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Short Communication

Characterization of three halide methyltransferases in *Arabidopsis thaliana*

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Abstract Methyl chloride and methyl bromide, which contribute to the destruction of the stratospheric ozone layer, are mainly emitted from natural sources. It was recently reported that tropical and subtropical plants were the largest sources of methyl chloride. Furthermore, the involvement of the gene *HARMLESS TO OZONE LAYER (HOL)* in methyl halide emissions from *Arabidopsis thaliana* was demonstrated. However, neither the physiological significance of the methyl chloride emission nor the biochemical properties of HOL, denoted as AtHOL1 in our study, have been reported yet. We identified two additional isoforms-*AtHOL2* and *AtHOL3*-from *Arabidopsis* and characterized them together with *AtHOL1*. *AtHOL1* was ubiquitously expressed during development, and its expression level was the highest among the three. The phylogenetic tree suggested that AtHOL1 homologous proteins were distributed throughout the plant kingdom. Biochemical analyses showed that the three recombinant AtHOL proteins were functional and had distinct levels of the S-adenosyl-L-methionine-dependent methyltransferase activities. Although a study of *AtHOL1*-disrupted mutants had shown that *AtHOL1* primarily controlled the production of methyl halide, our study suggested that the activation of *AtHOL2* and *AtHOL3* genes also contribute to the methyl halide emissions from *Arabidopsis*.

Key words: *Arabidopsis thaliana*, methyl halide, S-adenosyl-L-methionine-dependent methyltransferase activity.

The most abundant halohydrocarbons from natural sources are methyl chloride and methyl bromide, which catalyze the destruction of the stratospheric ozone layer. It has been estimated that methyl chloride and methyl bromide, respectively, are emitted at rates of 1,700–13,600 and 160–200 Gg per year into the stratosphere (WMO 2007). Oceans (Khalil et al. 1999), biomass burning (Lobert et al. 1999), and salt marshes (Rhew et al. 2002) were considered the major sources of these gases. Recently, a large amount of methyl chloride emission by tropical and subtropical plants was reported (Yokouchi et al. 2002; Hamilton et al. 2003). Hence, tropical and subtropical forests are now considered as major contributors to the global atmospheric budget of methyl chloride.

It was reported that methyl chloride transferase (MCT) from *Batis maritima* (Wuosmaa and Hager 1990; Ni and Hager 1998) and thiol methyltransferases (TMT) from *Brassica oleracea* (Attieh et al. 2000a; Attieh et al. 2000b; Attieh et al. 2002) possess S-adenosyl-L-methionine (SAM)-dependent methyltransferase activities and could synthesize methyl halide *in vitro* (Attieh et al. 2000a; Wousmaa and Hager 1990). The hypotheses concerning the physiological meaning of these enzymes include their involvement in salt tolerance (Ni and Hager 1999) or in metabolizing glucosinolate hydrolysis products such as thiols and thiocyanate (Attieh et al. 2000a). The *AtHOL1* gene was isolated from *Arabidopsis* as the MCT homolog, and the T-DNA insertion mutant analyses revealed that *AtHOL1* was involved in the synthesis of methyl halides including methyl chloride, methyl bromide, and methyl iodide (Rhew et al. 2003). However, biochemical analyses of AtHOL1 and its homologs in *Arabidopsis* have not been reported.

Using the amino acid sequence of AtHOL1 in the BLAST search of the *Arabidopsis* genome, we identified two additional isoforms, At2g43920 and At2g43940, on chromosome II and designated the genes *AtHOL2* and *AtHOL3*, respectively. AtHOL2 and AtHOL3 showed 82.9% and 54.2% amino acid similarity, respectively, to AtHOL1 (Figure 1A). The phylogenetic analysis showed that the nucleic acid sequences possibly encoding homologous proteins of AtHOL1 were distributed throughout the plant kingdom including dicot, monocot, and unicellular algae. *Oryza sativa*, of which the genome

Abbreviations: GC, Gas chromatography; MALDI-TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine
This article can be found at http://www.jspcmb.jp/

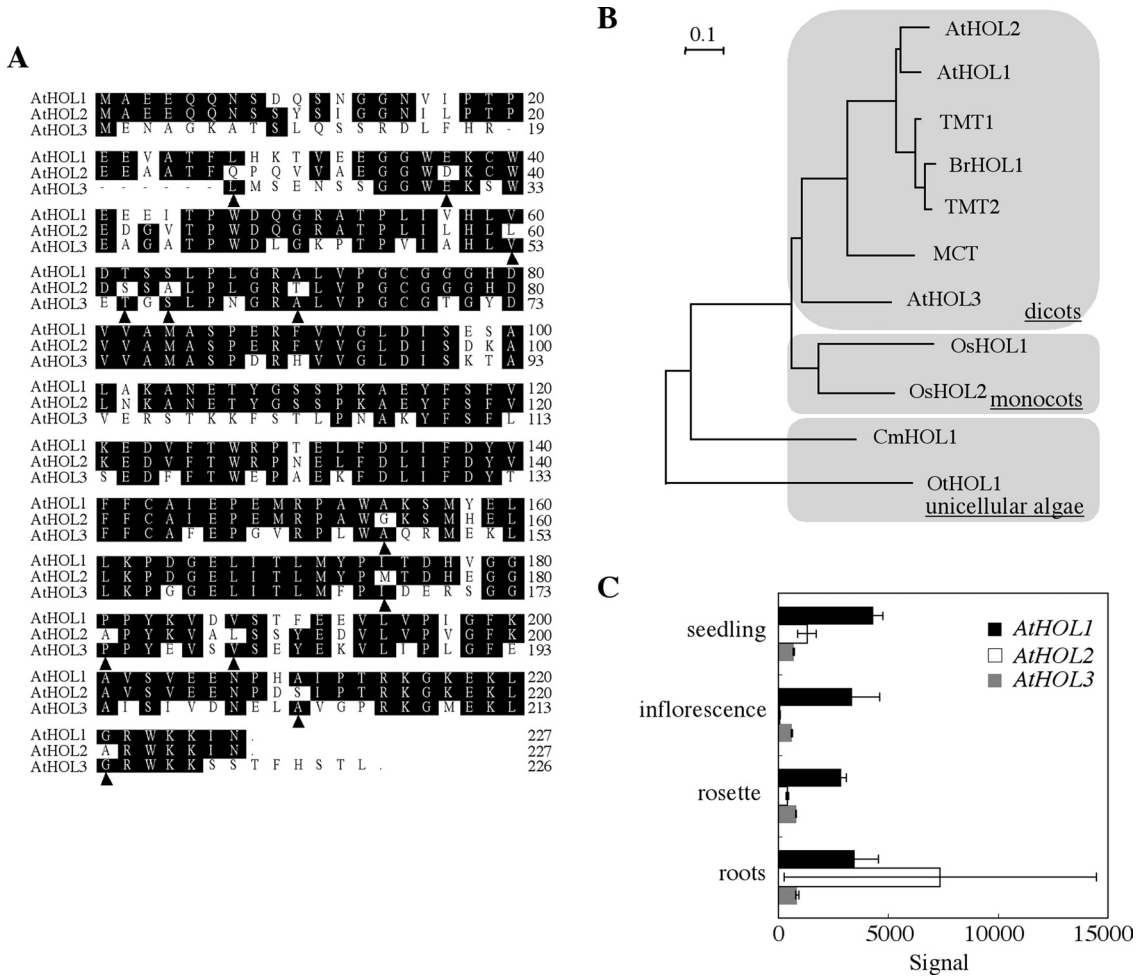

Figure 1. Properties of *AtHOL* genes. (A) Amino acid sequences of AtHOL1, AtHOL2, and AtHOL3. The deduced amino acid sequences of AtHOL1, AtHOL2, and AtHOL3 were aligned using ClustalW. Black boxes and the arrowheads denote amino acid identity and the amino acid residues that were only conserved in AtHOL1 and AtHOL3, respectively. (B) The phylogenetic tree of AtHOL1 homologous proteins. The deduced amino acid sequences of AtHOL1 homologs, of which complete cDNA sequences were determined, were selected using a BLAST search and aligned using ClustalW. The tree analysis was performed using the Njplot program. *Arabidopsis thaliana* (AtHOL1, AtHOL2, and AtHOL3; GenBank accession numbers NM_129953, NM_129954, and NM_180072, respectively), *Brassica oleracea* (TMT1 and TMT2; GenBank accession numbers AAK69760 and AAK69761, respectively), *Brassica rapa* (BrHOL1; GenBank accession number ABL86248), *Batis maritima* (MCT; GenBank accession number AAD26120), *Oryza sativa* (OsHOL1 and OsHOL2; GenBank accession numbers NP_001051867 and NP_001056843, respectively), *Ostreococcus tauri* (OtHOL1; GenBank accession number CAL52768), *Cyanidioschyzon merolae* (CmHOL1; GenBank accession number AP006501). (C) The digital northern analysis of *AtHOL* genes. The data were obtained from AtGenExpress at the GENEVESTIGATOR site. Signal intensities were averaged from more than two technical replicates.

had been sequenced in its entirety (International Rice Genome Sequencing Project 2005), has two homologous genes of *AtHOL1* (Figure 1B). This distribution suggested that the homologous genes of *AtHOL1* could have a common physiological significance in plants. To investigate the gene expression of each *AtHOL* gene, we analyzed available microarray data from the GENEVESTIGATOR database (https://www.genevestigator.ethz.ch/) (Zimmermann et al. 2004) (Figure 1C). *AtHOL1* was ubiquitously expressed during development; however, *AtHOL2* and *AtHOL3* were expressed at lower levels than *AtHOL1*.

Although it was predicted that three AtHOL proteins would possess the SAM-dependent methyltransferase activity, the enzymatic characterization of the proteins has not been reported. To determine whether each AtHOL protein has the enzymatic activity, we prepared and analyzed recombinant AtHOL proteins. Total RNA was extracted from *Arabidopsis thaliana* (ecotype Col-0) seven-day-old seedlings grown in constant light. Three *AtHOL* cDNAs (the GenBank accession numbers of *AtHOL1*, *AtHOL2*, and *AtHOL3* are NM_129953, NM_129954, and NM_180072, respectively) were prepared by PCR carried out using DNA polymerase KOD-plus (Toyobo) and the following primer sets: 5′-ATGGCTGAAGAACAACAAAA-3′ and 5′-CTCGAGCAACATTGAAACATACATAGAGCA-3′ for AtHOL1, 5′-ATGGCTGAAGAACAACAAAATTC-3′ and 5′-CTCGAGGCGTACGTAGACGCTGGT-3′ for AtHOL2, and 5′-ATGGAAAACGCCGGTAAAGC-3′

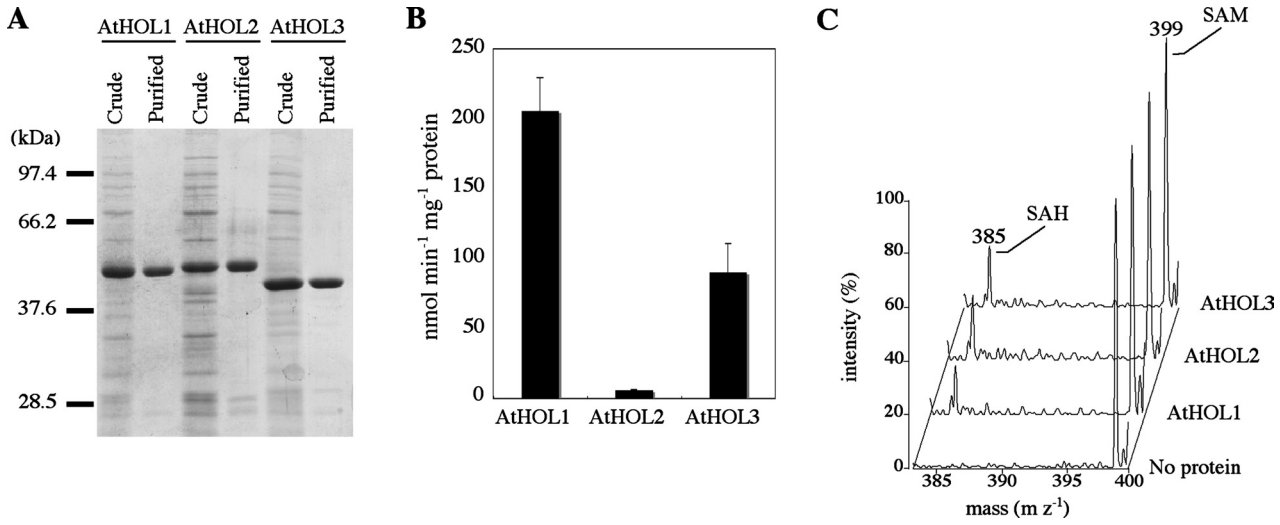

Figure 2. Characterization of the recombinant AtHOL proteins. (A) SDS-PAGE analysis of the recombinant AtHOL1, AtHOL2, and AtHOL3 GST fusion proteins. Soluble fractions of crude protein (Crude) and purified recombinant proteins (Purified) were electrophoresed on 10% acrylamide gel stained with Coomassie Brilliant Blue. The calculated molecular mass of the recombinant proteins, AtHOL1, AtHOL2, and AtHOL3, which include the GST tag and the extra amino acids, were 53.5, 53.3, and 53.3 kDa, respectively. The soluble protein contents were determined by the Bradford method (Bradford 1976), using the BioRad microassay procedure and BSA standard. (B) Iodide methyltransferase activity of recombinant AtHOL proteins analyzed using GC-ECD. The activities were determined by measuring produced CH_3I in the headspace of the assay mixture. The product was identified using a comparison of authentic CH_3I retention time. Headspace samples (200 μl) of the assay mixtures were injected in a 200×0.3 cm id stainless column packed with Porapak Q (Shimadzu, Kyoto, Japan) in GC-ECD. The column and injection temperatures were 160°C and 250°C, respectively, and the flow rate of the carrier gas (N_2) was 40 ml min^{-1}. The reaction rate was linear for at least 120 min (data not shown). The enzymatic activity values were expressed as those of native proteins without the conjugated GST tags. The activities of control samples without proteins were below the detectable limit (data not shown). Data are means±SE. (n=3). (C) MALDI-TOF-MS analyses of SAH produced by AtHOL proteins. SAM-dependent methyltransferase activities of recombinant proteins were analyzed using MALDI-TOF-MS in positive reflection mode. 1 μl of prepared samples containing 0.25μl of the assay mixture, 0.25 μl water and 0.5μl CHCA (5 mg ml^{-1} in 50% acetonitrile/50% trifluoroacetic acid) matrix solution were applied to the sample plate. Intensities of produced SAH were normalized by those of SAM.

and 5′-CTCGAGTGACAAATTCACAAAACATCCA-3′ for *AtHOL3*. cDNA fragments amplified for each *AtHOL* were cloned in-frame with an N-terminal glutathione S-transferase (GST) tag into an expression vector pDEST15-T that was modified to possess a thrombin recognition site at the C-terminus of GST in pDEST15 (Invitrogen). These recombinant proteins digested with thrombin had 15 extra amino acids (GSTSLYKKAGSEFAL) at the N-terminus of each AtHOL protein. Each recombinant protein was expressed in *Escherichia coli* (BL21) and purified from the soluble fractions using glutathione-Sepharose 4B (GE Healthcare). The purified proteins were stored in a buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM DTT, 30% glycerol) at −20°C. After 15 days, the recombinant proteins AtHOL1, AtHOL2, and AtHOL3 retained 60%, 40%, and 90%, respectively, of the iodide methyltransferase activity. The purity and molecular weights of the recombinant proteins were confirmed by SDS-PAGE analysis (Figure 2A) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS; Kratos Axima-CFR, Shimadzu) (data not shown). Since the recombinant proteins methylated iodide with greater efficiency than chloride (data not shown), we used iodide to examine the halide methyltransferase activity. The assay mixture volume was 200 μl containing 0.5 mM SAM, 0.1 mM KI, 0.1 M Tris acetate (pH 7.5), and 0.5 μg of each protein. The mixture was incubated in a 2 ml glass vial sealed with a screw cap fitted with a Teflon-lined septum and maintained on a shaker (150 rpm) at 25°C for 30 min. Produced CH_3I was analyzed using gas chromatography equipped with an electron capture detector (GC-ECD; GC-9A, Shimadzu) and quantified by peak area. The GC-ECD analyses showed that the recombinant AtHOL proteins have the iodide methyltransferase activity and the activities of AtHOL1, AtHOL2, and AtHOL3 were 205±24, 5.8±0.8, and 90±21 nmol $min^{-1} mg^{-1}$ protein, respectively (Figure 2B). Although AtHOL2 showed high amino acid similarity to AtHOL1, the iodide methyltransferase activity of AtHOL2 was very low in comparison to that of AtHOL1. An alignment of the three amino acid sequences indicated that twelve amino acid residues were conserved in AtHOL1 and AtHOL3 but not in AtHOL2 (Figure 1A). Among the twelve, a residue (Pro-181 in AtHOL1) was also conserved in thiopurine methyltransferases (TPMT) that are HOL-related proteins in animals. A crystallographic analysis of human TPMT indicated that the Pro residue was localized at the substrate-binding pocket (Wu et al. 2007) implying that the lower activity of AtHOL2 was due to

the mutation of this Pro to Ala residue.

To confirm that recombinant AtHOL proteins transfer the SAM methyl group to halide ions, we analyzed S-adenosyl-L-homocysteine (SAH) produced by SAM-dependent methylation using MALDI-TOF-MS (Figure 2C). The assay mixture volume was 10 μl containing 0.5 mM SAM, 5 mM KI, 0.1 M Tris acetate (pH 7.5), and 2 μg of each recombinant protein that was digested with thrombin and purified using the glutathione-Sepharose 4B. The mixture was incubated in a 1.5 ml tube at room temperature for 6 h. As a result of MALDI-TOF-MS analysis, SAH was detected from all assay mixtures incubated with each recombinant AtHOL protein. This result indicated that the three AtHOL proteins were actually SAM-dependent methyltransferases.

The distinct levels of the methyltransferase activities of each AtHOL recombinant protein, together with the distinct gene expression levels of the *AtHOL* genes, implied that the three AtHOL isoforms have independent physiological roles. The highest levels of both activity and gene expression of *AtHOL1* supported the recent report that showed that the T-DNA insertion *AtHOL1*-disrupted mutant plants have dramatically lower methyl halide emissions in contrast to wild-type plants (Rhew et al. 2003). The authors of the report discussed that *AtHOL1* primarily controlled the production of methyl halide in *Arabidopsis*. However, AtHOL2 and AtHOL3 recombinant proteins also possessed the methyltransferase activity, especially AtHOL3 showed methyltransferase activity about half of that of AtHOL1. Therefore, it would be important to elucidate the factors that activate the *AtHOL2* and *AtHOL3* genes, not only the *AtHOL1* gene, for an understanding of methyl halide emissions from *Arabidopsis*.

Although it was indicated that some plants have several isoforms of the enzyme, so far there is no report that has examined all isoforms in a single plant species. Analyzing all isoforms in *Arabidopsis thaliana* and *Oryza sativa*, of which the genomes were sequenced in their entirety, will provide a basis for understanding the physiological significance of this enzyme family.

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