Over-expressing At *JMT* in *Nicotiana attenuata* creates a metabolic sink in the JA pathway:

Consequences for flower development, jasmonate production and defense activation

Diploma thesis

submitted by

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Main Abbreviations

AOS ALLENE OXIDE SYNTHASE

as-lox3 *N.attenuata* line transformed with a fragment of the *LOX3* gene in antisense orientation to silence its expression

ir-coi1 *N.attenuata* transformed line transformed with a fragment of the *COI1*gene in inverted repeat orientation to silence its expression

Ctrl. experimental control, untreated plant

FAC fatty acid-amino acid conjugates

JATs jasmonates

JA jasmonic Acid

JA-Ile jasmonoyl-L-isoleucine

JA-OH (11- and 12-) hydroxy-jasmonic acid

JMT *S*-ADENOSYL-l-METHIONINE:JASMONIC ACID METHYLTRANSFERASE

M. sexta *Manduca sexta*

MeJA methyl jasmonate

N. attenuata *Nicotana attenuata*

OS oral secretions collected from 3^{th} - and 4^{th} instar *Manduca sexta* larvae

OPDA 12-oxo-phytodienoic acid

ov-jmt-1 *N. attenuata* line 1 over-expressing the *Arabidopsis thaliana* JMT gene

ov-jmt-2 *N. attenuata* line 2 over-expressing the *Arabidopsis thaliana* JMT gene

TPI trypsin proteinase inhibitors

TD Threonine deaminase

WT Wild type

W+OS wounding with a fabric pattern wheel and application of 20µl 1:10 diluted *Manduca sexta* oral secretions

W+W wounding with a fabric pattern wheel and application of 20µl (bidest) water

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1. Abstract

1.1 English abstract

Inducible defenses against herbivorous threats as well as different developmental processes are known to be regulated by jasmonic acid (JA) and its metabolites, collectively referred to as jasmonates (JATs). However, the specific function of endogenous methyl jasmonate (MeJA) has still to be clarified. Here we demonstrated that the over-expression of *Arabidopsis thaliana JASMONYL METHYL TRANSFERASE* (At *JMT*) in the solanaceous species *Nicotiana attenuata* reprograms JA biosynthesis and signaling. ov-jmt leaves elicited by the wounding and application of *Manduca sexta* larvae oral secretions (W+OS) accumulated significantly higher *ALLENE OXIDE SYNTHASE* (AOS) transcript levels than WT did. Constitutive MeJA levels were unchanged in the leaves of ov-jmt plants but significantly increased in their flowers, which had substantially impaired style elongation and constitutive defenses. After 1 hr W+OS elicitation resulted in 50-fold higher MeJA levels in ov-jmt leaves compared to in WT leaves. This excess of MeJA strongly reduced the availability of JA and its conversion to other JATs. Constitutive and W+OS-induced TPI and DTG levels, two direct defense markers, were strongly down- regulated in the leaf tissues of ov-jmt plants, which made them more vulnerable to caterpillar attack. Local, but not systemic, TPI activity could be restored in ov-jmt leaves almost to WT levels by JA-Ile complementation. These findings suggest that MeJA is not an active JAT signal mediating local or systemic defense in *N. attenuata.* In addition, this study highlights the over-expression of At *JMT* as a useful tool with which to investigate yet unknown functions of different JATs in defense and flower development.

1.2 Deutsche Zusammenfassung

Es ist bekannt, dass induzierbare Abwehrmaßnahmen gegen Pflanzenfresser als auch unterschiedliche Entwicklungsprozesse durch Jasmonsäure (JA) und ihre Stoffwechselprodukte, die Jasmonate (JATs), gesteuert werden. Die spezifischen Wirkungsweisen endogenen Methyljasmonats (MeJA) blieben bisher jedoch ungeklärt. In dieser Studie zeigen wir, dass die Überexpression der *Arabidopsis thaliana JASMONYL METHYLTRANSFERASE* (At *JMT*) in dem Nachtschattengewächs *Nicotiana attenuata,* sowohl Biosynthese als auch Signalwege der JA reprogrammiert. Blätter *JMT*-überexprimierender Pflanzen (ov-jmt) akkumulierten nach Induktion durch mechanische Verwundung sowie Applikation oraler Sekrete von *Manduca sexta* Larven (W+OS), vergleichsweise höhere Transkriptlevel für *ALLENE OXIDE SYNTHASE* (AOS) als Wildtyp-Pflanzen (WT). Waren die konstitutiven MeJA-Konzentrationen der Blättern von ov-jmt-Pflanzen kaum verändert, so wurden in deren Blüten, welche auch stark verkürzte Griffel und reduzierte Abwehr aufwiesen, deutlich höhere Werte gemessen als in WT-Blüten. In Blättern von ov-jmt-Pflanzen führte die Elizitierung durch W+OS nach einer Stunde zu 50-fach höheren MeJA-Gehalten als bei WT-Pflanzen. Diese exzessive MeJA-Bildung verursachte neben einer gravierenden Verknappung freier Jasmonsäure auch einen starken Rückgang ihrer Umsetzung zu anderen JATs. TPIs und DTGs, zwei kennzeichnende pflanzliche Abwehrsubstanzen, waren in Blättern der Transformanten unter konstitutiven und induzierten (W+OS) Bedingungen stark herab reguliert und verursachten so eine gesteigerte Anfälligkeit für Raupenfraß. Lokal aber nicht systemisch konnte die TPI-Aktivität in ov-jmt-Pflanzen, durch Applikationen von JA-Ile annähernd auf WT-Werte komplementiert werden. Diese Ergebnisse weisen darauf hin, das MeJA selbst in *N. attenuata* keine aktive Signalfunktion für die Vermittlung lokaler oder systemischer Abwehr innehat. Des Weiteren hebt diese Studie die Möglichkeiten hervor, welche durch die Überexpression von At *JMT* als nützliches Werkzeug für die Erforschung bislang unbekannter Funktionen unterschiedlicher Jasmonate in Abwehr und Blütenentwicklung geboten sind.

2. INTRODUCTION

Plant induced defenses

Plants have evolved an elaborate matrix of defense responses to protect themselves from insects. In addition to constitutive defense barriers such as trichomes or thick secondary walls, plants also use defense strategies activated specifically when insects start actively feeding on a leaf (Karban and Baldwin, 1997). These induced responses include direct defenses, such as the production of amino-acid-degrading enzymes (Chen et al., 2005), anti-digestive proteinase inhibitors (Johnson et al., 1989; Ryan, 1990; Zavala et al., 2004), and toxic or repelling chemicals that render plant tissues less suitable as food for herbivores (Duffey and Stout, 1996; Steppuhn et al., 2004), as well as indirect defenses based on volatile emissions that increase the attractiveness of attacked plants to natural enemies of their herbivores (De Moraes et al., 1998; Kessler and Baldwin, 2001).

JATs are master signals in plant´s induced defenses

Plants use intimately connected signaling transduction pathways to adaptively tailor their defensive status. Although ethylene (Voelckel et al., 2001; von Dahl and Baldwin, 2007; Leon-Reyes et al., 2009), salicylic acid (Rayapuram and Baldwin, 2007), and other small signaling molecules (e.g. NO, H_2O_2) are fulfilling regulatory functions, their contribution is relatively minor in comparison to that of jasmonic acid (JA) and its cyclic precursors and derivatives, collectively referred to as jasmonates (JATs). JATs promote plant defense responses to many insect herbivores, such as grasshoppers (e.g. *Acrididae* spp. Fig.1A), caterpillars (e.g. *Manduca quinquemaculata*, Fig.1B), beetles (e.g. *Thyeocoridae* spp., Fig.1C), leafhoppers (e.g. *Empoasca* spp., Fig.1D), spider mites (e.g. *Tetranychous urdicae*), or mirid bugs (e.g. *Tubiocoris notatus*).

Genetically silencing JA biosynthetic and signaling genes in all plant species examined to date, including *Nicotiana* (*attenuata*), the model of this study, became much more susceptible to herbivores in glasshouse or natural conditions (for review see (Browse and Howe, 2008)).

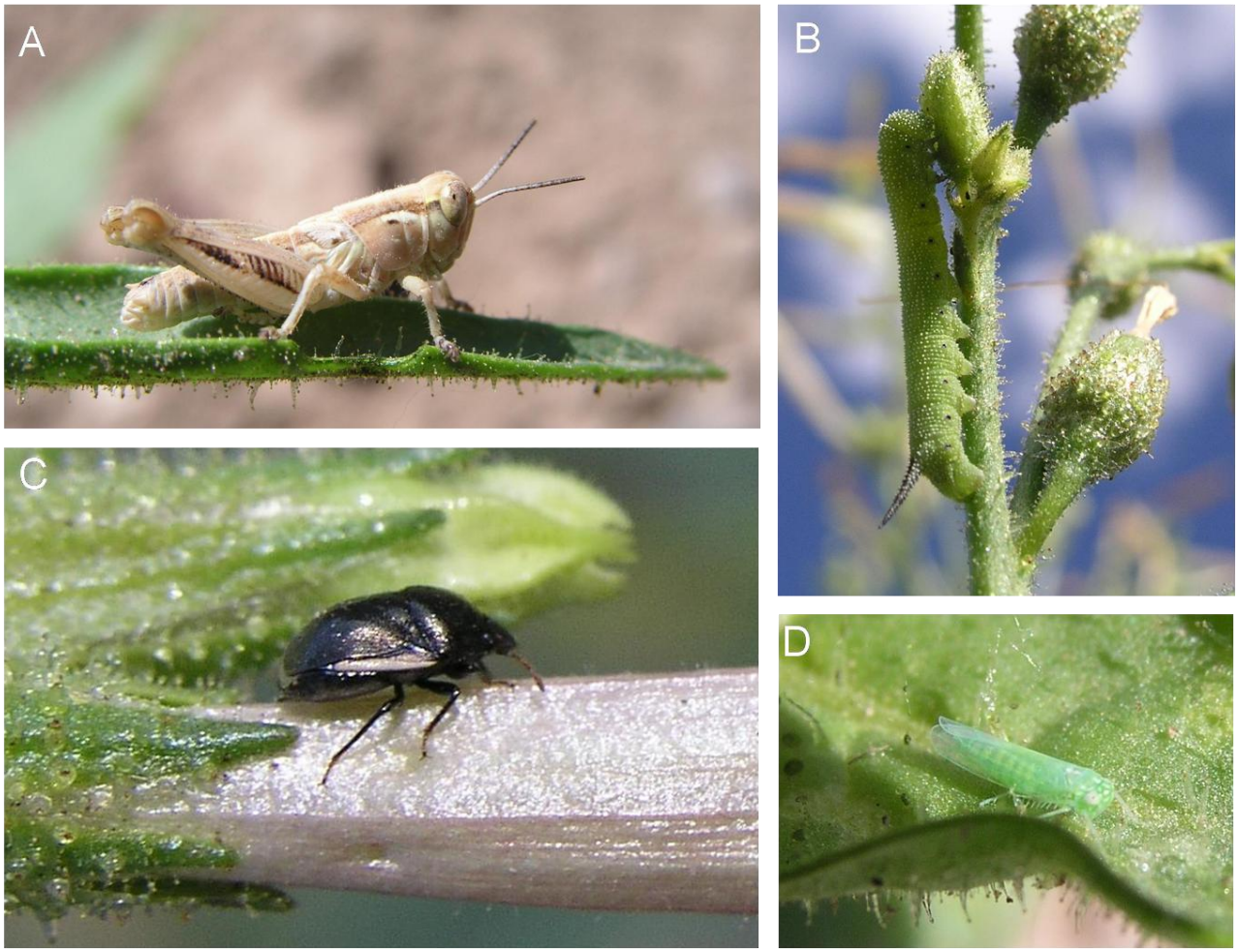

Figure 1. Some herbivores attacking *Nicotiana attenuata* in its natural environment. (A) *Acrididae spec*. (grasshopper) **(B)** *Manduca quinquemaculata* (tomato hornworm) **(C)** *Thyeocoridae* spp. (Negro bug) **(D)** *Empoasca* spp. (Leafhopper) (Lytle Field Station, UT, U-S-A., May 2009)

The JA biosynthetic and signaling pathway

According to the basic model (for review see (Wasternack, 2007), JA biosynthesis starts with the release of linolenic acid (C18:3) from chloroplast membranes after the activation of specific lipases. In *N. attenuata*, a recent study has demonstrated that this process requires intact WOUND-INDUCED PROTEIN KINASE (*WIPK*) signaling and is mediated by the homologue At *DAD1(DEFECTIVE ANTHER DEHISCENCE1)* (Kallenbach M., Alagna F., Baldwin I.T., Bonaventure G., unpublished results). Following C18:3 hydroperoxidation by 13-lipoxygenase enzymes (13-LOX) and its cyclization via the combined actions of ALLENE-OXIDE SYNTHASE (AOS) and ALLENE-OXIDE CYCLASE (AOC) proteins, 12-oxo-phytodienoic acid

(OPDA) is synthesized and transported to the peroxisome. Here, OPDA is converted to JA by OPDA REDUCTASE (OPR) proteins and a β-oxidation complex. Many routes for JA metabolism have been identified, among the major ones are: *(i)* methylation of the carboxylic function to yield MeJA by *S*-ADENOSYL-L-METHIONINE:JASMONIC ACID METHYLTRANSFERASE (JMT) enzymes (Seo et al., 2001); *(ii)* hydroxylation at C-12 (or C-11) (Miersch et al., 2008) or *(iii)* the amide-linked conjugation of the carboxyl group to isoleucine and other amino acids by JAR (JASMONATE RESISTANT) (Staswick and Tiryaki, 2004) proteins, yielding jasmonoyl-L-Ile (JA-Ile) and other jasmonoyl-amino acid conjugates, respectively.

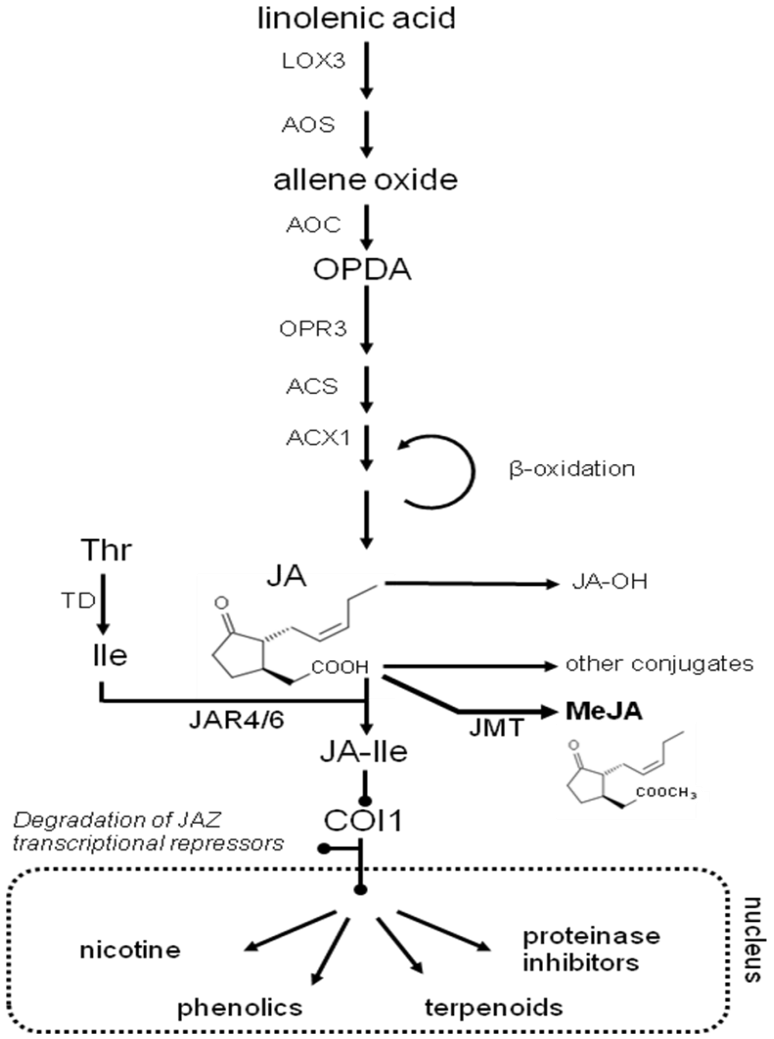

Figure 2. A simplified scheme of the JA bisosynthetic and signaling pathway in *Nicotiana attenuata.* Gene and chemical names are reported in the main text, except: ACS (1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE) and ACX1 (ACYL-COENZYME A OXIDASE1). Normal arrows represent biosynthetic steps; circle-ended lines represent signaling steps in the JA-cascade.

To date, the activity of only one JAT has been proved at the molecular level. (+)-7-iso-JA-Ile has been recently confirmed as the endogenous signal interacting with COI1 (CORONATINE INSENSITIVE1 (Xie et al., 1998). This interaction promotes the degradation by the 26S proteasome of transcriptional repressors called jasmonate zim domain (JAZ) proteins (Chini et al., 2007). Pull-down experiments performed for COI1 and JAZ1 have not demonstrated significant binding activity for JA, OPDA and MeJA (Thines et al., 2007). Since not all COI1-dependent responses are impaired in the *jar1* mutant (Zhang and Turner, 2008) – whose ability to produce JA-Ile is diminished, other JATs might have signaling functions.

JAs-based systemic signaling

Many plant induced responses are found not only in damaged leaves but also in undamaged tissues far from the initial sites of insect attack. In grafting experiments performed with JA biosynthetic tomato mutants -- *suppressor of prosystemin-mediated responses2* (*spr2*), *acyl-CoA oxidase* (*acx1*) -- and a JA response mutant -- *jasmonic acid insensitive* (*jai1*) -- systemic signaling was observed to require both the biosynthesis of JA at the site of wounding and the ability to perceive a JA signal in remote tissues (Li et al., 2002; Li et al., 2005). Consistent with the role of JAT in vascular based systemic signaling, increasing evidence indicate that JA biosynthesis is mainly restricted to specific cell types within the vascular system (Stenzel et al., 2003). However, the mechanisms by which JATs may act non-cell autonomously remain unknown.

The model system: **Nicotiana attenuata - Manduca sexta**

N. attenuata is a wild tobacco species growing native in post-fire soils in the Great Basin Desert in the United States of America (Fig. 1 a). From the seed bank, this annual diploid plant species germinates synchronized by yet unknown components of wood-smoke. In its natural environment *N.attenuata* is host to a large herbivore community that differs in number and distribution from year to year. By constantly adaptating its defense status to this unpredictable herbivore threat, *N.attenuata* has evolved fine-tuned regulatory mechanisms to control not only the amplitude (Skibbe et al., 2008) but also spatial and temporal JAT production (Skibbe et al., 2008; Stork et al., 2009)

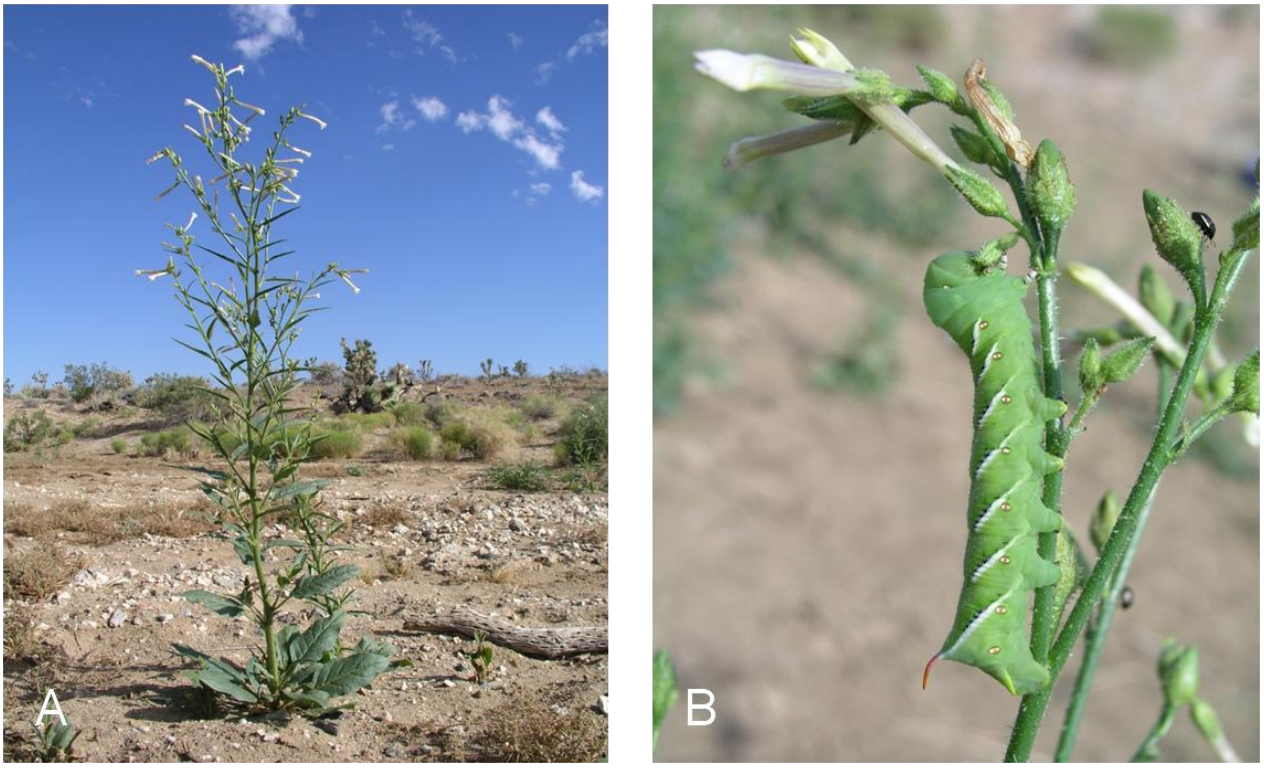

Figure 3. Modelsystem *Nicotiana attenuata* and *Manduca sexta.*
(A) *Nicotiana attenuata* in its natural habitat, the Great Basin Desert in Utah (U.S.A.). **(B)** Caterpillar of lepidopteran *Manduca sexta* (tobacco hornworm) feeding on flower buds of *N.attenuata* in its natural habitat. (UTAH, U.S.A., May 2009)

Like other Solanaceous plants, *N. attenuata* produces a set of defensive alkaloids. The major alkaloid in *N. attenuata* is nicotine, a neurotoxic compound naturally occurring in members

of the *Nicotiana* genus. Diterpene glucosides (Jassbi et al., 2008) and trypsin proteinase inhibitors (Zavala et al., 2004) are other defensive traits deployed under attack. However, the caterpillar of the lepidopteran *Manduca sexta* (Fig. 2B) is specialized to be able to feed on nicotine-containing plants. Over the last decade the sequence of events activated in *N. attenuata* during *M. sexta* attack has been intensely studied at the gene, protein and metabolite levels. Most of these changes, shown to be reproducible when mechanically wounded leaves are treated with the oral secretions of *M. sexta* larvae (OS), are regulated by fatty acid-amino acid conjugates (FACs). These highly potent defense elicitors present in OS of feeding *M.sexta* larvae greatly amplify JA production and dependent responses when applied to the wounded leave sites (Wu et al., 2007; Gaquerel et al., 2009).

The role of MeJA in plant induced defenses: what is real?

MeJA is a fragrant compound initially isolated from the flowers of *Jasminum grandiflorum* (Demole, 1962). To date, this JA metabolite ubiquitously distributed in the plant kingdom (Meyer et al., 1984) has been till date the most commonly used plant defense elicitor. In clear contrast, few studies have investigated downstream responses controlled by endogenous MeJA formation. A body of evidences suggests that MeJA itself is probably not a signaling molecule. A recent study from our group has shown that the inducing effect of MeJA on *TPI* expression requires the de-esterification of MeJA by jasmonate methyl esterase (JME) enzymes into free JA (Wu et al., 2008). In the same way, the airborne priming effect of volatile MeJA requires intact de-esterification and Ile conjugation activities in neighbouring plants (Tamogami et al., 2008). In addition to its postulated role as a volatile inter-plant signal, MeJA has also been assumed to act as an internal long-distance signal. Even though, C^{11} labelling experiments have revealed MeJA translocation in both phloem and xylem systems (Thorpe et al., 2007), nothing clear is known about its importance for systemic defense activation.

The transgenic over-expression of *Arabidopsis JMT* (ov-jmt) has been documented as a convincing means of increasing endogenous MeJA production (Seo et al., 2001). This over-expression caused a significant decline in seed production in *Arabidopsis* (Cipollini, 2007) and in grain yield in rice (Kim et al., 2007). Strikingly, the aforementioned *Arabidopsis* transgenic plants also constitutively expressed JA-responsive genes such as *VSP* (*VEGETATIVE STORAGE PROTEIN2)* and *PDF1.2* (*PLANT DEFENSINE1.2*) and enhanced resistance to the pathogenic

fungus *Botrytis cinerea* (Seo et al., 2001). However it is worth mentioning that the authors of this study did not monitor potential production-reprogrammings of other JATs. Taken together, and in light of the major advances on JA-Ile signalling, these data ask for a re-assessment of the role of MeJA during herbivory, and in particular a detailed characterization of JAT and defense activation in lines over-expressing *JMT*.

Reported results

Here we over-expressed *At* JMT in *N. attenuata* which resulted in a 50-fold increase of herbivory-induced MeJA levels, while compromising the formation of other jasmonates, especially its isoleucine conjugate (JA-Ile). Flowers from ov-jmt plants compared to those of WT, had reduced pistil lengths, partially open corollas and diminished nectar production, traits previously linked to JA signalling. Induced levels of *AOS*, a key jasmonate bionsynthetic transcript, were increased in ov-*jmt* lines compared to in WT plants, while those of *TPI* and *TD* were strongly decreased, suggesting that different JATs independently regulate genes related to defense- and JA-biosynthesis. Alterations in the production of major defense metabolites (for example TPI and DTGs) and the increase in vulnerability to insect attack of ov-jmt lines were similar to those detected in as-lox3, which lack the total jasmonate panoply. Moreover, the local activation of TPI, taken as an example, was almost, but not completely, restored by a JA-Ile treatment.

3. Results

3.1 Over-expressing At JMT *in* N.attenuata

Transgenic *N. attenuata* lines expressing At *JMT* under the control of a 35S promoter (P_{CaMV}) were generated by *Agrobacterium tumefaciens*-mediated transformation as described by (Kruegel et al., 2002) (Fig. 4-A). The transformation vector pRESC2JMT contained the full-length sense orientation of At *JMT* and the hygromycin resistance gene *hptII* as a selectable marker. Two T2 homozygous and independently transformed lines, ov-jmt-1 and ov-jmt-2, were selected by segregation analysis for hygromycin resistance. Southern blot analysis, performed with an hptII-specific probe, showed an insertion of the transgene for ov-jmt-1 line (Fig. 4B).

To ensure that At *JMT* was successfully expressed constitutively, we used Northern blot to analyze RNA extracted from the two selected transgenic lines using an At *JMT*-specific probe (Fig. 4C) As expected, high levels of At *JMT* transcripts were detected in the two ov-jmt-lines but not in WT plants.

We next verified that over-expressing At *JMT* increased the methylation of JA into MeJA. Therefore, MeJA levels were quantified in midvein tissues, where JATs accumulate at highest concentrations (Fig. 3D). Constitutive MeJA levels in ov-jmt-1 and ov-jmt-2 lines did not differ significantly from those in WT background. But, MeJA levels elicited 1 h after induction by wounding and applying *M.sexta* oral secretions (W+OS) were around 100 times higher in ov-jmt-1 ($P = 0.008$) and 90 times in ov-jmt-2 ($P = 0.001$) plants compared to levels in WT plants. No detectable amounts of MeJA were measured in the headspace of induced leaves during this period of time (data not shown).

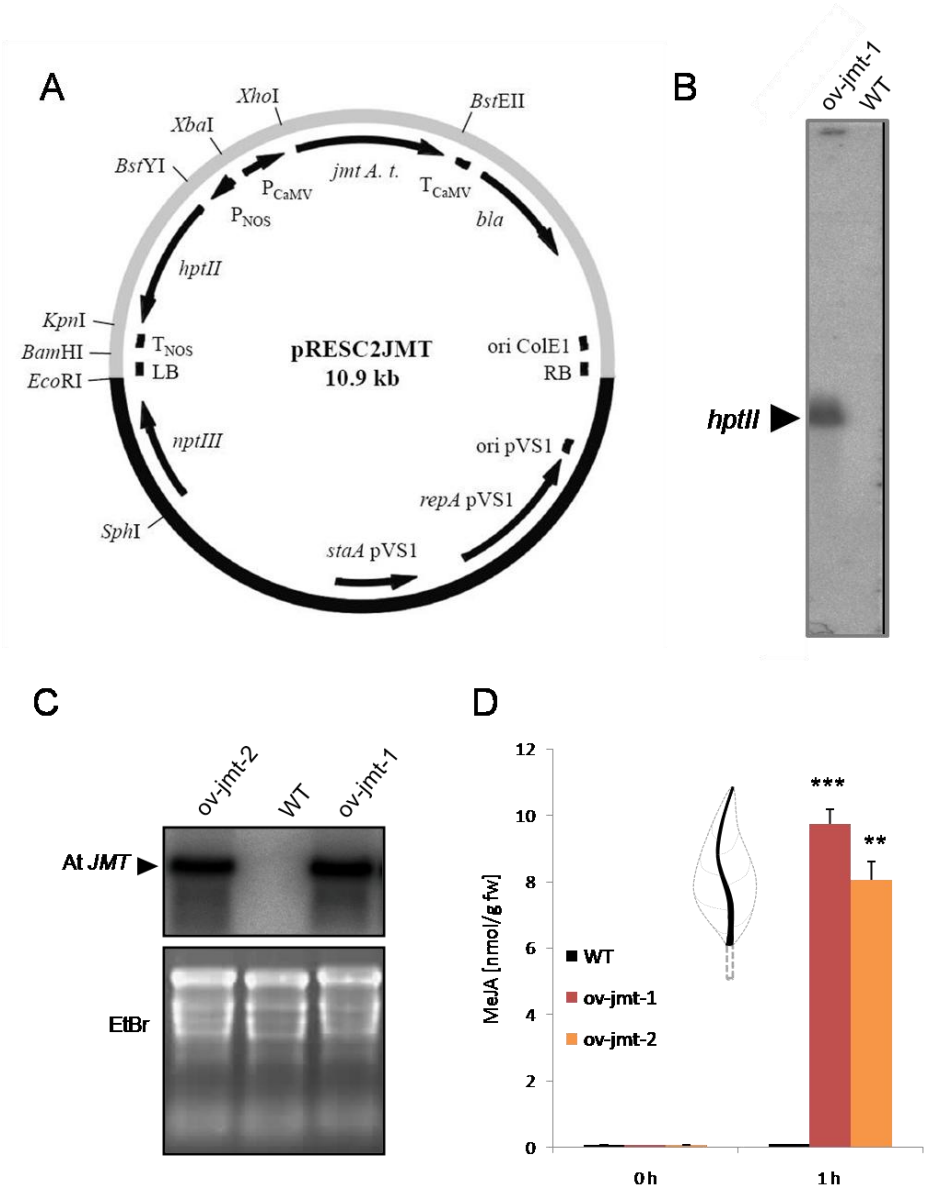

Figure 4. Production of transgenic lines over-expressing At *JMT*.
(A) Over-expression vector pRESC2JMT used to generate of transgenic ov-*jmt* plants. Transferred T-DNA with right and left borders (RB, LB) is shown in gray. T-DNA contains the *Arabidopsis thaliana JMT* (*jmt A.t.*) fragment under control of the *Cauliflower Mosaic Virus* (CaMV) constitutive expression promoter 35S. **(B)** Southern blot of WT and ov-*jmt*-1 genomic DNA of rosette-stage leaves. The blot was hybridized with a ^{32}P-labeled probe specific for the hygromycin phosphotransferase gene *hptII*. **(C)** RNA gel-blot analysis of WT and ov-jmt total RNA of rosette-stage leaves. Blots were hybridized with the ^{32}P-labeled At *JMT*-probe. Ethidium bromide (EtBr) staining of total RNA was performed to verify that equal RNA loading had occured. Top band, *JMT* mRNA; bottom band, 18s rRNA loading control. **(D)** Methyl-jasmonate accumulation of leaf midveins after wounding and application of *M. sexta* oral secretions. Asterisks represent significant differences between WT and ov-jmt lines (N = 5, unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

In contrast to what has been reported in soybean (Xue and Zhang, 2007), over-expressing At *JMT* in *N. attenuata* did not alter normal root elongation. Exogenously applied high concentrations of MeJA in the growing media are well-known to inhibit root development. This inhibitory effect is COI1-dependent and necessitates MeJA de-esterification and conversion into JA-Ile. (Xie et al., 1998; Staswick and Tiryaki, 2004)Compared to in WT, the root elongation of mutants impaired in JA-Ile formation, like the *jasmonate resistent1* (*jar1*), is therefore less inhibited by exogenous doses (20 respectively 50 µM) of MeJA. In agreement with favored MeJA production to the detriment of endogenous JA-Ile production, similar effects were observed for the root elongation of ov-jmt seedlings (Fig. 5A-C). The root length of treated ov-jmt-1 and ov-jmt-2 seedlings was between the root lengths of WT and *COI1*-silenced (ir-coi1) plants or a cross between ir-coi1-and ov-jmt-1 (ov-jmt-1 x ir-coi1) plants.

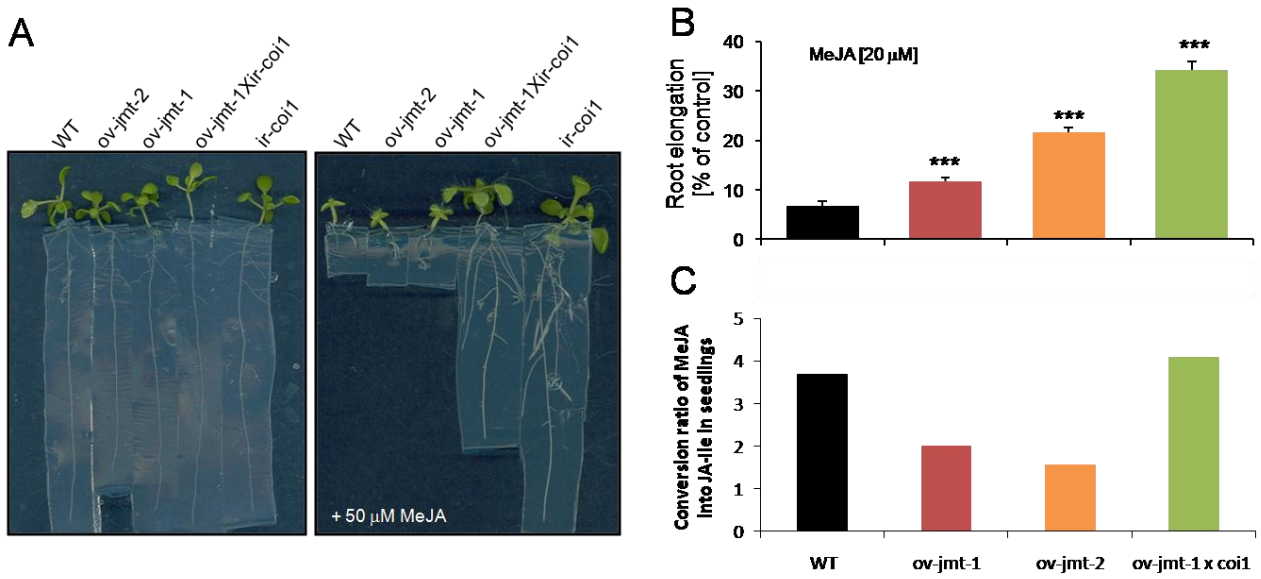

Figure 5. Over-expressing At JMT decreased JA perception.
(A) Root elongation in 5 kinds of 21 day-old seedlings: in WT; in ov-jmt lines -1 and -2; in ir-co1 (a line silenced for the JA-Ile receptor Na COI1); and in a line obtained from a cross between ov-jmt-1 and ir-coi1. Root elongation in ov-jmt lines was indistinguishable from that in WT under control conditions (left side) **(B)** Measuring the effect of MeJA inhibition on root elongation is the classical screen for alterations in JA-Ile formation or perception. Root elongation in ov-jmt lines was significantly less sensitive to MeJA application than was root elongation in WT plants **(C)** MeJA conversion into JA-Ile was calculated by dividing JA-Ile levels of seedlings grown on 20µM MeJA GB5 media by values measured in control seedlings. Asterisks represent significant differences between WT and ov-jmt lines. (n=10, unpaired t-test; ** P < 0.001, *** < 0.0001)

3.2 Characterization of ov-jmt flowers

Leaf shape, rosette size and stalk elongation were indistinguishable in the two ov-jmt lines and WT plants (Fig. 6A-B). However, flowers of ov-*jmt*-1 and ov-*jmt*-2 showed obvious phenotypic differences when compared with WT flowers. Corollas did not open completely and the lengths of styles were seriously reduced. When corollas with adnate stamen were removed, apparent nectaries at the base of ovaries in ov-jmt flowers were not bright orange as in WT flowers, but more whitish and yellowish (Fig. 6C-E).

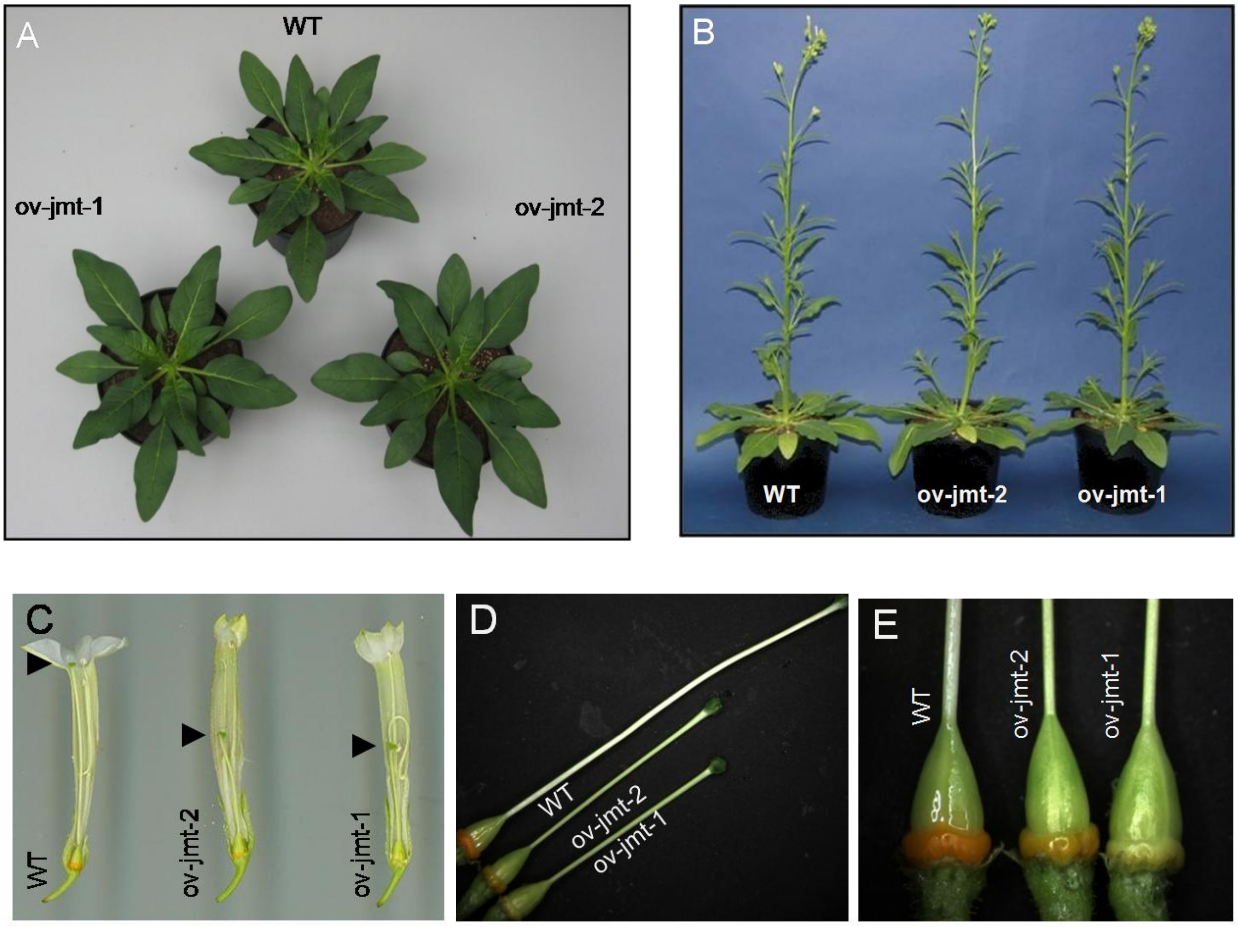

Figure 6. *N. attenuata* Over-expressing At *JMT* was indistinguishable from WT plants but showed altered flower morphology. (A) Plants of lines over-expressing At *JMT* (ov-jmt-1 and ov-jmt-2) and WT at rosette (30 days-old) **(B)** and flowering stages (45 days-old) **(C)** WT, ov-jmt-2 and ov-jmt-1 flowers longitudinally cut, showed partially closed ov-jmt corollas. **(D)** Ovaries with basal nectaries and style with stigma at top end. Styles of ov-jmt flowers were significantly shorter. **(E)** Nectaries showed different coloration.

We investigated whether these morphological alterations were correlated with changes in JAT composition. As expected, the corolla+stamen and the pistil+sepals samples collected from ov-jmt-1 flowers contained 5 (P = 0.007) and 13 times (P = 0.002) more MeJA, respectively, than did those from WT flowers. Conversely, large alterations of the JAT profile were detected in ov-jmt-1 flowers. Similarly large JAT levels were detected in ov-jmt-2 (data not shown). JA-Ile values detected in the corolla+stamen and the pistil+sepals samples from ov-jmt-1 were 30 % (P = 0.00001) and 3 % (P = 0.002), respectively, as large as samples from WT plants. JA and OH-JA-Ile concentrations in ov-jmt-1 pistil+sepals were not significantly changed compared to concentrations of WT flowers, but were significantly decreased in corolla+stamens extracts.

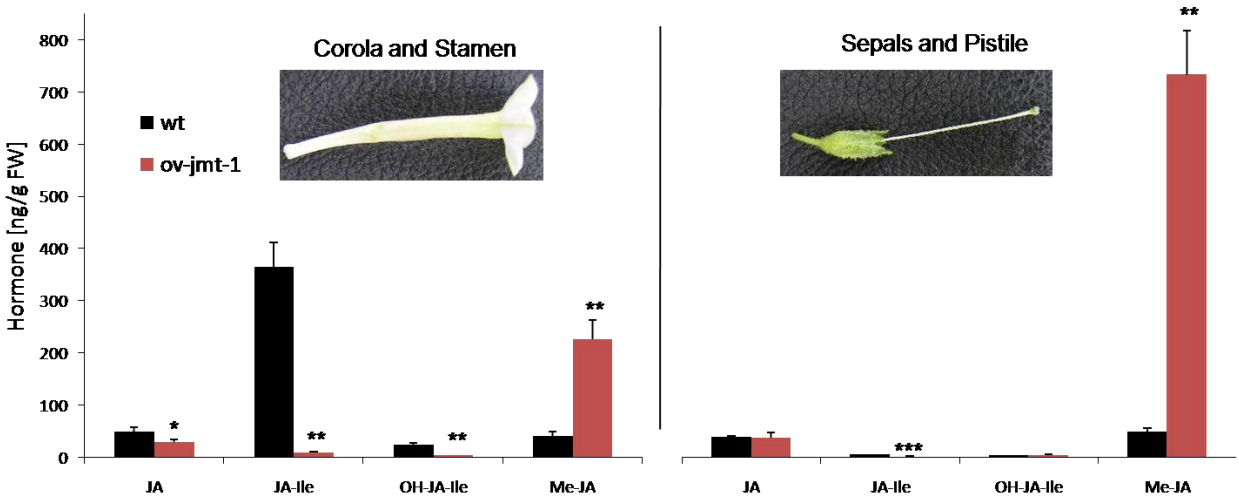

Figure 7. Comparison of flower morphology and JAT composition in WT and ov-jmt flower parts. JAT of flower parts. Mean + SE (n=5). Over-expressing At *JMT* decreased constitutively JA-Ile and increased MeJA-levels. Asterisks represent significant differences between WT and ov-jmt lines (N = 4, unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

We also measured TPI activity, a constitutive marker for JA -signaling, as well as the volume and sugar content of nectar collected from WT and ov-jmt flowers (Fig. 8A-C). Constitutive TPI activity detected in the protein extracts of complete ov-jmt-1 flowers was significantly reduced (P = 0.015) compared to that of WT. Nectar produced by ov-jmt -flowers contained more sugar compared to nectar produced by WT flowers but was found in significantly lower amounts (P = 0.0035).

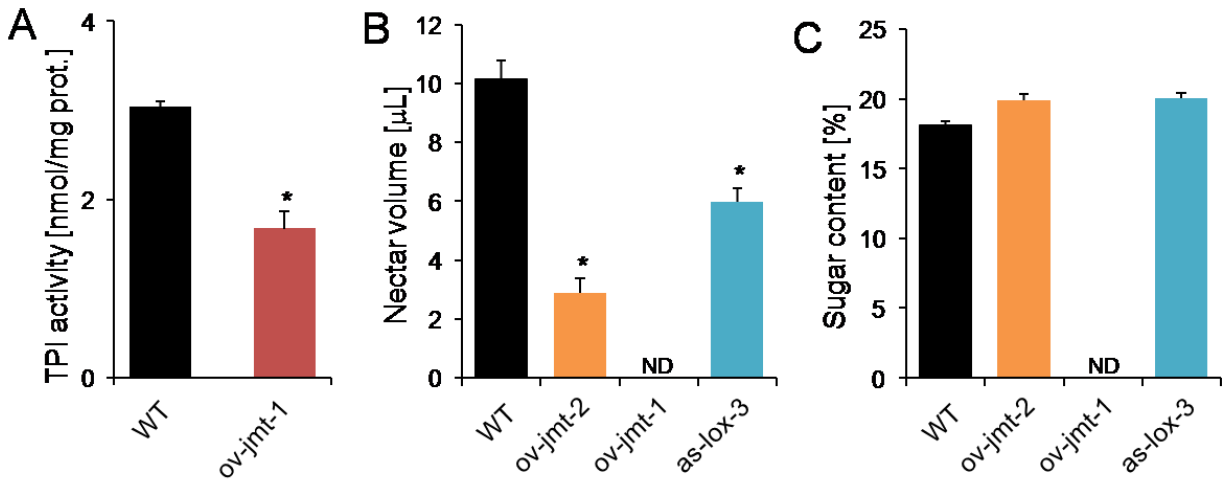

Figure 8. Over-expressing At JMT altered flower parameters.
(A) ov-jmt flowers showed decreased constitutive TPI compared to WT flowers, **(B)** reduced nectar production and **(C)** increased sugar content of nectar. ((A) N = 4; (B, C) N = 22, unpaired t-test; * P < 0.05)

Constitutive TPI activity in complete ov-jmt-1 flowers was significantly reduced compared to in WT flowers. Nectar volumes in three transgenic lines impaired in JATs (ov-jmt 1 and 2, as-lox-3) were much higher than those in WT flowers, but sugar concentrations were lower. as-lox3 was thereby the sole exception of the three lines, that did not show any phenotypic alteration in flower development beside reduced nectar production.

3.3 Over-expressing At JMT *compromised local and systemic JA metabolite production*

We explored the consequences of over-expressing At *JMT* for the temporal and spatial distribution of different JATs – JA, MeJA, JA-Ile, OH-JA and OPDA, the precursor of JA – after W+OS induction. To that end, locally and systemically induced leaves of ov-jmt and WT plants were collected at distinct time points (0, 30, 60, 120, 240 min). Leaves were dissected for petiole, midvein and lamina tissues. All JATs were consistently more abundant in vascular than in leaf lamina tissues, except JA-Ile, whose highest values were detected in the leaf lamina 1 h after induction. Even though constitutively more abundant in ov-jmt lines than in WT background, OPDA levels dropped quickly after induction to ~ 0 in all tissues of all genotypes. JA attained its highest levels in WT 1 h after induction in local vascular tissues but also started to increase systemically in petioles after 4 h. In clear contrast, local and systemic JA concentrations were severely reduced in ov-jmt plants. For instance, petiole, midvein and lamina samples of ov-jmt plants harvested after 1 h contained 88, 90 and 75 % less JA respectively than did WT (Fig. 9A-F).

However, comparing MeJA production in ov-jmt lines to MeJA concentrations found in WT, a reverse pattern for JA allocation can be observed. MeJA peaked highest at 0.5h in local midvein tissues of ov-jmt leaves and showed levels 70 fold higher than compared to in WT midveins. In ov-jmt leaves MeJA levels also increased in systemic tissues, peaking at 1 h, whereas MeJA levels in WT tissues never rose significantly above low constitutive levels. Differences in JA-Ile production across the genotypes were similar to those detected for JA. For instance JA-Ile values determined after 1 h in the lamina of ov-jmt-1 were 10 times less than in WT. The slight systemic increases detected in WT tissues were absent in ov-jmt lines. OH-JA levels increased after elicitation in all genotypes levels but to a lower extent in ov-jmt plants. In WT OH-JA levels increased in parallel to declining JA levels, whereas in ov-jmt lines they increased in parallel to declining MeJA levels (Fig. 10 A-I). In addition to these differences in JAT levels, slight modifications of SA and ABA patterns were also detected (not shown).

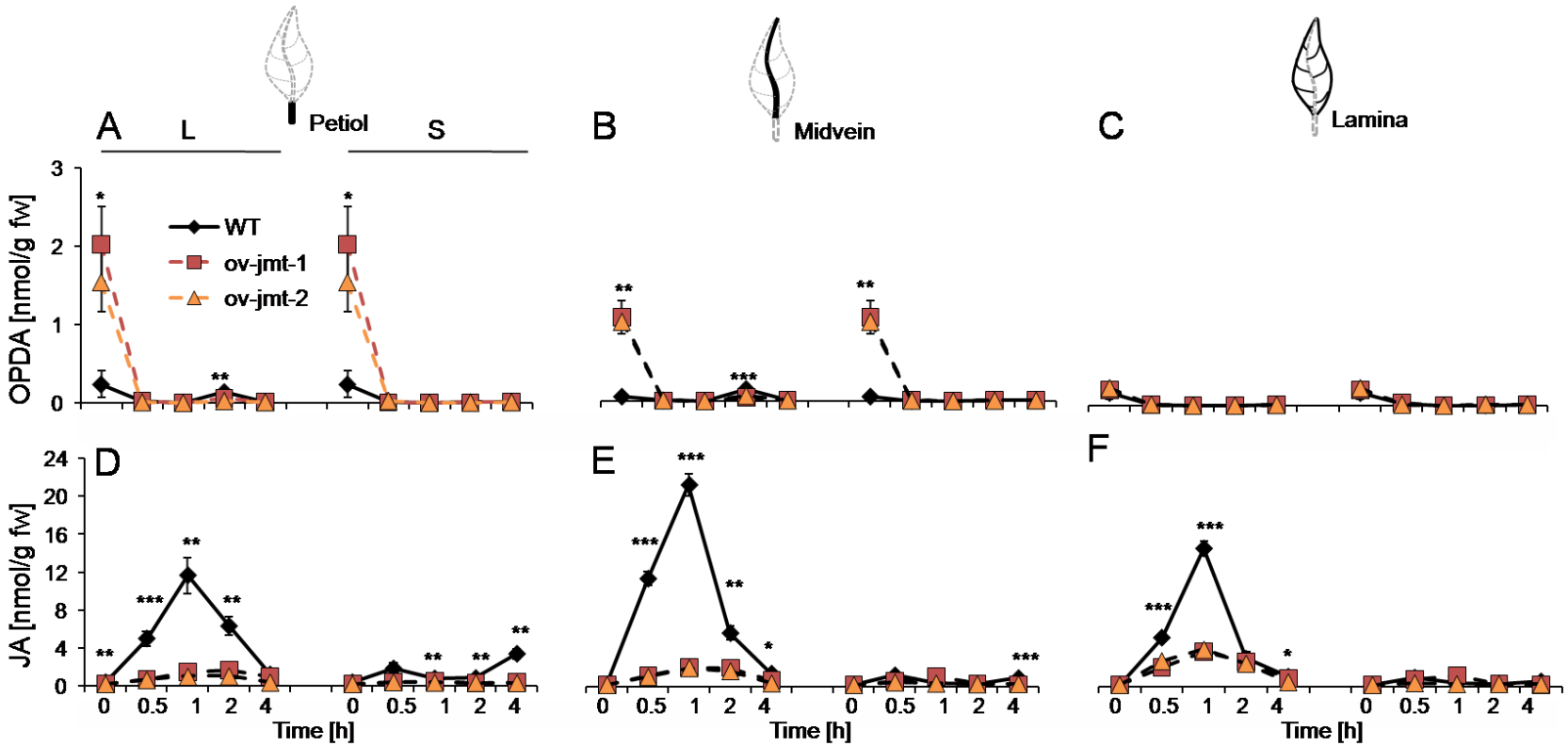

Figure 9. Dynamics of OPDA and JA leaves in locally and systemically induced leaves. Mean ± SE (n=5) levels of OPDA **(A – C)** and JA **(D – F)** expressed in nmol/g FW in petiole **(A, D),** midvein **(B, E)** and lamina **(C, F)** tissues from rosette leaves harvested from wild type plants (WT, black diamonds) and lines over-expressing At JMT (ov-jmt-1, open squares; ov-jmt-2, open triangles). One fully expanded leaf (local leaf, L) per plant was wounded with a fabric pattern wheel and treated with *M. sexta*'s oral secretions. The orthostichous leaf (growing with a minimal angular distance) above the treated leaf was considered as systemic leaf (**S**). Leaves were harvested at specific times after elicitation, immediately dissected and flash-frozen. Asterisks represent significant differences WT and ov-jmt -1 (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

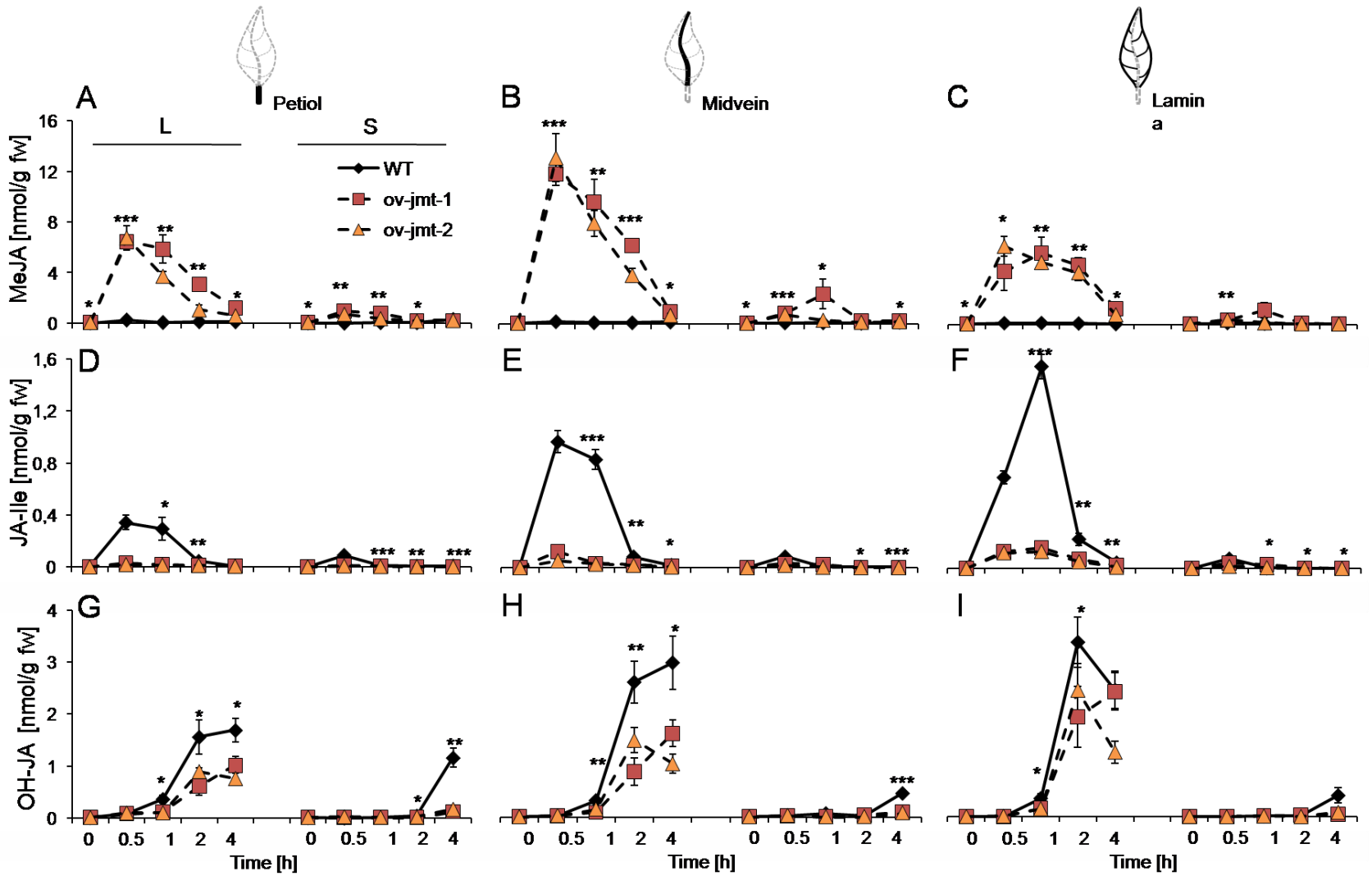

Figure 10. Dynamics of MeJA, JA-ile and JA-OH in locally and systemically elicited leaves. Mean ± SE (n=5) levels of MeJA **(A – C)**, JA-Ile **(D – F)** and JA-OH **(G – I)** expressed in nmol/g FW in petiole **(A, D, G),** midvein **(B, E, H)** and lamina **(C, F, I)** tissues from rosette leaves harvested from wild type plants (WT, black diamonds) and lines over-expressing At JMT (ov-jmt-1, open squares; ov-jmt-2, open triangles). One fully expanded leaf (local leaf, **L**) per plant was wounded with a fabric pattern wheel and treated with OS. The orthostichous leaf (growing with a minimal angular distance) above the treated leaf was considered as systemic leaf (**S**). Leaves were harvested at specific times after elicitation, immediately dissected and flash-frozen. Asterisks represent significant differences WT and ov-jmt-1 (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

3.4 ov-jmt plants were strongly susceptibleble to M. sexta attack

To examine the susceptibility of ov-jmt lines to insect attack, we compared the growth of *M. sexta* larvae feeding on ov-jmt, as-lox3 and WT plants (Fig. 11).

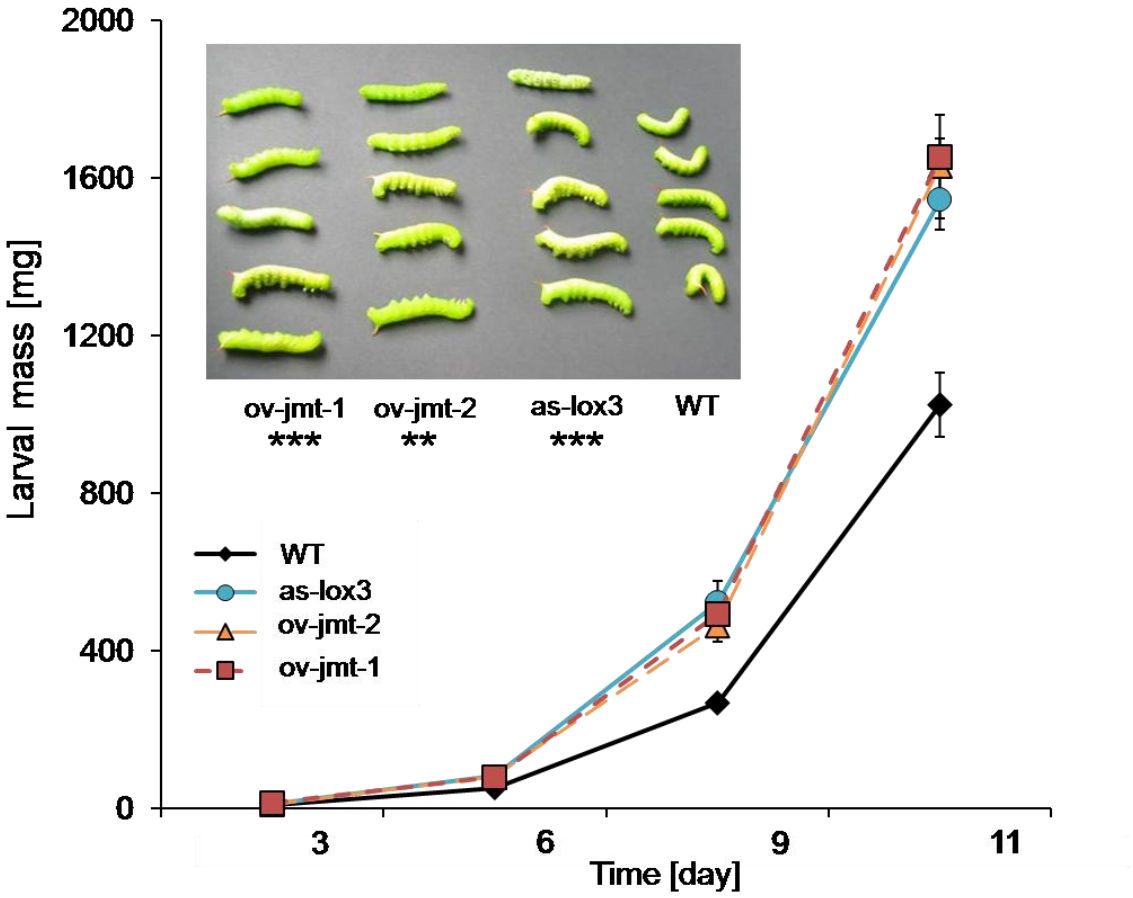

Figure 11. *M. sexta* larvae performed significantly better on ov-jmt lines than those on WT. Mean ± SE (n=25) mass of M. sexta larvae after 3, 6, 9 and 11 days of feeding on 25 replicate wild-type plants (WT, black diamonds), as-lox3 plants (open circles), and lines over-expressing At JMT (ov-jmt-1, open squares; ov-jmt-2, open triangles). The picture inserted shows representative 11 day-old larvae collected on each genotype. Asterisks represent significant differences between WT and ov-jmt lines (unpaired t-test; ** P < 0.001, *** < 0.0001).

As reported (Halitschke and Baldwin, 2003), *M. sexta* larvae that fed on as-lox3 plants, a mutant with altered herbivore defense caused by impaired jasmonate production, gained significantly more mass than on WT plants. Caterpillars that fed on ov-jmt plants grew as big as those that fed on as-lox3. The average weight of 11-day-old caterpillars that fed on ov-jmt-1, ov-jmt-2 and as-lox3 lines was 61 ($P = 4*10^{-8}$), 60 ($P = 0.0003$) and 51 % ($P = 2.5*10^{-5}$), respectively, above that of caterpillars that fed on WT plants.

3.5 Direct defense activation is impaired in ov-jmt and partly restored by JA-Ile

JA-Ile formation is critical for the induction of direct defenses in *N. attenuata*. We observed that ov-jmt plants, preferentially conducting JA metabolites towards methylation into MeJA, showed strongly affected formation of other JA metabolites, especially JA-Ile. Therefore, we next investigated the production of direct defense compounds by ov-jmt plants. As in flowers, constitutive TPI activity in ov-jmt leaves was strongly reduced compared to WT. Diterpene glucosides (DTGs) were also constitutively less abundant in ov-jmt compared to in WT leaves (Fig. 12A). Locally and systemically induced leaves were analyzed 3 days after induction by W+OS. W+OS-induced TPI and DTG levels were significantly lower in ov-jmt compared to in WT leaves, and nearly no systemic induction of TPI was detected in the two ov-jmt lines (Fig. 12 A-B). This effect was more pronounced in ov-jmt-1plants.

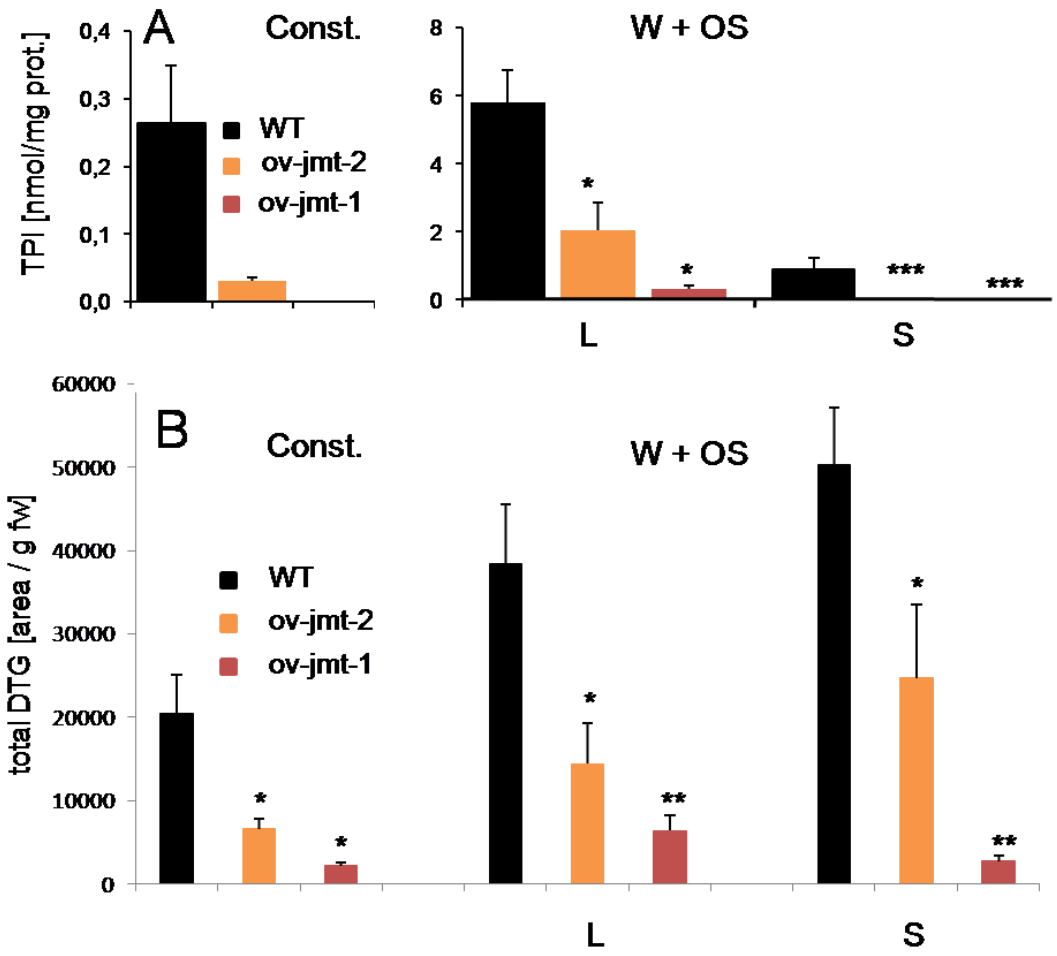

Figure 12. Over-expressing *At* JMT strongly reduced TPI and DTG accumulation in local and systemic leaf tissues after W+OS treatment. Mean + SE (n=5) **(A)** TPI activity and **(B)** DTG accumulation in rosette leaves from wild-type (WT, black bars) and lines over-expressing At JMT (ov-jmt-1, white bars; ov-jmt-2, gray bars) harvested from untreated plants (**A**,**B** const.: constitutive) or 3 days after that one fully expanded leaf per plant was wounded by a fabric pattern wheel (W) and treated with *M. sexta's* oral secretions (OS) The orthostichous leaf (growing with a minimal angular distance) above the treated leaf (**L**) was considered as systemic leaf (**S**). Asterisks represent significant differences between WT and ov-jmt lines (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

Using TPI as a marker, we next tested whether complementing wounded leaves with JA or JA-Ile restored local and systemic TPI activity in ov-jmt lines to levels detected in WT. Confirming W+OS-elicitation results, locally and systemically induced ov-jmt leaves harvested after wounding and applying water (W+W) showed significantly less TPI activity than did WT leaves.

When JA was applied instead of water (W+JA), TPI activity increased locally in WT but not in ov-jmt leaves which resulted in more pronounced differences in TPI activity of genotypes (P = 0.0058; P = $2*10^{-5}$). Unlike the W+W treatment, JA-Ile application (W+JA-Ile) amplified the local TPI activity in both WT and ov-jmt lines (Fig. 12A-C). This suggested that JA-Ile was the local signal lacking in ov-jmt plants. In contrast, differences still remained in systemic tissues.

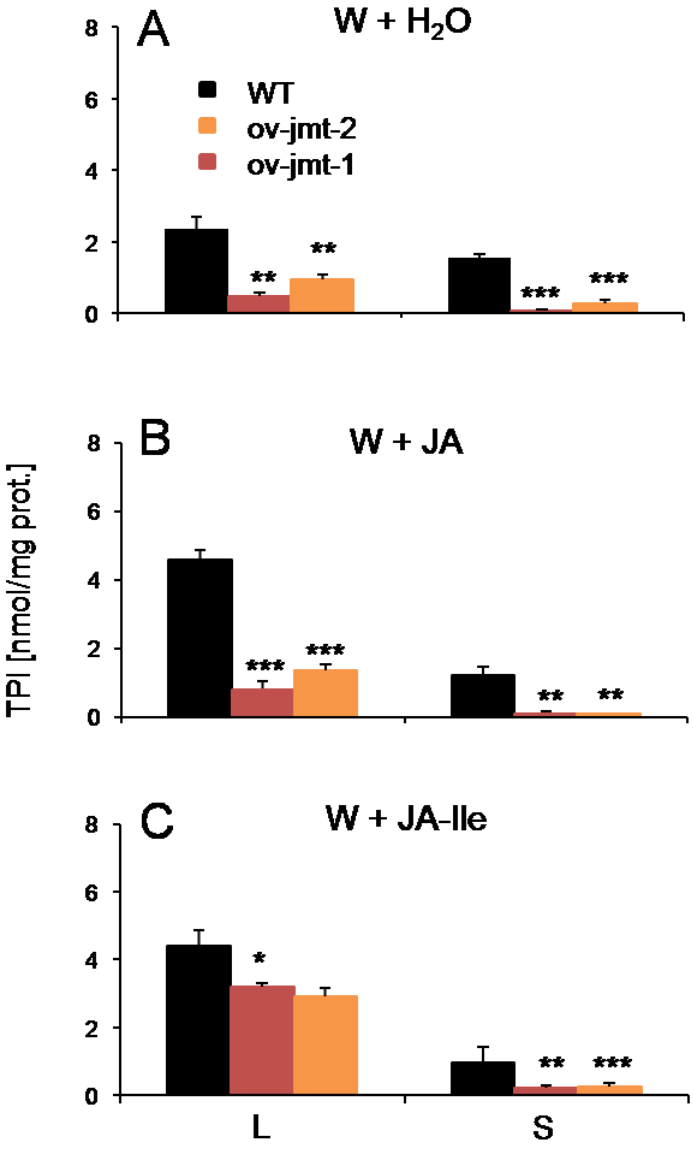

Figure 13. Local deficiency of TPI activity in ov-jmt plants was partly restored by JA-Ile but not by JA. Mean + SE (n=5) TPI activity in locally and systemically induced rosette-stage leaves from wild-type (WT, black bars) and lines over-expressing At *JMT* (ov-jmt-1, red bars; ov-jmt-2, orange bars) harvested from tissues 3 days after one fully expanded leaf per plant was wounded by a fabric pattern wheel (W) and treated with distilled water **(A)**, JA (0.1µmoles) **(B),** or JA-Ile (0.1 µmoles) **(C)**. The orthostichous leaf (growing with a minimal angular distance) above the treated leaf (**L**) was considered as systemic leaf (**S**). Asterisks represent significant differences between WT and ov-jmt lines (n = 5 unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

3.6 Over-expressing JMT *differentially affected genes related to JA biosynthesis and defense*

We analyzed transcriptional alterations in ov-jmt lines. We focused on *AOS*, a JA biosynthetic gene as well as on two defense-related ones, *TPI* and *TD*; the latter one controls the availability of isoleucine (Ile) (Kang et al., 2006) and also acts as an amino-acid-degrading defense after ingestion (Chen et al., 2005) (Fig. 14 A-C). Consistent with the above results, after 4 h TPI expression was significantly lowered by 75 % (P =0.004) locally, respectively, 76 % (P = $3*10^{-5}$) systemically in ov-jmt-1 leaves compared to in WT. A similar pattern was observed for TD expression. But surprisingly, induced expression levels detected for *AOS* were generally higher – 5 and 3 times more in local tissues at 0.5 and 4 h – in ov-jmt-1 than in WT plants. Similar patterns were observed for ov-jmt-2 line (not shown).

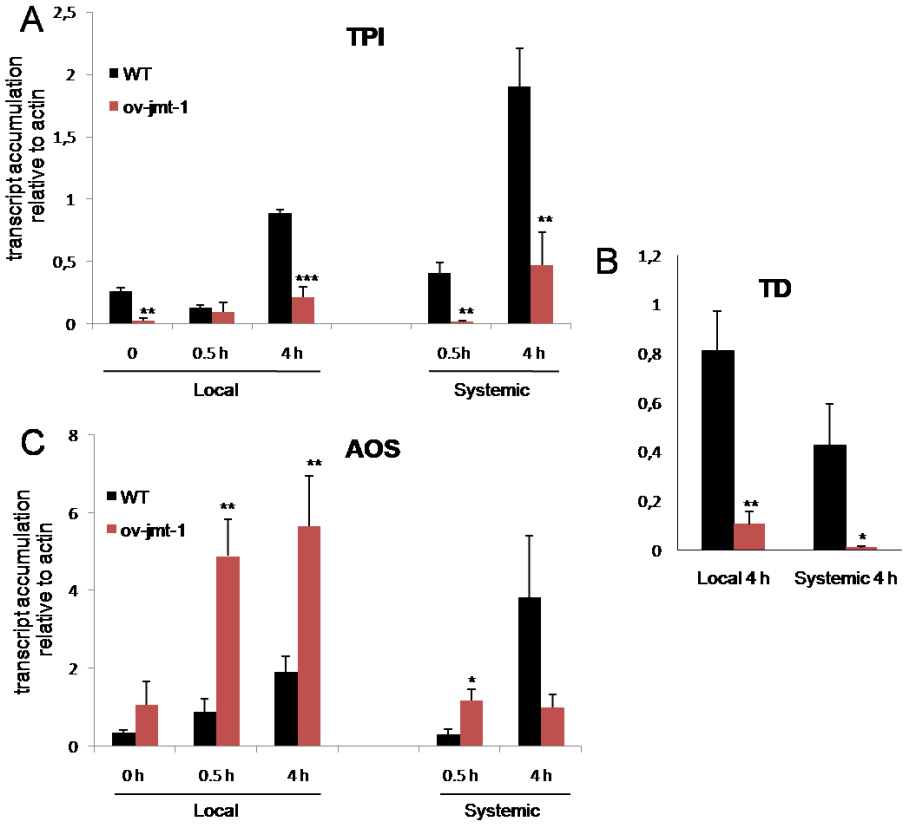

Figure 14. **Over-expression of At *JMT* alters levels of different transcripts before and after treatment with *M. sexta*'s oral secretions (OS) in locally and systemically induced tissues.** Mean + SE (n=5). Accumulation of TPI **(A)** and TD **(B)** transcripts were reduced locally and systemically in ov-*jmt*-1 compared to in WT. **(C)** AOS transcription is locally stronger but systemically less induced in ov-*jmt*-1 than in WT plants. Asterisks represent significant differences between WT and ov-jmt lines (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

4. Discussion

JA-dependent signalling controls several aspects of plants´ development, physiology and defense. Here we report the phenotypic and molecular characterization of two independently transformed *N. attenuata* lines over-expressing At *JMT,* a transferase responsible for MeJA formation. In this study we showed that the reduced availability of JA for conversions to JATs others than MeJA impaired based defense activation, altered flower morphology and reprogrammed transcript accumulations. A working model is shown in Figure 15.

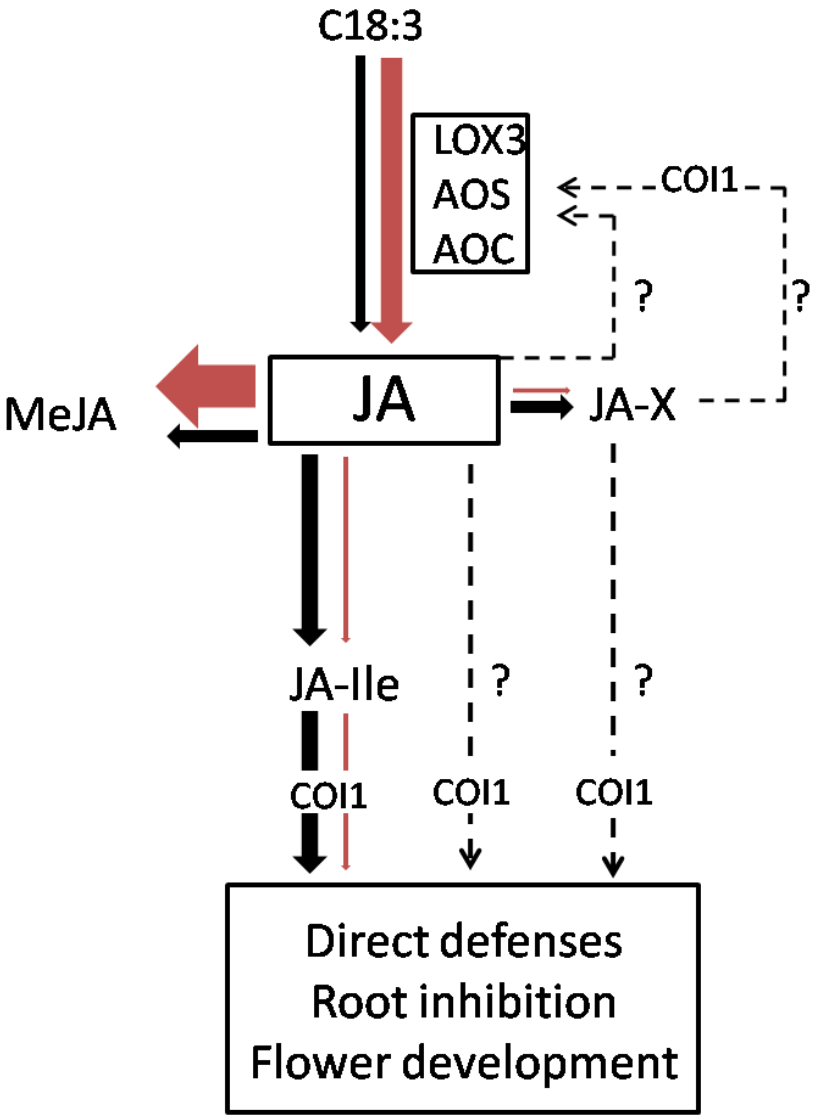

Figure 15. Possible model of JAT composition and signaling in *N. attenuata* ov-jmt and WT plants. Black arrows describe compound fluxes and induction paths in the WT background, red arrows those in the ov-jmt background. Dashed lines indicate possible functions of JATs.

Over-expressing At* JMT *disturbs the JAT balance regulating floral parameters

We observed that the lengths of the styles in ov-jmt flowers were reduced by 50 % compared to in WT flowers. Nevertheless, the increased distance between stigma and anthers of ov-jmt flowers did not obviously alter seed capsule production (data not shown). Normal flower development is known to be dependent on intact JA biosynthesis and signalling. Among others, the two JA biosynthetic *Arabidopsis* mutants *dad1* and *opr3* are male sterile due to a delay in anther development and dehiscence as well as reduced filament elongation (Ellis and Turner, 2002). Moreover, the *Arabidopsis* and *N. attenuata coi1* mutants impaired in JAT perception are also male sterile (Feys et al., 1994; Paschold et al., 2007). In contrast, in tomato, COI1 is essential for proper female fertility (Li et al., 2004), indicating the diversity of species-specific JAT functions in flower development. Till now, the mode of JAT action at the flower level has just been a part of the larger research body exploring JA signalling. Only recently has, *AGAMOUS*, a master switch in floral development, been shown to regulate *DAD1*, which encodes a lipase required required for the release of linolenic acid, the initial substrate for JA production (Ito et al., 2007). Moreover, an elegant comparative analysis of the stamen transcriptome of both JA-treated and untreated *opr3* mutants has revealed that JAT transduction is mediated by two specific MYB transcription factors (Mandaokar et al., 2006). Testing whether these molecular players are deregulated in ov-jmt flowers would be of major interest for further research.

Over-producing MeJA strongly reduced JA-Ile and OH-JA-Ile levels in ov-jmt pistil and sepal tissues, while slightly altering JA levels. A recent study on *N. attenuata* has just revealed the influence of JA-Ile concentrations on style elongation and, interestingly, described the opposite of our observations. Thus, when silencing systemin production in ir-sys lines, (Berger and Baldwin, 2009) reported longer flower styles than in WT plants. These changes were accompanied by a down-regulation of *TD,* resulting in lower JA-Ile amounts. It is noteworthy that in the latter study levels of other JAT were unchanged, suggesting that concomitantly altering more than one JAT and/or the over accumulation of MeJA is responsible for the observed ov-jmt flower phenotype. Interestingly, *N. attenuata* as-lox3 transformants having levels of each JAT, including JA-Ile, reduced to half of those in WT, do not display any obvious floral alteration, except a reduction in nectar production (Fig. 8 B). Altogether these data tend to reinforce the idea formulated by Wasternack et. al (2007) that a signature in oxylipines rather than the action of a single JAT controls the development of floral organs.

MeJA was not a defense signal in Nicotiana attenuata

Comparing to *JMT* over expressing transformants in *Arabidopsis* (3 fold higher MeJA levels) and soybean (2,5 fold), *N. attenuata´*s over-expression of At *JMT* did not result in significantly increased constitutive levels of MeJA. The observation that 1 h after induction ov-jmt leaves showed up to 50 fold higher MeJA levels compared to WT leaves, suggests that substrate limitation is the bottle neck for MeJA formation*. N. attenuata* ov-jmt lines had strongly reduced direct defenses (Figs. 8A and 12) and were as vulnerable to insect attack as *as-lox3* plants (Fig. 12) which are mute in total JAT production. Typical anti-herbivore defense compounds such as TPIs and DTGs were notably found to be less abundantly induced both locally as well as systemically in leaves of ov-jmt compared to WT plants (Fig. 12A, B) This interruption in JA-based signalling indicates that MeJA itself is not a bioactive defense signal. This confirms recent data demonstrating that the traditional eliciting activity of MeJA during application in a lanolin paste or as an airborne signal requires de-esterification and conjugation to Ile (Wu et al., 2008).

The decrease in defense expression observed in *N. attenuata* ov-jmt lines contrasts strikingly with data from *Arabidopsis* transgenic ov-jmt lines showing constitutive defense activation and enhanced resistance to fungal pathogens (Seo et al., 2001; Cipollini, 2007). The fact that *N. attenuata* accumulates constitutively high OPDA values is noteworthy. Indeed, this cyclopentanone has been shown to partly control defense expression in *Arabidopsi*s and to mediate resistance to dipteran *Bradysia impatiens* and the fungus *Alternaria brassicola* in *Arabidopsis* opr3, which is unable to convert OPDA to JA. Whether defenses are sustained by high OPDA accumulation in *Arabidopsis* ov-jmt plants remains to be explored.

JA-Ile was the critical but not unique bioactive JAT

The defenseless phenotype of ov-jmt plants largely mimics that of *N. attenuata* lines silenced for *JAR4* and *JAR6,* the two tobacco homologues for *JAR1* (Kang et al., 2006). *JAR4* and *JAR6* encode the JA amino acid conjugate enzymes critical for JA-Ile production. Supporting this, were the severe alterations we observed in JA-Ile formation in ov-jmt plants due to the diversion of free JA units towards methylation. JA-Ile has already been shown to partly restore TPI production in as-lox3 plants, suggesting that other lox3-dependent JAT complement

the activity of JA-Ile. In our studies, applying JA-Ile onto ov-jmt wounded leaves recovered most of the local, but not systemic, TPI expression. Altogether, these data reinforce the critical role played by JA-Ile as the pivotal switch in JA signaling, but suggest that other JATs whose production is antagonized by At *JMT* over expression also fulfill significant functions in systemic JA signaling.

Defense and JA-biosynthetic genes were independently regulated in ov-jmt

The expression of most defense-related genes is traditionally reported to be positively correlated with that of JA-biosynthetic ones. By monitoring induced transcript levels in leaves from ov-jmt plants, we observed an opposite pattern. Transcripts for *TD* and *TPI*, known to be regulated by JA-Ile, were in comparison to WT tissues strongly down regulated in ov-jmt leaves; the JA biosynthesis gene *AOS* was constitutively up-regulated. In agreement with latter findings, *AOS* expression has been observed to be significantly induced in *Arabidopsis* ov-jmt transformants. Taken together, these data together suggest that in *N. attenuata* WT plants the regulation of these two gene categories is coupled during herbivory, but mediated by two different JAT signals. Previous data published by Wang et al. (2008) have indicated that the JA positive feedback loop, though COI1-dependent, was independent from JA-Ile since induced levels of *AOS* and *AOC* transcripts were not altered in *JAR4/JAR6*-silenced plants. Considering these findings, our results suggest that a metabolite whose production is favored in ov-jmt plants, e.g. MeJA, or OPDA, up-regulates *AOS*. Alternatively, the formation of a strong transcriptional repressor could be impaired in ov-jmt lines. OH-JA for example, whose levels are decreased by 50 % in ov-jmt, has already been shown to repress the expression of *AOS*. Whether complementation experiments with OH-JA application in ov-jmt restore *AOS* levels to those found in WT will need to be tested.

In conclusion, over-expressing At *JMT* in *N. attenuata* creates a metabolic sink in JA-based defense signalling. This transformant offers new perspectives for future dissections of the distinct roles of different JATs in plants` developmental and defense processes.

5. Experimental procedures

5.1 Plant material

Nicotiana attenuata Torr. Ex Wats. (Solanaceae) originated from a field collection of an isolated 1000-plant population in 1998, near the Apex Mine in south-western Utah (UT). Seeds of all lines and from the 30th generation of self-pollinated greenhouse-grown plants were sterilized and treated with diluted liquid smoke solution before being germinated on Gamborg B5 Media (Krügel et al., 2002). After 10 d at 30°C with 16 h light and 8 h of darkness in a growth chamber, all seedlings were transferred for 10 more days in soil containing Teku pots (Waalwijk, Netherlands). Adjacent seedlings were planted into 1 L pots and transferred into the greenhouse to grew at 26-28°C and 65% humidity under 16 h day light (Philips Sun-T Agro 400 or 600 Watt sodium lights).

5.2 Insect rearing and plant treatments

Eggs of *Manduca sexta* were ordered from North Carolina State University (Raleigh, NC, USA) and kept in a growth chamber (Snijders Scientific, http://www.snijders-tilburg.nl) at 26°C 16-h light, 24°C 8-h dark until the larvae hatched. For *M. sexta* performance assays, neonates were directly placed on the fully developed leaves of rosette-stage plants. *M. sexta* oral secretions (OS) were collected from 3rd- to 4th-instar larvae reared on *N. attenuata* WT plants and diluted 1:10 (v/v). For all treatments plants were randomly assigned to different treatment groups and the youngest fully expanded leaf on the plant (source-sink transition leaf) was treated. To simulate of *Manduca sexta* feeding, a fabric pattern wheel was rolled over leaves, causing rows of punctured wounds. Then 20µl of oral secretions (OS, diluted 1:10, v/v) were immediately applied onto the wounded leaves (Fig. 16 A-B). For complementation assays, wounded leaves were either treated with 0.1 µM JA or JA-Ile and, controls were treated with an equivalent volume of water.

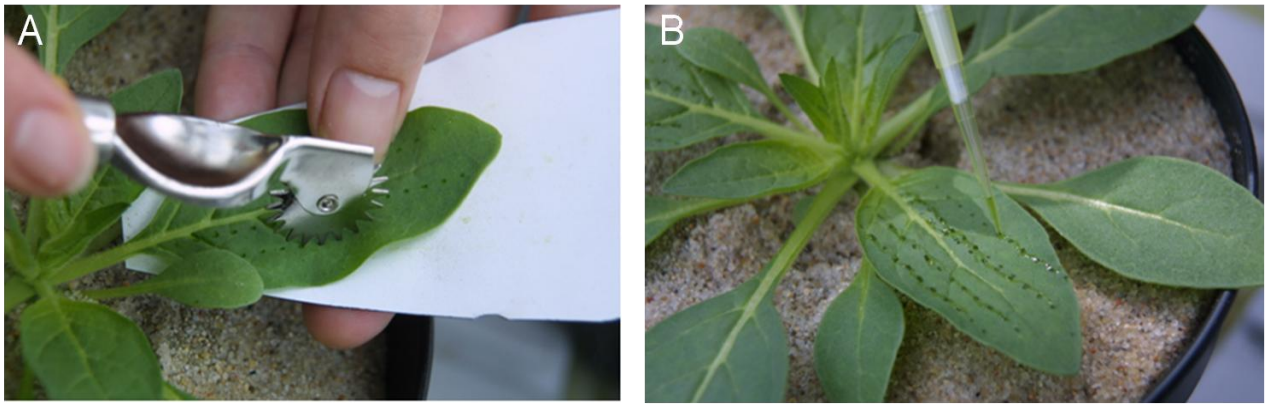

Figure 16. Plant treatments.

(A) Mechanically wounding a leaf (W) with a fabric pattern wheel. **(B)** *M.sexta* oral secretions (OS) applied to wounded leaf (Photos by Danny Kessler)

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5.3 Nucleic acid analysis

DNA was extracted from the leaf tissue of fully developed plants using the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1985) with the following modification: after the second chloroform/isoamyl alcohol extraction, a two-thirds volume of ice-cold isopropanol was added to the supernatant and the sample was incubated for 30 min. After being centrifuged briefly, the supernatant was discarded and the pellet was dissolved in high-salt Tris-EDTA. The solution was then incubated with 100 ng.µl^{-1} RNAse (30 min at 37°C) followed by another extraction with chloroform/isoamyl alcohol. DNA samples (10 µg) were digested with *Eco*RV restriction enzyme; size fractionated in a 0.8% (w/v) agarose gel and Southern blotted onto a nylon membrane (GeneScreen Plus; Perkin Elmer, **http://www.perkinelmer.com**). Fragments of *hptII* were amplified by PCR and used as probes for Southern hybridization. The probes were labeled with α-^{32}P (Rediprime® II DNA labeling system; Amersham Biosciences, **http://www.amersham.com).qwsd**

For Northern blotting analysis, total RNA was extracted via TRI reagent following the protocol from TIGR http://www.tigr.org/tdb/potato/images/SGED_SOP_3.1.1.pdf) and 10 µg per sample size-fractionated by 1.2% agarose-formaldehyde gel electrophoresis. The RNA was then blotted onto a nylon membrane (GeneScreen*Plus*; NEN-DuPont, Boston) following the

manufacturer´s protocol. For a loading control ethidium bromide fluorescence was used. Blots were pre-hybridized (1h, 42°C, Ultrahyb hybridization buffer, Ambion) after UV-crosslinking and subsequently hybridized overnight with α-^{32}P labeled At *JMT* probes (for sequences see appendices). The blots were washed at 60°C once for 15 min with 2x SSC, 0.1% SDS, and then twice for 20 min with 0.1x SSC, 0.1% SDS. Blots were exposed for 24 h on a phosphoimage film (Fuji Film) and the signals obtained read by a BAS-reader (Fuji Film).

The RNA extraction method for transcript analysis was similar to this of Northern blotting. cDNA was synthesized from 150 ng RNA using MultiScribe® reverse transcriptase (Applied Biosystems, **http://www.appliedbiosystems.com**). Quantitative real-time PCR (ABI PRISM®7000; Applied Biosystems) was conducted using the qPCR® core reagent kit (Eurogentec, **http://www.eurogentec.be**) using gene-specific primers and double fluorescent dye-labeled probes. Relative gene expression was calculated using a 200-fold dilution series of cDNAs. Actin, which is not regulated under our experimental conditions, served as the endogenous control gene (for sequences see appendices).

5.4 Jasmonate analysis

In liquid nitrogen flash-frozen leaves and dissected tissues – leaf lamina sectors, midvein and petiole (200mg) – were homogenized to powder by shaking them in 2ml reaction tubes containing 2 steel beads (5mm) with Genogrinder©. Samples were extracted by being shaken with 1ml ethyl acetate – containing D_2-JA, $^{13}C_2$-MeJA and $^{13}C_6$-JA-Ile – for 10 min. Amounts of internal standards (IS) were adjusted to match the expected endogenous jasmonate levels of different tissues: labeled JA and MeJA 100 ng each, labeled JA-Ile 20 ng for midvein and petiole samples 40 ng, for lamina samples 200 ng. After centrifugation at 4°C and maximum speed, supernatants were transferred to new 2ml reaction tubes, and remaining pellets were re-extracted with 0,5 ml of pure ethyl acetate. After centrifugation second and first supernatants were combined and evaporated completely at 30°C in a vacuum concentrator. Residues were resuspended in 70% methanol by shaking for 10 min and than centriguged at 4°C and maximum speed before being analyed. A 10µl aliquot of the resulting extract was analyzed by reverse-phase HPLC coupled to a mass spectrometry (RP-HPLC/ESI-MS/MS). Jasmonates were separated from extracts at a flow rate of 100µl/min on a Pursuit C8 column (3 µm, 150 x 2 mm, Varian) using a binary solvent system (A: 0.05% v/v formic acid in de-ionized water; B: 0.05% v/v formic acid in methanol) in gradient mode. Multiple reaction monitoring (MRM) was conducted

on a 1200L MS/MS system (Varian, Palo Alto, CA, USA), operated in negative (JA, JA-Ile, 12/11-OH-JA, 12/11-OH-JA-Ile, 12-COOH-JA-Ile) or positive (MeJA) ionization mode. Parent-ion/daughter-ion selections and collision energies were set as follows: 213/59 (D2-JA), 209/59 (JA), 225/59 (12/11-OH-JA), 328/136 (13C6-JA-Ile), 322/130 (JA-Ile), 338/130 (12/11-OH-JA-Ile), 352/130 (12-COOH-JA-Ile). MRM for 12/11-OH-JA and 12/11-OH-JA-Ile returned two separate peaks for each pair of hydroxylated region-isomers: retention time (RT) for 12-OH-JA= 5.31 min, RT11-OH-JA = 5.56 min, RT12-OH-JA-Ile = 5.67 min, RT11-OH-JA-Ile = 5.96 min. For these analytes, peak areas were combined prior to quantification. Jasmonate quantities were calculated according to the formula: analyte product ion peak area x (IS concentration/IS product ion peak area). D2-JA was used as IS for 12/11-OH-JA while 13C6-JA-Ile was used as IS for 12/11-OH-JA-Ile and 12-COOH-JA-Ile.

5.6 Measurement of nectar volumes and sugar concentrations

Nectar of all flowers was collected by inserting a standardized 25 µl glass capillary into the corolla until it reached the base of the nectaries. To obtain complete nectar volumes, capillaries were held with one hand, the corolla tube with the other hand, then, against the counter pressure of the inserted capillary, corolla tubes were removed. Nectar volume was measured in millimeters in the capillary and converted into microliters. Sugar concentrations were measured with a portable refractometer (Optech,Sliedrecht, the Netherlands) with a range of 0% to 32% and a resolution of 0.2%, by blowing the nectar out of the capillary directly on the measuring surface.

5.7 Root elongation inhibition assay

Seedlings germinated on MeJA-free Gamborg B5 Petri dishes were transferred 6 days after germination on culture plates with Gamborg B5 media containing 20 µM MeJA. As control plates with 0.01% ethanol were used since MeJA was diluted in equal volumes of ethanol. For 15 days the plates were placed in a growth chamber at 30°C with 16 h light and 8 h of darkness. Plates were evaluated by comparing the root lengths of seedling on control plates (set as 100%) with those on MeJA plates.

5.8 Caterpillar feeding assay

Plants at rosette stage were randomized in the greenhouse and a 1-day-old neonate of *M .sexta* was seat on the transition leaf of each plant. After 3, 6, 9 and 11 days, caterpillars were weighted and average weights calculated.

5.9 Analysis of direct defense traits

Leaf tissue (100-150 mg) was homogenized as before mentioned for Jasmonate analysis and analyzed for DTGs and TPI activity 3 days after elicitation by treating puncture wounds with *M. sexta* OS. In complementation experiments, leaves were elicited by puncture wounding and applying water, 0.1 µmol JA or 0.1 µmol JA-Ile. The accumulation of secondary metabolites was analyzed by high-performance liquid chromatography (HPLC) as previously described (Halitschke and Baldwin, 2003). TPI activity was analyzed by the radial diffusion assay described by (Van Dam et al., 2001).

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7. Appendices

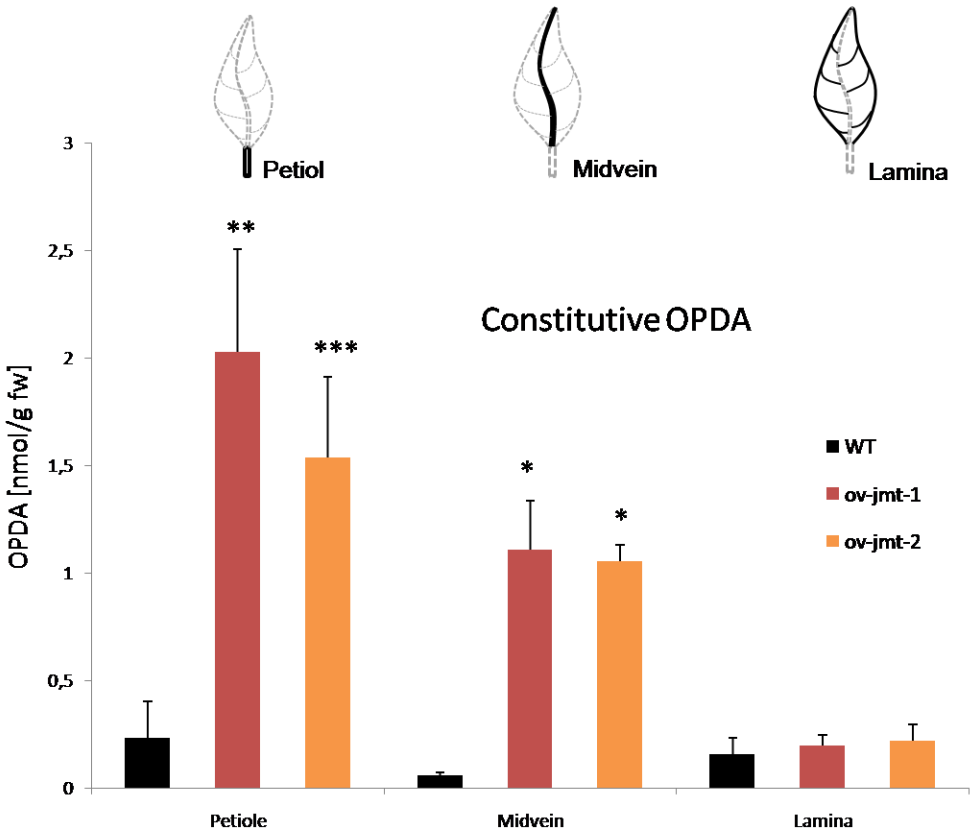

Figure 17. Over-expressing At *JMT* increases constitutive OPDA values in vascular tissues. Mean + SE (n=5) Petiole and Midvein extracts show significantly increased OPDA values compared to similar WT tissues . Asterisks represent significant differences WT and ov-jmt lines (unpaired t-test; * P < 0.05; ** < 0.001, *** < 0.0001).

Sequence of used At *JMT* primers: forward primer 5´-CTAGGCAGAAGAGTAATGGAC-3´;
reverse primer, 5´-GTGAAGGCTCCGGCGAGG-3´

Sequence of At *JMT*:

ATGGAGGTAATGCGAGTTCTTCACATGAACAAAGGAAACGGTGAAACGAG
TTATGCCAAGAACTCCACCGCTCAGAGTAACATAATATCTCTAGGCAGAA
GAGTAATGGACGAGGCCTTGAAGAAGTTAATGATGAGCAATTCAGAGATT
TCGAGCATTGGAATCGCCGACTTAGGCTGCTCCTCCGGTCCGAACAGTCT
CTTGTCCATCTCCAACATAGTTGACACGATCCACAACTTGTGTCCTGACC
TCGACCGTCCTGTCCCTGAGCTCAGAGTCTCTCTGAACGACCTCCCTAGC
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GTTTTGTGTCGGCCGTCCCAGGTTCGTTCTACGGACGTTTGTTTCCTCGC
CGGAGCCTTCACTTTGTGCATTCTTCTTCTAGTTTACATTGGTTATCTCA
GGTTCCATGTCGTGAGGCGGAGAAGGAAGACAGGACAATAACAGCTGATT
TAGAAAACATGGGGAAAATATACATATCAAAGACAAGTCCTAAGAGTGCA
CATAAAGCTTATGCTCTTCAATTCCAAACTGATTTCTGGGTTTTTTTGAG
ATCGCGATCTGAGGAGTTGGTCCCGGGAGGCCGAATGGTTTTATCGTTCC
TTGGTAGAAGATCACTGGATCCCACAACCGAAGAGAGTTGCTATCAATGG
GAACTCCTAGCTCAAGCTCTTATGTCCATGGCCAAAGAGGGTATCATCGA
GGAAGAGAAGATCGATGCTTTCAACGCTCCTTACTATGCTGCGAGCTCCG
AAGAGTTGAAAATGGTGATAGAGAAAGAAGGGTCATTTTCGATCGATAGG
CTTGAGATAAGTCCGATTGATTGGGAAGGTGGGAGTATCAGTGAGGAGAG
TTATGACCTTGTAATAAGGTCCAAACCCGAAGCCCTAGCTAGTGGCCGAA
GAGTGTCTAATACCATAAGAGCTGTGGTCGAGCCGATGCTAGAACCTACT
TTCGGTGAAAATGTGATGGACGAGCTTTTTGAAAGGTATGCAAAGATCGT
GGGAGAGTACTTCTATGTAAGCTCGCCACGATACGCTATTGTTATTCTTT
CGCTCGTTAGAACCGGTTGA

Probes and primers used for Taqman:

Gene	Forward primer	Reverse primer	Taqman probe
AOS	TCAACACATGAGCGAAACCC	GATCATTAGCCGAGTTTAAATCAGC	CATTATTATGGAGAAACTCGACCGGTCACC
TPI	TCAGGAGATAGTAAATATGGCTGTTCA	ATCTGCATGTTCCACATTGCTTA	CCTTGCTCTCCTCCTCTTATTTGGAATGTCT
TD	TAAGGCATTTGATGGGAGGC	TCTCCCTGTTCACGATAATGGAA	TTTTTAGATGCTTTCAGCCCTCGTTGGAA
Actin	GGTCGTACCACCGGTATTGTG	GTCAAGACGGAGAATGGCATG	TCAGCCACACCGTCCCAATTTATGAGG

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Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, das ich die vorliegende Arbeit selbstständig und nur unter der Verwendung der aufgeführten Quellen und Hilfsmittel angefertigt habe.

Jena, den 25.06.2009

Michael Stitz