely related

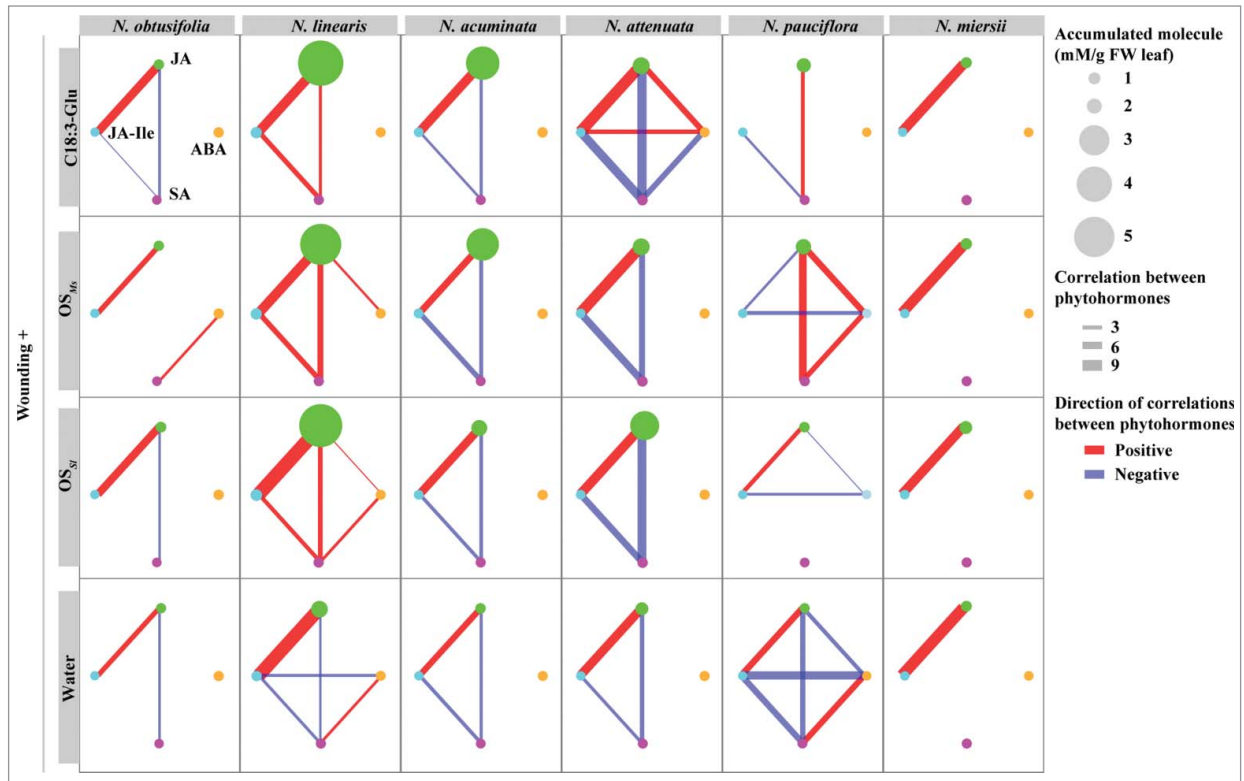
*Nicotiana* species,<sup>11</sup> we specifically investigated 2 questions: 1) how conserved are the relationships among HAE-induced phytohormones from closely related plant species? 2) can HAE elicitation regulate the relationships among phytohormones within a species?

We calculated the pair-wise correlations among the accumulations of 4 phytohormones (JA, JA-Ile, SA and ABA) within 2 hours of being induced by different HAEs in leaves of 6 closely related *Nicotiana* species.<sup>11</sup> Since our aim was to investigate the HAE-induced phytohormonal associations, we excluded the un-induced samples (control samples at 0 h). We found that while ABA did not correlate with other phytohormones in most of species, JA-Ile and SA showed interesting patterns of correlations with JA. JA-Ile was positively correlated with JA among most of the *Nicotiana* species (Fig. 1) and among different HAE elicitation. The highly conserved positive correlation between HAE-induced endogenous JA and JA-Ile likely reflects their biosynthetic relationship in which JA-Ile is a conjugation product of JA and isoleucine (Ile).<sup>13</sup> The only exception was *N. pauciflora* induced by C18:3-Glu (FAC) and *Manduca sexta* oral secretion (OS<sub>MS</sub>), which showed no or slight negative correlation between JA and JA-Ile. This is mainly due to the increased JA levels at later time points (2 h after elicitation), but no increase in JA-Ile levels,<sup>11</sup> indicating that the genes related to the metabolisms of JA and JA-Ile, such as jasmonate-resistant (JAR)<sup>14</sup> or

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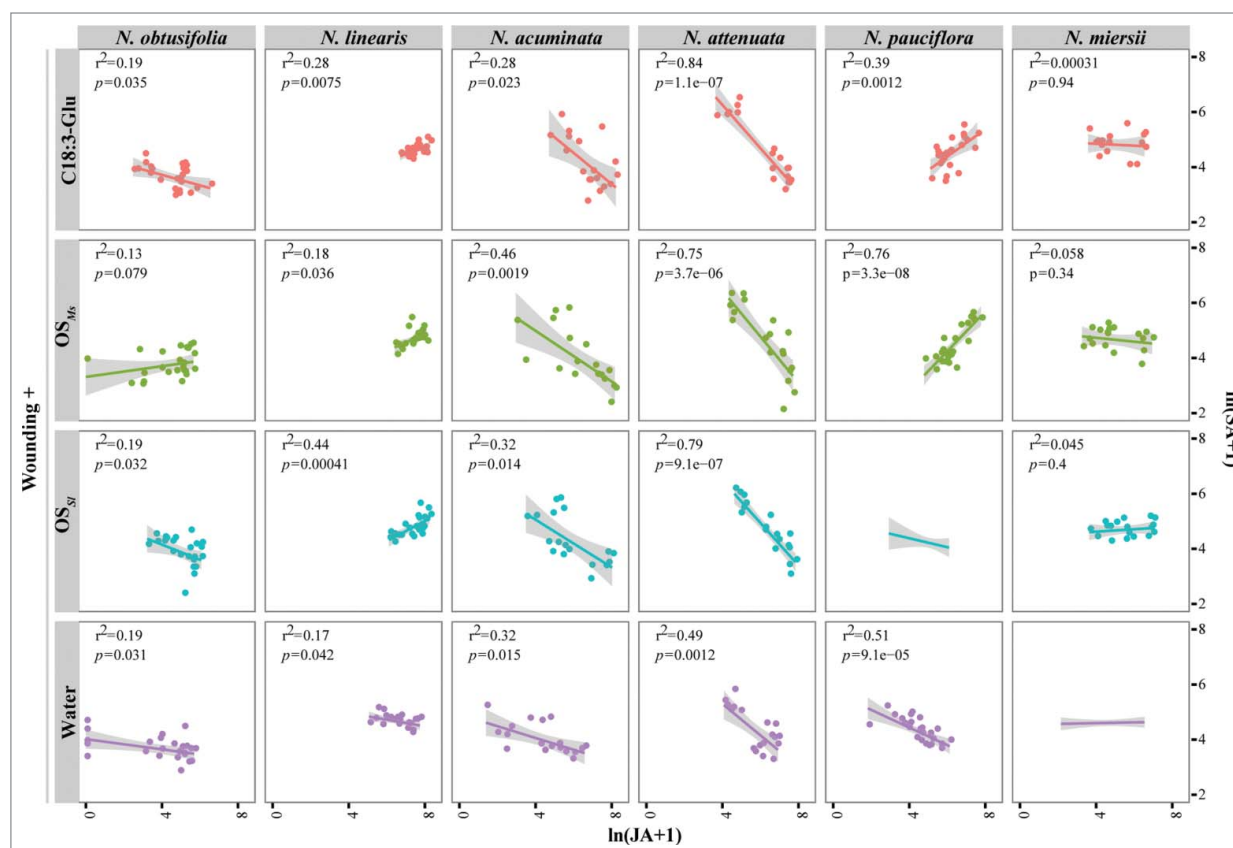
**Figure 1.** Correlations among the accumulations of 4 HAE-induced phytohormones. Each column represents different species, and each row represents treatments with different HAEs. FAC: C18:3-Glu, the most active fatty acid conjugate found in *Manduca sexta* oral secretion;  $OS_{Ms}$ : *M. sexta* oral secretion;  $OS_{Sl}$ : *Spodoptera littoralis* oral secretion. Within each plot, the circles represent different phytohormone molecules. Green: JA; light blue: JA-Ile; pink: SA; orange: ABA. The size of the circle indicates the total amount of accumulated JA within 2 h of elicitation. The correlated phytohormones ( $P < 0.05$ , linear regression) were connected with lines, with color and thickness referring to the direction and significance of the correlation, respectively. Blue color indicates negative correlations and red color indicates positive correlations. The thickness of the lines indicates the value of  $-\log_{10}(P\text{-value})$ , while the  $P$ -value was calculated using linear regression on 2 different phytohormones that were logarithmic transformed as  $f(x) = \ln(x + 1)$ .

jasmonoyl-L-isoleucine hydrolase (JIH)<sup>15</sup> are regulated by FAC or  $OS_{Ms}$  in *N. pauciflora* at later time points. However, further experiments to measure the HAE-induced regulations of these candidate genes are needed to falsify this hypothesis.

SA's correlation with JA was found to be highly variable. When treated with only wounding and water, all species except *N. miersii* showed a strong antagonistic JA-SA relationship (Fig. 2, bottom row). Interestingly, HAE inductions regulated the correlation of JA-SA differently among the different species. After HAE elicitation, while the correlation between JA and SA remained negative in *N. obtusifolia*, *N. acuminata* and *N. attenuata*, results consistent with previous studies using transgenic approaches,<sup>7,16</sup> this negative relationship was reversed in *N. linearis* and *N. pauciflora* (Fig. 2, second and fourth column). Interestingly, the regulations on JA-SA correlation within each species were largely consistent among different HAEs (Fig. 2).

The observed variations on JA-SA relationships have 3 implications. 1) Consistent with the patterns found among different *Asclepias* spp.,<sup>12</sup> HAE-induced JA and SA accumulations in *Nicotiana* can be regulated independently. Although at a qualitative level, the induction of JA and SA were mostly positively correlated - when JA is induced, SA is usually also induced<sup>11</sup> - at

quantitative level, however, no consistent relationships between JA and SA were found among species (Fig. 1). This is likely due to the fact that JA and SA were induced at different times. For example, after HAE elicitation, the highest JA accumulated was at 30 minutes in most of studied *Nicotiana* species, except *N. pauciflora*, however, from our data the highest SA accumulation was at 2 h in most of species.<sup>11</sup> 2) The antagonistic associations between wound-induced JA and SA elicitation can be rewired when HAE are introduced into wounds. The fact that the negative correlations between JA and SA, representing the general JA-SA antagonism frequently reported in plants,<sup>8</sup> can be either enhanced or reversed in different *Nicotiana* species after HAE elicitations (Fig. 1 and 2) indicates that other factors induced by HAE in *Nicotiana*, such as ethylene might contribute to the regulations of the antagonistic effects between JA and SA.<sup>17-19</sup> Further investigations on HAE induced ethylene levels would provide insights into how did HAE regulate the association between JA and SA. 3) The HAE-induced JA and SA association can evolve rapidly among closely related plant species, since closely related *Nicotiana* species can have different induced JA-SA associations (Fig. 2). Although the antagonism between JA and SA are thought to have evolved early in the evolutionary



**Figure 2.** The HAE-induced JA-SA correlations among 6 closely related *Nicotiana* species. Each column represents different species, and each row represents treatments with different HAEs. The color indicates the different HAEs. Vermillion: C18:3-Glu, the most active fatty acid conjugate found in OS<sub>Ms</sub>; green: *Manduca sexta* oral secretion (OS<sub>Ms</sub>); light blue: *Spodoptera littoralis* oral secretion (OS<sub>Sl</sub>); purple: water; The amount of JA and SA were  $f(x) = \ln(x + 1)$  transformed. Linear regression and r-square were shown in each plot; the ribbon represents 95% confidence interval.

history of angiosperm,<sup>8</sup> the breakdown of antagonisms was also reported in *Zea mays*<sup>20</sup> and milkweed.<sup>8,12</sup> Our study further suggests that such deconstructions of the antagonistic relationships between JA and SA might be more frequent than previously thought.

In summary, our data showed that among closely related *Nicotiana* species, while HAE-induced positive association between JA and JA-Ile is mostly conserved, the induced association between JA and SA can be regulated by HAE elicitation and can evolve rapidly among closely related species.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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