

RESEARCH PAPER

Pectin methylesterase *NaPME1* contributes to the emission of methanol during insect herbivory and to the elicitation of defence responses in *Nicotiana attenuata*

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Abstract

Pectin methylesterases (PMEs) catalyse the demethylation of pectin within plant cell walls, releasing methanol (MeOH) in the process. Thus far, PMEs have been found to be involved in diverse processes such as plant growth and development and defence responses against pathogens. Herbivore attack increases PME expression and activity and MeOH emissions in several plant species. To gain further insights into the role of PMEs in defence responses against herbivores, the expression of a *Manduca sexta* oral secretion (OS)-inducible PME in *Nicotiana attenuata* (*NaPME1*) was silenced by RNA interference (RNAi)-mediated gene silencing. Silenced lines (*ir-pme*) showed 50% reduced PME activity in leaves and 70% reduced MeOH emissions after OS elicitation compared with the wild type (WT), demonstrating that the herbivore-induced MeOH emissions originate from the demethylation of pectin by PME. In the initial phase of the OS-induced jasmonic acid (JA) burst (first 30 min), *ir-pme* lines produced WT levels of this hormone and of jasmonyl-isoleucine (JA-Ile); however, these levels were significantly reduced in the later phase (60–120 min) of the burst. Similarly, suppressed levels of the salicylic acid (SA) burst induced by OS elicitation were observed in *ir-pme* lines even though wounded *ir-pme* leaves contained slightly increased amounts of SA. This genotype also presented reduced levels of OS-induced trypsin proteinase inhibitor activity in leaves and consistently increased *M. sexta* larvae performance compared with WT plants. These latter responses could not be recovered by application of exogenous MeOH. Together, these results indicated that PME contributes, probably indirectly by affecting cell wall properties, to the induction of anti-herbivore responses.

Key words: Defence, herbivory, jasmonic acid, *Manduca*, methanol, *Nicotiana*, pectin methylesterase, proteinase inhibitor.

Introduction

Pectin methylesterases (PMEs, EC 3.1.1.11) are important cell wall enzymes involved in modelling the pectin matrix. Pectin is a major compound of plant cell walls and accounts for about one-third of all the macromolecules in dicotyledonous species. Different types of polysaccharides that are rich in galacturonic acid form the backbone of pectin: homogalacturonan (HG), xylogalacturonan (XG), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGI) (Willats *et al.*, 2001; Vorwerk *et al.*, 2004). HG is a linear polymer of 1,4-linked α -D-galacturonic acid that is synthesized in the Golgi apparatus and deposited in the

cell wall, with most (70–80%) of its galacturonic acid units methylated at the C-6 carboxyl group (O'Neill *et al.*, 1990). PMEs catalyse the C-6 demethylation of HG within plant cell walls. In this process, methanol (MeOH) is released and negatively charged carboxyl groups are created which can associate with other HG chains by calcium cross-links, making the cell wall more rigid. However, PME activity can also contribute to cell wall loosening and degradation by promoting the activity of cell wall hydrolases such as endopolygalacturonases (Micheli, 2001; Willats *et al.*, 2001). Thus, PMEs can

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Abbreviations: JA, jasmonic acid; JA-Ile, jasmonyl-isoleucine; MeOH, methanol; OS, *M. sexta* oral secretions; PME, pectin methylesterase; SA, salicylic acid; TPI, trypsin proteinase inhibitor.

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regulate the texture and mechanical properties of plant cell walls in opposite ways.

PMEs belong to large gene families, for example, in *Arabidopsis thaliana*, 66 PME genes have been predicted (Pelloux *et al.*, 2007). PMEs are involved in diverse processes associated with growth and development, such as cell wall remodelling, root tip elongation, seed germination, fruit ripening, and pollen tube growth (reviewed in Pelloux *et al.*, 2007). Moreover, PMEs play a role in plant defence against many pathogenic bacteria and fungi. For example, in *Solanum tuberosum*, differences in susceptibility to *Erwinia carotovora* correlate with the degree of methylation (DM) of pectin, with resistant genotypes having a higher DM of pectin than the susceptible genotypes (Marty *et al.*, 1997). Likewise, *A. thaliana* plants with reduced PME activity have a higher DM of pectin and are more resistant to fungal infection by *Botrytis cinerea* (Lionetti *et al.*, 2007). PMEs are also involved in different processes during viral infection. *Nicotiana tabacum* PMEs promote the movement of tobacco mosaic virus by binding to the virus-encoded movement proteins necessary for the dispersal of the virus (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Chen and Citovsky, 2003).

There are indications that PMEs may also play a role in defence against herbivores. In several plant species, PME mRNA and protein levels are up-regulated after herbivore attack (Divol *et al.*, 2005; Schmidt *et al.*, 2005; Giri *et al.*, 2006; von Dahl *et al.*, 2006). In addition, emissions of MeOH are increased (Peñuelas *et al.*, 2005; von Dahl *et al.*, 2006) and demethylation of pectin by PMEs was suggested to be the source of the emitted MeOH during herbivory (von Dahl *et al.*, 2006). Based on these observations, it was postulated that PME may participate in the induction of defence responses during insect herbivory either by generating MeOH as a signal molecule or via changes in the structural properties of the cell wall. To test this hypothesis, a herbivore-inducible PME gene (*NaPME1*) was silenced in *N. attenuata* plants and diverse plant responses to insect herbivores were studied.

Materials and methods

Plant growth and generation of ir-pme lines

Seeds of an inbred line of *N. attenuata* Torr. ex. Wats. and of the transformed *ir-pme* lines were germinated on Petri dishes containing Gamborg's B5 medium as described by Krügel *et al.* (2002). Plates were incubated in a growth chamber (Percival, Perry, IA, USA) at 26 °C/16 h light (155 $\mu\text{mol s}^{-1} \text{m}^{-2}$)–24 °C/8 h dark. After 10 d, seedlings were planted into Teku pots (Waalwijk, The Netherlands) and transferred into 1.0 l pots after an additional period of 10 d. Plants were grown in the greenhouse under high-pressure sodium lamps (800–1000 $\mu\text{mol s}^{-1} \text{m}^{-2}$) with a day/night ratio of 16 h (26–28 °C)/8 h (22–24 °C) and 45–55% humidity. Experiments were conducted when plants were at the rosette stage, shortly before stem elongation.

Transgenic *N. attenuata* (*ir-pme*) lines silenced in the expression of PME were constructed using the vector pRESC5PME1 (Supplementary Fig. S1A available at *JXB* online). This vector contains two 431 bp fragments of *NaPME1* (accession no. DQ115979; von Dahl *et al.*, 2006) which were PCR amplified using the primer pairs PME3-31 (5'-GCGGCGGAGCTCGGTTTCGAAAGCTTTGCTG-3') and PME4-31 (5'-GCGGCGCTCGAGCTTTCACATTCCTACTTCC-3'), and PME5-19 (5'-GGTTCGAAAGCTTTGCTG-3') and PME6-31 (5'-GCGGCGCCATGGCTTTCACATTCCTACTTCC-3'). This 431 bp fragment corresponded to the pectinesterase superfamily domain in plant PMEs. Transgenic plants were generated via *Agrobacterium*-mediated transformation as described by Krügel *et al.* (2002). Transformants were selected on Gamborg's B5 plates supplemented with hygromycin (0.025 mg ml⁻¹).

Elicitation treatments

To mimic herbivore feeding, the youngest or second youngest fully expanded rosette leaves were wounded with a fabric pattern wheel, causing four rows of punctured wounds on each side of the midrib. The wounds were immediately treated with 20 μl of water (w+w) or 20 μl of *Manduca sexta* oral secretions (OS) diluted 1:1 (v/v) with water (w+OS). Control plants remained untreated. *Manduca sexta* OS were collected from larvae reared on *N. attenuata* wild-type (WT) plants. Depending on the experiment, leaves were harvested at different time points after the treatments and immediately frozen in liquid nitrogen. Leaf samples were stored at –80 °C until further processing.

The average amount of MeOH emitted after *M. sexta* OS elicitation from a WT leaf was 0.58 $\mu\text{l min}^{-1}$ and the treatment leads to an immediate increase of MeOH emissions. Hence, ~8.7 μl of MeOH are released per leaf within the first 15 min after elicitation, when MeOH emissions are known to be at their maximum (von Dahl *et al.*, 2006). Therefore, the amount of methanol (9 μl) applied to the wounds of one leaf (w+OS+MeOH) represents realistic exposure.

For the induction of defence compounds in systemic leaves, one freshly hatched *M. sexta* larva was placed on the youngest, fully expanded rosette leaf, and clip cages were attached to restrict larval movement. After 5 d of feeding, the youngest, fully expanded leaf adjacent to the attacked leaf was harvested and analysed.

Southern and northern blot analysis

Genomic DNA of the WT and *ir-pme* lines was isolated by the cetyltrimethylammonium bromide method (CTAB) as described in Rogers and Bendich (1994). DNA samples (10 μg) were digested with different enzymes (see below) overnight and separated on a 0.8% (w/v) agarose gel using standard conditions. DNA was blotted onto Gene Screen Plus Hybridisation Transfer membranes (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) using the capillary transfer method. RNA was extracted using the

Trizol reagent (Sigma, Taufkirchen, Germany) according to the manufacturer's instructions. RNA samples (15 µg) were denatured in loading buffer at 68 °C for 10 min, separated on a 1.2% (w/v) formaldehyde-agarose gel, and blotted onto nylon membranes following standard conditions. Equal loading was controlled by staining with ethidium bromide.

For Southern blot analysis, gene-specific probes for NaPME1 and the hygromycin resistance gene *hptII* were generated by PCR using the primer pairs PME3-31 (5'-GCGGCGGAGCTCGGTTTCGCAAAGCTTTGCTG-3') and PME4-31 (5'-GCGGCGCTCGAGCTTTCACATTCCTACTTCC-3'), and HYG1-18 (5'-CCGATCGGACGATTGCG-3') and HYG3-20 (5'-CGTCTGTCGAGAA-GTTTCTG-3'), respectively. To determine the number of T-DNA insertions, genomic DNA from *ir-pme* lines was digested with *EcoRV* and hybridized with the *hptII* probe. In addition, genomic DNA from WT plants was digested with *DraI*, *SspI*, *HindIII*, and *EcoRI*, and hybridized with the *PME1* probe to determine the copy number of *PME1* in the *N. attenuata* genome.

For northern blot analysis, the probe to detect *PME* expression was generated using the primer pair PME1_F (5'-GGCCTCAGTTGCACTTGTAAT-3') and PME1_R (5'-GGCAATGCTGCTGCTGTTTTAC-3'). DNA probes were labelled with [α -³²P]dCTP (Perkin Elmer Life and Analytical Sciences) using a random prime labelling kit (Rediprime II, Amersham Pharmacia) according to the manufacturer's instructions.

PME and trypsin proteinase inhibitor (TPI) activity assays

PME activity was measured using a gel diffusion assay as described in Downie *et al.* (1998) and modified by Bourgault and Bewley (2002). *TPI* activity was measured using a gel diffusion assay as described in van Dam *et al.* (2001).

Methanol analysis

MeOH emissions of single leaves were measured with a proton transfer reaction mass spectrometer (PTR-MS; IONICON GmbH, Innsbruck, Austria) as described in von Dahl *et al.* (2006). The youngest rosette leaf was cut off at the petiole and inserted into a glass flow chamber. MeOH emissions were measured for 5–10 min to determine control emissions. Leaves were then wounded and MeOH release recorded for 10 min. Afterwards wounds were treated with 1:1 diluted *M. sexta* OS, and MeOH emissions were measured for an additional 10 min. The amount of MeOH released was calculated from the integrated areas of the two measurement periods after wounding and after application of OS. The calibration curve was obtained by repeated injections of 0.25, 0.35, 0.5, and 1 µl of MeOH into the flow chamber. MeOH emissions are expressed as µmol MeOH min⁻¹ cm⁻² leaf area. Leaf area was determined using Sigma Scan Pro 5 (SPSS) on scans of leaves.

Protein kinase activity assay

Protein kinase activity assays were performed as described by Zhang and Klessig (1997) and modified by Wu *et al.* (2007).

Phytohormone and nicotine analysis

For jasmonic acid (JA), jasmonyl-isoleucine (JA-Ile), and salicylic acid (SA) quantification, 200 mg of leaf material was extracted with 1 ml of ethylacetate containing 200 ng of [¹³C₂]JA, [D₄]SA, and [¹³C₆]JA-Ile as internal standards. After centrifugation at 13 000 rpm for 20 min at 4 °C, the supernatant was transferred into a fresh tube and the sample was re-extracted with 1 ml of ethylacetate. The combined supernatants were dried and samples dissolved in 0.5 ml of 70% (v/v) MeOH. A 0.2 ml aliquot of the supernatant was transferred into glass vials and analysed by HPLC-(ESI)-MS/MS as described in Wang *et al.* (2007).

To quantify ethylene emissions, the second youngest, fully expanded rosette leaf was wounded and treated with 1:1 diluted *M. sexta* OS. After the treatment, the leaf was cut off at the petiole and inserted into a 250 ml glass vessel. Ethylene was allowed to accumulate for 5 h. The headspace of the vessel was flushed into a laser photo-acoustic spectrometer (INVIVO, Adelzhausen, Germany) and ethylene concentration measured as described in von Dahl *et al.* (2007). Ethylene emissions are expressed as nl h⁻¹ g⁻¹ fresh mass. Nicotine content of leaves was determined as described in Keinänen *et al.* (2001).

Manduca sexta performance assays

One *M. sexta* neonate was put on each plant and caterpillars were weighed after 3, 5, 7, 9, and 11 d with a microbalance. In a second experiment, caterpillars were kept in small plastic containers and were fed with leaf material from plants that had been sprayed twice a day with a 5% (v/v) MeOH/H₂O solution. Spraying started 4 d before the start of the experiment and was continued until the end of the experiment. Each spraying delivered 0.1 ml of MeOH, the amount released in 6 h by an attacked *N. attenuata* leaf (von Dahl *et al.*, 2006). Control plants were sprayed with water. Leaf material without midribs was excised from each plant from each treatment group, weighed, and placed into small plastic containers (10.5×8×4 cm). One neonate was put into each container, which was then closed with a perforated lid. All boxes contained a moist filter paper and were placed in an incubator and maintained at 75% humidity at 26 °C with a 16:8 h light:dark cycle. Leaf material was first replaced after 3 d and then changed every 2 d. At the end of day 9, total dry mass of larvae and leaves was recorded after drying at 65 °C for 3 d. The efficiency of conversion of ingested food (ECI) [(larval mass gain)/(leaf mass consumed)] was calculated according to Waldbauer (1968). Total protein content in leaves was quantified according to Bradford (1976).

Statistics

Data were analysed with the software StatView (Abacus Concepts, Inc., Berkeley, CA, USA). Analysis of variance

(ANOVA) with Bonferroni corrected *post hoc* test or *t*-tests was used. To ensure homogeneity of variances, data were log transformed if necessary. Multiple plants were used in single experiments, and genotype and treatment were included as factors in the ANOVA.

Results

Generation of transgenic plants silenced in *NaPME1*

A putative PME gene from *N. attenuata* (*NaPME1*) previously described (von Dahl *et al.*, 2006) was identified based on its strong up-regulation at the mRNA level after *M. sexta* attack and *M. sexta* OS elicitation (Schmidt *et al.*, 2005; von Dahl *et al.*, 2006). The *NaPME1* mRNA starts to accumulate immediately after *M. sexta* OS elicitation and remains at higher than control levels for at least 24 h (von Dahl *et al.*, 2006). To study the function of this gene in anti-insect responses in *N. attenuata*, a 431 bp fragment of the *NaPME1* cDNA was cloned in an inverted orientation and used to generate stable transformed lines (*ir-pme*) by inverted repeat gene silencing (see Materials and methods). Two homozygous independently transformed *ir-pme* lines (434-7 and 457-8) were selected and used for all experiments. To determine the number of T-DNA insertions, genomic DNA from *ir-pme* lines was digested with *EcoRV* and hybridized with a probe specific for the hygromycin phosphotransferase gene *hptII*. Line 457-8 contained a single T-DNA insertion and, in line 434-7, a left border over-read occurred, producing a single band with the same size (2.4 kb) as the pRESC5PME1 plasmid DNA fragment (Supplementary Fig. S1B at *JXB* online). After *EcoRI* digestion and hybridization with a probe specific for *NaPME1*, line 434-7 presented three bands: one corresponding to the endogenous gene and two to the transgene, suggesting that the T-DNA had been inserted twice (in a tandem array) at a single site in the plant genome (Supplementary Fig. S1C). Southern blot analysis was also used to determine the copy number of *PME1*. The blot revealed that *PME1* is a single copy gene in the genome of *N. attenuata* (Supplementary Fig. S1C).

The morphology and development of *ir-pme* lines were similar to those of the WT at all stages of the plant's life cycle (Supplementary Fig. S2 at *JXB* online, and data not shown).

PME activity and MeOH emissions are reduced in ir-pme lines

It has been previously shown that *N. attenuata* plants emit large amounts of MeOH when attacked by *M. sexta* larvae and it has been proposed that the source of this MeOH is the result of PME activation (von Dahl *et al.*, 2006). Thus, the *PME1* transcript levels, PME activity, and the release of MeOH in *ir-pme* lines were determined after they had been elicited with *M. sexta* OS. Control plants were left untreated. Transcript levels were measured in leaves of WT and *ir-pme* lines using northern blot analysis (Fig. 1A). In WT plants, *NaPME1* transcripts were detectable in both

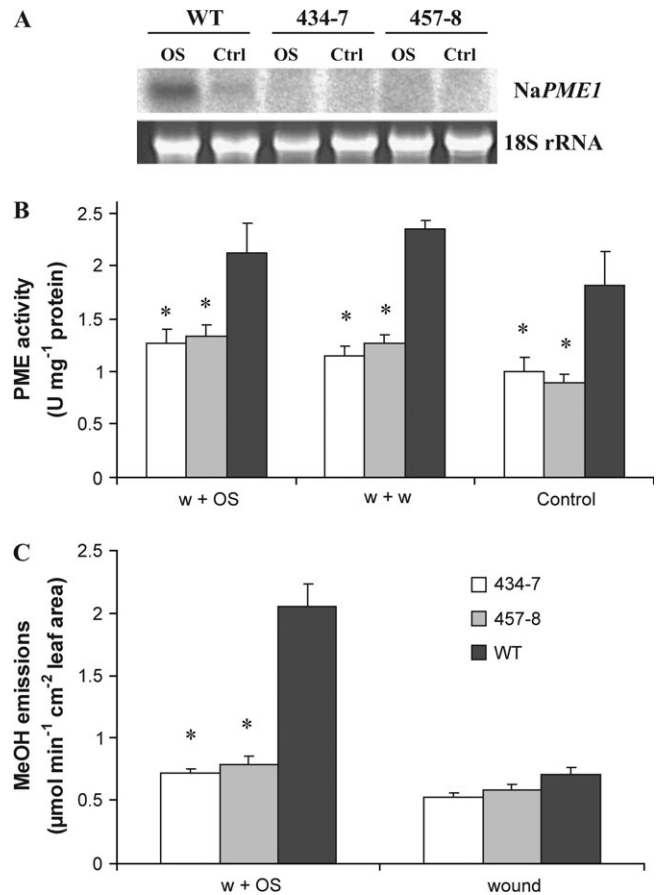


Fig. 1. *PME1* transcript levels, PME activity, and MeOH emissions are reduced in *ir-pme* lines. (A) Northern blot analysis of *NaPME1* mRNA in WT plants and *ir-pme* lines (434-7, 457-8) 6 h after wounding+OS treatment (OS) and in control leaves (Ctrl). (B) Mean (\pm SE) PME activity in *N. attenuata* leaves of WT plants and *ir-pme* lines 4 h after elicitation. Leaves were wounded and the wounds treated with either water (w+w) or 1:1 diluted *M. sexta* oral secretions (w+OS). Control plants remained untreated ($n=4-5$). (C) Mean (\pm SE) methanol emissions of single *N. attenuata* leaves of WT plants and *ir-pme* lines after wounding alone (wound) or subsequent application of 1:1 diluted *M. sexta* OS (w+OS) ($n=3-5$). Asterisks represent significant differences from the WT (ANOVA).

control and treated leaves. As expected, OS treatment increased *PME1* transcript levels several fold after 6 h. In contrast, in leaves of *ir-pme* lines, no *NaPME1* transcripts were detectable in either control or treated plants (Fig. 1A).

Secondly, PME activity was quantified in control, wounded, and OS-elicited WT and *ir-pme* lines. PME activity was significantly reduced by 40–50% in *ir-pme* lines compared with WT plants (Fig. 1B, two-way ANOVA, $F_{2,35}=26.34$, $P<0.001$) in non-induced plants, 4 h after wounding and 4 h after OS elicitation. The two wound treatments slightly increased PME activity in all genotypes compared with non-induced plants (Fig. 1B). PME activity was also quantified at 40 min after the treatments and differences between genotypes similar to those observed at 4 h were detected (data not shown).

Finally, MeOH emissions of single leaves from WT and *ir-pme* lines were quantified using PTR-MS. MeOH emissions were recorded both after wounding and after applying OS to the wounds. As expected, treating the leaves of WT plants with OS caused a higher release of MeOH than wounding alone (Fig. 1C, paired *t*-test, $P < 0.001$). In contrast, no MeOH burst occurred in *ir-pme* lines after either wounding or application of OS. On average, WT plants emitted three times more MeOH than *ir-pme* lines (Fig. 1C, ANOVA, $F_{2,8}=51.49$, $P < 0.001$).

Silencing of NaPME1 affects phytohormone dynamics after OS elicitation

To evaluate the influence of NaPME1 on direct defences against herbivores, local induction of different defence and defence-related responses (early and late signalling events) was compared in leaves of WT and *ir-pme* lines.

Manduca sexta larvae attack or OS elicitation causes a transient increase in JA and the amino acid conjugate JA-Ile which is larger than the increase caused by wounding alone (Schittko *et al.*, 2000; Kang *et al.*, 2006). The levels of these phytohormones were quantified in control and treated leaves (30, 60, 90, and 120 min after wounding or OS elicitation). In line 457-8, JA accumulated at significantly reduced levels (Fig. 2A, ANOVA, $F_{2,11}=5.49$, $P=0.02$) after 30 min of induction, and in line 434-7 JA accumulated at significantly reduced levels after 60 min compared with the WT. At 90 min after OS treatment, leaves of both *ir-pme*

lines contained 40% less JA than WT leaves (Fig. 2A, ANOVA, $F_{2,11}=8.31$, $P < 0.01$). A similar profile was observed for JA-Ile accumulation, with lower levels at 90 min in both *ir-pme* lines compared with WT (Fig. 2B, ANOVA, $F_{2,11}=8.27$, $P < 0.01$). In contrast, the accumulation of both JA and JA-Ile did not differ between WT and *ir-pme* lines after wounding alone (Fig. 2A, B, two-way ANOVA: JA, $F_{2,59}=0.29$, $P=0.75$; JA-Ile, $F_{2,59}=0.34$, $P=0.72$).

Additional phytohormones such as ethylene (ET) and SA are known to tune defence responses against herbivores. Therefore, the levels of SA were quantified in leaves 30, 60, 90, and 120 min after wounding and OS elicitation, and in leaves of untreated plants. SA content in leaves harvested 30, 60, and 90 min after wounding and application of either water or OS did not differ from constitutive levels in WT and *ir-pme* lines (data not shown). However, 120 min after elicitation, SA levels varied among genotypes and treatments. Levels of SA were higher in wounded *ir-pme* lines compared with the WT (Fig. 2C, ANOVA, $F_{2,12}=4.84$, $P=0.03$) and OS elicitation caused an increase in SA levels in WT plants but not in *ir-pme* lines (ANOVA, $F_{2,12}=5.81$, $P=0.02$). In the latter, SA levels remained similar to the levels in wounded leaves.

ET emissions were also measured in WT and *ir-pme* lines 5 h after OS treatment using a photo-acoustic spectrometer. No differences were observed between genotypes (Fig. 2D, ANOVA, $F_{2,12}=1.03$, $P=0.39$).

One of the earliest responses to herbivore attack in *N. attenuata* is the activation of mitogen-activated protein

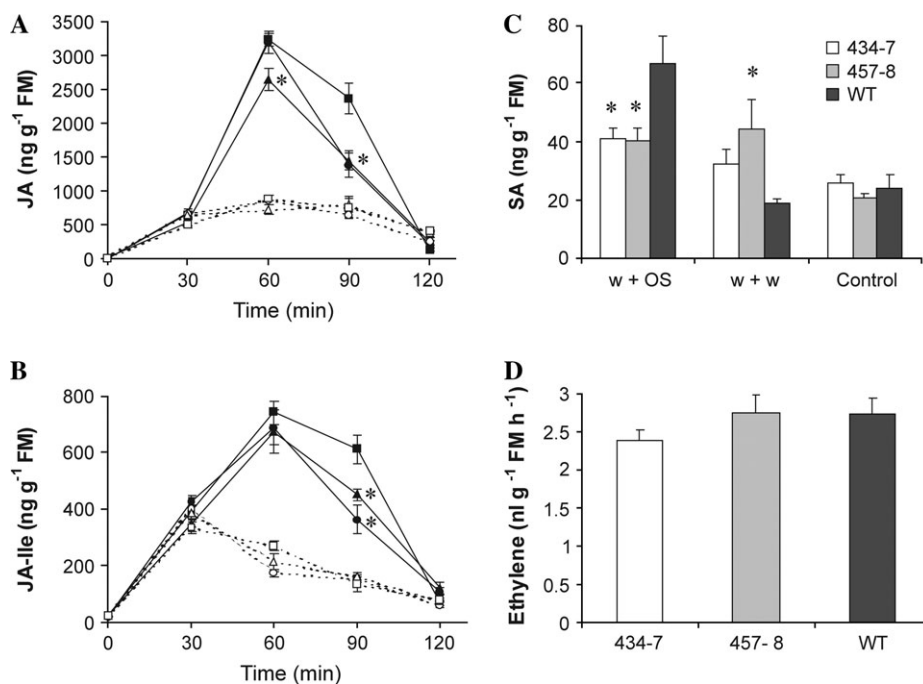


Fig. 2. Silencing NaPME1 affects phytohormone dynamics after OS elicitation. Mean (\pm SE) JA (A) and JA-Ile (B) levels in *N. attenuata* leaves of WT plants (squares) and *ir-pme* lines 434-7 (circles) and 457-8 (triangles) 30, 60, 90, and 120 min after elicitation. Leaves were wounded and the wounds treated with either water (w+w, dashed lines) or 1:1 diluted *M. sexta* oral secretions (w+OS, solid lines). Control plants remained untreated ($t=0$ min) ($n=4-5$). (C) Mean (\pm SE) SA levels of WT plants and *ir-pme* lines 120 min after elicitation ($n=5$). (D) Mean (\pm SE) ethylene emissions of single leaves after wounding and application of OS ($n=5$). Asterisks represent significant differences from the WT (ANOVA).

kinases (MAPK) such as SA-induced protein kinase (SIPK) (Wu *et al.*, 2007). To test SIPK activity, leaves were harvested 10 min after OS elicitation corresponding to the peak of SIPK activity in *N. attenuata* (Wu *et al.*, 2007). SIPK activity increased dramatically after OS treatment in all genotypes; however, no differences between WT and *ir-pme* lines were detected (Supplementary Fig. S3 at *JXB* online).

TPI activity is reduced in *ir-pme* lines after OS induction

The increase in JA and JA-related compounds after herbivore attack or OS elicitation leads to the production of defence compounds such as TPIs within a few days (van Dam *et al.*, 2001; Halitschke and Baldwin, 2005). TPI activity was monitored over several days after OS elicitation and in untreated leaves (day 0, Fig. 3A). TPI levels in *ir-pme* lines were reduced at 2 d and 4 d, and amounted only to 60% of WT levels after 6 d (Fig. 3A, two-way ANOVA, $F_{2,48}=5.96$, $P < 0.01$).

As described above, *M. sexta* attack or *M. sexta* OS elicitation immediately increases MeOH emissions in WT plants but not in *ir-pme* lines. To test whether the observed differences in the induction of TPI in *ir-pme* lines were caused by the reduced MeOH production in this genotype, TPI activity was quantified in OS-elicited leaves of WT and *ir-pme* lines supplemented with exogenous MeOH. The amount of MeOH added to the wounds represented the amount of MeOH released by a single WT leaf after OS treatment within the first 15 min (see Materials and

methods). MeOH emissions are known to be highest during this interval (von Dahl *et al.*, 2006). Therefore, the conditions used for these experiments reflect physiological MeOH amounts generated by the plant. MeOH treatment did not restore TPI activity to WT levels in *ir-pme* lines (Fig. 3B, two-way ANOVA, $F_{1,53}=2.24$, $P=0.14$).

To test whether PME is involved in systemic signalling of defence responses, TPI activity and levels of nicotine were also measured in systemic leaves of WT and *ir-pme* lines. Leaves were harvested 5 d after a *M. sexta* larva had started feeding on the adjacent leaf. TPI activity was slightly reduced in systemic leaves of line 457-8 but not in line 434-7 compared with the WT (Fig. 3C, ANOVA, $F_{2,55}=10.02$, $P < 0.001$). Nicotine levels of systemic leaves did not differ between lines after *M. sexta* attack (Fig. 3D, ANOVA, $F_{2,56}=0.88$, $P=0.42$).

Silencing of NaPME1 enhances *M. sexta* performance

To test whether the observed differences in the production of jasmonates and SA and in the induction of TPI activity affect the performance of *M. sexta* larva, caterpillar performance bioassays were conducted in the glasshouse. Neonates were put on either WT plants or *ir-pme* lines and their weight was recorded every 2–3 d for a total of 11 d. *Manduca sexta* larvae feeding on *ir-pme* lines gained more body mass after 11 d than those feeding on WT plants (Fig. 4, ANOVA, $F_{2,23}=5.23$, $P=0.01$).

A second experiment to assess caterpillar performance was done with *M. sexta* larvae and leaf material in closed

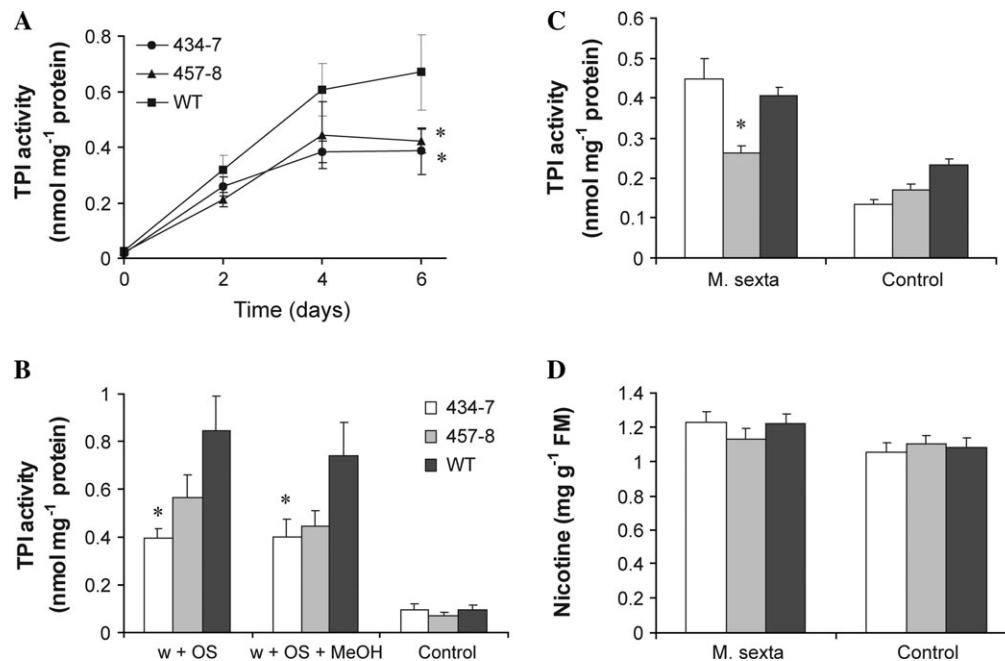


Fig. 3. TPI activity is reduced in *ir-pme* lines after OS elicitation. (A) Mean (\pm SE) TPI activity in *N. attenuata* leaves of WT plants (squares) and *ir-pme* lines 434-7 (circles) and 457-8 (triangles) 2, 4, and 6 d after elicitation. Leaves were wounded and the wounds treated with 1:1 diluted *M. sexta* oral secretions. Control plants remained untreated ($t=0$ d) ($n=5$). (B) Mean (\pm SE) TPI activity in leaves of WT plants and *ir-pme* lines 4 d after the treatment. Leaves were wounded and the wounds treated with *M. sexta* OS diluted 1:1 in either water (w+OS) or methanol (w+OS+MeOH). Control plants remained untreated ($n=9-10$). Mean (\pm SE) TPI activity (C) and nicotine levels (D) in systemic leaves 5 d after *M. sexta* larva feeding on an adjacent leaf (*M. sexta*). Control plants were not attacked ($n=19-20$). Asterisks represent significant differences from the WT (ANOVA).

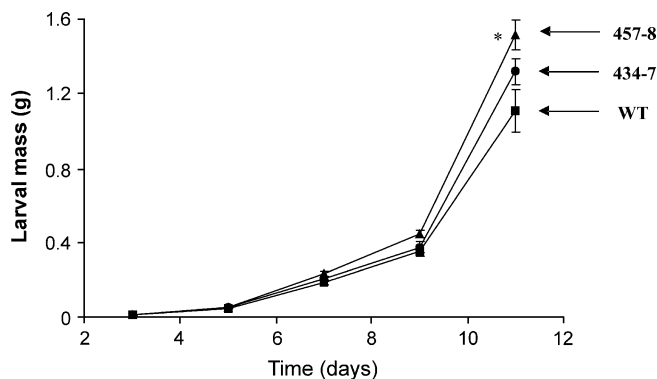


Fig. 4. Silencing of NaPME1 enhances *M. sexta* performance. Mean (\pm SE) larval mass of *M. sexta* larvae feeding on either the WT (squares) or *ir-pme* lines 434-7 (circles) and 457-8 (triangles) ($n=8-9$). Asterisks represent significant differences from the WT (ANOVA).

cages (Waldbauer, 1968). To test the effect of MeOH on *M. sexta* performance, plants were sprayed twice a day with either water or a 5% MeOH solution delivering 100 μ l of MeOH at each spraying, the amount released in 6 h by an attacked plant (von Dahl *et al.*, 2006). Supplementing *ir-pme* lines with exogenous MeOH did not affect larval growth or ECI compared with water-treated *ir-pme* lines (Supplementary Fig. S4 at *JXB* online). However, larvae feeding on *ir-pme* lines were more efficient in utilizing food than those feeding on the WT (Supplementary Fig. S4B, two-way ANOVA, $F_{2,69}=4.18$, $P=0.02$). Total protein content of leaves, which influences caterpillar performance, did not differ between WT and *ir-pme* lines (data not shown).

Discussion

The observation that *M. sexta* OS induces the transcript levels of NaPME1 raised the question of whether this enzyme is involved in anti-herbivore defence responses, via either MeOH production, modification of the food ingested by the caterpillar, and/or changes in the cell wall structure and phytohormone production.

Plants release a considerable amount of MeOH into the atmosphere which is mainly produced during cell wall modification by the demethylation of pectin catalysed by PMEs (Fall and Benson, 1996; Seco *et al.*, 2007). Interestingly, herbivore attack causes an increase in MeOH emissions when compared with non-induced plants (Peñuelas *et al.*, 2005; von Dahl *et al.*, 2006). For example, *N. attenuata* increases MeOH emissions when attacked by *M. sexta* larvae or elicited with *M. sexta* OS. It was previously postulated that this enhanced MeOH emission originates from the demethylation of pectin by the action of PMEs (von Dahl *et al.*, 2006). By silencing NaPME1, an OS-inducible PME in *N. attenuata*, it is here demonstrated that this enzyme participates in the enhanced production of MeOH after *M. sexta* OS elicitation. Because NaPME1 expression was silenced using a cDNA fragment corre-

sponding to the conserved pectinesterase domain, the possibility could not be ruled out that other members of the NaPME family also contribute to this reaction and accounted therefore for a fraction of the reduced MeOH emitted in *ir-pme* plants after elicitation and the \sim 50% decrease in PME activity in the same genotype. It has been postulated that induced MeOH production during insect herbivory may mediate defence responses and interactions between plants and insects (Peñuelas *et al.*, 2005; von Dahl *et al.*, 2006). Moreover, studies performed with *A. thaliana* showed that MeOH affects the expression of hundreds of genes including stress- and defence-related genes (Downie *et al.*, 2004). Thus, the generation of transgenic plants partially impaired in MeOH emission provides a very good system to test the above-mentioned hypotheses. Transgenic *ir-pme* lines showed no alterations in growth or morphology compared with the WT both in the greenhouse and in field trials in their native habitat in Utah, suggesting that NaPME1 is not involved in growth or developmental processes and may be specific for defence.

When analysed by a gel diffusion assay, total PME activity in leaves of WT plants was not enhanced by OS elicitation compared with wounding (after 40 min and 4 h), whereas MeOH emission increased several fold within 10 min. These results suggested that PME activity was rapidly and differentially affected by OS treatment and that this control occurred at the post-translational level. The total PME activity quantified after protein extraction in a gel diffusion assay may not reflect this rapid and probably transient activation of PME but rather the total activity induced at later time points by the transcriptional activation of NaPME1 and perhaps other PME family members.

In *ir-pme* lines, TPI activity in *M. sexta* OS-induced leaves was reduced by \sim 40% compared with the WT after 6 d of the treatment. Supplying the wounds of *ir-pme* leaves with MeOH in quantities that mimicked the MeOH released from an attacked WT leaf did not restore TPI activity to WT levels. These results suggested that MeOH as a signal molecule does not directly participate in the induction of TPI in *M. sexta* OS-elicited leaves. It is possible, however, that supplementation of the wounds with exogenous MeOH does not efficiently mimic the release of MeOH by PME activity in attacked leaves which occurs in small quantities for several hours.

In addition to functioning in within-plant signalling, MeOH could also mediate interactions between plants and insects (Peñuelas *et al.*, 2005; von Dahl *et al.*, 2006). Other volatile organic compounds (VOCs) released by plants after herbivore attack such as green leaf volatiles (GLVs) and terpenoid volatiles have been extensively studied and play important roles in plant-herbivore interactions (Kessler and Baldwin, 2001; Halitschke *et al.*, 2004). Whether MeOH could act in a similar manner needs further investigation. Little is known about the perception of MeOH by insects in this context, but it has been shown, for example, that bark beetles are attracted by alcohols such as ethanol (EtOH) and MeOH, which are used in host location (Byers, 1992). *Manduca sexta* larvae feeding on *ir-pme* lines gained more

body mass than those feeding on WT plants. Supplementing *ir-pme* lines with exogenous MeOH did not reduce larval growth compared with untreated *ir-pme* lines, suggesting that the better performance of the *M. sexta* larvae on this genotype is independent of MeOH signalling. Changes in the kinetics of accumulation of jasmonates and SA but not ET were observed after *M. sexta* OS induction in *ir-pme* lines compared with the WT. Both JA and JA-Ile levels were significantly reduced in the late phase of the jasmonate burst after elicitation but not before. *Manduca sexta* OS-induced SA levels were significantly reduced by 40% in both *ir-pme* lines compared with WT SA levels 120 min after the treatment. Interestingly, wounded *ir-pme* leaves contained 2-fold higher levels of SA than WT leaves. In *A. thaliana*, perturbation of the cell wall structure or homeostasis by mutations in cellulose synthase genes leads to deregulation of JA biosynthesis and thereby defence responses (Ellis *et al.*, 2002; Ko *et al.*, 2006). These previous studies established a strong link between cell wall structure/homeostasis and JA biosynthesis and stress responses. In *Arabidopsis* and poplar (*Populus trichocarpa*), wounding and JA also up-regulate the transcript levels of several PME genes (Cheong *et al.*, 2002; Pelloux *et al.*, 2007), and induced changes in the physical properties of plant cell walls may affect elicitor perception or early signalling events in the plant's defence responses (Vorwerk *et al.*, 2004; Pelloux *et al.*, 2007). The actual mechanisms underlying the changes in phytohormone dynamics in *ir-pme* lines remain unknown at present.

Lower JA and JA-Ile levels could partially contribute to the mechanisms underlying the reduced levels of inducible TPI activity in this genotype, but additional mechanisms may also operate. These mechanisms may be diverse. For example, PME can contribute to the generation of oligogalacturonides (OGAs) by promoting the activity of endopolygalacturonases, which degrade pectin. These small pectin fragments are known to elicit defence responses in plants against pathogens (Cote and Hahn, 1994; Shibuya and Minami, 2001) and are involved in the induction of proteinase inhibitors in tomato (Bishop *et al.*, 1981; Doares *et al.*, 1995). Moreover, in the wild strawberry *Fragaria vesca* the ability of OGAs to induce plant defences depends on their DM, which is reduced by PME activity (Osorio *et al.*, 2008). Therefore, reduced PME activity in *ir-pme* lines could affect the structure of OGAs, making them less efficient in mediating defence responses. A second possible scenario could be related to the production of free negatively charged carboxyl groups by induced PME activity. It is known that cations such as Ca²⁺ bind to these free groups, and silencing the expression of PME in *Solanum tuberosum* changes the accumulation and partitioning of Ca²⁺. Cell walls of these plants bind less Ca²⁺ than WT cell walls and the total content of Ca²⁺ is reduced in leaves of these transgenic plants (Pilling *et al.*, 2004). Extracellular Ca²⁺ influx into the cytosol is generally observed as one of the earliest responses upon herbivore attack (Maffei *et al.*, 2004) and therefore it is plausible that changes in cell wall-associated Ca²⁺ pools in *ir-pme* lines

could diminish the induction of defence responses in *N. attenuata*. Additionally, PMEs may also play a role in the metabolism of the larva's ingested food, thereby negatively influencing its performance. This is supported by the observation that *M. sexta* larva's feeding on *ir-pme* lines were more efficient in utilizing food than those feeding on the WT. Finally, changes in the structure of pectin in the cell wall of *ir-pme* plants should also be considered as one factor potentially influencing the efficiency of food utilization by *M. sexta* caterpillars. Future investigations will focus on the elucidation of the actual mechanisms affected in *ir-pme* lines and leading to reduced responses to insect herbivores.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Plant transformation vector pRESC5PME1 and Southern blot analysis of *ir-pme* lines and the WT.

Fig. S2. Growth parameters of WT and *ir-pme* lines.

Fig. S3. In-gel MAPK activity assay of WT and *ir-pme* lines.

Fig. S4. Nutritional indices of *M. sexta* larvae feeding on WT and *ir-pme* lines.

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