

Characterization of a Methyl Jasmonate Specific Esterase in Arabidopsis

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Abstract Methyl jasmonate (MeJA)-specific methyl esterase of Arabidopsis (AtMJE) was identified and characterized. AtMJE has high substrate specificity to MeJA compared to other related substrates, methyl indole-3-acetate (MeIAA) and methyl salicylate (MeSA). Through enzyme kinetics analysis, we found AtMJE has similar level of substrate affinity to JA carboxyl methyltransferase (AtJMT). However, AtMJE has 10 times lower catalytic efficiency than AtJMT at low substrate concentrations. *AtMJE* gene expression was suppressed for 2 h after MeJA treatment, even though its expression recovered and was induced to maximum level within 8 h after treatment. *AtMJE* overexpressing plants (*AtMJEox*) showed enhanced MeJA methyl esterase activity demonstrating esterase activity of AtMJE *in vivo*. *AtMJEox* plants responded differentially to JA and MeJA in root growth. MeJA in the media could be a source for more JA production in *AtMJEox* plants, which resulted in root growth inhibition. In contrast, *AtMJEox* plants grown on JA containing media showed similar root growth inhibition as wild-type. These results show that AtMJE functions in altering JA/MeJA ratios in Arabidopsis and increased JA, because the conversion of MeJA to JA enhances JA responsive gene expression.

Keywords jasmonic acid · jasmonate carboxyl methyltransferase · methyl jasmonate · methyl jasmonate methyl esterase

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Introduction

Jasmonates (JAs) are a group of lipid-derived compounds found in all plant species. JAs consist of the central compound jasmonic acid (JA) and its derivatives, including carboxyl methylated JA (MeJA) and JA conjugated to amino acids (Delker et al., 2006; Wasternack, 2007; Browse, 2009a). JAs are important hormonal regulators involved in diverse developmental processes such as seed germination, root growth, flowering, fruit ripening, and senescence (Creelman and Mullet, 1997; Creelman and Rao, 2002; Browse, 2009b; Linkies and Leubner-Metzger, 2012). In addition, JAs activate plant defense mechanisms in response to insect-driven wounding, pathogen infection, drought, low temperature and high salt conditions (Wasternack and Parthier, 1997; Reymond and Farmer, 1998; Wasternack and Hause, 2002; Browse, 2009a; Koo and Howe, 2009).

MeJA is a major volatile derivative of JA and is used as an elicitor of plant defense responses (McConn et al., 1997; Baldwin, 1998). MeJA is synthesized from JA by JA carboxyl methyltransferase (AtJMT) (Seo et al., 2001), and JA is regenerated from MeJA by methyl esterase (MJE), a hydrolyzing enzyme (Stuhlfelder et al., 2004; Wu et al., 2008). MeJA is proposed to have a role in intra- and inter-plant signalings (Seo et al., 2001; Baldwin et al., 2006; Kessler et al., 2006; Tamogami et al., 2012). For instance, the increase of MeJA level without affecting JA level induced JA responsive genes including *VSP* and *PDF1.2* (Seo et al., 2001). Furthermore, overexpressing *AtJMT* changes flower morphology in tobacco and rice (Kim et al., 2009; Stitz et al., 2011). However MeJA must be de-methylated in order to elicit plant defense responses, because MeJA itself is an inactive molecule for inducing defense responses (Wu et al., 2008). Taken together, these results indicate that MeJA has an active role in regulating JA signaling.

Understanding the role of MeJA esterase is essential in understanding the role of MeJA in plants. Two MJEs have been cloned and characterized in tomato (LeMJE) (Stuhlfelder et al.,

2004) and in tobacco (NaMJE) (Wu et al., 2008). *LeMJE* expression is suppressed by MeJA treatment, which suggests that *LeMJE* can affect the level of JA-isoleucine conjugate (JA-Ile), a signal molecule used in JA response which affects JA responsive gene expression. Silencing of *NaMJE* reduced the expression of phenylalanine ammonia lyase (*NaPAL1*) and threonine deaminase (*NaTD*) as well as reduced resistance to the *Manduca sexta* larvae. These results demonstrate that the regeneration of JA from MeJA is an important step for JA signal transduction. In addition, MeJA overproduction has been used to suppress JA signaling in plants, because MeJA appears to be an inert molecule that does not elicit JA-induced defense responses; therefore, the production of MeJA becomes a metabolic sink (Stitz et al., 2011). These results indicate that the management of JA/MeJA conversions is a useful tool to study the role of JAs through manipulating the level of active JAs for specific plant physiologies *in vivo*.

Methyl salicylate (MeSA) and methyl indole-3-acetate (MeIAA) were also discovered to be volatile methyl esters that are converted from the major plant hormones SA and indole-3-acetic acid (IAA), respectively. MeSA has been found to be a critical mobile signal molecule that is required for systemic acquired resistance to pathogen attack (Kumar and Klessig, 2003; Forouhar et al., 2005; Park et al., 2007). IAA is also methylated on its carboxyl group by IAA carboxyl methyltransferase (IAMT) (Zubieta et al., 2003; Qin et al., 2005), whereas MeIAA is hydrolyzed by MeIAA esterase (*AtMES17*) (Yang et al., 2008). Even though MeSA is believed to be a mobile signal, both MeSA and MeIAA themselves are functionally inactive and need to be hydrolyzed in order to form active molecules (Koo et al., 2007; Yang et al., 2008). The role of MeSA as a mobile signaling molecule was demonstrated by altering expression of MeSA esterase (SABP2) and SA methyltransferase (Park et al., 2007).

The inter-conversion between JA and MeJA could be an important step to control JA signaling in plants. Therefore, characterization of the enzyme responsible for the reverse reaction of JMT can provide information concerning JA metabolism and its signaling. Manipulation of JA/MeJA balance would provide more chance to understand the role of MeJA. In this report, we characterized a MeJA-specific esterase in *Arabidopsis* and showed this gene can be used to manipulate JA/MeJA ratios in plants.

Materials and Methods

Plant growth. *Arabidopsis thaliana* Columbia ecotype (Col-0) was used as wild-type and background for the transgenic plants. Seeds were stored at 4°C for 2 days for synchronized germination and grown in a chamber maintained at 23°C with 16 h/8 h light/dark cycles. Half strength of Murashige and Skoog salt was used for culture on medium with 1% (w/v) of phyto-agar. Four weeks old plants grown in soil were used for wounding and the Northern blot analysis. For wounding stress experiments the 5th and 6th leaves were injured with a hemostat up to five times.

Protein expression and purification. For expression of the *AtMES* proteins in *Escherichia coli*, the *AtMES* cDNA were amplified from full length cDNA clones obtained from Arabidopsis Biological Resource Center (ABRC; www.arabidopsis.org) by polymerase chain reaction (PCR) with specific primer sets (Supplemental Table 1). PCR products were digested with *Bam*HI and ligated into the expression vector pGEX-5X-1 (Amersham Pharmacia Biotech Inc., USA), and then introduced into *E. coli* BL21 (DE3) pLysS. Protein purification was conducted as described previously (Seo et al., 2001). To generate *AtMJE* overexpressor, the full length *AtMJE* cDNA was re-amplified with primer set 5'-GGGACAAGTTTGTACAAAAAAGCAGGCT-3' and 5'-GGG GACCACTTTGTACAAGAAAGCTGGGT-3', recombined into the pDONR201 (Invitrogen), and subcloned into pGWB17 vector. **MeJA esterase activity assay.** Thin-layer chromatography (TLC) screening and silylation-based gas chromatography-mass spectroscopy (GC-MS) quantification methods were conducted as described by Koo et al. (2008). Various methyl esters at the concentration of 2 mM were reacted with purified enzymes in 50 mL of Tris-HCl (pH 7.5) buffer containing 5 mM of potassium chloride for 30 min. Ten microliters of 1 M HCl and diethyl ether were added for immediate extraction. The diethyl ether phase was concentrated for either TLC loading or silylation reaction. All methyl esters and acids were developed on TLC plates (Silica gel 60 F254, Merck, Germany) using a hexane : ethyl acetate : acetic acid (70:30:1) solvent mixture. JA/MeJA and IAA/MeIAA were visualized by burning with 95% ethanol containing 2.5% (v/v) anisaldehyde, 1% (v/v) acetic acid and 3.5% (v/v) sulfuric acid. SA/MeSA and cinnamic acid/methyl cinnamate were visualized under UV light (254 nm). For GC-MS analysis, reaction products were silylated with the reagent BSTFA + TMCS (SUPELCO, USA) at 80°C for 30 min. GC-MS equipment and detailed monitoring parameters were described previously (Koo et al., 2008). For plant extract reactions, about 100 µg of two weeks old *Arabidopsis* roots were harvested, and root extracts were incubated with 2 mM of MeJA in 50 mM of Tris-HCl (pH 7.5) buffer with 5 mM of KCl at 37°C for 30 min. Reaction products were silylated and analyzed by GC-MS with chemical ionization (CI)/selected ion monitoring (SIM) mode. Methyl *o*-anisic acid (MeOAA) was used for internal standard and the reaction product, silylated OAA (OAA-TMS) was detected at 11.40 min.

Results

Selection and cloning of *AtMJE* candidates. To identify the *MJE* in *Arabidopsis*, candidate genes were selected through a homology search of protein sequences using the tobacco salicylic acid-binding protein 2 (SABP2) and the tomato MJE. Previously, Yang et al. (2008) analyzed substrate specificity of 20 methyl esterase candidates (MESs) to identify MeIAA esterase. According to the phylogenetic analysis with protein sequences, ten *Arabidopsis* candidates and the tomato MJE were assigned to subfamily 1. A

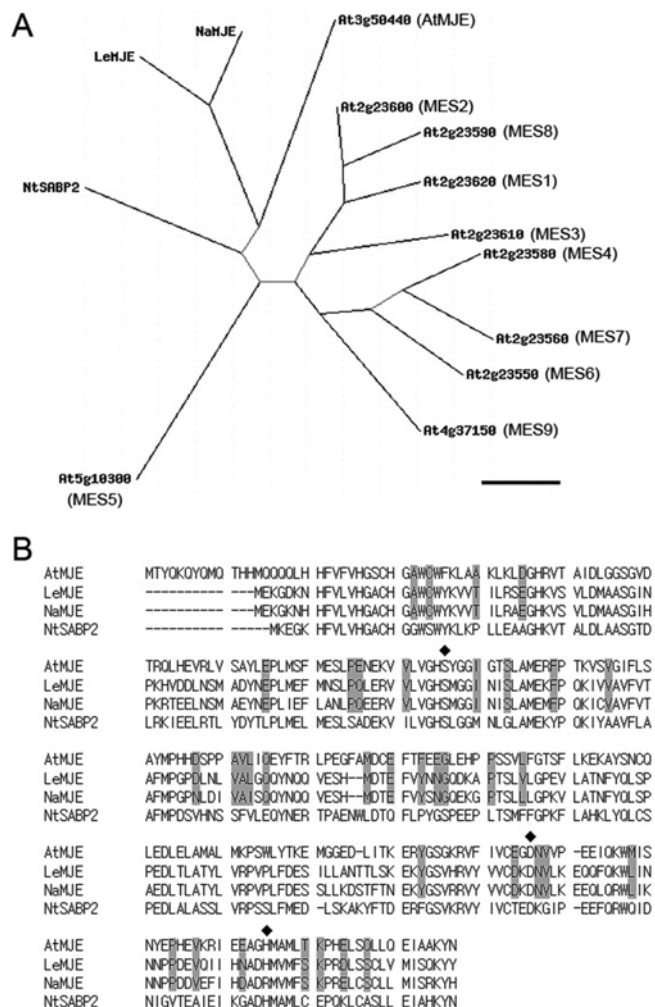


Fig. 1 Amino acid sequence alignment. A. Ten methyl esterase candidates that were previously aligned to identify MeIAA esterase were grouped again to select out *AtMJE*. Bar indicates 0.1 distances between each sequences. Distance 1 is set as no sequence similarity. Dashed line indicates below 10% differences. B. AtMJE sequence aligned with LeMJE, NaMJE, and tobacco methyl salicylate esterase, SABP2. The shaded residues are the only conserved amino acids in MeJA esterases.

more detailed analysis was carried out in the present study by using the Clustal W program (<http://www.genebee.msu.su/>), wherein the coding protein of gene At3g50440 grouped closer to LeMJE and NaMJE than the 9 other esterase candidates (Fig. 1A). The amino acid sequence alignment is shown in Figure 1B, where putative MJE- (or MES10, At3g50440) specific residues are shaded. To test esterase activity of the candidate, cDNA clones for the eight selected candidates were obtained from the ABRC, subcloned into the *E. coli* expression vector pGEX-2T (GE Healthcare, UK), and purified (Supplemental Table 1).

Screening AtMJE by TLC method. To test substrate specificity of the eight cloned candidates, MeJA, MeIAA, and MeSA were tested as substrates for carboxyl methyl esterase activity.

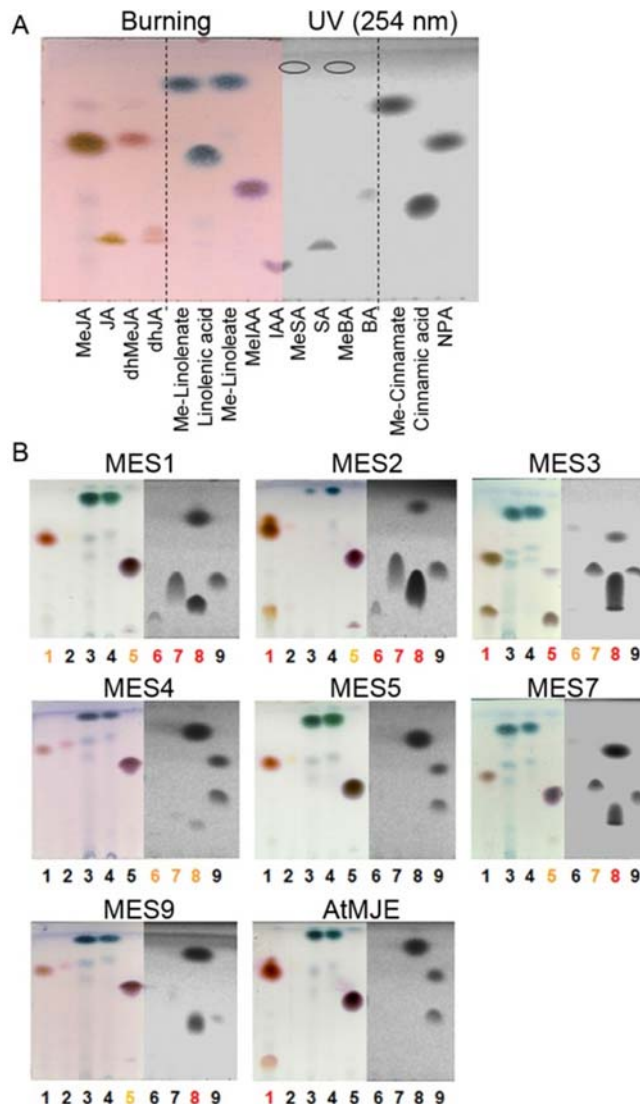


Fig. 2 Methyl carboxyl esterase activities of eight different *Arabidopsis genes in vitro*. A. The various reference chemicals were loaded on TLC and visualized by burning with burning solution (left, stained) or UV light (254 nm) irradiation. B. 1 μg of each purified proteins were incubated with methyl esters for 30 min. The products were separated by ether extraction, and the ether phases were loaded on TLC plates. The methyl esters used for substrates are as follows: lane 1, Jasmonate methylester; 2, Dihydro jasmonate methylester; 3, Linolenate methylester; 4, Linoleate methylester; 5, Indole acetate methylester; 6, Salicylate methylester; 7, Benzoate methylester; 8, Cinnamate methylester; 9, Nitrophenylenediamine (NPA). Red and orange numbers represent relatively strong and weak product signal observations, respectively.

Additionally, other JA and SA derivatives were also used to test substrate specificity of the candidate carboxyl methyl esterases. Specifically, linoleate methylester and linolenate methylester were tested as JA analogs, and benzoate methylester and cinnamate methylester were tested as SA analogs. According to the TLC analysis, MES10 showed strong MeJA esterase activity, and JA

Table 1 Relative esterase activities to three different substrates

	AGI	MeJA	MeIAA	MeSA
MES1	At2g23620	2.2	<1	5.3
MES2	At2g23600	2.9	1.2	2.7
MES3	At2g23610	8.2	35	1.3
MES9	At4g37150	1.0	3.9	<1
MES10	At3g50440	100	<1	<1

spot was detected on TLC, but SA and IAA spots were not detected on the reaction with MeSA and MeIAA, respectively (Fig. 2), whereas the other candidates showed relatively broad range of substrate specificities. MES1 (At2g23620), MES2 (At2g23600), and MES3 (At2g23610) have esterase activity to all three different types of substrates, MeJA, MeSA, and MeIAA (Fig. 2). The remaining four candidates MES4, MES5, MES7, and MES9 also had a broad range of substrate specificity, but relatively much weaker product spots were observed compared to MES1, MES2, MES3, and MES10 at the same reaction condition. **Substrate specificity of AtMJE.** GC-MS based quantification was attempted to specify reaction products as well as quantitative measurement of enzyme activity. Five candidates, MES1, MES2, MES3, MES9, and MES10, which were selected from the TLC analysis were reacted with MeJA, MeSA, and MeIAA, and the product acids were silylated with TMS for GC-MS analysis. The levels of reaction products were calculated relative to that of JA produced from reaction with MES10 from MeJA, which was set as the 100% reaction (Table 1). All five candidates have esterase activity towards MeJA, but they were less than 10% of the AtMJE activity. Furthermore, MES1, MES2, MES3, and MES9 have relatively higher hydrolysis activities towards both MeSA and MeIAA compared to MeJA. Therefore none of the candidates except putative MES10 have substrate specificity to MeJA. The production of JA from MeJA by AtMJE was considerably higher compared to its production of SA and IAA. Less than 1% of SA and IAA were detected at the reaction with MeSA and MeIAA, respectively. Overall, these results tell us that the substrate specificity and productivity of the MES10 on MeJA was considerably higher than other tested enzymes, indicating that MES10 is the AtMJE, an enzyme responsible for the specific hydrolysis of MeJA in Arabidopsis.

Catalytic Efficiency of AtMJE is lower than AtJMT. The kinetic parameters of AtMJE were analyzed and compared with

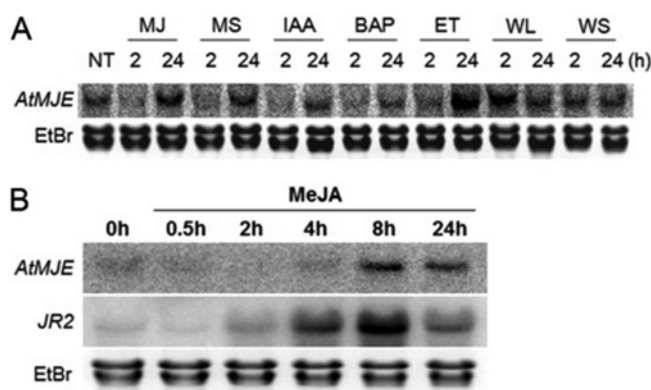


Fig. 3 Gene expression patterns of *AtMJE* in response to various stimulations. A. The responses of *AtMJE* to various plant hormones. MJ, methyl jasmonate 100 μ M; MS, methyl salicylate 100 μ M; IAA, Indole-3-acetic acid 100 μ M; BAP, benzyl aminopurine 100 μ M; ET, ethephon 100 μ M; WL, wounding local; WS, wounding systemic. B. Temporal expression pattern of the *AtMJE* gene in response to MeJA treatment. The treated leaves were collected at the indicated time after treatment. Ethidium Bromide (EtBr) stained rRNA is shown to indicate equal loading of total RNA.

other known methyl esterases and carboxyl methyltransferases (Table 2). The K_m and the k_{cat}/K_m values of AtMJE were found to be two times higher than those of the tomato MeJA esterase, LeMJE, which had four times higher K_m but similar k_{cat}/K_m values compared to tobacco MeSA esterase, SABP2. Therefore AtMJE is expected to be in the similar range of enzyme activity with LeMJE and SABP2. On the other hand, the K_m value was similar with AtJMT, but the k_{cat}/K_m value was ten times lower than that of AtJMT. These values indicate that the equilibrium would shift toward MeJA rather than JA in Arabidopsis when both protein levels are the same.

AtMJE basal expression and phenotype of AtMJE overexpressor. *AtMJE* was highly induced by MeJA/JA and mechanical wounding stimulus (Fig. 3A). This gene expression pattern was similar with other JA-responsive genes (Seo et al., 2001; Song et al., 2009). However, the inducing time point was relatively later than JA-responsive gene *JR2*, which was induced 2 h after MeJA treatment (Fig. 3B). These patterns indicate that *AtMJE* transcription may be regulated by general MeJA/JA signaling pathway that control expression of JA inducible genes. However, *AtMJE* shows suppressed expression in response to MeJA treatment at early time

Table 2 Kinetic parameter comparison between methyltransferases and methyl esterases of JA and SA in various plants

		Methyltransferase			Methyl esterase			References
		Name	K_m (μ M)	k_{cat}/K_m ($s^{-1} \mu M^{-1}$)	Name	K_m (μ M)	k_{cat}/K_m ($s^{-1} \mu M^{-1}$)	
JA/MeJA	Arabidopsis	AtJMT	38.5	0.4	AtMJE	34.5	0.03	Seo et al. (2001)
	Tomato				LeMJE	15	0.016	Stuhlfelder et al. (2004)
SA/MeSA	Arabidopsis	AtBSMT1	16	0.004				Chen et al. (2003)
	Tobacco				SABP2	8.6	0.05	Wu et al (2008)
	Rice	OsBSMT1	78	0.0003				Koo et al. (2007)

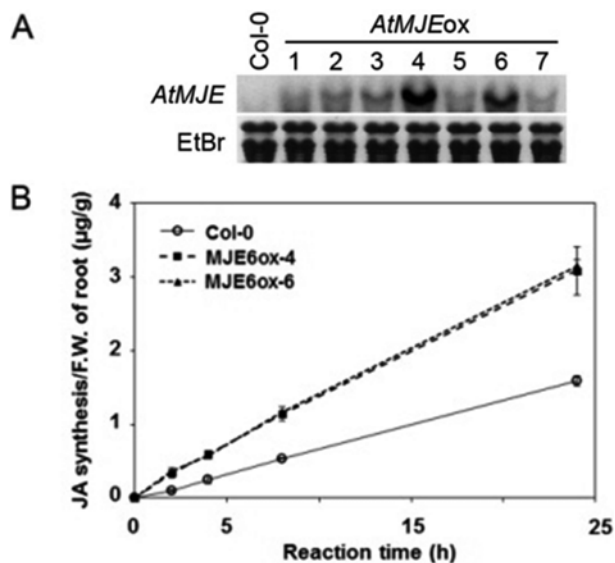


Fig. 4 Reactivity of the *AtMJE* overexpressor to MeJA. A. Northern blot showing the expression levels of the *AtMJE* gene in seven independent *AtMJEox* overexpressor lines. B. The root extract of overexpressor lines *AtMJEox-4* and *AtMJEox-6* were incubated with 2 mM of MeJA. The amount of JA produced was analyzed from 2 to 24 h after incubation.

points, from 30 min to 4 h (Fig. 3B). A similar early suppression pattern was also shown in *AtMJE* expression in response to MeSA, IAA, and ethylene treatments (Fig. 3A). However *AtMJE* gene was induced just 2 h after mechanical wounding, which indicates that wounding signal transmittance is transferred through a different pathway than the MeJA responsive pathway.

***AtMJE* protein expressed in Arabidopsis is functional.** To test its MJE activity in Arabidopsis, the *AtMJE* overexpressor was prepared by subcloning the *AtMJE* cDNA gene downstream of a 35S promoter in the binary vector pGWB and transforming the construct into Arabidopsis. Two independent overexpressors, *AtMJEox-4* and *AtMJEox-6*, were selected, and a single site gene insertion was confirmed by genomic Southern blot analysis (Fig. 4A, Supplemental Fig. 1). Root extracts from the two independent overexpressor lines, *AtMJEox-4* and *AtMJEox-6* produced twice the amount of JA when compared to Col-0 plants during all time points measured (Fig. 4B). These results show that cloned *AtMJE* protein expressed in Arabidopsis is functional as was expressed in *E. coli*.

The *AtMJE* overexpressor shows a hypersensitive response to MeJA. Root growth assays were used to observe the phenotypic changes resulting from *AtMJE* overexpression. Root growth inhibition is one of phenotypes that is associated with exposure of plants to JAs (Staswick et al., 1992). *AtMJEox-4* and Col-0 plants were grown on either MeJA or JA containing MS media. When grown on MeJA-containing medium, the *AtMJEox-4* root length was approximately 40% shorter than that of the Col-0 plant after 2 weeks of growth. However, on JA-containing medium, *AtMJEox-4* plants showed similar root growth inhibition observed in Col-0

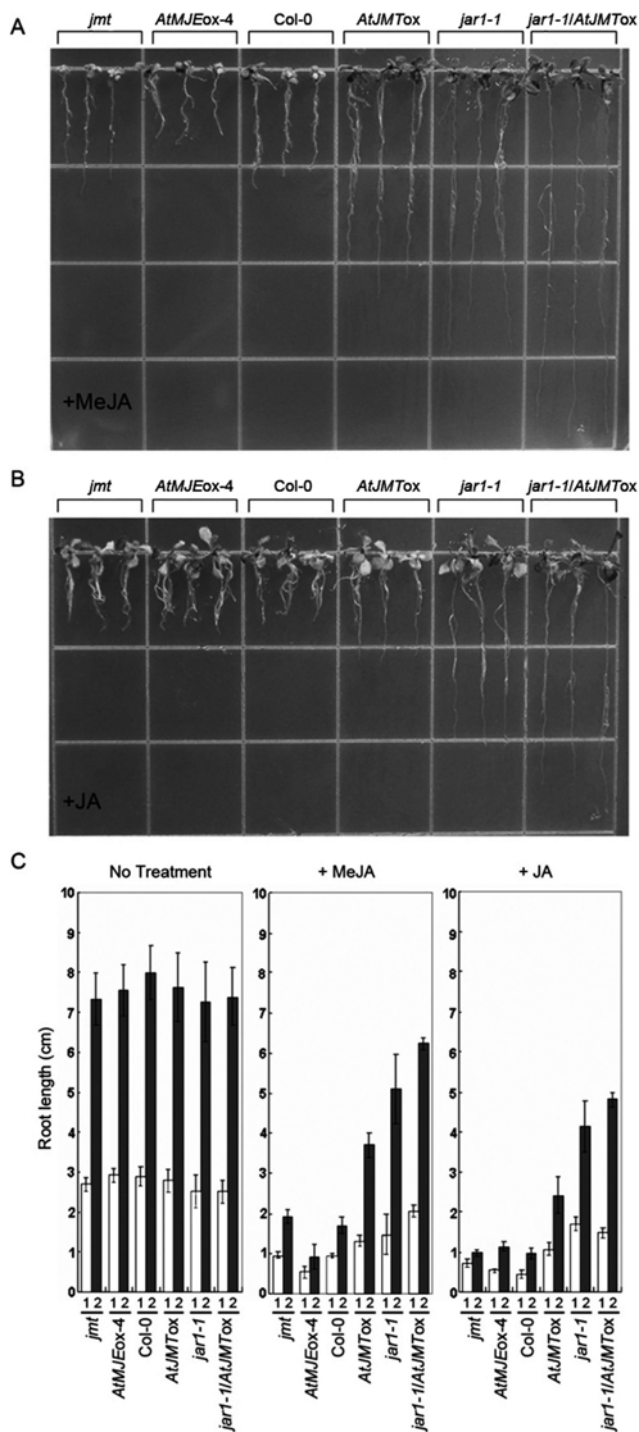


Fig. 5 Inhibitory root growth of *AtMJEox-4*. Various JA mutants were grown on 50 µM of MeJA-(A) or JA-(B) containing media. The pictures were taken 2 week after germination. C. Root growth was measured at 1 (open) and 2 weeks (shade) after germination. Error bars are standard deviations of six replicates.

plants. In other words, *AtMJEox-4* showed hypersensitivity compared to wild-type when grown on MeJA, but similar phenotype to Col-0

0 when grown on JA medium (Fig. 5). Root length of *AtJMT*-overexpressing plants (*AtJMTox*), however, was longer than those observed in Col-0 roots on both MeJA and JA containing medium, which indicates that *AtJMTox* is less sensitive to JA. These results indicate that AtMJE functions as a MeJA esterase and has a role in managing the MeJA and JA ratio in Arabidopsis. Root growth inhibition could be an effect of JA rather than MeJA. The root growth assay was also carried out with both the *jar1-1* and the *jar1-1/AtJMTox* mutants, which are unable to synthesize JA-Ile. Both mutants showed an insensitive phenotype upon exposure to both MeJA and JA, which supports that JA-Ile is the active molecule required for inhibiting root growth (Staswick and Tiryaki, 2004).

Discussion

A highly specific MeJA methyl esterase, AtMJE, was identified in Arabidopsis. Based on sequence similarity of the candidates to the known methyl esterases from other species, AtMJE is predicted to be a MeJA-specific methyl esterase in Arabidopsis. The catalytic activity of AtMJE is over ten times higher than that of MES3, which is the next highest JA productive enzyme. Furthermore, the most appropriate substrate for MES3 was MeIAA, which has at least four times higher affinity to this enzyme (Table 1).

AtMJE showed similar substrate affinity to that of AtJMT; however, catalytic efficiency at low concentration of substrate was ten times lower than that of AtJMT, suggesting that AtMJE protein might be several times more abundant compared to AtJMT, because JA and MeJA are present in similar amount in Arabidopsis (Seo et al., 2001).

The transcript level of *AtMJE* was down regulated during MeJA treatment; however, the expression levels slowly recovered and peak expression occurred 8 h after initial treatment. This result was similar to expression patterns of tomato MJE when treated with MeJA (Stuhlfelder et al., 2004). In comparison, the *AtJMT* gene is strongly induced between 2 and 8 h after MeJA treatment like other JA responsive genes, *JR2*, *LOXII*, and *JAR1* (Seo et al., 2001), indicating *AtJMT* gene expression was opposite to the *AtMJE* expression around 2 h after MeJA treatment. As result, the direction in this reversible metabolic pathway between JA and MeJA is supposed to move forward to MeJA at the early time period in Arabidopsis leaves. In other words, Repression of *AtMJE* gene expression in response to MeJA enhances the increase of MeJA production by AtJMT. It is not yet known how *AtMJE* is repressed and whether *AtMJE* has evolutionarily conserved gene expression patterns with *LeMJE* in response to MeJA treatment. It is still possible that an unknown transcriptional regulation mechanism could exist and regulate the expression timing of *AtJMT* and *AtMJE*.

In *Arabidopsis*, JAs are known to inhibit root elongation (Staswick et al., 1992). This effect is mostly observed in JA- and MeJA-treated seedlings. However, according to a study of the

Arabidopsis *jar1-1* mutant, JA-Ile is an active ingredient required for root growth inhibition (Staswick and Tiryaki, 2004). *AtMJEox* showed hypersensitive root growth inhibition in response to MeJA but not in response to JA, which indicates that overexpression of *AtMJE* leads to increased production of active JA. Higher permeability of neutral charged MeJA also could contribute to increase MeJA concentration in root cells. This result shows altered expression level of AtMJE could change the inhibition of JA related root growth.

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