Running head: Jasmonate dynamics in 35S-jmt plants

Corresponding author: Emmanuel Gaquerel
Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Hans Knöllstr. 8 Jena 07745, Germany.

E-mail address: egaquerel@ice.mpg.de

Tel: +49 (0)3641 571131, Fax: +49 (0)3641 571102

Journal Area: Plants interacting with other organisms
Ectopic expression of *AtJMT* in *Nicotiana attenuata*. Creating a metabolic sink has tissue-specific consequences for the jasmonate metabolic network and silences downstream gene expression

Michael Stitz, Klaus Gase, Ian T. Baldwin, Emmanuel Gaquerel*

Max Planck Institute for Chemical Ecology, Department of Molecular Ecology
Hans-Knöll Straße 8 07745 Jena, Germany

*Corresponding author email: egaquerel@ice.mpg.de; fax +49-3641-571102

The author responsible for distribution of materials integral to the findings presented in this article is: Ian T. Baldwin (baldwin@ice.mpg.de).
Financial source: Max Planck Society

Corresponding author:
Emmanuel Gaquerel
E-mail: egaquerel@ice.mpg.de; fax +49-3641-571102

The author responsible for distribution of materials integral to the findings presented in this article is: Ian T. Baldwin (baldwin@ice.mpg.de).
To create a metabolic sink in the jasmonic acid (JA) pathway, we generated transgenic *Nicotiana attenuata* lines ectopically expressing *Arabidopsis thaliana* JA O-methyltransferase (*AtJMT*, 35S-*jmt*) and additionally silenced in other lines the *N. attenuata* methyl jasmonate (MeJA) esterase (35S-*jmt*/*ir-mje*) to reduce the de-esterification of MeJA. Basal jasmonate levels did not differ between transgenic and wild type (WT) plants; however, after wounding and elicitation with *Manduca sexta* oral secretions (W+OS), the bursts of JA, jasmonoyl-isoleucine (JA-Ile) and their metabolites that are normally observed in the lamina, midvein and petiole of elicited WT leaves were largely absent in both transformants but replaced by a burst of endogenous MeJA that accounted for almost half of the total elicited jasmonate pools. In these plants, MeJA became a metabolic sink which affected the jasmonate metabolic network and its spread to systemic leaves, with major effects on 12-oxo-phytodieonic acid, JA and hydroxy-JA in petioles and on JA-Ile in laminas. Alterations in the size of jasmonate pools were most obvious in systemic tissues, especially petioles. Expression of *threonine desaminase* and *trypsin proteinase inhibitor*, two JA-inducible defense genes, was strongly decreased in both transgenic lines without influencing the expression of JA biosynthesis genes which were uncoupled from the W+OS-elicited JA-Ile gradient in elicited leaves. Taken together, this study provides support for a central role of the vasculature in the propagation of jasmonates and new insights into the versatile spatio-temporal characteristics of the jasmonate metabolic network.

**Keywords:** jasmonic acid O-methyltransferase, methyl jasmonate, metabolic sink, systemic signaling, jasmonates, *Nicotiana attenuata*. 
INTRODUCTION

Plant cells, both those proximal to a wound site as well as those in more distal locations respond to tissue damage with large-scale changes in their transcription and metabolism, changes which help to promote survival of the entire plant (Reymond et al., 2000; Yan et al., 2007; Koo and Howe, 2009). Oxylipins, derived from fatty acid oxidation, play a central role in these responses: some function as direct defenses (Weber et al., 1999), others elicit the expression of defense related genes (Browse, 2005; Gfeller et al., 2010), or act as synomones in tritrophic interactions (Allmann and Baldwin, 2010). Jasmonic acid (JA) and its different derivatives collectively referred to as jasmonates, are the oxylipins whose biosynthesis and functions have been best characterized. They regulate responses to biotic and abiotic stresses (Baldwin et al., 1994; Devoto and Turner, 2005; Browse and Howe, 2008), but they also control different developmental processes throughout the life cycle of higher plants, including seed dormancy, flower morphogenesis and fruit formation (Creelman and Mullet, 1995; Hause et al., 2000; Stintzi and Browse, 2000; Li et al., 2004).

Initial steps in jasmonate biosynthesis occur in plastids and are associated with the quick biochemical activation of specific lipases and oxidizing enzymes upon tissue damage. Trienoic fatty acids released from lipid membranes are first dioxygenated by 13-lipoxygenase enzymes to form the oxylipin 13-HPOT. The combined catalytic action of allene oxide synthase (AOS) and allene oxide cyclase (AOC) converts 13-HPOT to cis-OPDA (12-oxo-phytodienoic-acid). The next catalytic steps require the export of OPDA from plastids and its import into the peroxisome where it is reduced by OPDA reductase enzymes (OPR). Three subsequent cycles of \( \beta \)-oxidation finally yield JA, which is transported to the cytoplasm and serves as a precursor for the synthesis of a broad range of JA derivatives (Gfeller et al., 2010). Our understanding of JA biosynthesis is largely biased towards the transcriptional regulation of JA biosynthesis genes (Howe and Schilmiller, 2002; Paschold et al., 2008) and several transcription factors modulating the accumulation of JA (Chung et al., 2008; Skibbe et al., 2008). However, fatty acid precursor availability is another essential determinant of the flux and metabolism of the JA pathway. Moreover the nature of the rate-limiting steps may also be context dependent. For instance, over expression of \textit{AOS} in Arabidopsis and \textit{Nicotiana tabacum} or \textit{AOC} in tomato did not increase basal JA levels, but amplified the JA burst that is elicited in response to mechanical wounding (Laudert et al., 2000; Stenzel et al., 2003; Miersch et al., 2004). JA levels in leaves of many plant species increases within a few minutes after mechanical damage, which is too rapid to result entirely from the transcriptional regulation of JA biosynthesis genes. In
Nicotiana attenuata, the model of this study, this early metabolic control is at least partly provided by the biochemical activation of a specific glycerolipase, NaGLA1, which supplies fatty acid substrate for herbivory-induced JA accumulation (Kallenbach et al., 2010).

Multiple competing enzymatic reactions controlling JA metabolism have been described. The resulting jasmonate signatures have been shown to differ between different tissues (Hause et al., 2000; Glauser et al., 2008) which suggest that different tissues may differentially metabolize JA. In tomato, much of the wound-induced JA synthesis has been proposed to take place in vascular tissues where JA biosynthesis enzymes seem to be preferentially located (Hause et al., 2003; Wasternack, 2007). Formation of JA’s amino acid conjugates such as jasmonoyl-isoleucine (JA-Ile) is mediated by JAR in Arabidopsis and its homologues in other plant species (Staswick et al., 2002; Staswick and Tiryaki, 2004; Kang et al., 2006). JA-Ile promotes the interaction of the F-box protein Coronatine insensitive1 (COI1) with jasmonate ZIM-domain (JAZ) repressors and in turn, their targeting for proteolytic degradation in the proteasome (Chini et al., 2007; Thines et al., 2007). Less is known about the potential signaling functions of the different jasmonates produced upon wounding and whether the different reactions controlling their production also help regulating JA-Ile homeostasis.

Other modifications of JA include methylation to methyl jasmonate (MeJA), which increases JA’s volatility and capacity to cross membranes. Over-expression of \textit{O-methyltransferases} targeting for instance auxin, gibberellins or salicylic acid, has been used to manipulate hormonal homeostasis and conversely uncover new signaling outcomes (Qin et al., 2005; Varbanova et al., 2007). In Arabidopsis, the expression of jasmonic acid \textit{O-methyltransferase} (\textit{AtJMT}) responsible for MeJA formation is developmentally regulated. Similar to JA biosynthesis and defense related genes, \textit{AtJMT} is also induced upon wounding or in response to JA application (Seo et al., 2001). Arabidopsis plants over-expressing \textit{AtJMT} contain elevated levels of MeJA and normal levels of JA but display constitutively up-regulated expression of defense and JA biosynthesis related genes (Seo et al., 2001).

The approach most commonly used to analyze jasmonate production \textit{in planta} entails silencing or knock-out mutations in JA biosynthesis genes. However, these loss-of-function approaches, while useful for the analysis of downstream consequences of interrupting specific steps in the JA pathway, \textit{per se} cannot fully unravel the multiple control mechanisms that enable plants to constantly adjust their JA synthesis to its signaling output. Here, we redirect JA metabolism in \textit{N. attenuata} towards the formation of MeJA, without disrupting the whole JA pathway, by ectopically expressing \textit{AtJMT} (35S-\textit{jmt}). We further reduced the de-
esterification of MeJA in 35S-jmt plants by additionally silencing in other lines the expression of *N. attenuata* methyl jasmonate esterase (NaMJE) and examined the consequences of these genetic manipulations on jasmonate kinetics, metabolic networks, pools and downstream gene expression.
RESULTS

Generation of transgenic plants

In Arabidopsis thaliana (At), the homologue of Brassica campestris and Brassica rapa NTR1 (Nectarin1) genes has been shown to encode for a jasmonic acid O-methyltransferase (AtJMT, At1g19640) (Seo et al., 2001). The AtJMT gene-product methylates JA, but not OPDA, and its constitutive expression resulted in strongly increased basal endogenous MeJA levels (Seo et al., 2001). To re-route JA metabolism and evaluate the consequences for the regulation of the complete octadecanoid pathway, we generated stable transgenic lines expressing AtJMT in sense orientation under the control of the CaMV 35S promoter. Two independently transformed homozygous 35S-jmt lines (35S-jmt-1 and 35S-jmt-2) with comparable alterations in floral style elongation (see below, Fig. 1B) were selected. The first line, 35S-jmt-1, harbored a single T-DNA insertion, the second one, 35S-jmt-2, harbored two detectable T-DNA insertions as revealed by Southern-blot analysis (Fig. S1). To prevent MeJA de-esterification, we further created transgenic lines ectopically expressing AtJMT in which the expression of N. attenuata methyl jasmonate esterase (NaMJE) was additionally silenced. NaMJE had been previously cloned and functionally characterized in our group (Wu et al., 2008). As for 35S-jmt plants, two independently transformed homozygous 35S-jmt/ir-mje lines with similar alterations in flower morphology (35S-jmt/ir-mje-1 and 35S-jmt/ir-mje-2) were selected. The first line, 35S-jmt/ir-mje-1, harbored a single T-DNA insertion, the second line 35S-jmt/ir-mje-2 harbored three T-DNA insertions (Fig. S1).

All transgenic lines were indistinguishable from WT plants during rosette stage growth under controlled growth conditions (Fig. 1A). As is commonly reported for many mutants and transgenic lines altered in JA metabolism or signaling (Wasternack, 2007), flowers of 35S-jmt and 35S-jmt/ir-mje morphologically differed from those produced by WT plants. For all transgenic lines selected, style elongation was reduced to approximately half of that in WT flowers and the opening of the corolla limbs was impaired (Fig. 1B). Transcript measurements with gene-specific primers confirmed high relative expression levels of AtJMT in 35S-jmt-1 and 35S-jmt/ir-mje-1 but not in untransformed WT plants (Fig. 1C). After wounding and application of Manduca sexta oral secretions (W+OS), the expression of NaMJE was comparably high in 35S-jmt-1 and WT leaves and reduced by 95% in 35S-jmt/ir-mje-1 leaves.
35S-jmt and 35S-jmt/ir-mje plants have altered JA methylation and MeJA demethylation activities

We next verified that transgenic lines had deregulated JA methylation and MeJA demethylation activities. To assess AtJMT activity \textit{in vivo}, we infiltrated 0.5 µg JA into WT, 35S-jmt-1 and 35S-jmt/ir-mje-1 leaf tissues and quantified increases in MeJA levels over a 45 min time period (Fig. 2A). Consistent with the low extent of damage inflicted by the infiltration technique, jasmonate (JA, MeJA and JA-Ile) levels remained relatively low – barely exceeding basal levels – in the leaves of all three genotypes after infiltration of a control solution (‘Mock infiltration’, Fig. 2A, 2B and S2A). MeJA levels increased in JA-infiltrated leaf discs of 35S-jmt-1 and 35S-jmt/ir-mje-1 – respectively up to 60-fold and 170-fold the levels of mock-infiltrated leaves – but remained as low as after mock infiltration in WT leaves. The formation of other JA derivatives such as JA-Ile was antagonized compared to in WT leaves (Fig. S2B). Consistent with different de-esterification rates, MeJA accumulation was greater in 35S-jmt/ir-mje-1 – attaining at 45 min 150 ng, 167-fold WT levels – than in 35S-jmt-1 plants – which attained at 45 min 30 ng, 32-fold WT levels.

The impairment of MeJA de-methylation in 35S-jmt/ir-mje-1 was verified by measuring, as described above, the turnover of infiltrated MeJA (Fig. 2B). For simplicity, we do not describe here accumulation patterns of JA metabolites produced after MeJA de-esterification (Fig. S2B). While MeJA infiltration into WT and 35S-jmt-1 leaf laminas resulted in a rapid (0.5 min after infiltration) increases of free JA levels, the release of JA was, at the same time, reduced by ~ 80% in MeJA-infiltrated leaf areas of 35S-jmt/ir-mje-1 (Fig. 2B): JA levels in 35S-jmt/ir-mje-1 attained 45 min after infiltration only 23 % of the levels observed in WT and translated into lower amounts of JA derivatives (Fig. S2B). JA levels in MeJA-infiltrated 35S-jmt leaves were lower by up to 30 % compared to WT and the MeJA-to-JA conversion efficiencies did not differ among the three sampling times. These observations suggested that de-esterified MeJA was quickly re-methylated in 35S-jmt-1 leaves.

To evaluate the importance of this reaction in both 35S-jmt-1 and 35S-jmt/ir-mje-1, we performed the same experiment as above using 0.25 µg of synthetic MeJA labeled with $^{13}$C on the two first carbons and on the methylester group ([1, 2, 13-$^{13}$C]MeJA). Similar differences were detected between 35S-jmt/ir-mje-1, 35S-jmt-1 and WT plants during [1, 2, 13-$^{13}$C]MeJA infiltration (Fig. 2C), consistent with the above-mentioned results. High resolution mass measurement of [1, 2, 13-$^{13}$C]MeJA-infiltrated leaf discs revealed the production of a $m/z$ signal at 227.155 characteristic for [1, 2-$^{13}$C]MeJA (absolute $\Delta m/z \text{calculated}-m/z \text{measured} = 3.2$ ppm) formed by re-methylation of the de-esterified [1, 2, 13-$^{13}$C]MeJA in 35S-jmt-1 and 35S-jmt/ir-
AtJMT ectopic expression depletes W+OS-induced JA levels

From the above results, we concluded that both transformants had increased JA methylation activities and that the flux of compounds through the methyl transferase out-competed that through the intact MJE activity in 35S-jmt and the residual MJE activity in 35S-jmt/ir-mje-1. In N. attenuata, applying M. sexta oral secretions to puncture wounds in leaves (W+OS) is known to elicit a transient JA burst which wanes to basal levels in approximately 2 h. In comparison to JA and other jasmonates, the accumulation of MeJA remains marginal in resting tissues as well as after W+OS elicitation (von Dahl and Baldwin, 2004). To confirm that AtJMT activity depleted W+OS-induced JA pools, we compared JA and MeJA basal and maximal levels (1 hour after W+OS elicitation) in leaf lamina of WT and the two 35S-jmt and 35S-jmt/ir-mje lines (Fig. 3). 35S-jmt and 35S-jmt/ir-mje plants failed to accumulate JA after W+OS elicitation: JA levels in these transformants were reduced to ~ 27 and 30 % respectively of those in WT. In contrast, although MeJA levels in WT were about as low as before elicitation (0.03 nmol g⁻¹ FW⁻¹), they reached 2.9 nmol g⁻¹ FW⁻¹ in 35S-jmt and 7.9 nmol g⁻¹ FW⁻¹ in 35S-jmt/ir-mje after W+OS elicitation. This shift of the JA flux towards methylation did not translate into increased MeJA emissions – less than 0.01 % of endogenous maximum MeJA levels could be recovered from in the headspace of 35S-jmt and 35S-jmt/ir-mje leaves (Fig. S3).

Re-routing JA metabolism in 35S-jmt and 35S-jmt/ir-mje has profound, but differential, effects on jasmonate spatio-temporal accumulation patterns

We examined large-scale alterations in the jasmonate profiles caused by the depletion of the W+OS-induced JA pools (Fig. 4). For each plant, the lamina of one fully expanded leaf close to the rosette base was treated by W+OS on both sides of the midvein and then used as tissue source for the analysis of local responses. Undamaged younger leaves, growing orthostichous to the treated leaf, were selected to examine the systemic responses. Changes in JA metabolism in both treated and systemic leaves were examined after dissection of the leaf into lamina, midvein and petiole. To get a broad perspective on jasmonate production, we
quantified by LC-MS/MS/MS a wide range of the previously reported JA metabolites (Table SI).

Formation of OPDA, the JA precursor, increased in a consistent biphasic manner in the leaf laminas of all 3 genotypes after a single W+OS elicitation (Fig. 4). The second phase in OPDA accumulation occurred in all three genotypes after the waning of the JA burst. Importantly, OPDA levels attained similar levels in WT, 35S-jmt and 35S-jmli/r-mje-1 plants in response to elicitation, indicating that the manipulation of JA metabolism in these two transformants did not modify upstream components of the JA pathway. As observed above, maximum JA levels, detected 1 h after W+OS elicitation in 35S-jmt-1 and 35S-jmli/r-mje-1 plants represented only 49% and 35% of those detected in WT, respectively. The impaired JA burst in both transgenic lines was mirrored by an enhanced and longer lasting MeJA burst that waned more slowly in 35S-jmli/r-mje-1. Similar to JA, MeJA reached its highest levels – ~4.1 nmol g⁻¹ FW⁻¹ in laminas of both 35S-jmt-1 and 35S-jmli/r-mje-1 plants compared to less than 0.05 nmol g⁻¹ FW⁻¹ in WT plants – 1 h post elicitation.

In agreement with the reported high substrate specificity of AtJMT, we did not detect the presence of methylated forms of JA-Ile and OPDA in all tissues examined. Interestingly, decreases in JA-Ile levels were much more pronounced than those detected for JA or other JA-amino acid conjugates including the 12- and 11-hydroxylated derivatives of JA-Ile and 12-COOH-JA-Ile (Fig. S4). The maximum JA-Ile levels in both 35S-jmt-1 and 35S-jmli/r-mje-1 laminas were reduced by ~95% compared to those in WT. Formation of 12/11-OH-JA has been proposed to account for the waning of the JA burst. JA hydroxylation after W+OS elicitation was delayed in 35S-jmt-1 and 35S-jmli/r-mje-1 leaf laminas; 12- and 11-OH-JA levels – here the sum of both is reported as OH-JA – attained WT-maximum levels in the two transgenic lines 4 to 5 h after elicitation, values which are attained in WT laminas after 2 h.

With the exception of MeJA, the accumulation of all other jasmonates was also strongly reduced in the midveins and petioles of treated 35S-jmt-1 and 35S-jmli/r-mje-1 leaves. In the case of OPDA, significant reductions were exclusively detected in petioles where OPDA attained its maximal levels. JA levels in midveins of WT exceeded those measured in leaf laminas and petioles. Levels for JA were those most severely depleted also in the midveins – representing only 19% and 12% respectively, of those detected in WT midveins after 1 h. MeJA levels at this sampling time explained approximately half of the reduction in free JA levels. Interestingly, the increase in MeJA levels was less apparent in the petiole of treated leaves of both lines and accounted only for ~30 and 20% of the detected reduction in JA. JA-Ile accumulation in vascular tissues followed a different response than...
that of JA; JA-Ile was most abundant at the wound sites in WT while strongly reduced in transgenic lines and less abundant in tissues of midveins and especially petioles. As seen before, accumulation of OH-JA in midveins and petioles of the two transgenic lines was also delayed compared to WT plants.

**Jasmonate profiles of systemic petioles most closely match those of elicited leaves**

We examined whether alterations in herbivory-induced jasmonate accumulations also spread to systemic leaves (Fig. 5). For simplicity, we highlight here only the main differences between treated and systemic leaves. Absolute jasmonate levels detected in systemic leaves were except for OPDA, depending on the tissue type, 10 to 60 times lower than in treated leaves and the relative decreases in transgenic lines compared to those in WT were also less pronounced. While JA and especially JA-Ile were the two jasmonates of elicited leaves most severely affected by the redirection of the JA flux towards MeJA in the transgenic lines, the major alterations in systemic leaves were in decreasing order detected for: JA (88 to 92 % reductions of peaking levels), OPDA (84 to 91 %) and OH-JA (53 to 82 %). Unlike in treated leaves, in systemic leaves the highest levels for each jasmonate were consistently detected in petioles, especially for JA, OH-JA and OPDA – for this latter jasmonate maximum levels quantified 5 h post elicitation even exceeded those measured in the three tissues of the treated leaves. JA-Ile was the only jasmonate showing the rapidly waxing and waning profile of the typical JA and JA-Ile bursts of elicited leaves, but its accumulation was less affected in the systemic tissues of transgenic lines than previously observed in the elicited tissues. This observation also correlated with a less pronounced MeJA formation in systemic tissues of transgenic plants.

A hierarchical clustering analysis (HCA) performed on tissue-specific jasmonate profiles further confirmed that although jasmonate accumulation in local and systemic tissues largely behaved independently, only the profile of the systemic petiole WT samples clustered together with that of elicited WT samples (Fig. 6A). Co-expression networks represent alternative and powerful statistical approaches to visualize large-scale modifications in a metabolic cluster (Han, 2008). The co-expression network drawn from major jasmonates measured here confirmed the pattern observed in the HCA: a large uncoupling between jasmonate clusters calculated for elicited and systemic tissues (Fig. S5). In WT plants, a high degree of connectivity, depicting important correlations among the different jasmonates, was seen for the systemic tissues and interestingly only elicited JA and JA-Ile levels, particularly from petioles displayed significant correlations with the jasmonates measured in systemic
tissues. As expected, the topology of this network was strongly distorted in the two transgenic lines (Fig. S5). Most importantly, the topology of the network of the two transgenic lines almost perfectly overlapped, suggesting that silencing of NaMJE provided likely little additional effect to the AtJMT-mediated alterations.

**AtJMT ectopic expression affects jasmonate pools in a tissue-dependent manner**

To determine whether the observed changes in jasmonate levels and the distortions of the jasmonate co-expression network resulted only from the diversion of JA metabolism towards MeJA or also involved deregulation of the jasmonate biosynthetic capacities, we calculated the sum of the 5 main abundant jasmonates detected in the individual tissues for each time point (Fig. 6B). As expected, jasmonate pools of elicited leaf laminas of 35S-jmt-1 and 35S-jmt/ir-mje-1 plants clearly differed in their relative composition from that of WT counterparts, but the sum of jasmonate levels in transgenic plants attained comparable maximum levels after 1h as in WT and vanished more slowly. Important differences in the relative composition and size of the jasmonate pools of local vascular tissues of transgenic lines were also observed when comparing with those of WT plants. But more important, for this comparison, maximum jasmonate pools elicited in midveins and petioles of the two transgenic lines were significantly decreased compared to those of WT – respectively by 44 % ( P < 0.001) and 60 % ( P < 0.001) in 35S-jmt-1 and by 60 % ( P < 0.001) and 54 % (P = 0.014) in 35S-jmt/ir-mje-1.

As expected from the individual kinetics of Fig. 4 and 5, the size of the jasmonate pools elicited in systemic lamina tissues was considerably smaller than in local tissues (~ 0.15 nmol g⁻¹ FW⁻¹ vs more than 8.5 nmol g⁻¹ FW⁻¹ 1 h post elicitation) and did not significantly differ between the two transgenic lines and WT. Compared to WT tissues, relative reductions in jasmonate pools of transgenic plants were more pronounced in systemic than in local vascular tissues. These decreases were most severe in petiole samples of systemic leaves – 85 % reduction in 35S-jmt-1 ( P = 0.03) and 91 % reduction in 35S-jmt/ir-mje-1 ( P = 0.03) after 2 h – and from a quantitative standpoint mainly attributable to decreases in OPDA and JA formation (Fig. 5) which did not directly result from AtJMT-catalyzed MeJA production. This result was best visualized when the relative differences in total jasmonate production in transgenic lines vs WT were plotted for the different tissue types from the local to the distal lamina (Fig. S6).

**AtJMT ectopic expression differentially affects JA biosynthesis and JA-dependent direct defense gene expression**
We conclude from the above results that, at least at the elicitation sites, AtJMT ectopic expression caused a redirection of the JA flux towards MeJA without deregulating the entire JA biosynthetic machinery. To evaluate this conclusion, we quantified transcript levels of central JA biosynthesis genes in W+OS elicited leaf laminas. We also measured the expression of two direct defense and JA-inducible genes, NaTD (Kang et al., 2006) and NaTPI (Horn et al., 2005), as references for the output of jasmonate signaling in the two transgenic lines. For both gene categories, transcriptional analyses were performed at times at which maximum transcript levels have been observed in previous studies (Halitschke and Baldwin, 2003; Halitschke et al., 2004). Maximum levels NaLOX3, NaAOS and NaOPR3 transcripts were similar in all three genotypes (Fig. 7). In contrast, the relative abundance of the two highly JA-induced defense transcripts of NaTD and NaTPI significantly differed before and after W+OS elicitation (Fig. 7), suggesting an uncoupling in the transcriptional regulation of JA biosynthesis and the expression of these two direct defense genes in the transgenic lines.
DISCUSSION

This study demonstrates that ectopically expressing AtJMT in N. attenuata is sufficient to create a strong metabolic sink in the endogenous JA pathway which out-competes endogenous catalytic reactions controlling the availability, bioactivity and catabolism of free JA (Fig. 8). Individual accumulation patterns, especially those of JA-Ile, total jasmonate pools and networks were altered with different degrees of intensity and in a tissue-dependent manner in transgenic lines. This strong redirection of JA metabolism impaired the accumulation of defense-related transcripts but did not affect transcript levels of JA biosynthesis genes in transformed plants.

Creating a metabolic sink in the JA pathway

Methylation is one of the catalytic reactions used by plants to adjust their pools of active hormones to environmental conditions. Conversely, manipulating the scale of this reaction and characterizing its consequences at the metabolic and organismic level has been shown to be a valuable approach to uncover new signaling outputs and to revisit the importance of homeostatic control over metabolite fluxes derived from hormonal signals (Qin et al., 2005; Varbanova et al., 2007; Tieman et al., 2010). By ectopically expressing Arabidopsis JA-specific O-methyltransferase (AtJMT), we engineered a massive redirection of the JA flux towards the formation of MeJA. MeJA hyper-accumulation occurred at the expense of other jasmonates and only after W+OS elicitation, which confirmed that substrate availability, i.e. induced JA formation, was the limiting step for MeJA production in both 35S-jmt and 35S-jmt/ir-mje transformants. Substrate availability exerts an important regulatory function within the JA pathway. For instance, constitutive over-expression of AOS, in tobacco and in Arabidopsis (Laudert et al., 2000), or AOC, in tomato, increased JA and OPDA levels in leaves only after mechanical wounding and in the case of AOC in tomato also dramatically affected the levels and relative ratios of developmentally regulated jasmonates in the different organs of transgenic flowers (Miersch et al., 2004). Our results demonstrate that substrate availability constrains the output of JA metabolism and that its influence varies amongst different leaf tissues.

The expression ‘metabolic sink’ has previously been used when diverting or inactivating the metabolic and/or signaling outputs of biosynthetic pathways (Yao et al., 1995; Li and van Eck, 2007). Decreases in herbivory-induced jasmonate levels reported here that result from the depletion of JA levels by AtJMT, were as large or larger than those of RNAi-based approaches previously used in our group to silence NaLOX3 (Halitschke and Baldwin,
2003) or NaJAR4 and NaJAR6 (Wang et al., 2008) expression. This metabolic sink did not deregulate jasmonate biosynthetic capacities, since no effects were seen in the transcript abundance of JA biosynthesis genes and the accumulation of upstream elements of the JA pathway, such as OPDA. However, we cannot fully rule out that changes had occurred in the activity of enzymes, since in *N. attenuata*, critical steps within the JA pathway are not transcriptionally regulated (Kallenbach et al., 2010). The strength of the metabolic diversion was sustained in 35S-*jmt* by the re-methylation of de-esterified MeJA; this recycling reaction may also explain SA metabolism patterns in *SA-O-methyltransferase (SAMT)* over-expressing lines (Tieman et al., 2010).

In agreement with the low amounts of MeJA accumulating in WT leaves after OS elicitation (von Dahl et al., 2004 and this study), JA methylation activity measured in vivo was extremely low compared to that of the transgenic lines and the re-methylation of de-esterified MeJA did not occur in WT leaves infiltrated with MeJA. The relative contribution of re-methylation to MeJA fate was more important in 35S-*jmt* than in 35S-*jmt/ir-mje* leaves, which highlights the importance of considering both JA methylation as well as MeJA stability when analyzing JA and MeJA accumulation patterns.

**Jasmonate-specific consequences of the metabolic sink**

Arabidopsis plants over-expressing AtJMT contain elevated MeJA levels and normal levels of JA, however the consequences on other jasmonates were not examined (Seo et al., 2001). Our analysis revealed that MeJA accumulation occurred at the expense of other JA-dependent reactions and that the degree of depletion varied among the different jasmonates. Compartmentalization of the enzymes controlling JA synthesis and metabolism within the different cellular organelles may be responsible for these differences. JMT is predicted, since the amino acid sequence lacks an apparent organ-specific transit signal peptide, to act within the cytosol and therefore to strongly compete with the formation of JA-Ile by JAR enzymes which have been shown to be localized in the cytosol (Hsieh et al., 2000). Additionally, the almost complete depletion of the JA-Ile pool may in part have resulted from the reduced NaTD expression which mediates Ile formation (Kang et al., 2006). The contribution of NaTD down-regulation to the depletion of JA-Ile pools may also explain why less pronounced alterations were seen for other JA-amino acid conjugates than for JA-Ile (Fig. S4). An explanation for not having observed developmental alterations resembling those of *N. attenuata* transgenic lines strongly silenced for NaTD expression (Kang et al., 2006) could
likely be that the metabolic sink created in the two transgenic backgrounds affected only inducible levels of expression of this gene and not its developmental regulation.

**Tissue-specific spread of jasmonate bursts and the role of petioles**

Different jasmonates reach their maximum levels in different tissues (Hause et al., 2000; Glauser et al., 2008) and importantly JA-Ile, the bioactive jasmonate, accumulates preferentially at wound sites and was the only jasmonate with a clear “burst” character in distal laminas, as previously shown (Koo et al., 2009). As previously reported in Arabidopsis (Koo et al., 2009) and *N. attenuata* (Wang et al., 2008), JA and JA-Ile in systemic leaves were, depending on the tissue type, 10 to 60-fold lower than in treated tissues. Our results are consistent with a central role played by vascular tissues in JA biosynthesis and the spread of jasmonate bursts. Clustering and network analyses highlighted the particularity of jasmonate profiles of petioles. The jasmonate profiles in petioles resembled those detected in treated leaves and the gradual increases in certain jasmonates preceding JA-Ile accumulation in distal laminas were only observed in unelicited petioles.

Calculation of jasmonate pools at each time point and for the distinct tissues revealed that *AtJMT* ectopic expression translated, only in vascular tissues, into significant reductions of total jasmonate pools, with the largest effects in petioles. We did not find any evidence for significantly higher *AtJMT* expression in vascular which may have directly contributed to the larger depletions of jasmonate pools seen in petioles and midveins. We rather think that these decreases of total jasmonate pools arose both from alterations in jasmonate translocation as revealed from PEX analysis (Fig. S7) and from alterations in *de novo* synthesis. This in turn implies that petioles of both local and systemic leaves may play an important role in the amplification of jasmonate synthesis and in sustaining the spread of jasmonate bursts towards distal tissues. Recent studies have reopened the discussion about jasmonate formation and translocation in vascular tissues (Mielke et al., 2011). Testing this interesting hypothesis would demand an analysis of JA biosynthesis at the transcriptional and enzymatic levels in midvein and petiole tissues of 35S-*jmt*-1 and 35S-*jmt*/ir-*mje*-1 plants.

**JA biosynthesis and direct defense transcript levels are uncoupled in transgenic lines**

JA biosynthesis, at least at the elicitation sites, was not repressed by the sink created within JA metabolism. This hypothesis was verified at the transcriptional level by the absence of differences, before and after induction, for central genes of the JA biosynthesis pathway (Fig. 7). In other plants species, over-expression of *AOS* or *AOC* has also been shown to not
affect significantly the expression of other genes of the JA pathway (Harms et al., 1995). However, these results contrast with studies in Arabidopsis 35S-\textit{jmt} plants reporting constitutively higher transcript levels for JA biosynthesis genes (Seo et al., 2001; Jung et al., 2003). Similarly, a positive feedback effect on SA biosynthesis mediated either by the increase of MeSA itself or by a disruption in the control of the SA-related pool or flux has been observed in \textit{SAMT} over-expressing tomato plants (Tieman et al., 2010). We cannot therefore completely exclude the possibility that the flux of compounds through the JA pathway may have been accelerated in \textit{N. attenuata} in a transcription-independent manner which could not be captured by quantifying individual metabolites. We indeed may have underestimated MeJA levels, which according to the petiole exudate measurements (Fig. S7) and literature reports, may likely be quickly translocated within the plant’s phloem (Thorpe et al., 2007).

The absence of deregulation of JA biosynthesis gene expression, despite strong reduced JA-Ile levels suggests that the transcriptional positive feedback of the JA pathway may be independent of the JA-Ile gradient established after simulated herbivory. Wang et al. (2008) suggested from microarray analyses comparing \textit{N. attenuata} lines silenced for \textit{NaLOX3} and JA-Ile forming genes (\textit{NaJAR4} and \textit{NaJAR6}) that \textit{NaAOS}, \textit{NaAOC} and \textit{NaOPR3} expression was not directly linked to JA-Ile synthesis. In contrast, the abundance of \textit{NaTD} and \textit{NaTPI} transcripts, which have been consistently shown by microarray analysis to be among the most highly JA and JA-Ile inducible genes in \textit{N. attenuata}, was strongly impaired in both transformants and this effect was also observed in systemic lamina tissues of 35S-\textit{jmt}-1 plants (Fig. S8). Such uncoupling between the expression of JA biosynthesis genes from that of “late”-induced defense-related genes has been demonstrated and extensively studied at the temporal and spatial levels in tomato (Howe et al., 2000; Ryan, 2000; Strassner et al., 2002). Moreover, tomato mutants differentially affected in these two classes of genes, as were our transgenic lines, have been reported. For instance, in the \textit{def-1} mutant which is deficient in wound-induced JA accumulation and in turn impaired in \textit{PI} expression, \textit{AOS} expression, as well as that of \textit{LOX-D} involved in JA biosynthesis, is not affected (Howe et al., 2000).

Tomato plants silenced for \textit{Leucine Aminopeptidase A} also show specific alterations in late wound-/JA-responsive gene expression, although JA biosynthesis and perception appears to be intact in these lines (Fowler et al., 2009). Such uncoupling may originate in the aforementioned tomato and \textit{N. attenuata} transformants from alterations downstream of COI1 signaling, for instance from specific deregulations of JAZ levels, since activation of the
expression of these transcriptional repressors, like that of the two aforementioned gene
classes, involves COI1 (Chung et al., 2008; Paschold et al., 2008).

To summarize, ectopically expressing AtJMT in *N. attenuata* dramatically depletes
herbivory-induced jasmonate metabolism in elicited and systemic tissues. These results
support the need for future work analyzing more deeply alterations in defense-related
metabolic processes in AtJMT expressing lines and their consequences on the plant’s
ecological performance.

**MATERIAL AND METHODS**

**Plant material and growing conditions**

Wild-type *Nicotiana attenuata* Torr. ex Watson from an inbred line in its 30\(^{th}\)
generation were used for all experiments. The original seeds were collected in 1988 from an
isolated population at the DI ranch in southwestern Utah, USA. Before germination on agar
plates containing Gamborg B5 media, all seeds were sterilized and incubated with diluted
smoke and 0.1 M GA\(_3\), as described in Kruegel et al. (2002). Plants were grown with a
day/night cycle of 16 h (26°C–28°C) / 8h (22°C–24°C) under supplemental light from Master
Sun-T PIA Agro 400 or Master Sun-TPIA Plus 600-W sodium lights (Philips).

For the generation of 35S-*jmt* plants, the complete cDNA sequences of *AtJMT* (*JA O-
methyltransferase*) and of the *hptII* hygromycin resistance genes were inserted into the
pRES2 transformation vector, both in sense orientation under the control of the CaMV 35S
promoter. To generate 35S-*jmt/ir-mje* transgenic lines, plants were transformed with a vector
that contained additionally to the cDNA sense constructs of *AtJMT* and *hptII*, a cDNA
fragment in inverted orientation of *N. attenuata* methyl jasmonate esterase (Accession
number EU196055) (Wu et al., 2008). Vectors were inserted into *N. attenuata* WT plants’
genome using *Agrobacterium tumefaciens*-mediated transformation (Kruegel et al., 2002).

Homozgyosity of the resulting T\(_2\) plants was determined by screening for the resistance to the
antibiotic hygromycin and the number of insertions was determined as described in (Gase et
al., 2010), by southern blot hybridization of genomic DNA using a PCR fragment of the *hptII*
gene as a probe (Fig. S1).

**Plant treatments**

For all experiments, plant treatments were randomly assigned among rosette stage
plants and the first fully elongated leaf (+1 position) was treated. *Manduca sexta* feeding was
simulated by wounding the leaf lamina with a fabric pattern wheel on both sides of the midrib and immediately applying 20 µl of *M. sexta* OS (diluted 1:10 in water) to the fresh wounds (W+OS); this procedure which is referred to as OS-elicitation, provides a convenient means of accurately standardizing herbivore elicitation of *N. attenuata* plants and allowing for detailed kinetic analyses of the elicitation process. *M. sexta* oral secretions (OS) were collected from 3rd - 4th-instar larvae reared on *N. attenuata* WT leaves as described in Roda *et al.* (2004). Eggs of the tobacco hornworm *M. sexta* were obtained from North Carolina State University (Raleigh, NC, USA).

For jasmonate profiling, leaves of similar size and at the same developmental stage were harvested after W+OS treatment and, before being flash-frozen, rapidly dissected with a scalpel into three distinct tissue types: petioles (the vascular tissue connecting leaf laminas to the plant’s shoot), midveins (the leaf’s major vein acting as a vascular manifold) and the lamina (expanding right and left of the midvein). Petioles were flash-frozen immediately after being detached (less than 10 seconds). The midvein and the right and left sections of the leaf lamina were dissected and flash-frozen within ~ 10 seconds. The treated leaf was analyzed for local responses. The untreated leaf growing on the same plant with a minimal angular distance above the treated leaf and therefore orthostichous to the treated leaf was considered the systemic leaf.

**Phytohormone Analysis**

Approximately 150 mg of frozen tissues were homogenized in a mortar and further pulverized to a fine powder by shaking with 2 steel beads (5 mm) in 2 mL reaction tubes using a Genogrinder® (SPEX Certi Prep, Metuchen, NJ) at a frequency of 1200 strokes/minute for 40 seconds. Jasmonates were extracted by shaking for 3 min with 1 mL ethylacetate containing internal standards (IS) for JA (9,10-2H2-dihydro-jasmonic acid), JA-Ile (jasmonyl-[13C6]isoleucine) and MeJA ([1, 2, 13-13C]methyl jasmonate, synthesized by esterification of [1, 2-13C]JA with 13C methanol as previously described in Zhang and Baldwin, 1997). Samples were analyzed as previously described by Wang *et al.* (2007). Briefly, 15 µL of the resulting extracts were analyzed for jasmonates using a Varian 1200L liquid chromatography-MS/MS system (Varian, Palo Alto, CA, USA) working with an electrospray ionization source (ESI). Negative or positive ionization mode was used depending on the jasmonate structure (Table SI). JA IS was used as internal standard for the quantification of 12 and 11-hydroxy-jasmonic-acid (OH-JA) and 12-oxo-phytodieonic acid (OPDA) and concentrations were adjusted with response factors: peak response factors for
OPDA (1.28) and OH-JA (1.06) calculated versus the JA IS (Table SI) were obtained by measurement of dilution series of pure OPDA, OH-JA and JA IS dissolved into a leaf matrix. The JA-Ile IS was used to perform relative quantification of 12/11-hydroxy-JA-Ile (12/11-OH-JA-Ile), 12-carboxy-JA-Ile (12-COOH-JA-Ile), JA-glutamine (JA-Gln) and JA-valine (JA-Val) and their levels expressed in relative amounts (Fig. S4) since no response factors could be calculated for these compounds for which no authentic standard was available.

Elemental formulas of [1, 2-13C]MeJA and 12-COOH-JA-Ile were verified by ultra-high pressure liquid chromatography time-of-flight MS using the method and calculation settings described in Gaquerel et al. (2010).

Collection of petiole exudates

The petiole exudates (PEX) collection protocol was modified from previously published methods (King and Zeevaart, 1974; Maldonado et al., 2002; Park et al., 2007). WT, 35S-jmt-1 and 35S-jmt-2 rosette stage plants of similar size were used for petiole exudate collection. For one replicate, three leaves of similar developmental stage were gently detached with a scalpel at the very base of their petioles and then subsequently re-cut in 1 mM EDTA solution (pH 7.5) to prevent callose deposition and closure of phloem vessels. Leaf laminas were mechanically wounded with a fabric pattern wheel and *M. sexta* oral secretions were applied to the wound sites (W+OS). Petioles of three induced leaves of one genotype were then immersed into a fresh 1.5 mL 1 mM EDTA solution (pH 7.5). After 2.5 h the collection solution was renewed and PEX collected for another 2.5 h combined with the first fraction. PEX were freeze dried and dissolved in 70% methanol prior to the analysis for jasmonates.

**in vivo JMT and MJE enzyme activity assays**

To examine AtJMT and NaMJE activities, we infiltrated with a syringe JA, MeJA or [1, 2, 13-13C]MeJA (labeled with 13C on the three first carbons counting from the methylester group) dissolved in 1% DMSO in distilled water into leaves of rosette stage plants. Concentrations of the solutions were for JA of 12.5 µg/mL, for MeJA of 12.5 µg/mL and for 13C3-MeJA of 6.25 µg/mL. The amounts of infiltrated substrate per leaf discs were of 0.5 µg for JA and MeJA and of 0.25 µg for 13C3-MeJA. To evaluate the effect of the infiltration on endogenous jasmonate production, similar volumes of 1% DMSO solution (Mock infiltration) were infiltrated with a syringe into leaves of equal size. After 0.5, 10 and 45 min, a leaf disc of 0.4 cm² was removed from the infiltrated leaf area with a cork borer and immediately flash-frozen in liquid nitrogen until analyses for jasmonates.
Quantitative real-time PCR analysis

Total RNA from five biological replicates per line was extracted as described in Linke at al. (2002). RNA extracts were treated with DNase using the DNA-free™ Kit from Ambion (Applied Biosystems/Ambion, Austin). cDNA was synthesized from 500 ng RNA using SuperScript II Reverse Transcriptase (Invitrogen, Germany) and a poly-T primer. Quantitative real-time PCR (qRT-PCR, Stratagene 500 Mx3005P, Waldbronn, Germany) was conducted with 30 ng cDNA using the core reagent kit (Eurogentec, http://www.eurogentec.be) and pairs of gene specific primers (Table SII). qPCR products were either detected by gene-specific double fluorescent dye-labeled TaqMan® probes or after reaction with SYBR Green (qPCR Core Kit for SYBR Green I; Eurogentec, Köln, Germany). Relative gene expression was calculated using a 200-fold dilution series of cDNAs synthesized from RNA samples of the same experiment and normalized, according to Pfaffl et al. (2002), by the expression value of the *N. attenuata ACTIN* gene.

Statistical analysis

Most statistics were performed with StatView (Abacus Concepts Inc., http://www.statview.com). Jasmonate pools were calculated in each tissues of local and systemic leaves after W+OS elicitation by summing, for each time point, the average (n = 5) OPDA, JA, MeJA, OH-JA and JA-Ile levels of individual leaf samples. Jasmonate pools were then presented as bar charts and analyzed by Student’s t-test.

The hierarchical clustering analysis was performed with the TIGR MeV 3.1 software (http://www.tm4.org/mev.html) on autoscaled data using as expression vector, the average levels of each jasmonate (OPDA, JA, MeJA, OH-JA and JA-Ile) at the different time points in a particular tissue of local or systemic leaves (20 variables). The data matrix consisted of 20 variables x 18 samples (3 tissue types x 2 leaf position x 3 genotypes). The Pearson correlation was used as clustering metric and the complete linkage aggregation algorithm as clustering method.

For the network analysis, Pearson correlation coefficients were calculated among the different jasmonates using the mean of all biological replicates at each time point and for each tissue harvested after dissection from treated and systemic leaves independently. Significance levels for correlation coefficients (r) were determined following the number of metabolite pairs (n) using the equation $t = r \times (n - 2)^{0.5} / (1 - r^2)^{0.5}$. Correlations corresponding to a coefficient with P < 0.05 from a distance matrix calculations were transposed into a pairwise
format and visualized using Networks cartography and Pajek software version 1.22 (http://vlado.fmf.uni-lj.si/pub/networks/pajek/). The distance used to rebuild the network was 1 – the absolute value of the correlation coefficient with the Fruchtermann-Reingold three-dimensional algorithm.

ACKNOWLEDGEMENTS

We thank Dr. Matthias Schoettner, Mario Kallenbach and Christian Hettenhausen for help with analytical equipments, Dr. Markus Hartl for fruitful discussions, Prof. Claus Wasternack for providing the 11/12-OH-JA for the determination of the OH-JA/JA response factor, and the Max Planck Society for funding. We thank the two reviewers and the handling editor (Gregg Howe) for insightful comments. Dedicated to the memory of Dr. Jean-Pierre Salaün (1944-2011).

SUPPLEMENTAL MATERIAL

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

**Table SI.** Multiple reaction monitoring conditions for jasmonate profiling in *N. attenuata*.

**Table SII.** Sequences of gene-specific primers used for qRT-PCR.

**Figure S1.** Southern blot analysis of 35S-*jmt* and 35S-*jmt*/ir-*mje* lines.

**Figure S2.** Conversion of infiltrated JA and MeJA into JA-Ile is outcompeted by AtJMT activity in 35S-*jmt*-1 and 35S-*jmt*/ir-*mje*-1.

**Figure S3.** Measurement of volatile MeJA in the headspace of W+OS-induced leaves.

**Figure S4.** Levels of JA-Ile metabolites, JA-Gln and JA-Val are differentially affected by AtJMT activity in different tissues of W+OS-treated leaves.

**Figure S5.** The topology of the jasmonate metabolic network is largely and similarly altered in 35S-*jmt* and 35S-*jmt*/ir-*mje* leaves.

**Figure S6.** Decreases in W+OS-induced total jasmonate pools of transgenic plants are more severe in petiole tissues.

**Figure S7.** MeJA is the most abundant jasmonate in the petiole exudates of W+OS-treated 35S-*jmt*-1 and 35S-*jmt*-2 leaves.

**Figure S8.** Transcript levels of direct defense genes *NaTPI* and *NaTD* are strongly decreased locally and systemically by AtJMT ectopic expression.

REFERENCES

Allmann S, Baldwin IT (2010) Insects betray themselves in nature to predators by rapid isomerization of green leaf volatiles. Science **329**: 1075-1078


Qin GJ, Gu HY, Zhao YD, Ma ZQ, Shi GL, Yang Y, Pichersky E, Chen HD, Liu MH, Chen ZL, Qu LJ (2005) An indole-3-acetic acid carboxyl methyltransferase regulates Arabidopsis leaf development. Plant Cell 17: 2693-2704


Staswick PE, Tiryaki I, Rowe ML (2002) Jasmonate response locus *JAR1* and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell 14: 1405-1415


Functional analysis of a tomato salicylic acid methyl transferase and its role in
synthesis of the flavor volatile methyl salicylate. Plant Journal 62: 113-123

Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochov R, Yu F,
Jikumaru Y, Ross J, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya
Arabidopsis GAMT1 and GAMT2. Plant Cell 19: 32-45

von Dahl CC, Baldwin IT (2004) Methyl jasmonate and cis-jasmine do not dispose of the
herbivore-induced jasmonate burst in Nicotiana attenuata. Physiologia Plantarum 120:
474 - 481

JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic
acid-amino acid conjugates play different roles in herbivore resistance of Nicotiana
attenuata. Plant Physiology 146: 904 - 915

silencing two JAR family members impairs levels of trypsin proteinase inhibitors but
not nicotine. Planta 226: 159 - 167


MeJA function as a signal without being hydrolyzed to JA? Planta 227: 1161-1168

downstream mediator in the growth repression limb of the jasmonate pathway. Plant
Cell 19: 2470-2483

Yao KN, Deluca V, Brisson N (1995) creation of a metabolic sink for tryptophan alters the
phenylpropanoid pathway and the susceptibility of potato to Phytophthora infestans.
Plant Cell 7: 1787-1799

Zhang ZP, Baldwin IT (1997) Transport of [2-C-14]jasmonic acid from leaves to roots
mimics wound-induced changes in endogenous jasmonic acid pools in Nicotiana
sylvestris. Planta 203: 436 - 441

FIGURE LEGENDS

Figure 1. N. attenuata plants ectopically expressing AtJMT (35S-jmt) or additionally
silenced for the expression of NaMJE (35S-jm/tir-mje) are indistinguishable from WT
during rosette stage growth but produce flowers with altered morphology.

(A) N. attenuata plants ectopically expressing AtJMT (35S-jmt-1) and additionally
silenced for NaMJE (35S-jm/tir-mje-1) do not differ from WT plants during rosette stage (~30
days-old). (B) Flowers of 35S-jmt and 35S-jm/tir-mje plants have short styles (white arrows
indicate stigma position) and closed corollas compared to WT plants. (C) Relative transcript
abundance (mean ± SD, n = 5) of AtJMT and NaMJE in leaf laminas of WT, 35S-jmt and
35S-jm/tir-mje-1 plants 1 h after mechanical wounding and application of Manduca sexta
oral secretions to the wounds (W+OS). No AtJMT expression was detected (n.d.) in WT tissues
after W+OS elicitation. *NaMJE* relative transcript levels in 35S-*jmt*/ir-*mje*-1 leaf laminas after W+OS elicitation were reduced to ~ 5% of WT levels but were unchanged in 35S-*jmt*-1.

**Figure 2.** *N. attenuata* 35S-*jmt* and 35S-*jmt*/ir-*mje* plants have altered JA-methylation and MeJA-demethylation activities.

Leaf discs (0.4 cm²) were infiltrated with a control solution (Mock) and 0.5 µg of JA (A), unlabeled MeJA (B) or 0.25 µg synthetic MeJA labeled with 13C ([1, 2, 13-13C]MeJA) (C). JA methylation (A), MeJA demethylation (B) and the re-methylation of the subsequently released JA (C) were analyzed by quantifying 0.5, 10 and 45 min increases (mean ± SD, n = 5) in MeJA (JA → MeJA), JA (MeJA → JA) and [1, 2,-13C]MeJA levels after infiltration in the elicited leaves. After JA infiltration, 35S-*jmt*-1 and 35S-*jmt*/ir-*mje*-1 leaves showed larger MeJA accumulations than WT which came at the expense of other JA metabolites (**Fig. S2**). MeJA de-esterification was strongly impaired in 35S-*jmt*/ir-*mje*-1 and only slightly reduced in 35S-*jmt*-1 plants. **Left panel of (C):** high resolution time-of-flight MS measurement of (calculated m/z = 228.158) [1, 2, 13-13C]MeJA-infiltrated leaf areas – zoom-in of the spectral range m/z 227 to 229 – revealed the production of a m/z signal at 227.155 characteristic for 13C₂-MeJA ([1, 2,-13C]MeJA, calculated m/z = 227.155) formed by re-methylation of the de-esterified 13C₃-MeJA in 35S-*jmt*-1 and 35S-*jmt*/ir-*mje*-1, but not in WT leaf samples. 13C atoms are circled. **Right panel of (C):** [1, 2,-13C]MeJA levels. Asterisks represent significant differences between WT and transgenic lines (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001). n.d.: not detected.

**Figure 3.** AtJMT-catalyzed MeJA formation depletes W+OS-induced JA levels.

Mean (± SD, n = 5) constitutive and induced JA (A) and MeJA (B) levels (nmol g⁻¹ FW⁻¹) after mechanical wounding and application of *Manduca sexta* oral secretions to the puncture wounds (OS-elicitation) in laminas of WT, 35S-*jmt* (35S-*jmt*-1, 35S-*jmt*-2) and 35S-*jmt*/ir-*mje* (35S-*jmt*/ir-*mje*-1, 35S-*jmt*/ir-*mje*-2) plants. Prior to elicitation, JA and MeJA levels in leaf laminas of 35S-*jmt* and 35S-*jmt*/ir-*mje* lines did not differ from those in WT. The JA burst observed in WT leaf laminas after W+OS elicitation was largely absent in 35S-*jmt* and 35S-*jmt*/ir-*mje* plants but was mirrored, due to AtJMT activity, by a large burst of methyl jasmonate (MeJA). Asterisks represent significant differences between WT and transgenic lines (unpaired t-test; * P < 0.05, ** < 0.001, *** < 0.0001).
Figure 4. Ectopic expression of \( \text{AtJMT} \) in 35S-\textit{jmt} and 35S-\textit{jmt/ir-mje} has profound, but differential, effects on spatio-temporal accumulation patterns of jasmonates in lamina, midribs and petioles.

Mean (± SD, n = 5) of jasmonate levels – OPDA, JA, MeJA, JA-Ile and OH-JA (the sum of 12- and 11-OH-JA) – in WT, 35S-\textit{jmt}-1 and 35S-\textit{jmt/ir-mje}-1 leaves after OS elicitation (W+OS). Leaves were harvested 0 to 5 h after elicitation, dissected into leaf lamina, midvein and petiole (see schematics above line graphs) and analyzed separately. Different jasmonates attained their maximum levels in different tissues post-elicitation. MeJA hyper-accumulation occurred at the expense of JA-Ile and JA accumulation but only retarded the accumulation of OH-JA. \( \text{AtJMT} \) ectopic expression also had different consequences on the accumulation of JA-Ile and other JA-amino-acid conjugates (Fig. S4). Asterisks (35S-\textit{jmt}-1) and plus signs (35S-\textit{jmt/ir-mje}-1) represent significant differences between WT and transgenic lines (unpaired t-test; */+ P < 0.05, **/++ < 0.001, ***/+++ < 0.0001).

Figure 5. Alterations in W+OS elicited jasmonate levels in systemic unelicited leaves of 35S-\textit{jmt} -1 and 35S-\textit{jmt/ir-mje}-1 are most pronounced in petioles and differ from those observed in elicited leaves.

Mean (± SD, n = 5) levels jasmonates– OPDA, JA, MeJA, JA-Ile and OH-JA (the sum of 12- and 11-OH-JA) – in WT, 35S-\textit{jmt}-1 and 35S-\textit{jmt/ir-mje}-1 leaves after OS elicitation (W+OS). Untreated leaves growing on the same plants as in Figure 4 with a minimal angular distance above the treated leaf, and hence orthostichous to the treated leaf, were considered systemic leaves. Leaves were harvested 0 to 5 h after elicitation, dissected into leaf lamina, midvein and petiole (see schematics above line graphs) and analyzed separately. All jasmonates attained their maximum levels in petioles. Accumulation of JA, OPDA and OH-JA was largely reduced in systemic petioles, although MeJA formation was less pronounced than in elicited tissues (Fig. 4). Jasmonate levels detected in systemic leaves were, except for OPDA and depending on the tissue type, 10 to 60 times lower than in treated leaves. Asterisks (35S-\textit{jmt}-1) and plus signs (35S-\textit{jmt/ir-mje}-1) represent significant differences between WT and transgenic lines (unpaired t-test; */+ P < 0.05, **/++ < 0.001, ***/+++ < 0.0001).

Figure 6. Alterations in jasmonate pools and relative composition are tissue-dependent and most pronounced in petioles.
Hierarchical clustering analysis performed, using the Pearson correlation as metric, on tissue-specific jasmonate signatures using vectors defined by the different levels reached at the different sampling times by a given jasmonate in a given tissue. Jasmonate profiles in systemic petioles relate the most to the signatures elicited after OS elicitation (W+OS) of treated leaves. The structure of locally elicited jasmonate signatures in WT is not retained in 35S-jmt-1 and 35S-jmt/ir-mje-1 leaves as revealed by the clustering. (B) Mean (± SD, n = 5) summed levels of the five most abundant jasmonates (OPDA, JA, MeJA, JA-Ile and OH-JA) detected at 0 to 5 h after W+OS elicitation in local (upper panel) and systemic (lower panel) leaf tissues (lamina, midvein and petiole) of WT, 35S-jmt-1 and 35S-jmt/ir-mje-1. Major quantitative changes in jasmonate pools were detected in midveins and petioles (Fig. S6). Asterisks represent significant differences between tissues of WT and transgenic plants (unpaired t-test; * P < 0.05, ** < 0.001, *** < 0.0001).

Figure 7. Transcript levels of JA biosynthesis and two direct defense genes are differentially affected by AtJMT ectopic expression.

Mean (± SD, n = 5) transcript abundance (relative to NaACTIN) of JA biosynthesis genes in leaf lamina – allene oxide synthase (NaAOS), lipoxygenase3 (NaLOX3), 12-oxophytodienoic acid reductase (NaOPR3) – does not differ between WT and 35S-jmt-1 and 35S-jmt/ir-mje-1 plants before and after OS elicitation (W+OS). In contrast, constitutive and W+OS-induced transcript abundance of threonine deaminase (NaTD) and trypsin proteinase inhibitor (NaTPI), two direct defense genes regulated by the JA signaling pathway, were decreased in 35S-jmt-1 and 35S-jmt/ir-mje-1 compared to those in WT plants. OS-induced leaf laminas were harvested when peak expression levels have been reported for the selected genes. Asterisks represent significant differences between WT and transgenic lines (NaAOS, NaTD, NaLOX3, NaOPR3 unpaired t-test, * P < 0.05; NaTPI Welch’s-test, * P < 0.05). n.d.: not detected.

Figure 8. Model of OS-induced JA metabolism and signaling in leaf lamina of 35S-jmt and 35S-jmt/ir-mje plants.

AtJMT ectopic expression in concert with NaMJE silencing creates a powerful metabolic sink in the JA pathway that outcompetes herbivory-induced JA and JA-Ile bursts, and in turn, compromises NaCOI1-mediated activation of NaTD and NaTPI expression but not that of JA biosynthesis genes. Font size and arrows’ thickness are proportional to the...
intensity of metabolite fluxes and activation of gene expression in leaf laminas tissues after simulated *M. sexta* herbivory.
Figure 1. *N. attenuata* plants ectopically expressing *AtJMT* (35S-jmt) or additionally silenced for the expression of *NaMJE* (35S-jmt/ir-mje) are indistinguishable from WT during rosette stage growth but produce flowers with altered morphology. (A) *N. attenuata* plants ectopically expressing *AtJMT* (35S-jmt-1) and additionally silenced for *NaMJE* (35S-jmt/ir-mje-1) do not differ from WT plants during rosette stage (~30 days-old). (B) Flowers of 35S-jmt and 35S-jmt/ir-mje plants have short styles (white arrows indicate stigma position) and closed corollas compared to WT plants. (C) Relative transcript abundance (mean ± SD, n = 5) of *AtJMT* and *NaMJE* in leaf laminas of WT, 35S-jmt and 35S-jmt/ir-mje-1 plants 1 h after mechanical wounding and application of *Manduca sexta* oral secretions to the wounds (W+OS). No *AtJMT* expression was detected (n.d.) in WT tissues after W+OS elicitation. *NaMJE* relative transcript levels in 35S-jmt/ir-mje-1 leaf laminas after W+OS elicitation were reduced to ~5% of WT levels but were unchanged in 35S-jmt-1.
Leaf discs (0.4 cm²) were infiltrated with a control solution (Mock) and 0.5 µg of JA (A), unlabeled MeJA (B) or 0.25 µg synthetic MeJA labeled with 13C ([1, 2, 13-13C]MeJA) (C). JA methylation (A), MeJA demethylation (B) and the re-methylation of the subsequently released JA (C) were analyzed by quantifying 0.5, 10 and 45 min increases (mean ± SD, n = 5) in MeJA (JA → MeJA), JA (MeJA → JA) and [1, 2, 13C]MeJA levels after infiltration in the elicited leaves. After JA infiltration, 35S-jmt-1 and 35S-jmt/ir-mje-1 leaves showed larger MeJA accumulations than WT which came at the expense of other JA metabolites (Fig. S2). MeJA de-esterification was strongly impaired in 35S-jmt-1 and only slightly reduced in 35S-jmt-1 plants. Left panel of (C): high resolution time-of-flight MS measurement of (calculated m/z = 228.158) [1, 2, 13-13C]MeJA-infiltrated leaf areas – zoom-in of the spectral range m/z 227 to 229 – revealed the production of a m/z signal at 227.155 characteristic for 13C2-MeJA ([1, 2, 13C]MeJA, calculated m/z = 227.155) formed by re-methylation of the de-esterified 13C3-MeJA in 35S-jmt-1 and 35S-jmt/ir-mje-1, but not in WT leaf samples. 13C atoms are circled. Right panel of (C): [1, 2, 13C]MeJA levels. Asterisks represent significant differences between WT and transgenic lines (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001). n.d.: not detected.
Figure 3. AtJMT-catalyzed MeJA formation depletes W+OS-induced JA levels.
Mean (± SD, n = 5) constitutive and induced JA (A) and MeJA (B) levels (nmol g⁻¹ FW⁻¹) after mechanical wounding and application of Manduca sexta oral secretions to the puncture wounds (OS-elicitation) in laminas of WT, 35S-jmt (35S-jmt-1, 35S-jmt-2) and 35S-jmt/ir-mje (35S-jmt/ir-mje-1, 35S-jmt/ir-mje-2) plants. Prior to elicitation, JA and MeJA levels in leaf laminas of 35S-jmt and 35S-jmt/ir-mje lines did not differ from those in WT. The JA burst observed in WT leaf laminas after W+OS elicitation was largely absent in 35S-jmt and 35S-jmt/ir-mje plants but was mirrored, due to AtJMT activity, by a large burst of methyl jasmonate (MeJA). Asterisks represent significant differences between WT and transgenic lines (unpaired t-test; * P < 0.05, ** < 0.001, *** < 0.0001).
Figure 4. Ectopic expression of \textit{AtJMT} in 35S-\textit{jmt} and 35S-\textit{jmt/ir-mje} has profound, but differential, effects on spatio-temporal accumulation patterns of jasmonates in lamina, midribs and petioles.

Mean ($\pm$ SD, $n = 5$) of jasmonate levels – OPDA, JA, MeJA, JA-Ile and OH-JA (the sum of 12- and 11-OH-JA) – in WT, 35S-\textit{jmt}-1 and 35S-\textit{jmt/ir-mje}-1 leaves after OS elicitation (W+OS). Leaves were harvested 0 to 5 h after elicitation, dissected into leaf lamina, midvein and petiole (see schematics above line graphs) and analyzed separately. Different jasmonates attained their maximum levels in different tissues post-elicitation. MeJA hyper-accumulation occurred at the expense of JA-Ile and JA accumulation but only retarded the accumulation of OH-JA. \textit{AtJMT} ectopic expression also had different consequences on the accumulation of JA-Ile and other JA-amino-acid conjugates (Fig. S4). Asterisks (35S-\textit{jmt}-1) and plus signs (35S-\textit{jmt/ir-mje}-1) represent significant differences between WT and transgenic lines (unpaired t-test; */+ $P < 0.05$, **/+ < 0.001, ***/+ < 0.0001).
Figure 5. Alterations in W+OS elicited jasmonate levels in systemic unelicited leaves of 35S-jmt-1 and 35S-jmt/ir-mje-1 are most pronounced in petioles and differ from those observed in elicited leaves.

Mean (± SD, n = 5) levels jasmonates—OPDA, JA, MeJA, JA-Ile and OH-JA (the sum of 12- and 11-OH-JA) – in WT, 35S-jmt-1 and 35S-jmt/ir-mje-1 leaves after OS elicitation (W+OS). Untreated leaves growing on the same plants as in Figure 4 with a minimal angular distance above the treated leaf, and hence orthostichous to the treated leaf, were considered systemic leaves. Leaves were harvested 0 to 5 h after elicitation, dissected into leaf lamina, midvein and petiole (see schematics above line graphs) and analyzed separately. All jasmonates attained their maximum levels in petioles. Accumulation of JA, OPDA and OH-JA was largely reduced in systemic petioles, although MeJA formation was less pronounced than in elicited tissues (Fig. 4). Jasmonate levels detected in systemic leaves were, except for OPDA and depending on the tissue type, 10 to 60 times lower than in treated leaves. Asterisks (35S-jmt-1) and plus signs (35S-jmt/ir-mje-1) represent significant differences between WT and transgenic lines (unpaired t-test; */+ P < 0.05, **/+ < 0.001, ***/+ < 0.0001).
Figure 6. Alterations in jasmonate pools and relative composition are tissue-dependent and most pronounced in petioles.

(A) Hierarchical clustering analysis performed, using the Pearson correlation as metric, on tissue-specific jasmonate signatures using vectors defined by the different levels reached at the different sampling times by a given jasmonate in a given tissue. Jasmonate profiles in systemic petioles relate the most to the signatures elicited after OS elicitation (W+OS) of treated leaves. The structure of locally elicited jasmonate signatures in WT is not retained in 35S-jmt-1 and 35S-jmt/ir-mje-1 leaves as revealed by the clustering. (B) Mean (± SD, n = 5) summed levels of the five most abundant jasmonates (OPDA, JA, MeJA, JA-Ile and OH-JA) detected at 0 to 5 h after W+OS elicitation in local (upper panel) and systemic (lower panel) leaf tissues (lamina, midvein and petiole) of WT, 35S-jmt-1 and 35S-jmt/ir-mje-1. Major quantitative changes in jasmonate pools were detected in midveins and petioles (Fig. S6). Asterisks represent significant differences between tissues of WT and transgenic plants (unpaired t-test; * P < 0.05, ** < 0.001, *** < 0.0001).
Figure 7. Transcript levels of JA biosynthesis and two direct defense genes are differentially affected by AtJMT ectopic expression.

Mean (± SD, n = 5) transcript abundance (relative to NaACTIN) of JA biosynthesis genes in leaf lamina – allene oxide synthase (NaAOS), lipoxygenase3 (NaLOX3), 12-oxo-phytodienoic acid reductase (NaOPR3) – does not differ between WT and 35S-jmt-1 and 35S-jmt/ir-mje-1 plants before and after OS elicitation (W+OS). In contrast, constitutive and W+OS-induced transcript abundance of threonine deaminase (NaTD) and trypsin proteinase inhibitor (NaTPI), two direct defense genes regulated by the JA signaling pathway, were decreased in 35S-jmt-1 and 35S-jmt/ir-mje-1 compared to those in WT plants. OS-induced leaf laminas were harvested when peak expression levels have been reported for the selected genes. Asterisks represent significant differences between WT and transgenic lines (NaAOS, NaTD, NaLOX3, NaOPR3 unpaired t-test, * P < 0.05; NaTPI Welch’s-test, * P < 0.05). n.d.: not detected.
Figure 8. Model of OS-induced JA metabolism and signaling in leaf lamina of 35S-\textit{jmt} and 35S-\textit{jmt}/ir-\textit{mje} plants.

\textit{AtJMT} ectopic expression in concert with \textit{NaMJE} silencing creates a powerful metabolic sink in the JA pathway that outcompetes herbivory-induced JA and JA-Ile bursts, and in turn, compromises \textit{NaCOI1}-mediated activation of \textit{NaTD} and \textit{NaTPI} expression but not that of JA biosynthesis genes. Font size and arrows’ thickness are proportional to the intensity of metabolite fluxes and activation of gene expression in leaf laminas tissues after simulated \textit{M. sexta} herbivory.