Biochemical characterization of the jasmonic acid methyltransferase gene from wasabi (Eutrema japonicum)

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Received May 12, 2020; accepted June 22, 2020 (Edited by M. Mizutani)

Abstract Methyl jasmonate and jasmonic acid play important roles as signaling molecules in regulating plant development and stress-related responses. Previous studies have shown that jasmonic acid carboxyl methyltransferase (JMT), which belongs to the SABATH methyltransferase gene family, catalyzes the transfer of methyl groups from S-adenosyl-L-methionine to the carboxyl groups of jasmonic acid. In the present study, we used RNA-seq analysis to identify a putative JMT gene, EujJMT, in wasabi (Eutrema japonicum). The EujJMT proteins showed the highest similarity (89% identity) to JMT proteins of Brassica rapa. Functional characterization of a recombinant EujJMT protein expressed in Escherichia coli showed the highest level of activity with jasmonic acid among the different carboxylic acids tested. The apparent $K_m$ value of EujJMT using jasmonic acid as substrate was 62.6 $\mu$M, which is comparable to the values of known JMTs. Phylogenetic analysis suggested that EujJMT shares a common ancestor with the JMTs of Arabidopsis and Brassica species and that the strict substrate specificity toward jasmonic acid is conserved among Brassicaceae JMTs.

Key words: carboxyl methyltransferase, jasmonic acid, plant evolution, substrate specificity.

Methyl jasmonate (MeJA) and its free acid jasmonic acid (JA) are compounds widely distributed throughout the plant kingdom and are involved in diverse biological processes, such as seed germination, flower and fruit development, and leaf senescence (Huang et al. 2017). In addition, MeJA is thought to be an important airborne signal that mediates plant-to-plant communication for defense responses (Cheong and Choi 2003). JA is synthesized from esterified or free linolenic acid in plants, mainly via the octadecanoid pathway. It is then further converted to its methyl ester by the action of S-adenosyl-L-methionine (SAM): jasmonic acid carboxyl methyltransferase (JMT) (Seo et al. 2001a) (Figure 1A). JMTs are members of the plant ‘SABATH’ methyltransferase family, which was named after the first three enzymes—salicylic acid methyltransferase (SAMT), benzoic acid methyltransferase (BAMT), and theobromine synthase—discovered in the family. Other SABATH methyltransferases include indole acetic acid methyltransferase (IAMT), gibberellic acid methyltransferase (GAMT), benzoic acid and salicylic acid methyltransferase (BSMT), farnesoic acid methyltransferase (FAMT), nicotinate methyltransferase (NaMT), and cinnamate/p-coumarate carboxyl methyltransferase (CCMT). Most SABATH family proteins display strict substrate specificities (Chaiprasongsuk et al. 2018).

Although a number of JMT-like genes have been extensively investigated in various plant species at the transcriptional level, to date, enzymatic activities of the proteins they encode have been reported in only a limited number of plant species: Oryza sativa, Arabidopsis thaliana, Picea abies, Populus trichocarpa, Cymbidium ensifolium, Fragaria vesca, Solanum lycopersicum, and Brassica rapa (Chaiprasongsuk et al. 2018; Huang et al. 2015; Preuß et al. 2014; Qi et al. 2016; Seo et al. 2001a, b; Tieman et al. 2010; Zhao et al. 2013). Recently, we identified the EjMT1 gene in loquat (Eriobotrya japonica), and subsequently discovered that the carboxyl methyltransferase coded by this gene has higher methylation activities against benzoic acid and its derivatives than against JA (Koeduka et al. 2016). EjMT1 and JMTs in several different species have exhibited different substrate specificity. For example, strawberry JMT (FvJMT) shows high specificity for JA and does not accept benzoic acid as a substrate, even

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This article can be found at http://www.jspcmb.jp/

Published online September 5, 2020

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though EjMT1 and FvJMT are similar to each other (65% identity) and are decidedly orthologs, which are distantly related to BSMT and BAMT proteins with benzoic acid methylation activity (Chen et al. 2003; Murfitt et al. 2000). *Arabidopsis* JMT (AtJMT) is also known to have high specificity for JA and have clustered with EjMT1 and FvJMT. On the other hand, tomato methyltransferase S3MT (specifically, protein cLEI3O14) shows high activities against jasmonic acid in addition to benzoic acid (Tieman et al. 2010). However, tomato S3MT is most closely related to AmBAMT from snapdragon and falls into a clade that does not contain JMT proteins, including EjMT1 and AtJMT. It is intriguing to investigate how plants have developed their enzymatic functions during their evolution. In order to address this question, it is important to isolate and characterize more JMTs from different plant species. In the present study, we identified a cDNA that encodes JMT in wasabi (*Eutrema japonicum*), one of the most popular Japanese spices, and functionally characterized the enzymatic properties (including substrate specificity and kinetic characteristics) of recombinant EujJMT when expressed in *Escherichia coli*.

In order to identify putative wasabi *JMT* genes, JMT protein sequence from *A. thaliana* was used as a protein query to search a previously constructed *E. japonicum* database (Mashima et al. 2019). This homology search identified one JMT-like sequence, and it was designated as EujJMT (DDBJ accession no. LC543983). The transcriptome analysis (RNA-seq), using RPKM values of EujJMT among different plant organs in the database, suggested that EujJMT was highly expressed in wasabi flower, followed by petiole, seedling, and leaf, but not expressed in the rhizome and the root (Figure 1B). The EujJMT protein was similar to the JMTs of *A. thaliana* and *B. rapa*, with 88 and 89% sequence identities, respectively. The multiple alignments of EujJMT with known JMTs are shown in Supplementary Figure S1. The deduced amino acid sequence of EujJMT contained the conserved SSSS-motif, which works as a substrate-binding site, and six active-site residues for SAM-binding.

In order to test the enzymatic activity of EujJMT in vitro, cDNA was amplified by PCR with the primers 5′-GCG CGG CAG CCA TATG GAA GTA ATG AGA ATT CTT CA-3′ and 5′-ACG GAG CTC GAA TTC TTA ACC CGT TTT AAC GAG TGA A-3′ (the underlined parts indicate a start and a stop codon, respectively) after the full-length of the EujJMT gene was synthesized by using gene blocks (gBlocks; Integrated DNA Technologies Inc., California, USA) and cloned into the pET28a vector by In-Fusion Cloning (TaKaRa Co., Shiga, Japan). The recombinant protein was expressed in *E. coli* and purified using a His affinity tag, as described in Koeduka et al. (2016). SDS-PAGE analysis showed that the recombinant EujJMT was successfully purified, although the apparent size of the protein on the gel seemed to be larger (the calculated size was 43.4 kDa) than expected (Figure 2A).

In order to determine whether EujJMT has methyltransferase activity, an enzyme assay was performed using crude proteins instead of purified enzymes as, for unknown reasons, we could not obtain sufficient amounts of the reaction products for GC-MS analysis. The enzyme assay contained 150 mM Tris-
HCl (pH 7.0), crude protein extracts (1.04 mg of total proteins), 1 mM SAM, and 1 mM JA in 1 ml of reaction mixture, and was incubated at 30°C for 24 h. The reaction product was extracted with 3 ml of ethyl acetate, concentrated under gaseous N₂, and analyzed using gas chromatography-mass spectrometry (GC-MS) (QP2010 Plus, Shimadzu, Kyoto, Japan) equipped with a DB-5 ms column (30 m length×0.25 mm diameter×0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA). The column temperature was programmed as follows: 100°C for 1 min, increasing by 10°C min⁻¹ to 170°C, and subsequently increasing by 20°C min⁻¹ to 240°C for 6 min with He as the carrier gas. The mass spectrometer was operated in the electron ionization mode with ionization energy of 70 eV and with ion source and interface temperatures of 240°C, with a continuous scan in the 60–300 m/z range. GC-MS analysis of a MT assay with EujJMT found several enzymatic products (Figure 3). These products were identified as MeJA stereoisomers since they produced the same MS spectra as the standard specimen of MeJA.

Substrate preferences of the purified EujJMT were tested using several carboxylic acids as substrates, as shown in Figure 2B. MT activity assay for EujJMT proteins was performed using a radiochemical method, according to Koeduka et al. (2016). The enzyme showed the highest activity toward JA, and exhibited subtle activity toward caffeic acid, salicylic acid, and cinnamic acid. In contrast, the enzyme displayed negligible activity to indole acetic acid and benzoic acid. These results suggested that EujJMT is highly specific for JA, and this substrate preference is consistent with those of JMTs found in the Brassicaceae family, which includes A. thaliana and B. rapa. Detailed characterization of
EujJMT revealed that the apparent $K_m$ value of the enzyme against JA was $62.6 \pm 11.7 \mu M$ (Figure 2C). This value was relatively higher than those of AtJMT ($38.5 \mu M$) and BrJMT ($38.0 \mu M$), but 2.8 times less than the apparent $K_m$ value ($175.7 \mu M$) of black cottonwood methyltransferase PtJMT with JA.

Phylogenetic analysis with previously reported SABATH protein sequences was performed using the neighbor-joining method with 1,000 bootstrap replications in the MEGA6 software (Tamura et al. 2013). Our results indicated that EujJMT belongs to the same clade as AtJMT and BrJMT, but in a separate clade from monocots and Rosaceae (Figure 4). We also found that EujJMT is distantly related to three JMTs from Norway spruce. Previous studies have indicated that most JMTs in distinct protein lineages, including FvJMT, CeJMT, and PaJMTs, have the strongest preference for JA among all JMTs, whereas several SABATH proteins, such as EjMT1, PtJMT, and tomato SMT (cLEI13O14), accept benzoic acid as a substrate in addition to JA. Therefore, it is likely that the ancestral proteins of JMTs have a strict specificity for carboxylic acids and that substrate promiscuity with catalytic activity for both benzoic acid and JA has occurred since the split of the specific protein lineages during plant evolution. To identify which amino acids and domains of JMTs are responsible for the specific formation of MeJA, further isolation of JMT proteins with different substrate specificity are required from different plant species.

Overall, our study provided biochemical evidence that deepened previous knowledge on high specificity toward JA among the species from the family Brassicaceae. This study presented the first identification of JMT from wasabi, which is one of the most traditional Japanese spices. The results of our study may be useful for future physiological studies related to MeJA/JA-mediated stress response in wasabi.

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