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ORIGINAL ARTICLE

Methyl jasmonate-elicited herbivore resistance: does MeJA function as a signal without being hydrolyzed to JA?

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Abstract Treatment with methyl jasmonate (MeJA) elicits herbivore resistance in many plant species and over-expression of JA carboxyl methyltransferase (JMT) constitutively increases JA-induced responses in *Arabidopsis*. When wild-type (WT) *Nicotiana attenuata* plants are treated with MeJA, a rapid transient endogenous JA burst is elicited, which in turn increases levels of nicotine and trypsin proteinase inhibitors (TPIs) and resistance to larvae of the specialist herbivore, *Manduca sexta*. All of these responses are impaired in plants silenced in lipoxygenase 3 expression (as*LOX3*) but are restored to WT levels by MeJA treatment. Whether these MeJA-induced responses are directly elicited by MeJA or by its cleavage product, JA, is unknown. Using virus-induced gene silencing (VIGS), we silenced MeJA-esterase (*NaMJE*) expression and found this gene responsible for most of the MeJA-cleaving activity in *N. attenuata* protein extracts. Silencing *NaMJE* in as*LOX3*, but not in WT plants, significantly reduced MeJA-induced nicotine levels and resistance to *M. sexta*, but not TPI levels. MeJA-induced transcript levels of threonine deaminase (*NaTD*) and phenylalanine ammonia lyase (*NaPAL1*) were also decreased in VIGS *MJE* (as*LOX3*) plants. Finally the performance of *M. sexta* larvae that fed on plants treated with JA or MeJA demonstrated that silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants. From these results, we conclude that the resistance elicited by MeJA treatment is directly elicited not by MeJA but by its de-methylated product, JA.

Keywords MeJA esterase (*NaMJE*) · Methyl jasmonate (MeJA) · Jasmonate (JA) · *Nicotiana attenuata* · *Manduca sexta*

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Introduction

Methyl jasmonate (MeJA), jasmonic acid (JA) and its amino acid conjugates, collectively referred to as jasmonates, are important cellular regulators mediating diverse developmental processes including root growth, pollen production, and plant resistance to insects and pathogens (Creelman and Mullet 1997; Kessler and Baldwin 2002). Jasmonates are synthesized in plants via the octadecanoid pathway (Creelman and Mullet 1997). Briefly, linolenic acid is oxygenated by lipoxygenase (LOX) and then converted to 12-oxo-phytodienoic acid (12-oxo-PDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). JA is synthesized from 12-oxo-PDA through reduction and three steps of β-oxidation, and then catabolized further to form its volatile counterpart, MeJA (Seo et al. 2001), and numerous conjugates including JA-isoleucine (JA-Ile, Staswick and Tiryaki 2004; Kang et al. 2006; Wang et al. 2007a, b). MeJA is one of the JA metabolites proposed to play an important role in inter- and intra-plant signaling (Farmer and Ryan 1990; Seo et al. 2001; Karban et al. 2000; Kessler et al. 2006; Baldwin et al. 2006). When plants are exposed to volatile MeJA, they quickly elicit a series of JA mediated defense responses and MeJA treatment is the most commonly used means of eliciting herbivore resistance in many different plant species (McConn et al. 1997; Baldwin 1998; Li et al. 2002). However, it is

still not known how plants elicit herbivore resistance traits in response to MeJA exposure.

When *Nicotiana attenuata* is attacked by herbivores, it produces both volatiles to recruit the herbivores' natural enemies (Kessler and Baldwin 2001; Mattiacci et al. 1995) and secondary metabolites that function as direct defenses, such as the neurotoxin nicotine (Baldwin 1999; Steppuhn et al. 2004), and trypsin proteinase inhibitors (TPIs, Zavala et al. 2004). JA signaling plays a central role in these responses. Silencing a key gene involved in supplying fatty acid hydroperoxides for JA biosynthesis, lipoxygenase 3 (*NaLOX3*), reduces the wound- and herbivore-induced accumulation of JA, but not the constitutive levels of JA. This JA deficiency inhibits the elicitation of direct (TPIs and nicotine) and indirect (volatiles) defenses and reduces *N. attenuata*'s resistance to attack by larvae of the specialist herbivore of *N. attenuata*, *Manduca sexta* (Halitschke and Baldwin 2003), and makes the plants susceptible to two new herbivores, the leaf-chewing beetle *Diabrotica undecimpunctata* and the piercing–sucking leafhopper *Empoasca* spp. (Kessler et al. 2004). Interestingly, when *NaLOX3*-silenced plants are treated with MeJA, their ability to produce nicotine and herbivore-resistance traits is fully restored (Halitschke and Baldwin 2003), suggesting that the exogenous MeJA treatment is sufficient to elicit most JA responses. However, these results raise an important question: does the exogenously supplied MeJA function directly as a signal or must it first be hydrolyzed to JA? Both JA and MeJA are elicitors of defense responses when applied exogenously, and they induce almost the same set of genes (Taki et al. 2005), but the nature of the endogenous signal remains unclear as both are rapidly interchangeable. Overexpression of JA carboxyl methyltransferase (JMT) in *Arabidopsis* increases endogenous MeJA levels 3-fold without altering JA levels and results in the constitutive expression of JA-responsive genes, including *VSP* and *PDF1.2* (Seo et al. 2001). These results suggest that MeJA rather than JA elicits systemically transmitted defense responses. Similarly, a long-standing debate about whether salicylic acid (SA) or its methyl ester, MeSA, was the elicitor of systemically acquired resistance to pathogen attack was recently resolved by the ingenious idea of grafting together combinations of plants altered in their expression of either the MeSA-esterase and SA-methyl transferases. MeSA was confirmed to be the critical mobile signal for SAR (Park et al. 2007).

The discovery of MeJA-esterase (MJE, Stuhlfelder et al. 2002, 2004), which hydrolyzes MeJA to JA, provides a means of determining whether MeJA is the elicitor of MeJA-elicited herbivore resistance. Since MeJA and JA treatment of plants is known to elicit endogenous JA production (Ziegler et al. 2001; Miersch and Wasternack 2000; Pluskota et al. 2007), the hypothesis is best tested in plants reduced in their endogenous JA production. We tested the hypothesis that MeJA-elicited herbivore resistance is actually elicited after de-esterification to JA. We used virus-induced gene silencing (VIGS) to silence MeJA esterase (*NaMJE*) transcripts in wild-type (WT) and *asLOX3* *N. attenuata* plants (Halitschke and Baldwin 2003) with a tobacco rattle virus-based system that had been optimized for *N. attenuata* (Saedler and Baldwin 2004) and measured defense responses, transcripts and herbivore performance in plants elicited with MeJA or JA treatments.

Materials and methods

Plant growth

We used seeds of the 21st generation of an inbred line of *N. attenuata* Torr. Ex Watts (synonymous with *Nicotiana torreyana*: Solanaceae) for transformation. Seed germination and plant growth were conducted as described by Krügel et al. (2002). In brief, seeds were sterilized and germinated on agar with Gamborg B5 (Duchefa http://www.duchefa.com) after soaking in a 1:50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 1 mM of gibberellic acid (GA_3). After 10 days, seedlings were planted into soil in Teku pots. Once established, plants were transferred to 1 L pots in soil and grown in a growth chamber at 22°C, under 16 h of light supplemented with Philips Sun-T Agro 400 Na lights (Philips, http://www.philips.com) or not.

Isolating *NaMJE*

A 312 bp fragment of the *NaMJE* cDNA sequence was cloned with primers (MJE30_for: 5′-GCTAGTTCATGG AGCTTGTC-3′ and MJE341_rev: 5′-TTAGGACCAGG CATGAAAGC-3′), the design of which was based on the sequence similarity of EST (DB679695) and LeMJE (AY455313). After the first round of PCR with AP primer (5′-GCCACGCGTCGACTAGTACTTTTTTTTT TTTTTTTT-3′) and primer MJE30_for, 3 terminal cDNA was amplified with primer MJE 49_for (5′-CACGGTGCA TGGTGTTGGTA-3′) and primer NAP (5′-GCCACGCGT CGACTAGTAC-3′). The 5 terminal cDNA was obtained with primer MJE0_for (5′-AGATGACATGGAAAAGG GT-3′) and primer MJE341_rev. All cDNA fragments were cloned into a pGEM-T EASY vector (Promega, http://www.promega.com) and sequenced.

Generation of VIGS plants

A 312 bp fragment of the *NaMJE* cDNA sequence, which was amplified by primers MJE30_for and MJE341_rev, was cloned into the pTV00. The pTV00 vector is a 5.5-kb plas-

mid with an origin of replication for *Escherichia coli* and *A. tumefaciens* and a gene for kanamycin resistance (Ratcliff et al. 2001). The *A. tumefaciens* (strain GV3101)-mediated transformation procedure was described previously (Saedler and Baldwin 2004). To monitor the progress of VIGS, we silenced phytoene desaturase, a gene that oxidizes and cyclizes phytoene to α- and β-carotene. These compounds are subsequently converted into the xanthophylls of the antenna pigments of the photosystems of plants, resulting in the visible bleaching of green tissues (Saedler and Baldwin 2004). When the leaves of phytoene desaturase-silenced plants began to bleach (5 weeks after germination), leaves of *NaMJE*-silenced (VIGS *MJE*) and empty vector-inoculated (EV) plants from both WT and *asLOX3* plants were used.

Caterpillar performance

Eggs of *M. sexta* were acquired from North Carolina State University (http://www.ncsu.edu) and kept in a growth chamber (Snijders Scientific, http://www.snijders-tilburg.nl) at 26°C 16 h light, 24°C 8 h darkness, until the larvae hatched. Freshly hatched neonates were placed directly on the source–sink transition leaves 3 days after MeJA treatment. In each treatment, 20 larvae were weighed after the indicated times.

Analysis of nicotine levels and TPI activity

Nicotine was extracted and quantified by HPLC as described in (Keinanen et al. 2001). Trypsin proteinase (TPI) activity was analyzed by radial diffusion activity as described in (van Dam et al. 2001).

JA and JA-Ile measurements

JA and JA-Ile were extracted and quantified by LC/MS as described in (Wang et al. 2007a). In brief, about 200 mg of leaf tissues from each sample was homogenized on a FastPrep homogenizer (http://www.thermo.com) with 1 mL of ethyl acetate spiked with 200 ng of 1,2-^{13}C-JA, D_4-SA and p-coumaric acid (PCA) in FastPrep tubes. After being centrifuged, the supernatants were transferred to fresh 2 mL tubes and evaporated on a vacuum concentrator. The residue was resuspended in 0.5 mL of 70% methanol (v/v) and centrifuged at maximum speed for 5 min. The supernatants were analyzed for JA, JA-Ile, and SA with a 1200L LC/MS/MS system (Varian, http://www.varianinc.com).

SYBR green real-time PCR assay (qPCR)

Total RNA was extracted with TRI Reagent (Sigma, http://www.sigmaaldrich.com) according to the manufacturer's instructions, and cDNA was prepared from 500 ng total RNA with multiScribeTM reverse transcriptase (Applied Biosystems, http://www.appliedbiosys.com). The primers for *NaMJE* mRNA expression detection by qPCR were as follows:

NaMJE forward primer:5′-GCTTTCATGCCTGGTCC TAA-3′,
NaMJE reverse primer:5′-GACCTTTCTCCTGTCCG TTG-3′,
NaTPI forward primer:5′-TCAGGAGATAGTAAATATG GCTGTTCA -3′,
NaTPI reverse primer:5′-ATCTGCATGTTCCACATTGC TTA-3′,
NaTD forward primer:5′-TAAGGCATTTGATGGGA GGC-3′,
NaTD reverse primer:5′-TCTCCCTGTTCACGATAATG GAA-3′,
NaPAL1 forward primer:5′-TTTGCATACGCTGATGA CGC-3′,
NaPAL1 reverse primer:5′-TGGAAGATAGAGCTGTTC GCG-3′,
NaActin forward primer:5′-GGTCGTACCACCGGTATT GTG-3′,
NaActin reverse primer:5′-GTCAAGACGGAGAATGGC ATG-3′.

Real-time PCR was performed on an ABI PRISM 7700 Sequence Detection System (qPCR Core Kit, Eurogentec, http://www.eurogentec.com) with *NaActin* for normalization and according to the manufacturer's instructions under the following cycle conditions: 10 min 95°C; 40 cycles: 30 s 95°C, 30 s 55°C.

MeJA esterase activity assay

Around 200 mg leaf sample was ground in liquid nitrogen, and total proteins were extracted with buffer (100 mM Tris, 5% polyvinyl polypyrrolidone, 0.2% phenylthio urea, and 0.5% diethyl dithio carbamate, pH 7.6). A standard enzyme incubation mixture (total volume 50 μL) contained 10 μL total protein (2 μg), 2 μL MeJA (2.2 μg in 10% ethanol), and 38 μL 100 mM Tris (pH 7.6). Reactions were performed at 40°C as suggested as the optimum temperature by Stuhlfelder et al. (2002) for 0, 20, and 60 min and terminated by chilling on ice. One milliliter of ethyl acetate (spiked with internal JA standard $^{13}C_2$-JA 200 ng) was added quickly. The product JA from cleaved MeJA was quantified by LC/MS/MS as described in (Wang et al. 2007a). From the concentration of JA produced, we obtained the amount of cleaved MeJA. We also performed reactions for negative controls with only 10 μL total proteins of EV and VIGS *MJE* plants individually or only MeJA in Tris buffer at the same condition for 60 min, the results showed that the amount of JA produced by these reactions is under the detection limit of our LC/MS/MS.

Results

Silencing *NaMJE* dramatically reduces the hydrolysis of MeJA in protein extracts

The methyl jasmonate esterase (*NaMJE*, EU196055) cDNA was cloned by RT-PCR using primers designed from *MJE* in tomato (*LeMJE*). The predicted amino acid sequence of *NaMJE* shares 80% sequence identity with LeMJE (AY455313, Fig. S1). We used the VIGS system to silence *NaMJE* mRNA in WT and as*LOX3* plants. Plants were inoculated with *Agrobacterium*-harboring TRV constructs that contained an empty vector (EV) or a 312-bp *NaMJE* fragment (VIGS *MJE*). After 14 days, when the visible bleaching of green tissues had been observed for 3 days in plants inoculated with phytoene desaturase (PDS), a gene required for the biosynthesis of xanthophylls of the antenna pigments, *NaMJE* transcripts were analyzed with qRT-PCR in four replicate source–sink transition leaves of EV and VIGS *MJE* plants and normalized to levels of *NaActin*. The results show that VIGS *MJE* plants had only 16% of the *NaMJE* transcripts that EV plants had in both WT and as*LOX3* backgrounds (Fig. 1a).

To determine if the *NaMJE* we silenced was responsible for hydrolyzing MeJA to free JA, we measured the MeJA-cleaving activity of proteins extracted from leaves of EV and VIGS *MJE* plants (Fig. 1b). In proteins extracted from EV as*LOX3* plants, 45 µg MeJA was cleaved to free JA by 1 mg EV proteins after 20 min under standard conditions, while only 9 µg MeJA was cleaved by 1 mg of proteins extracted from VIGS *MJE* as*LOX3* plants (unpaired *t* test, $P = 0.0002$); after 60 min, the MeJA cleaved by VIGS *MJE* was only 16% of the amount that EV proteins had cleaved (unpaired *t* test, $P = 0.0003$). Dramatically reduced MeJA-cleaving activity was also found in VIGS *MJE* proteins in WT background (Fig. 1b). These results demonstrate that *NaMJE* is largely responsible for the MeJA-cleaving activity in *N. attenuata* leaves.

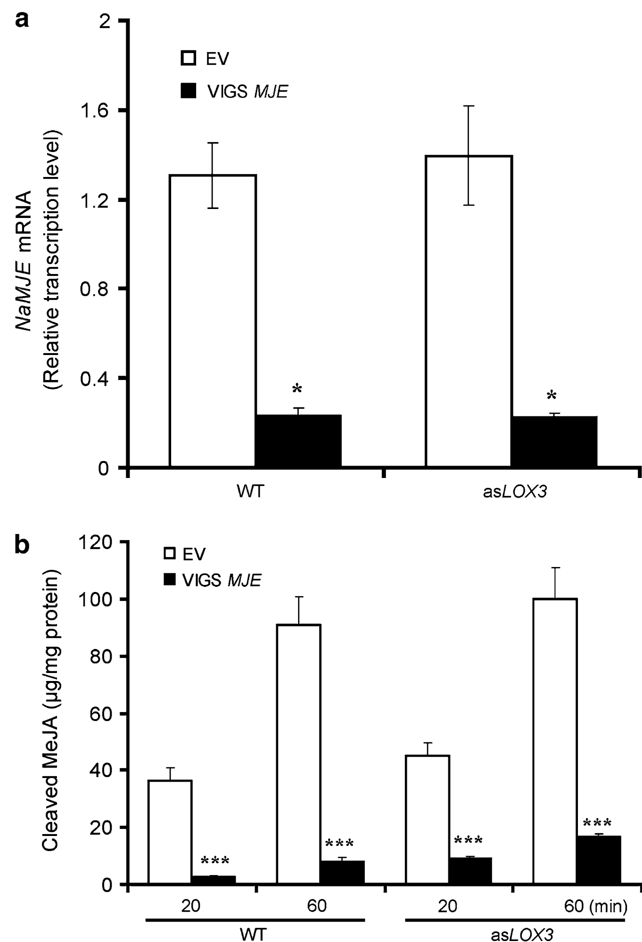

Fig. 1 Silencing *NaMJE* reduces the MeJA-cleaving activity. WT and as*LOX3* plants were inoculated with *Agrobacterium*-harboring TRV constructs, which contained an empty vector (EV) or a 312-bp *NaMJE* fragment (VIGS *MJE*). After 14 days, four replicate source–sink transition leaves were harvested. **a**: Mean (±SE) *NaMJE* transcript accumulation in source–sink transition leaves of EV and VIGS *MJE* plants from both WT and as*LOX3* backgrounds as analyzed with qRT-PCR normalized to levels of *NaActin*. **b**: Mean (±SE) amount of MeJA hydrolyzed to free JA in four replicate total crude protein samples prepared from four individual EV and VIGS *MJE*-innoculated plants (WT and as*LOX3* background) after incubation with MeJA for 20 and 60 min. *Asterisks* indicate the level of significant difference between EV and VIGS *MJE* plants (unpaired *t* test: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.005$)

Silencing *NaMJE* inhibits MeJA-induced resistance to *M. sexta* in as*LOX3* plants but not in WT plants

To determine if silencing *NaMJE* influenced MeJA-induced resistance to *M. sexta* larvae, the source–sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin paste (lanolin) or 75 µg MeJA in lanolin (MeJA). After 4 days, *M. sexta* larvae were placed on these leaves and allowed to feed for 12 days. As expected, larvae feeding on lanolin-treated as*LOX3* plants gained significantly more mass than those on lanolin-treated WT plants and MeJA treatment dramatically reduced the *M. sexta* larval mass on EV plants: larvae that fed on MeJA-treated plants gained only 60.5 and 54% of the mass of those that fed on lanolin-treated WT and as*LOX3* plants, respectively (Fig. 2). Larvae performed equally well on EV and VIGS *MJE* plants treated with lanolin in either WT or as*LOX3* genetic backgrounds (Fig. 2). However, silencing *NaMJE* completely inhibited MeJA-induced resistance in as*LOX3* plants: larvae that fed on MeJA-treated VIGS *MJE* plants gained the same mass as those that fed on plants treated with lanolin (Fig. 2), but gained 50% more mass than those that fed on EV plants treated with MeJA (Fig. 2; unpaired *t* test, $P = 0.04$). These results demonstrate that in *asLOX3* plants, silencing *NaMJE* is sufficient to block MeJA-induced resistance.

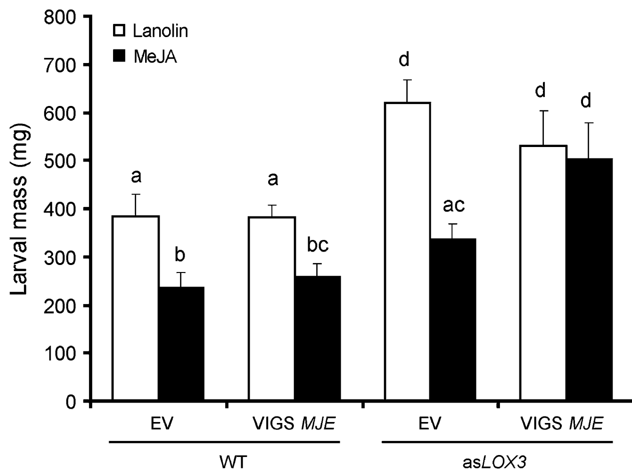

Fig. 2 Silencing *NaMJE* inhibits MeJA-induced resistance to *M. sexta* in as*LOX3* plants but not WT plants. Mean (±SE) mass of 18 replicate *M. sexta* larvae after 12 days of feeding individually on EV and VIGS *MJE* WT and as*LOX3* plants. The source–sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin or 75 µg MeJA in lanolin. After 4 days, *M. sexta* larvae were placed on these leaves. *Different letters* indicate statistically significant differences between treatments (Fisher's PLSD test; $P < 0.05$)

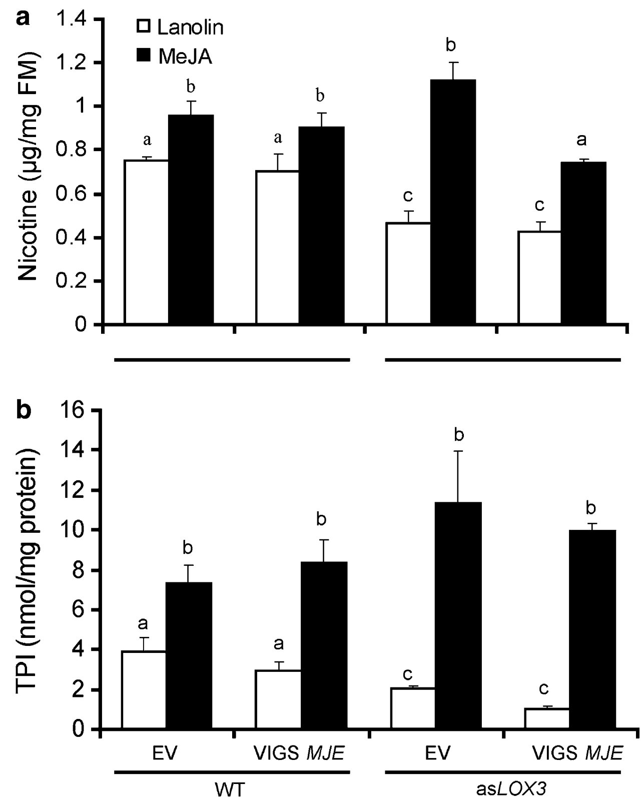

Fig. 3 Effects of silencing *NaMJE* on MeJA-induced levels of nicotine and TPI in WT and as*LOX3* plants. The source–sink transition leaves of EV and VIGS *MJE* plants were treated with lanolin or 75 µg MeJA in lanolin. After 3 days, four replicate leaf samples per treatment were harvested and analyzed for: **a** Mean (±SE) nicotine levels and **b** mean (±SE) TPI levels. *Different letters* indicate statistically significant differences among treatments (Fisher's PLSD test; $P < 0.05$)

Silencing *NaMJE* impairs MeJA-induced nicotine, *NaPAL1* and *TD* transcripts but not TPI responses in as*LOX3* plants

To understand why silencing *NaMJE* transcripts had such a strong effect on caterpillar performance in MeJA-induced *asLOX3* but not WT plants, we measured MeJA-induced nicotine and TPI levels. After being treated with MeJA for 3 days, all EV plants had significantly increased nicotine levels (Fig. 3a). In WT plants, VIGS *MJE* plants accumulated the same amount of nicotine as EV plants had after MeJA treatment. However, nicotine levels of MeJA-treated VIGS *MJE* as*LOX3* plants were only 66% of those in MeJA-treated EV plants (Fig. 3a; unpaired *t* test, $P = 0.01$).

MeJA treatment elicited the same amount of TPI activity in EV and VIGS *MJE* plants in both WT and as*LOX3* backgrounds (Fig. 3b), suggesting that silencing *NaMJE* had no effect on the elicitation of TPIs by MeJA. Measurements of TPI transcripts in as*LOX3* plants 8 h after MeJA treatment also revealed no significant differences between EV and VIGS *MJE* plants (unpaired *t* test, $P = 0.5$).

To better understand why silencing *NaMJE* in as*LOX3* plants had such a large effect on MeJA-induced herbivore resistance, we compared transcript levels of phenylalanine ammonia lyase (*PAL1*), a well-known gene marker of phenolic-based defense responses, in the source–sink transition leaves of EV and VIGS *MJE* plants after MeJA treatment. *PAL1* was dramatically induced in EV plants. After 4 h of MeJA treatment, levels of *PAL1* transcripts in VIGS *MJE* plants were only 21% of those in EV plants (Fig. 4a; unpaired *t* test, $P = 0.005$); after 8 h, levels were 25% of those in EV plants (Fig. 4a; unpaired *t* test, $P = 0.05$).

Threonine deaminase (*TD*) catalyzes the conversion of Thr to a-keto butyrate in Ile biosynthesis, and is strongly elicited in MeJA-elicited leaves in *N. attenuata* where it supplies the Ile required for JA-Ile production (Kang et al. 2006). MeJA treatments dramatically increased *NaTD* transcripts in EV plants; however, in VIGS *MJE* plants, *NaTD* transcript levels were only 9% of those in EV plants 8 h after MeJA treatment (Fig. 4b; unpaired *t* test, $P = 0.03$).

Silencing *NaMJE* inhibits MeJA-induced but not JA-induced resistance to *M. sexta* in as*LOX3* plants

If it is true that MeJA functions as a signal prior to being hydrolyzed to JA, silencing *NaMJE* should inhibit MeJA-induced resistance but have no effect on JA-induced resistance. Due to its polarity, JA does not elicit resistance when added to plants in a lipophilic lanolin paste. Therefore, aqueous solutions of JA and MeJA were added to standardized puncture wounds in leaves of EV and VIGS *MJE* as*LOX3*

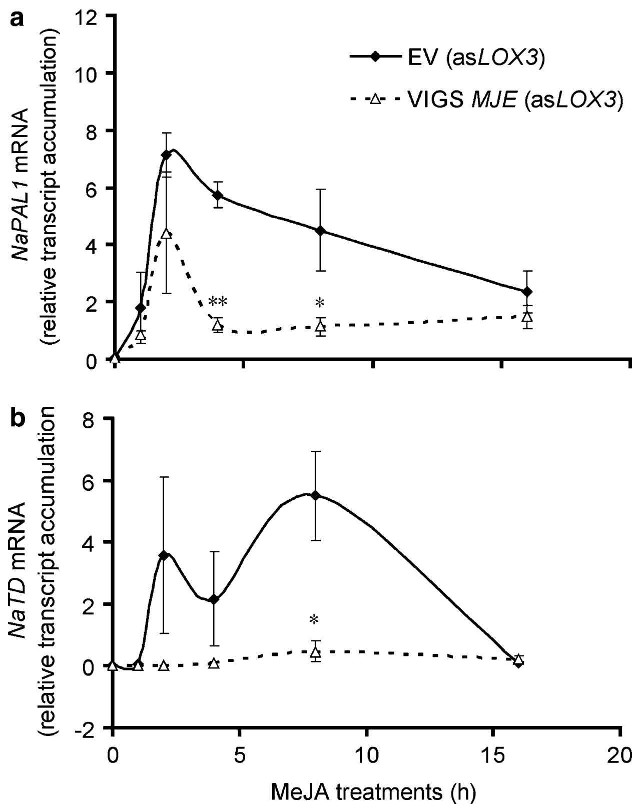

Fig. 4 Silencing *NaMJE* inhibited MeJA-induced *NaPAL1* and *TD* transcripts in as*LOX3* plants. Four replicate source–sink transition leaves of EV and VIGS *MJE* plants were treated with 75 µg MeJA in lanolin and harvested after 0, 1, 2, 4, 8, and 16 h. Mean (±SE) **a** *NaPAL1* and **b** *TD* transcription levels were analyzed with qRT-PCR normalized to levels of *NaActin*. *Asterisks* indicate the level of significant difference between EV and VIGS *MJE* plants, both treated with MeJA (unpaired *t* test: * $P < 0.05$; ** $P < 0.005$)

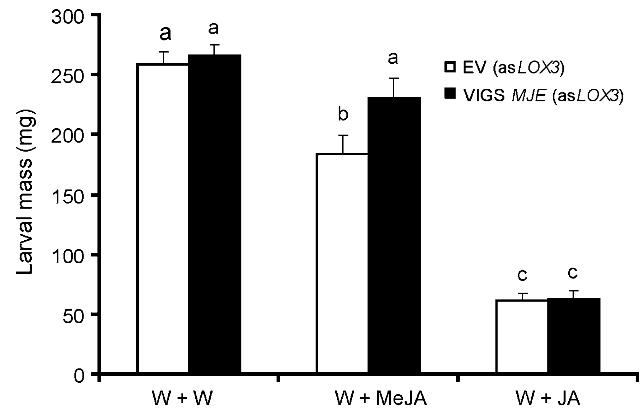

Fig. 5 Silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance to *M. sexta* in as*LOX3* plants The source–sink transition leaves of EV and VIGS *MJE* plants were wounded with a fabric pattern wheel; water (12.5% ethanol), or 0.25 µmol JA, or 0.25 µmol MeJA were immediately applied to the puncture wounds. After 2 days, 18 *M. sexta* larvae were placed individually on the source–sink transition leaves and weighed 15 days after the start of feeding. *Different letters* indicate statistically significant differences between treatments (Fisher's PLSD test; $P < 0.05$)

plants and we tested this hypothesis by measuring the performance of larvae. As expected, the mass of caterpillars that fed on EV plants treated with both JA and MeJA was dramatically reduced compared to the mass of those that fed on EV plants treated with water (Fig. 5). Larvae that fed on JA-treated VIGS *MJE* plants gained the same mass as those that fed on EV plants treated with JA; however, larvae gained significantly more mass when they fed on VIGS *MJE* plants than on EV plants treated with MeJA (Fig. 5).

Discussion

Staswick's pioneering work on MeJA-insensitive *jar1* mutant in *Arabidopsis* suggests that exogenously applied MeJA is first demethylated and then conjugated to Ile before it becomes an active inhibitor of root growth (Staswick et al. 1992; Staswick and Tiryaki 2004). Indeed, MeJA hydrolyzing enzyme activity occurs in all the plant species that have been examined to date (Stuhlfelder et al. 2002). It is also reported that MeJA was rapidly hydrolyzed to JA and further metabolized like JA when tobacco BY-2 cells were treated with MeJA (Swiatek et al. 2004). In *N. attenuata*, a model plant with extensively studied herbivore-induced responses, we also detected high levels of MeJA-cleaving activity (Fig. 1b). Using the sequence similarity with tomato *LeMJE* (Stuhlfelder et al. 2004), we cloned *NaMJE*, a MeJA esterase gene that shared very high sequence similarity with *LeMJE* (Fig. S1). By silencing the expression of this gene, we demonstrated that *NaMJE* is largely responsible for the MeJA-cleaving activity of *N. attenuata* leaves (Fig. 1b).

MeJA treatment is the most commonly used means of eliciting herbivore resistance in many different plant species (McConn et al. 1997; Baldwin 1998; Li et al. 2002). However, it is still not known how herbivore resistance traits are elicited by MeJA treatment. We determined whether MeJA functions as a signal prior to being hydrolyzed to JA by investigating MeJA-induced defense responses in both WT and JA-deficient *asLOX3* plants (Figs. S2, S3) with reduced MeJA-cleaving activity. Silencing *NaMJE* transcripts was sufficient to block most MeJA-induced responses in as*LOX3* but not WT plants, including the production of nicotine (Fig. 3), transcripts of *NaPAL1* and *NaTD* (Fig. 4). Importantly, the dramatic decreases in mass that are usually observed in larvae that feed on MeJA-treated EV plants disappeared when *NaMJE* transcripts were silenced (Fig. 2). Furthermore, silencing *NaMJE* inhibited MeJA-induced but not JA-induced resistance in as*LOX3* plants (Fig. 5). Why is silencing *NaMJE* sufficient to block most MeJA-induced responses in as*LOX* plants but not in WT plants? MeJA

treatment of plants is known to elicit endogenous JA production (Ziegler et al. 2001; Miersch and Wasternack 2000). Without any treatments, *asLOX3* plants have the same level of endogenous JA and JA-Ile as WT plants (Figs. S2a, S3). However, after MeJA treatments, *asLOX3* plants accumulate lower levels of JA than do WT plants (Fig. S2b). These results are consistent with the reports in Halitschke and Baldwin (2003) that *asLOX3* plants have the same basal levels of JA as WT plants do but accumulate half of the JA levels of WT plants after wounding, suggesting that the JA burst arises in part from de novo biosynthesis and in part from JA that is released from some unknown storage pool. Since *NaMJE* mRNA levels and MeJA cleaving activity are the same in EV (WT) and EV (*asLOX3*) plants, the higher levels of JA (Fig. S2b) elicited by MeJA in EV (WT) plants compared to EV (*asLOX3*) plants are likely due to the larger JA storage pool in WT plants. In light of these considerations, we conclude that most exogenous MeJA-induced herbivore responses are actually elicited after MeJA is demethylated to JA.

Trypsin proteinase inhibitors (TPIs) play a role as a direct defensive against herbivores in *N. attenuata* (Zavala et al. 2004). The activities and transcripts of TPIs can be dramatically induced by MeJA treatments. Preston et al. (2004) showed that the application of 5 μg MeJA was sufficient to elicit a significant increase in TPI activity, which suggested that TPIs are very sensitive to MeJA treatment, which may explain why even a reduction of 84% of the MeJA-cleaving activity was not sufficient to inhibit the MeJA-induced TPI response in as*LOX3* plants. Therefore from these results, we cannot determine whether the accumulations of TPIs were elicited by MeJA directly or after demethylation to JA.

Silencing *NaMJE* completely blocked the MeJA-induced resistance to *M. sexta* larvae in as*LOX3* plants (Fig. 3). Although the levels of MeJA-induced nicotine were significantly lower in VIGS *MJE* plants than in EV plants, the reduction in the nicotine response was not likely sufficiently large to account for the increase in herbivore performance (Steppuhn et al. 2004). Two additional possible explanations are suggested by the results: (1) the reduction in *NaPAL1* transcripts could reflect reductions in an unknown phenolic-based defense; (2) reduced *NaTD* transcripts may reflect decreased TD activity, which may function post-ingestively as an antinutritive defense that limits the supply of Thr needed for herbivore growth (Chen et al. 2005).

In summary, our results demonstrate that most herbivore-resistant responses are elicited by JA when plants were treated with exogenously applied MeJA.

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