Journal of Experimental Botany, Vol. 64, No. 4, pp. 935–948, 2013
doi:10.1093/jxb/ers372 Advance Access publication 10 January, 2013
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

ML3: a novel regulator of herbivory-induced responses in *Arabidopsis thaliana*

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Received 29 November 2012; Revised 6 December 2012; Accepted 10 December 2012

Abstract

ML (MD2-related lipid recognition) proteins are known to enhance innate immune responses in mammals. This study reports the analysis of the putative ML gene family in *Arabidopsis thaliana* and suggests a role for the *ML3* gene in herbivory-associated responses in plants. Feeding by larvae of the Lepidopteran generalist herbivore *Spodoptera littoralis* and larvae of the specialist herbivore *Plutella xylostella* activated ML3 transcription in leaf tissues. *ML3* loss-of-function *Arabidopsis* plants were compromised in the upregulation of herbivory-induced genes and displayed a semi-dwarf phenotype. Herbivory bioassays showed that larvae of *S. littoralis* fed on *ml3* mutant plants gained more weight compared to larvae fed on wild-type plants while larvae of *P. xylostella* did not show any significant difference. Virus-induced gene silencing of *ML3* expression in plants compromised in jasmonic acid (JA) and salicylic acid (SA) signalling revealed a complex role of *ML3* in JA/defence signalling affecting both JA- and SA-dependent responses. The data suggest that ML3 is involved in herbivory-mediated responses in *Arabidopsis* and that it has a potential role in herbivory-associated molecular pattern recognition.

Key words: *Arabidopsis thaliana*, herbivory, jasmonic acid, *Plutella xylostella*, salicylic acid, *Spodoptera littoralis*, VIGS.

Introduction

Plants have evolved during millions of years to defend themselves against a multitude of pathogenic and herbivorous attackers. Recognition of pathogenic microbes and insect herbivores by plants is a crucial step for subsequent activation of a cascade of defence responses. The Microbe-Associated Molecular Pattern (MAMP), the Herbivory-Associated Molecular Pattern (HAMP), and the elicitor-recognizing receptors present in plants are three key players involved in the triggering of plant defence (Felton and Tumlinson, 2008; Bonaventure *et al*., 2011). Recognition of pathogen invasion or herbivore infestation activates a first line of responses, including a common array of enhanced ion fluxes across the plasma membrane specific to the entry zone, subsequently activating various defence responses (Maffei *et al*., 2004; Wu *et al*., 2007).

Upon herbivory or pathogen attack, a number of volatile organic compounds are released, initiating a cascade of signalling events stimulating various defence responses that are capable of mounting resistance to the invaders. The plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the major players in the regulation of signalling networks involved in defence (Kessler and Baldwin, 2002; Pieterse and Dicke, 2007). In a majority of cases it has been observed that pathogens with a biotrophic lifestyle are

more sensitive to SA-dependent responses, whereas necrotrophic pathogens and herbivores are resisted by defences dependent on JA, ET, or both (Bostock, 2005; Glazebrook, 2005). There are exceptional examples for the activation of the above defence responses where certain unidentified regulatory mechanisms and attacker-induced signals eventually shape the attacker-specific defence response (De Vos *et al.*, 2005; Mur *et al.*, 2006).

Reverse genetics approaches to silence JA synthesis or JA perception in *Arabidopsis thaliana* and other species have rendered the plants more susceptible to insect attack (Mewis *et al.*, 2006; Bodenhausen and Reymond, 2007; Zarate *et al.*, 2007, Hind *et al.*, 2011). Levels of JA and its derivative methyl jasmonate (MeJA) are known to increase rapidly in plants in response to wounding and herbivore damage, subsequently activating downstream plant defence-related genes (Ziegler *et al.*, 2001; Chung *et al.*, 2008). Exogenous application of JA is also known to be sufficient to induce the expression of the JA signalling pathway genes, thereby improving plant resistance to herbivores. Previous transcriptomic profiling studies have indicated that JA signalling results in the induction of several defence-related genes (Reymond *et al.*, 2000; Yan *et al.*, 2007). In addition, various feeding deterrent compounds and volatile organic compounds are produced as subsequent survival strategies by the plant. Wounding of plant tissues is a primary event upon damage by chewing insects. Mechanical wounding has been used in many studies to mimic herbivory and to study HAMPs, the effects on gene expression, and emission of volatile organic compounds (Spiteller *et al.*, 2001; Schmelz *et al.*, 2006). However, it has been observed that plant responses upon herbivory or mechanical wounding often are not at the same level since mechanical wounding is not a continuous process. Furthermore, insect oral secretions can contain compounds with elicitor-like properties that trigger plants further (Roda *et al.*, 2004; Schmelz *et al.*, 2006). Recently it was also shown that oral secretions from certain insects are capable of suppressing wound-induced responses in *Arabidopsis* (Consales *et al.*, 2012) similar to effectors produced by certain pathogens to hamper pathogen-triggered plant immunity (Nicaise *et al.*, 2009).

While several MAMPs have been identified only a few HAMPs have been isolated and characterized. Herbivory-derived proteins such as glucose oxidase, alkaline phosphatase, and β-glucosidase have been reported to serve as elicitors (Mattiacci *et al.*, 1995; Eichenseer *et al.*, 1999a; Funk, 2001). It has also been reported that some herbivores produce a cocktail of effectors to suppress the plant-induced defence pathways, either by mimicking plant hormone responses or by masking the perception of HAMPs (Musser *et al.*, 2002; Zarate *et al.*, 2007). Glucose oxidase remains the sole identified salivary constituent of caterpillars reported to suppress recognition sites for activation of plant defence (Eichenseer *et al.*, 1999b, 2010). It has also been reported that lower molecular weight proteins act as potent elicitors. For example inceptin, a disulfide-bridged proteolytic fragment of a chloroplast ATP synthase isolated from *Spodoptera frugiperda* larvae, was found to initiate specific plant responses upon insect attack (Schmelz *et al.*, 2006). Recent isolation of HAMP elicitors has made it possible to identify and investigate binding proteins that could function as receptors triggering signal transduction pathways that subsequently activate appropriate defence responses in the plant. However, to date, only one HAMP receptor-like binding site has been identified (for volicitin) and this is known to be activated also by exogenous application of MeJA treatment, linking its role in JA signalling (Truitt *et al.*, 2004).

An earlier study used cDNA-AFLP-based transcript profiling to follow gene expression changes in oilseed rape (*Brassica napus*) leaves upon feeding by *Plutella xylostella*, MeJA treatment, and mechanical wounding (Bejai and Meijer, 2007). Transcripts belonging to the ML (MD2-related lipid recognition) gene family were found to be highly upregulated. ML is a novel domain identified in plants, animals, and fungi (Inohara and Nunez, 2002). In mammalian cells, ML proteins have been identified to be a binding partner of Toll-like receptor 4 (TLR4) that induce innate immunity responses after recognition of bacterial lipopolysaccharides (Shimazu *et al.*, 1999; Viriyakosol *et al.*, 2001). However, in plants, ML family genes are to date of unknown function. The present study therefore aimed to gather more information concerning the role of ML genes in plant defence, especially against herbivory, based on the induction observed earlier after wound-related stress. The oilseed rape relative *A. thaliana* was used as a powerful resource to explore the role of the ML gene family in herbivory-associated plant defence. This study examined the expression of all nine putative ML genes upon herbivory in plants under controlled growth conditions. Together with *in silico* analysis, ML3 seemed to be the most interesting candidate involved in wound stress responses. This study reports the further characterization of ML3, a putative MD2 lipopolysaccharide-recognition domain protein, and shows that it has functions related to JA signalling and could be a regulator for HAMP recognition. This study also evaluated the tolerance of *ml3* mutants to herbivore feeding and studied the function of ML3 in the JA-mediated defence pathway. Finally, the potential role of ML3 as a novel HAMP response regulator in plants is discussed.

Materials and methods

Plant material and growth parameters

Arabidopsis ecotype Col-0 was used as wild type for all experiments. Soil-grown plants were maintained in a growth chamber at 22 °C under 16/8 light/dark (200 μE m^{-2} s^{-1}). Seeds were sterilized in 50% bleach for 1 min and 70% ethanol for 30 seconds and washed four times with sterile water. Seeds collected from heterozygous *coi1-1* plants (Feys *et al.*, 1994) were germinated on MS medium containing 50 μM MeJA to select for JA-insensitive *coi1-1* homozygous plants. Seeds from the *jar1-1* mutant were germinated on MeJA containing MS medium (Staswick *et al.*, 1992). *ml3* T-DNA knockout homozygous *Arabidopsis* lines (Salk_059591C and Salk_091638) were generated through the SIGnAL project (http://signal.salk.edu/tabout.html) and obtained from the Nottingham *Arabidopsis* Stock Centre (Nottingham, UK). *ML3*- and T-DNA-specific primers were used to select the plants homozygous for the inserts. Analysis of the knockout lines was performed with PCR.

Phylogenetic analysis

The phylogenetic tree of nine *Arabidopsis thaliana* ML genes was constructed with the Neighbor Joining (NJ) method using MEGA 4 software (Molecular Evolutionary Genetics Analysis) available at http://www.megasoftware.net/index.html. Values on each node are percentage of bootstrap values (only values greater than 50 are shown). A bootstrap analysis was performed with 1000 replications.

Insects

Spodoptera littoralis eggs were obtained from the Department of Plant Biology, Faculty of Landscape Planning, Horticulture and Agricultural Sciences, SLU, Alnarp and incubated at 22 °C until they hatched. *P. xylostella* larvae were obtained from the Department of Ecology, Swedish University of Agricultural Sciences, Uppsala.

Herbivore bioassay

For the *S. littoralis* first instar larvae, newly hatched larvae were maintained on an artificial diet for 24 hours and on the second day groups of 4 larvae were placed on leaves of four 3-week-old *Arabidopsis* lines and allowed to feed for 10 days. Ten pots containing the four plants were used per *Arabidopsis* line. During the experimental period, those plants consumed by the larvae were replaced with new plants of the respective lines. After 10 days the larvae were removed from the plants and weighed. The experiment was repeated thrice, average larval weight per replicate was used in the statistical analysis. An analysis of variance (ANOVA) was performed in order to see if the larval weights differed depending on what plant line they fed upon.

P. xylostella larval performance was monitored as above by using the first instar larvae. ANOVA was used to analyse differences in the larval weights.

Quantitative real-time PCR analysis

S. littoralis second instar larvae were placed on the first leaf of Col-0 wild-type plants and allowed to feed. After 24 h of initiation of feeding, the larvae were removed and the damaged (local) leaves and the undamaged (systemic) leaves were snap frozen in liquid nitrogen and used for quantitative real-time PCR (qRT-PCR). Plants without any larvae feeding were used as a control. As a template, cDNA synthesized from total RNA was used. Total RNA was extracted using Plant RNeasy Mini Kit (QIAGEN, Germany) and followed by DNase I (Ambion, UK) treatment to remove any genomic DNA contamination. The first-strand cDNA was synthesized from 500 ng of each DNase-treated total RNA in 25 µl reaction volume using qscript cDNA synthesis kit (Quanta Biosciences) and then diluted up to 50 µl with 3 mM TE buffer (pH 7.5). Primer Express 2.0 software (PE Applied Biosystems, USA) was used to design the gene-specific forward and reverse primers to amplify by qRT-PCR: *ML3* (AT5G23820; forward primer 5'-GCGCCGAAAGACCTTAGAG-3', reverse primer 5'-AATAGAGACAAATGCAGGAGCTG-3'), *PR1a* (At2g14610; forward primer 5'-TGATCCTCGTGGGAATTATGT-3', reverse primer 5'-TGCATGATCACATCATTACTTCAT-3'), *LOX2* (At3g45140; forward primer 5'-CTTACCCGCGGATCTCATC-3', reverse primer 5′-ACTCCATGTTCTGCGGTCTT-3'), *VSP2* (At5g24770; forward primer 5'-GTTAGGGACCGGAGCATCAA-3' and reverse primer 5'-AACGGTCACTGAGTATGATGGGT-3'), At*MPK4* (At4g01370; forward primer 5'-CAAGCAGACGCATCACAGTT-3' and reverse primer 5'-AAAATTGAACGGCCTCACAC-3'), Tubulin transcript (At5g62700; forward primer 5'-CGATGTTGTTCGTAAGGAAGC-3' and reverse primer 5'-TCCTCCCAATGAGTGACAAA-3'), and *UBQ5* (At3g62270; forward primer 5'-CGATGGATCTGGAAAGGTTC-3' and reverse primer 5'-AGCTCCACAGGTTGCGTTAG-3'. ABI Prism 7000 sequence detection system and software (PE Applied Biosystems) was used. qRT-PCR reactions (20 µl) included SYBR Green PCR master mix (Applied Biosystems) supplemented with 5 µM primers and 1 µl cDNA as a template. As a negative control, reaction mixtures without cDNA were used. PCR reactions were performed using the following parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C in 96-well optical reaction plates (Applied Biosystems). The identities of the amplicons and the specificity of the reactions were verified by melting curve analysis. The data were analysed by comparative C_T method (Livak and Schmittgen 2001) with PCR efficiency correction. PCR efficiency was determined based on the slope of standard curves. The gene expression level obtained by qRT-PCR was normalized using the tubulin and *UBQ5* genes and subsequently the fold-differences in the transcript levels and mean standard error were calculated as described elsewhere (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). At least, two different RNA isolations from independent biological replicates and cDNA synthesis products were used for quantification and each cDNA was measured in triplicate.

Construction of the virus-induced gene silencing plasmid

The tobacco rattle virus (TRV)-derived binary vectors *pTRV1* (pYL192) and *pTRV2* (pYL156) have been described by Liu *et al.* (2002). To generate *pTRV2-ML3*, a 695 nt fragment of the *Arabidopsis ML3* gene was amplified from *Arabidopsis* genomic DNA using primers AtML3fwdKpn (5'-AAGGTACCATAAAATAGCAGACTTGCATAG-3') and AtML3revBam (5'-AAGGATCCCCATCTCTAAAGGCTACG-3'). The resulting products were cloned in antisense orientation into *Bam*HI/*Kpn*I-cut *pTRV2*. The construct was transformed into cells of *Agrobacterium tumefaciens* GV3101 using the freeze–thaw method (An *et al.*, 1988) with the modification that non-competent cells were used instead of chemically competent cells.

Plant growth and agroinfiltration

Wild-type *Arabidopsis* ecotype Col-0 and *NahG*, *jar1-1*, *coi-1*, *npr1* lines were grown in pots at 23 °C in a growth

chamber under a 16/8 photoperiod with 60% humidity. Two-week-old plants were used for virus-induced gene silencing (VIGS) infiltration. Inoculum of *Agrobacterium* containing *pTRV1*, *pTRV2*, *pTRV2-PDS*, or *pTRV2-ML3* were grown at 28 °C in 50 ml LB medium containing 25 mg l^{-1} gentamycin, 50 mg l^{-1} kanamycin, 10 mM MES, and 20 µM acetosyringone. The following day, *A. tumefaciens* cells were harvested by centrifugation and resuspended in infiltration media (10 mM $MgCl_2$, 10 mM MES, and 150 µM acetosyringone), adjusted to an OD_{600} of 1.5, and left at room temperature for 4 h. Agro-solution of *pTRV1* was mixed in 1:1 ratio with solutions of *pTRV2*, *pTRV2-PDS* (as controls for symptoms caused by the viral vector itself), or *pTRV2-ML3*. Agroinfiltration was performed with a needleless 1 ml syringe into two leaves each of 2- or 3-leaf-stage plants, infiltrating the entire leaf.

JA analysis

The quantitative analysis of jasmonic acid (JA) and its conjugate with isoleucine (JA-Ile) was performed using *A. thaliana* WT (Col-0) plants upon and without herbivory treatment as well as *ml3* mutant plants. About 25 mg fresh weight were homogenized in liquid nitrogen followed by homogenization using an MM 301 vibration mill (Retsch, Haan, Germany) at a frequency of 27 Hz for 3 min after adding 3-mm tungsten carbide beads (Retsch) in the presence of extraction solvent composed of cold 10% acidic methanol and 20 pmol of [$^2H_6$] JA and [$^2H_2$]JA-Ile as internal standards. The analytes were then concentrated using solid-phase extraction cartridges containing reversed-phase sorbent (Waters, Milford, MA, USA). Dried eluates were re-solubilized in the mobile phase and analysed by the Acquity UPLC System (Waters, USA) coupled to Xevo TQ MS (Waters, Micromass, UK). The data were then analysed using Masslynx 4.1 software (Waters, USA) and quantified by the standard isotope-dilution method.

Results

The ML gene family in Arabidopsis

Using the TAIR database, this study identified nine potential ML genes (At*ML1*–At*ML9*) in the *A. thaliana* genome. A phylogenetic tree revealed that At*ML3* and At*ML5* grouped together with 80% identity, followed by At*ML1* and At*ML4* that grouped together (70% identity), while the remaining genes were classified as singletons (Fig. 1). Since this study only concerns the ML gene family in *Arabidopsis*, ML is used as the gene name instead of At*ML*. In an *in silico* analysis using *Arabidopsis* microarray data available at Genevestigator, this study observed the ML genes to be differentially expressed throughout various tissues and developmental stages of the plant (Supplementary Fig. S1, available at *JXB* online). In addition, it was observed that *ML1*, *ML3*, and *ML6* were expressed at a very high level in different parts of roots. Interestingly, *ML3* was the only member in the gene family to be predominantly expressed in leaves, stem, and roots. Expression of *ML1* was observed at high

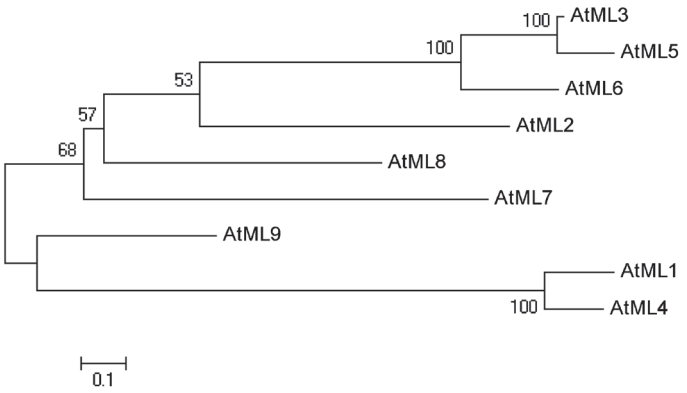

Fig. 1. Neighbour-joining phylogenetic tree of the *Arabidopsis thaliana ML* gene family. The tree was constructed using MEGA4 where bootstrap values (>50%) based on 1000 replications are shown at the branch nodes. The bar represents 0.1 substitutions per nucleotide position.

levels throughout different development stages of the plant, whereas *ML3* was highly expressed during the beginning of the growth cycle of the plant but subsequently decreased at later developmental stages (Supplementary Fig. S2). Transcript levels of the ML genes were found to be significantly increased only by biotic stimuli, especially by MeJA treatment mimicking wounding, as compared to abiotic stimuli although the degree of expression varied among the members of the ML gene family (Supplementary Fig. S3A). *ML3* was the only member found to be significantly highly expressed upon MeJA treatment.

A time-course analysis of *ML3* expression in *Arabidopsis* (Col-0) leaves upon MeJA treatment revealed an upregulation of *ML3* transcripts at 4 h and 24 h whereas a significant downregulation was observed at the same time intervals upon SA treatment (Supplementary Fig. S3B).

Expression of ML family genes upon herbivory

To test the expression of the ML genes upon herbivory, qRT-PCR analysis was performed on *Arabidopsis* leaves collected 24 h after feeding by first instar larvae of the generalist insect *S. littoralis* or second instar larvae of the specialist herbivore *P. xylostella*. The results showed differentially upregulated expression profiles among the nine ML genes (Fig. 2). Feeding by *Spodoptera* larvae increased expression of all ML genes except *ML8*, whereas feeding by *Plutella* larvae did not elicit the expression of *ML4, ML8*, or *ML9*. Interestingly, among all the ML genes that were upregulated, *ML3, ML5*, and *ML7* were observed to be especially highly expressed in the damaged leaves in both treatments. Upon herbivory, plants are known to accumulate defensive signals not only in the local leaves but also in the distal (systemic) leaves to promote a better defence response in tissues expected to become attacked later. Increased expression in the systemic leaves was observed for *ML1, ML2, ML3, ML5*, and *ML7* upon herbivory by *S. littoralis*. Intriguingly, in the *P. xylostella*-infested plants,

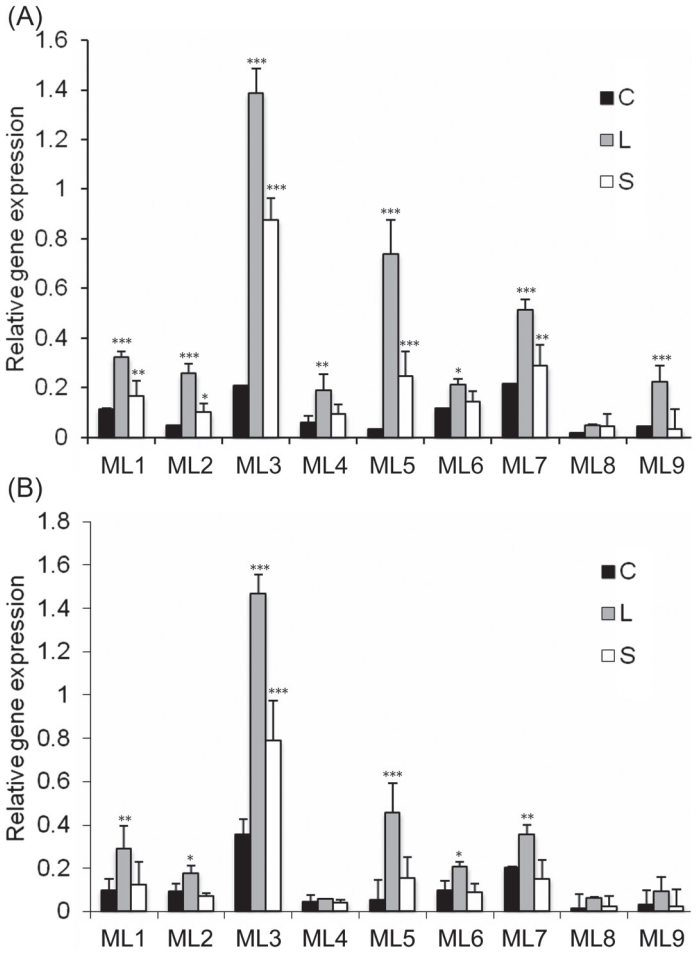

Fig. 2. Expression of ML genes in response to feeding by herbivores. (A) Three-week-old wild-type (Col-0) plants were challenged with second instar larvae of generalist herbivore *S. littoralis* larvae, then 24 h after initiation of larval feeding, samples were harvested and used for RNA extraction: C, control; L, damaged local leaves; S, undamaged systemic leaves. (B) Three-week-old wild-type (Col-0) plants were challenged with second instar larvae of specialist herbivore *P. xylostella* larvae, then 24 h after initiation of larval feeding, samples were harvested and used for RNA extraction. Error bars represent SE of three biological replicates. Asterisks indicate statistically significant differences to the respective control (Student's t-test; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

the systemic leaves did not show a difference in gene expression compared to the control except for *ML3*. This demonstrates that the activation of a systemic response of ML genes seems to be dependent on the attacker. *ML3* seems to be the only gene being activated to higher levels both locally and systemically upon feeding by both *S. littoralis* and *P. xylostella.*

Activation of ML3 expression in response to JA treatment

In order to investigate the effect of MeJA treatment on the expression of the ML genes, qRT-PCR analysis was performed on wild-type rosette leaves 6 h after treatment with MeJA. Results revealed a strong induction of *ML3* and *ML5* expression compared to *ML1*, *ML2*, *ML4*, *ML6*, *ML7*, *ML8*, and *ML9* (Fig. 3A). Furthermore, the expression level of *ML3* and *ML5* was significantly higher upon MeJA treatment than upon herbivory. Time-course studies following MeJA treatment revealed that expression of *ML3* was strongly induced within 1 h of treatment, peaked at 6 h and subsequently declined at later time points (Fig. 3B). *ML5* induction was much slower compared to *ML3* but reached a peak at 6 h and was downregulated at 9 h (data not shown).

Based on the above results, this study hypothesized that *ML3* could be playing a major role in wound-induced herbivore response. Henceforth, it proceeded to elucidate the functional role of *ML3* in plant defence.

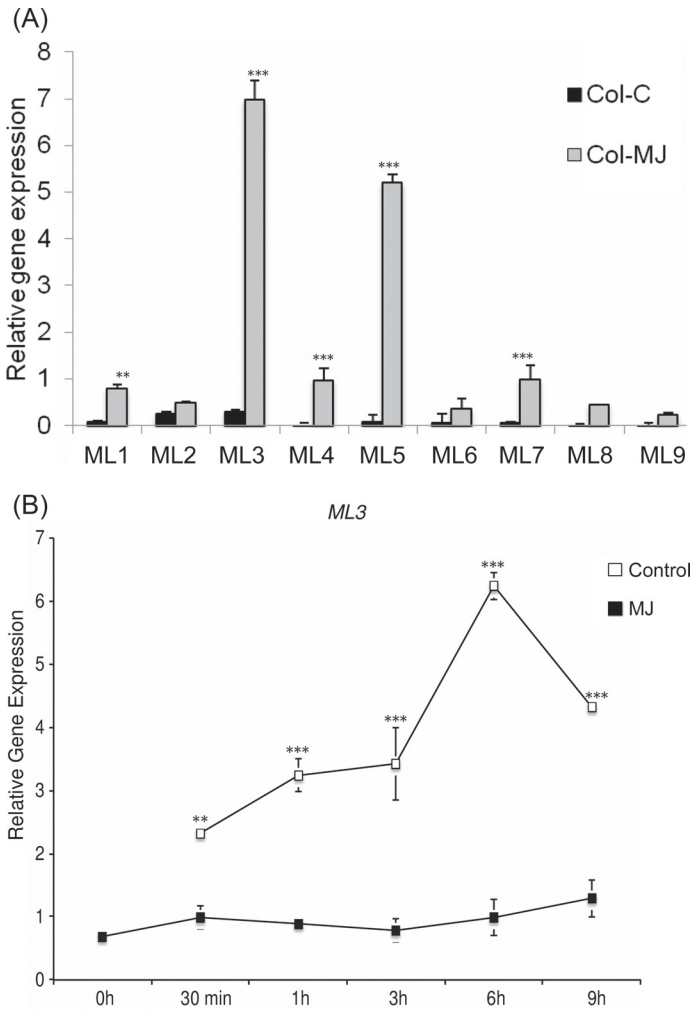

Fig. 3. Expression analysis of the ML gene family (A) and *ML3* (B) on 3-week-old wild-type (Col-0) plants treated with 30 μM MeJA. Leaves were harvested 6 h post treatment and used for RNA extraction and qRT-PCR. Error bars represent SE of three biological replicates. Asterisks indicate statistically significant differences to the respective control (Student's t-test; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

ml3 mutants have differential responsiveness to generalist and specialist herbivores

Using the SIGnAL database (http://signal.salk.edu/cgi-bin/atta), this study identified two T-DNA knockout lines for *ML3*, Salk_059591C and Salk_091638, and named them *ml3-1* and *ml3-2,* respectively. Both mutants showed a semi-dwarf phenotype when grown on soil under long-day conditions (Fig. 4A). Semi-quantitative reverse transcription PCR using *ML3* primers demonstrated a lack of *ML3* transcripts in both the insertion lines as opposed to the wild type (Fig. 4B).

The role of the *ML3* gene product in plant–herbivore interactions has not been reported earlier. This study showed that larvae of *S. littoralis* reared on *ml3* mutants gained more weight than larvae reared on wild-type (Col-0) plants (Fig. 5A and C). This observation was in contrast to the specialist herbivore *P. xylostella*, where no significant difference was observed between the larvae that fed on the *ml3* mutants or on the wild-type plants (Fig. 5B). This suggests a role for *ML3* in the regulation of plant defence against specific herbivore species.

ml3 mutants are compromised in JA-dependent responses

Lipoxygenases including *LOX2* in *Arabidopsis* are known to be involved in the synthesis of JA and are upregulated by herbivory (Reymond *et al.*, 2004). In order to study the level of activation of *LOX2* in *ml3* mutants, qRT-PCR was used to compare the expression of *LOX2* in herbivore-treated and untreated leaf samples of wild-type plants and the *ml3* mutants (Fig. 6). The level of *LOX2* expression in untreated leaves was the same in wild-type and *ml3* mutant plants. However, in the wild type, a 4-fold upregulation of *LOX2* was observed in the herbivore-treated leaves. In contrast, *ml3-1* and *ml3-2* plants showed only a 2-fold or less upregulation of *LOX2* expression upon herbivory compared to untreated mutant plants. This led to the hypothesis that JA-mediated *LOX2* expression is compromised in the *ml3* plants thus making the plants more appetizing for the larvae. Mitogen-activated protein kinase 4 (MPK4) has earlier been reported to be involved in JA signalling. At*MPK4* expression was compromised in the *ml3* mutants (Fig. 7) indicating that *ML3* acts upstream of mitogen-activated protein kinases. Endogenous JA and JA-Ile levels were significantly downregulated in the *ml3* mutants compared with the Col-0 wild type (Fig. 8). Upon herbivory, *ml3* mutants were able to accumulate only 0.5-fold JA-Ile levels compared to Col-0 that accumulated 5-fold higher levels.

Co-silencing of ml3 with defence signalling pathway mutants indicates role of JA pathway

Basal level of *ML3* expression was found to be lower in the *jar1-1* and *coi1* mutants compared to the Col-0 wild type. In contrast *ML3* was highly upregulated in leaves of 3-week-old *NahG* and *npr1* plants (Fig. 9A).

To evaluate the relevance of *ML3* in the JA signalling pathway, this study used VIGS to silence *ML3* in the signalling

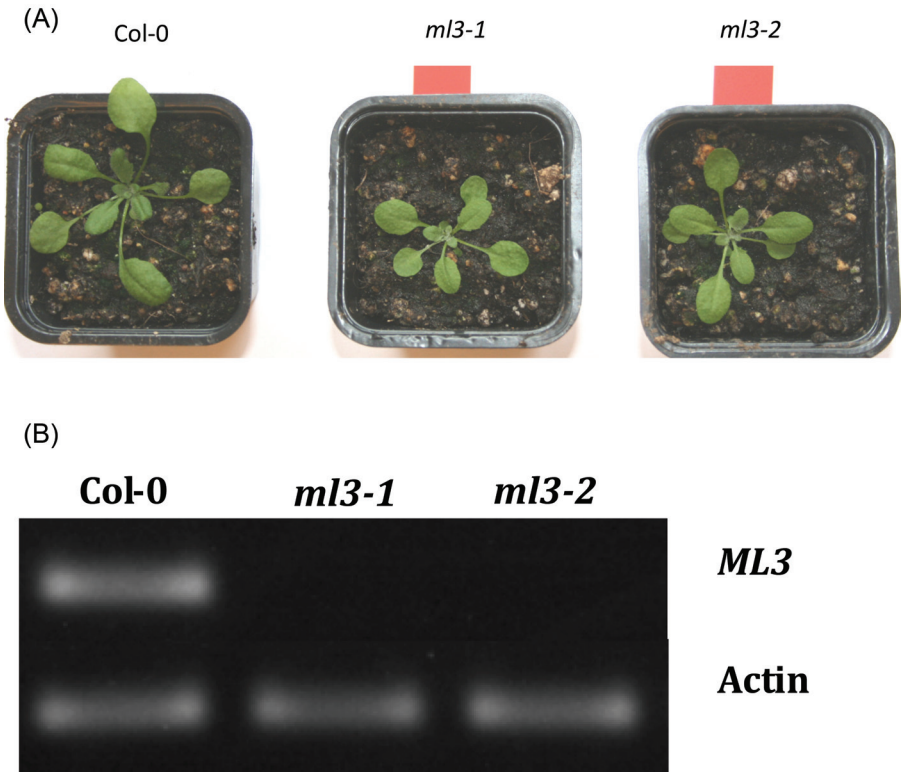

Fig. 4. (A) *Arabidopsis* wild-type (Col-0), *ml3-1*, and *ml3-2* plants 3 weeks after germination. (B) Reverse-transcription PCR analysis of *ML3* and *Actin* transcripts in Col-0, *ml3-1*, and *ml3-2* plants.

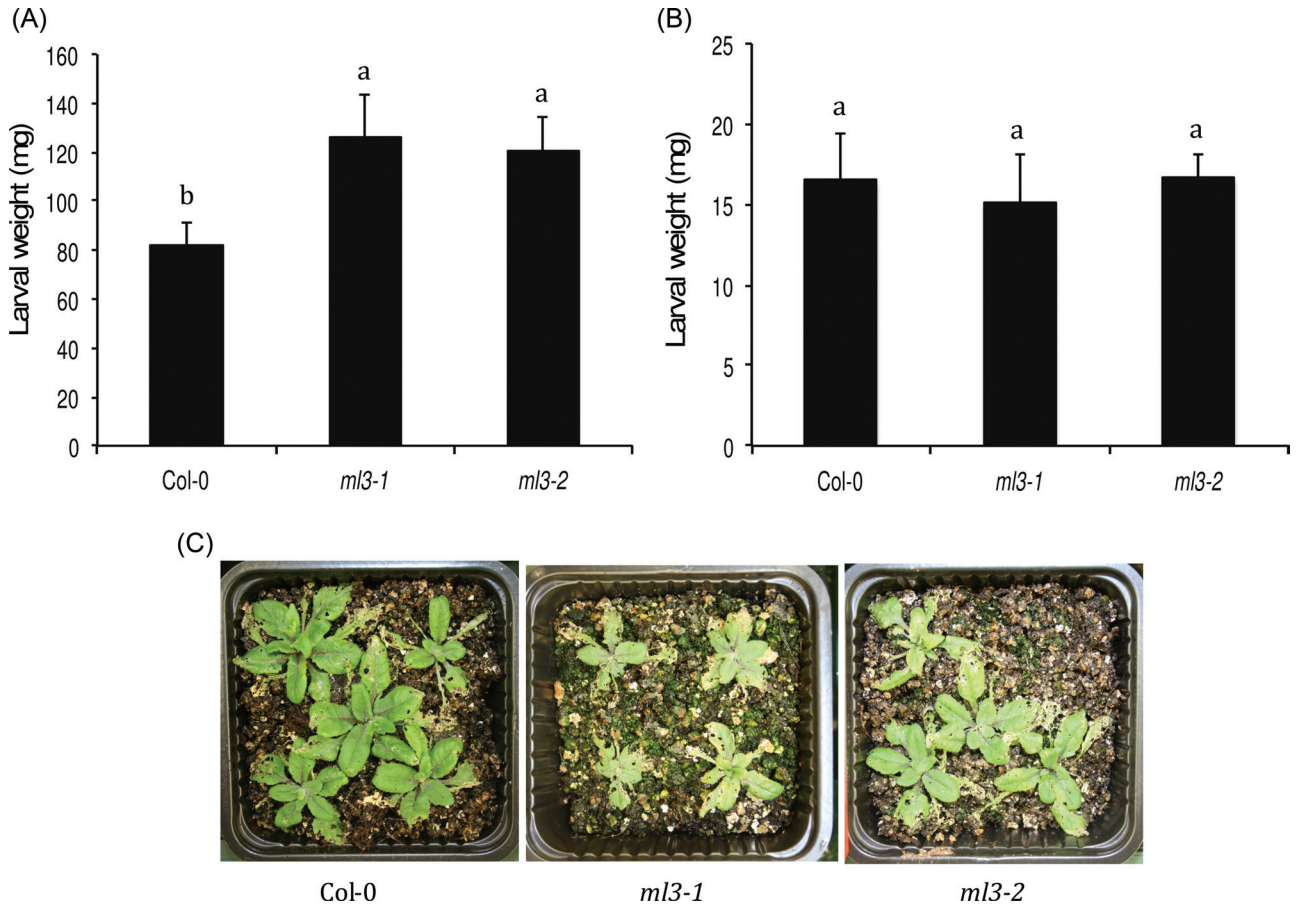

Fig. 5. Herbivory bioassay on *ml3-1* and *ml3-2* plants. (A, B) Larval weights of *S. littoralis* 15 days (A) and *P. xylostella* 6 days (B) after initiation of feeding. Values indicate the mean ± SE of three independent experiments. ANOVA was performed in order to see if the larval weights differed depending on what plant type they fed on. Different letters indicate significantly different means. (C) Representative *Arabidopsis* plants after feeding by *S. littoralis* larvae for 2 days.

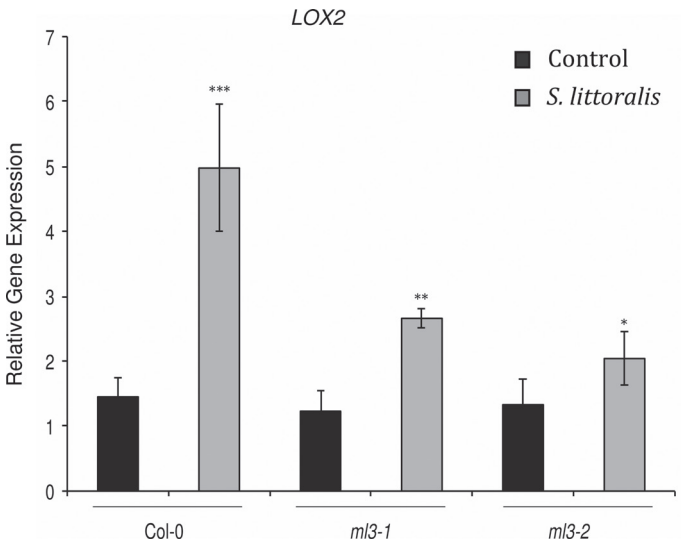

Fig. 6. Effect of herbivory on *LOX2* expression in the ml3 mutants. Three-week-old wild-type (Col-0), *ml3-1*, and *ml3-2* plants were exposed to first instar larvae of *S. littoralis* and leaves were collected 6 h later. Leaves from control plants were collected separately for qRT-PCR analysis. Error bars represent SE of three biological replicates. Asterisks indicate statistically significant differences to the respective control (Student's t-test; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

pathways mutants. A fragment of the *ML3* sequence was introduced into the RNA2 plasmid (pYL156) of the TRV-based VIGS vector (Liu *et al.*, 2002), subsequently transformed into *A. tumefaciens* and co-infiltrated with the RNA1 plasmid (pYL192) into *Arabidopsis* leaves, thus forming *pTRV-ML3*. The transgenic line *nahG* carries a bacterial salicylate hydroxylase that converts SA to catechol, preventing accumulation of SA and induction of SAR, was used to probe the SA pathway (Lawton *et al.*, 1995). To evaluate the importance of JA signalling pathway in response to herbivory, this study used the jasmonate-insensitive *coi1* mutant (Feys *et al.*, 1994). Marker lines for the JA pathway (*coi1-1*) and the SA pathway (*NahG*) were infiltrated with *pTRV-ML3* and 2 weeks later first instar larvae of *S. littoralis* were placed on the leaves and allowed to feed for 24 h. qRT-PCR analysis of the herbivory-damaged leaves was carried out to confirm silencing of the *ML3* gene (data not shown) and subsequently the mRNA was analysed for expression of the downstream marker genes *PR1* and *VSP2* to probe the SA and JA pathway, respectively.

When basal level of *PR1* expression was measured in the co-silenced plants, significantly high upregulated levels were found in the *coi1-1:pTRV-ML3* and *Col-0:pTRV-ML3* lines

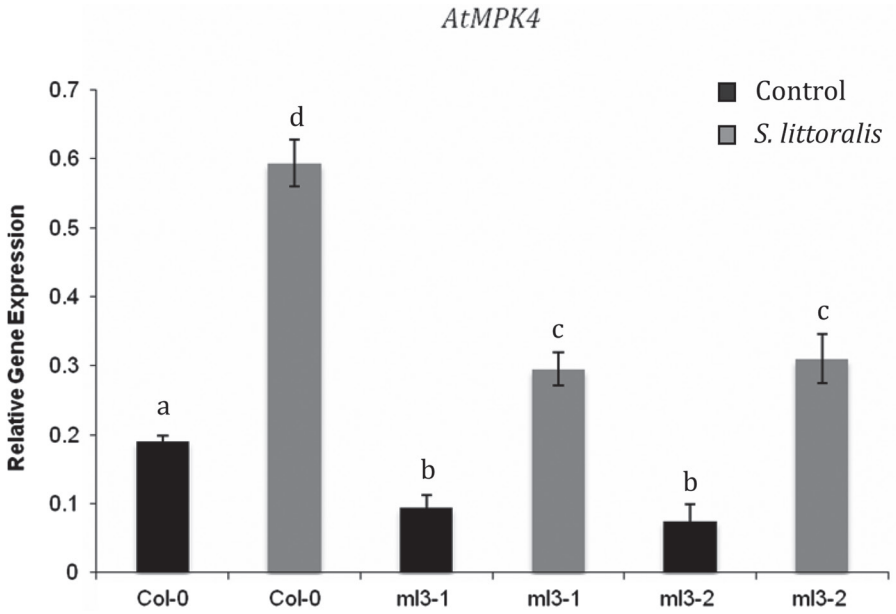

Fig. 7. Quantitative real-time PCR analysis of At*MPK4* basal level gene expression in the leaves of wild-type Col-0 and *ml3-1* and *ml3-2* mutants. Error bars represent SE of two biological replicates. The data presented is representative of the three independent experiments consisting of three biological replicates. One-way ANOVA with Fisher pairwise comparison ($P < 0.001$). Different letters indicate significantly different means.

compared to the *NahG:pTRV-ML3* lines (Fig. 9B). Upon herbivory, there was no significant change observed in the *NahG:pTRV-ML3* and *coi1-1:pTRV-ML3* co-silenced lines. Even though a 5-fold upregulated expression was observed in the *Col-0:pTRV-ML3* plants compared to the Col-0 control upon herbivory, no significant change was observed. However, the plants infiltrated with the empty vector showed no significant change in the expression.

VSP2 expression was downregulated in the *coi1-1* line as expected. A significant downregulation of *VSP2* was observed in the *coi1-1:pTRV-ML3* plants compared to the single mutant (Fig. 9C). Higher *VSP2* levels were found in the *NahG* and even more in the *NahG:pTRV-ML3* line, indicating a negative role of ML3 on JA responses at low levels of SA. Upon herbivory, *NahG:pTRV-ML3* plants showed a similar level of expression as compared to the wild type. Whereas, the *coi1-1* and *coi1-1:pTRV-ML3* did not show any significant change in expression upon herbivory. Taken together, the data obtained reveal that *ML3* plays a positive role in the JA-mediated defence pathway and a negative role in the SA pathway.

Coronatine-insensitive (*COI1*) encodes an LRR/F-box protein that confers specificity to the SCF type E3 ubiquitin ligase involved in the activation of JA signalling (Xu *et al.*, 2002). It has earlier been reported that wound-induced *LOX2* and JA levels fail to respond in *coi1* mutants upon herbivory (Moran and Thompson, 2001; Li *et al.*, 2004). Hence to further explore the role of *ML3* in wound-induced JA signalling, this study used VIGS to silence *ML3* in the JA mutant *coi1* (Supplementary Fig. S4). Interestingly, *Spodoptera* larvae fed more on the co-silenced *coi1:pTRV-ML3* line compared to the *coi1* mutants (Supplementary Fig. S5).

Discussion

Plants generate an array of defence responses upon herbivore attack. In turn, certain herbivores produce a cocktail of effectors that can suppress plant defensive pathways, mimic plant hormones, and mask the perception of HAMPs (Felton and Tumlinson, 2008). Previously, this study group identified some signature transcripts of genes involved in herbivory-induced responses in oilseed rape plants including ML orthologues (Bejai and Meijer, 2007). qRT-PCR analysis of the ML genes following feeding experiments indicated their role in induced defence to insect attack in *Arabidopsis* (Fig. 2). Based on this background information, this study was embarked upon with the goal of determining how the ML genes in *Arabidopsis* are affected in response to herbivory. Very little is known about ML proteins in plants although they are believed to serve in innate immunity responses based on homology with ML proteins in other species. However, recently the ML3 protein was found to be highly expressed in cell-wall fractions of 5-day old *Arabidopsis* hypocotyls, suggesting a role also in plant development (Irshad *et al.*, 2008).

The results show that *ML3* and *ML5* are induced to a higher level in *Arabidopsis* plants compared to the other ML genes in response to MeJA and feeding by both *S. littoralis* and *P. xylostella* larvae. *ML3* and *ML5* expression was observed to be induced also in systemic leaves upon feeding by *S. littoralis*, whereas only *ML3* showed a systemic response in response to *P. xylostella* herbivory. Therefore, this study chose to focus the work on *ML3* and conduct further studies to understand its mechanistic role upon herbivory. It is interesting to note that *ML4*, *ML8*, and *ML9* were not induced upon feeding by specialist larvae (*P. xylostella*) compared

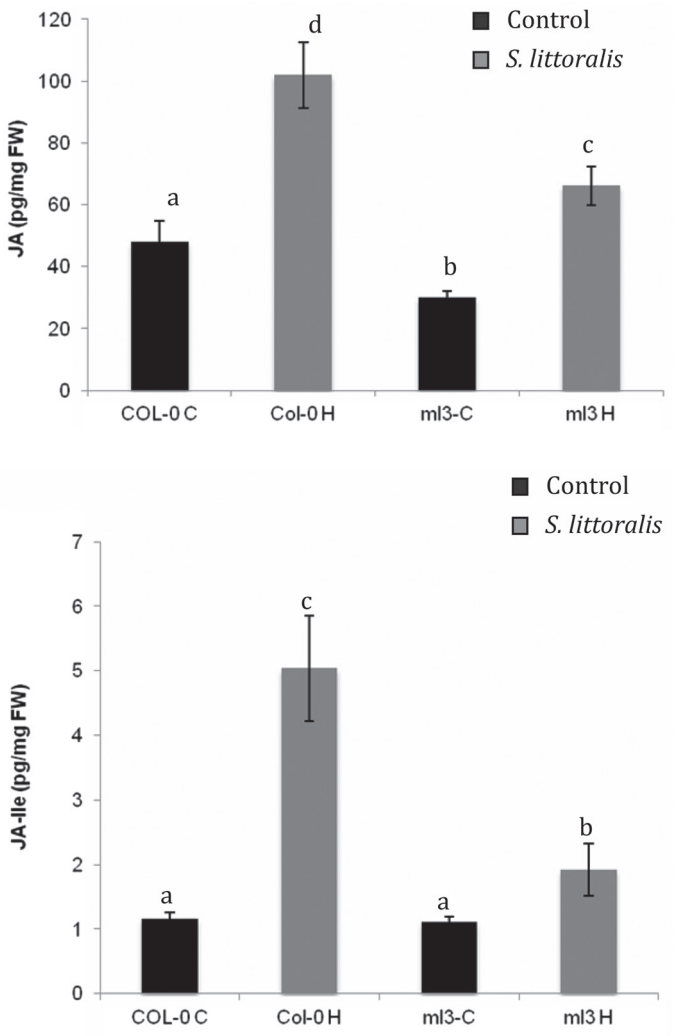

Fig. 8. JA and JA-Ile levels in non-infested Col-0 (control, C), herbivory-treated Col-0 (*S. littoralis* fed 6 h, H), non-infested *ml3* (mutant control, ml-C), and herbivory-treated *ml3* (*S. littoralis* fed 6 h, ml3-H). Error bars represent SE of three biological replicates. ANOVA with Fisher pairwise comparison ($P < 0.001$). Different letters indicate significantly different means.

to generalist larvae (*S. littoralis*). However, the ML genes showed differences in the amplitude of signals among the different treatments, with higher intensity observed after MeJA treatment. Previous studies have also shown that herbivory-mediated responses elicit similar changes in JA-dependent gene expression (De Vos *et al.*, 2005; Mithofer *et al.*, 2005). JA is the major hormone involved in the induced production of plant volatile organic compounds that attract natural enemies of the herbivores (Dicke and Van Poecke, 2002). In addition, JA-mediated defence responses produce secondary metabolites rendering the plants not appetizing or toxic to the herbivores feeding on them. In turn, studies have also shown that insect oral secretions may suppress the expression of host plant defences (Schittko *et al.*, 2001; Musser *et al.*, 2005; Consales *et al.*, 2012). Hence, it could be that specialist insects are able to suppress the HAMP-mediated systemic defence responses based on ML gene activation in plants in addition to disarming plant chemical defence (e.g. Ratzka *et al.*, 2002).

Herbivory-induced expression of ML genes is JA responsive

MeJA is a known defence elicitor that is often used to mimic the effects of herbivory-induced wounding (Baldwin and Hamilton, 2000; Pauwels *et al.*, 2009). This study used MeJA to simulate herbivore attack to observe the effect on *ML* expression. A time-course study for the expression of *ML3* after MeJA treatment in the wild-type Col-0 plants (Fig. 3B) showed an early induction already after 30 min after MeJA treatment and that reached a peak at 6 h. The results are in agreement with the *in vitro* stimuli data obtained from Genevestigator V3 (http://www.genevestigator.com, Hruz *et al.*, 2008). Previous studies have shown that *Arabidopsis* genes encoding JA biosynthetic enzymes are upregulated in response to wounding and JA treatment in a positive feedback manner (Reymond *et al.*, 2000, Devoto and Turner, 2005; Koo *et al.*, 2006) However, it should be kept in mind that although MeJA provided a qualitative response, mimicking larvae feeding, the quantitative responses were not the same demonstrating opportunities to further study the more complex events going on in a true plant–pest interaction.

This study analysed the expression of *ML3* in the JA and SA signalling compromised plants. The JA pathway-responsive mutants *jar1-1* and *coi1-1* showed reduced expression compared to wild type. In contrast, the expression of *ML3* was approximately 3-fold higher in the SA pathway defect *NahG* and *npr1* plants than in wild type. This led to the hypothesis that *ML3* may negatively regulate the SA signalling pathway while positively regulating the JA signalling pathway. As shown by VIGS, *Col-0:pTRV-ML3* plants showed a higher basal level of *PR1* expression and the levels did not change upon herbivory by *S. littoralis*. Col-0 plants infiltrated with the empty vector did not show any significant change in *PR1* expression. The co-silenced plant *coi1-1:pTRV-ML3* showed a 1.5-fold higher accumulation of *PR1* transcripts compared to the *coi1-1* mutant, whereas no significant differences were observed upon herbivory by *S. littoralis*. Similarly, JA mutants silenced for *ML3* did not display any significant change in *VSP2* expression compared to non-silenced plants. Previous studies have shown that JA and SA pathways are antagonistic to each other (Penninckx *et al.*, 1996; Gupta *et al.*, 2000). Upon herbivory by *S. littoralis*, the co-silenced *NahG:pTRV-ML3* plants did not accumulate *VSP2* transcripts. The increased levels of the *VSP2,* serving as a marker for the JA pathway, in the non-accumulating SA line *NahG:pTRV-ML3* led this study to hypothesize that the expression of *ML3* also antagonize SA effects. Upon herbivory, there was no significant change in the co-silenced *NahG:pTRV-ML3* compared to the control. Whereas the *NahG* transgenic line showed a 3-fold increase upon herbivory compared to the non-infested plants.

MPK4 is an important regulator of wound responses. In tobacco, *Nicotiana tabacum* and *N. attenuata*, *MPK4* is involved in JA signalling and the response to herbivore attack

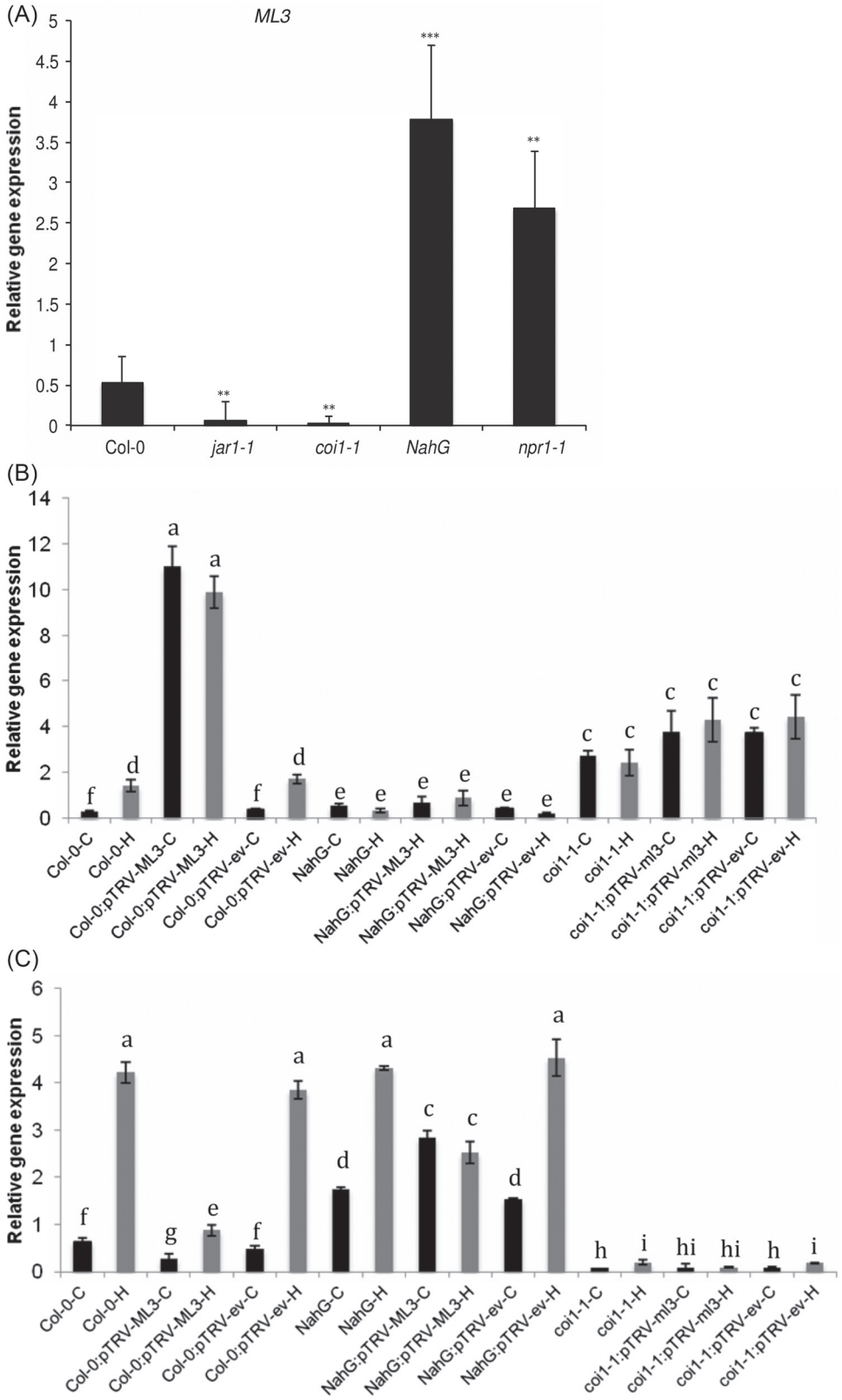

Fig. 9. (A) Expression of *ML3* in *A. thaliana* signalling mutants. Leaves were harvested from 3-week-old wild-type Col-0, jasmonic acid mutants (coi1-1, jar1-1), salicylic acid transgenic line (NahG), and salicylic acid mutant (npr1-1). Error bars represent SE of three biological replicates. Asterisks indicate statistically significant differences to the respective control (Student's t-test; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). (B) Expression of *PR1* in *A. thaliana* signalling mutants infiltrated with *ML3* VIGS constructs and 24 h after herbivory by first instar larvae of *S. littoralis*: ev, empty pTRV vector. Error bars represent SE of three biological replicates. ANOVA with Fisher pairwise comparison ($P < 0.001$). Different letters indicate significantly different means. (C) Expression of *VSP2* in *A. thaliana* signalling mutants infiltrated with *ML3* VIGS constructs and 24 h after herbivory by first instar larvae of *S. littoralis*. Error bars represent SE of three biological replicates. One-way ANOVA with Fisher pairwise comparison ($P < 0.001$). The different letters above the bars indicate if the mean values differ significantly among the treatments or not.

(Wu *et al.*, 2007). *Arabidopsis MPK4* mutants have been reported to be compromised in JA signalling (Petersen *et al.*, 2000; Brader *et al.*, 2007). In the present study, At*MPK4* expression was compromised in the *ml3* mutants compared to wild-type plants indicating that *ML3* acts upstream of mitogen-activated protein kinases. The results also indicate lack of redundancy of the ML gene family in this respect, pointing to ML3 as a major player for the herbivore response.

It was observed that co-silenced *coi1-1*:*pTRV-ML3* was more appetizing to the *Spodoptera* larvae compared to the *coi1-1* mutants (Supplementary Fig. S5). This supports that silencing of *ML3* attenuates JA responsiveness upon herbivory. VSPs are expressed in a *COI1*-dependent manner and are known to be elicited during herbivory (Benedetti *et al.*, 1995; Verhage *et al.*, 2011). In agreement with this, it has previously been demonstrated that the *coi1-1* mutant was highly favoured by the *Spodoptera exigua* larvae. This effect was correlated with a reduced production of glucosinolates, which has a role in defence against generalist herbivores (Mewis *et al.*, 2006; Verhage *et al.*, 2011). Similar results on herbivory by *S. littoralis* have been shown in other *Arabidopsis* signalling mutants like *pad2-1*, which accumulates lower levels of glucosinolates (Schlaeppi *et al.*, 2008).

In addition, analysis of the promoter region of *ML3* revealed the presence of a G-box or its variant T/G box, which are the binding targets of MYC2 (Supplementary Fig. S6). MYC2 in association with *JAZ* genes is known to play a central role in JA-regulated signalling (Chini *et al.*, 2007; Pozo *et al.*, 2008). Plants overexpressing a *35S:MYC2* construct showed an elevated expression of *ML3* and *ML7*, along with other JA-responsive genes, and downregulated expression in the jasmonate-insensitive (*jai3-1*) plants after JA treatment (Chini *et al.*, 2007).

In silico studies showed that *ML3* is co-expressed with the ATR1/MYB34 transcription factor (Supplementary Fig. S7). MYB34 is known to play a role in indole glucosinolate homeostasis (Celenza *et al.*, 2005). In addition, the present study observed that *ML3* is co-expressed with other jacalin lectin proteins and with PYK10, a myrosinase like protein associated with plant defence. Hence, it is also proposed that *ML3* might be involved in the activation of the glucosinolate/myrosinase system in plant defence against insects. Further studies are being carried out to find out if *ML3* plays a direct role in glucosinolate–myrosinase interaction.

ml3 mutants are compromised in the induction of wound-induced signals

Mechanical wounding has been previously observed to mimic herbivory-induced signalling in various plant species (Bell and Mullet 1993; Reymond *et al.*, 2000). *LOX2* is the isoform of the lipoxygenase family that is commonly used as a marker gene for JA biosynthesis (Bell *et al.*, 1995) being induced upon herbivory, mechanical wounding, and MeJA treatment (Chung *et al.*, 2008).

This study found the *ml3* mutants (*ml3-1* and *ml3-2*) to be semi-dwarf in phenotype and less responsive in *LOX2* expression upon herbivory. Further, this study explored if *ML3* was required to upregulate the expression of *LOX2* upon herbivory.

ML3 plays a role in plant defence against the generalist herbivore S. littoralis

The results showed that *ml3-1* and *ml3-2* plants were more favoured by generalist herbivore larvae (*S. littoralis*) than specialist larvae *(P. xylostella)*. This provides evidence that *ML3* plays a vital role in regulating plant processes that confer resistance to generalist insect herbivores. The increased susceptibility of the *ml3* mutants found might be explained by the fact that the mutants exhibit reduced responsiveness to the JA signalling pathway. Similar results have been reported where JAZ mutants, that are JA non-responsive, were found to be more favoured by *S. exigua* (Thines *et al.*, 2007; Chung *et al.*, 2008). It has also been published that induced defence against the generalist *S. littoralis* in *Arabidopsis* is due to the activation of the JA-mediated pathway (Stotz *et al.*, 2002; Reymond *et al.*, 2004; Consales *et al.*, 2012). Salivary components of specialist and generalist herbivores can have powerful effects on gene expression, by triggering or repressing genes in plants (Schittko *et al.*, 2001; Alborn *et al.*, 2003), leading to differential host responses to particular herbivore species (De Moraes *et al.*, 1998; Dicke, 1999). Specialist herbivores have been shown to be more tolerant to defence responses of their hosts compared to generalists having co-evolved mechanism to disarm the secondary metabolite-based defence or other strategies (Wittstock *et al.*, 2004; Müller *et al.*, 2010; Wittstock and Burrow, 2010).

Based on these results, this study proposes that ML3 is a positive regulator of HAMP signalling in *Arabidopsis*. Although the above results give ML3 a primary role as the regulator determining the specificity of herbivore perception, it is likely that additional protein components are required for the formation of a functional receptor complex and may influence downstream signalling. Since ML3 is linked to the MD2 lipid recognition domain, it is possible that ML3 might interact with lipopolysaccharide- or peptidoglycan-like signalling molecules during HAMP responses. Work is currently ongoing to identify potential herbivore effectors and HAMP recognition factors.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Heat map representation of the expression analysis of ML genes in different organs of *A. thaliana* based on microarray data available at Genevestigator.

Supplementary Fig. S2. Heat map representation of the expression analysis of the ML gene family at different growth stages of *A. thaliana* based on microarray data available at Genevestigator.

Supplementary Fig. S3. (A) Heat map representation of the expression analysis of ML genes in *A. thaliana* in response to hormonal treatments based on microarray data available at Genevestigator. (B) Expression analysis of ML gene family on 3-week-old wild-type (Col-0) plants treated with 30 µM MeJA and 5 mM SA.

Supplementary Fig. S4. Efficiency of ML3 VIGS in Col-0 and *coi1-1* plants.

Supplementary Fig. S5. Larval weights of *S. littoralis* 15 days after initiation of feeding.

Supplementary Fig. S6. The *ML3* promoter region.

Supplementary Fig. S7. Co-expression of *ML3* with other genes in the network.

Acknowledgements

The authors give sincere thanks to Prof. Barbara Ekbom, Prof. Christina Dixelius, and Prof. Daniel Hofius for helpful discussions. They thank Ms. Elisabeth Marling, Department of Plant Biology, SLU, Alnarp for providing the *S. littoralis* eggs and the artificial diet. They also thank Ms. Carol Hogfeldt for providing the *P. xylostella* larvae. Sincere thanks go also to Urban Petterson and Gunilla Swärdh for help with the maintenance of the plants in the growth chambers. The authors also thank two anonymous reviewers for their insightful comments. This work was supported by the Carl Tryggers Foundation, the Nilsson Ehle Foundation, the Helge:Axsson Johnsson Foundation, the Grant Agency of the Academy of Sciences of the Czech Republic (KAN200380801) and EU funding from the Operational Program Research and Development for Innovations (ED0007/01/01).

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