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Genetic Control of Methyl Halide Production in *Arabidopsis*

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Summary

Methyl chloride (CH₃Cl) and methyl bromide (CH₃Br) are the primary carriers of natural chlorine and bromine, respectively, to the stratosphere [1, 2], where they catalyze the destruction of ozone, whereas methyl iodide (CH₃I) influences aerosol formation [3] and ozone loss [4, 5] in the boundary layer. CH₃Br is also an agricultural pesticide whose use is regulated by international agreement [6]. Despite the economic and environmental importance of these methyl halides, their natural sources and biological production mechanisms are poorly understood. Besides CH₃Br fumigation, important sources include oceans, biomass burning, tropical plants, salt marshes, and certain crops and fungi [7]. Here, we demonstrate that the model plant *Arabidopsis thaliana* produces and emits methyl halides and that the enzyme primarily responsible for the production is encoded by the *HARMLESS TO OZONE LAYER (HOL)* gene. The encoded protein belongs to a group of methyltransferases capable of catalyzing the S-adenosyl-L-methionine (SAM)-dependent methylation of chloride (Cl⁻), bromide (Br⁻), and iodide (I⁻) to produce methyl halides [8–10]. In mutant plants with the *HOL* gene disrupted, methyl halide production is largely eliminated. A phylogenetic analysis with the *HOL* gene suggests that the ability to produce methyl halides is widespread among vascular plants. This approach provides a genetic basis for understanding and predicting patterns of methyl halide production by plants.

Results and Discussion

Arabidopsis thaliana belongs to the Brassicaceae family, of which several members have been shown to emit methyl halides [11, 12]. The genome of *Arabidopsis* has been sequenced in its entirety, and near-saturation mutant collections are available, thus providing an opportunity to investigate the genetic regulation of methyl halide production in plants. Using the amino acid sequence of a methyl chloride transferase from the salt marsh plant *Batis maritima* (BmMCT; GenBank accession number

AF084829) in a BLAST search of the *Arabidopsis thaliana* genome, we identified a possible ortholog of BmMCT on chromosome II (At2g43910). The predicted translational product of this gene is 66% identical to BmMCT. Comparison of the identified *Arabidopsis thaliana* genomic sequence to an mRNA sequence representing this gene (CERES mRNA 24370) revealed the presence of eight exons and seven introns (Figure 1A) with an open reading frame encoding a polypeptide of 227 amino acids (Figure 1B). We have designated the gene *HARMLESS TO OZONE LAYER (HOL)*, based on the expected effect of its loss of function. *HOL* mRNA was found in all above-ground tissues. However, the expression level in 5-day-old seedlings was considerably higher than in tissues from 4-week-old plants (Figure 1C). For the 4-week-old plants, *HOL* mRNA levels in reproductive tissue (flowers and green siliques) were lower than in vegetative tissue. *HOL* is not a single-copy gene, however. Analysis of the *Arabidopsis* genome sequence revealed the existence of a gene whose protein product is 83% identical to *HOL*. This gene (At2g43920), which is located immediately adjacent to *HOL*, is expressed at a much lower level than *HOL* in the tissues tested (data not shown).

In order to test the loss of function of the *HOL* gene, an insertion line with a T-DNA inserted 160 nucleotides into intron 2 was identified in the SALK T-DNA collection (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Homozygous mutants (*hol-1*) were isolated from a segregating T2 population, and only one insertion was detected according to a Southern blot analysis (see Figure S1 in the Supplemental Data available with this article online). A Northern blot analysis demonstrated that the presence of full-length *HOL* mRNA was dramatically reduced in homozygous *hol-1* plants compared to *Arabidopsis* plants of ecotype Columbia 0 (ColO) (Figure 1D). However, the mutant plants showed no obvious growth abnormalities.

We investigated the importance of *HOL* for methyl halide production in *Arabidopsis thaliana* by comparing methyl halide emissions of wild-type with those of *hol-1* plants. We quantified the methyl halide emissions from juvenile (10–17 days after germination) and adult (3–4 weeks after germination) *Arabidopsis* plants by sealing whole plants inside glass incubation chambers and measuring the headspace concentration over time (see the Supplemental Data). Juvenile plants (100–300 seedlings) were grown in agar in small glass jars, and each jar of juvenile plants was incubated separately during flux measurements. Adult plants were extracted from the potted soil in which they were grown, and their roots were immersed in deionized water or dilute halide solutions during incubations. These incubations typically included five adult plants exposed to consecutive stepwise increases of halide concentrations. Halide concentrations in the agar (10–15 μmol Cl⁻, 0.03–0.3 μmol Br⁻, and 5–12 nmol I⁻ per gram of agar) and the solution (25–50 μmol Cl⁻, 3–7 μmol Br⁻, and 3–7 nmol I⁻ per milliliter of solution) were purposefully low in order to minimize competitive inhibition, to allow simultaneous analyses of all three methyl halides, and to expose the

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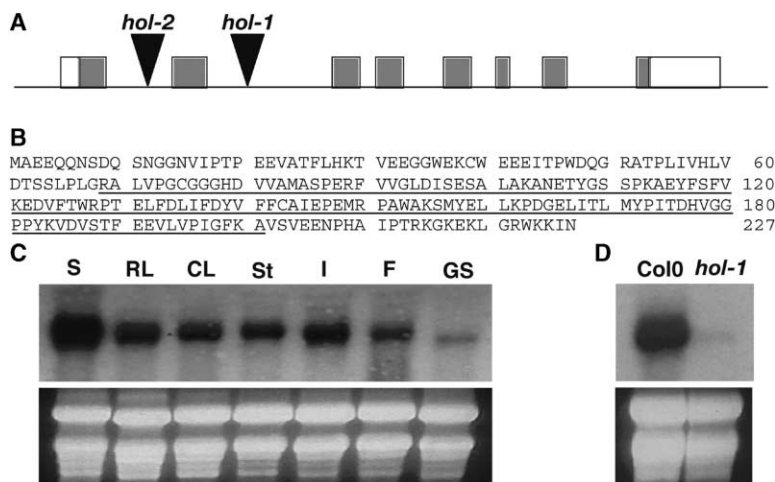


Figure 1. Organization and Regulation of the *HOL* Gene on *Arabidopsis* Chromosome II

(A) Genomic organization of the *HOL* gene. The boxes indicate exons with noncoding (white) and coding (gray) sequence. The black triangles point to the location of the T-DNA insertion in the *hol-1* mutant and the Ds insertion in the *hol-2* mutant.

(B) The amino acid sequence of *HOL*. The SAM binding methyltransferase domain is underlined.

(C) Northern blot of 10 µg total RNA from 5-day-old Col0 seedlings (S), rosette leaves (RL), cauline leaves (CL), stem tissue (St), inflorescence tissue (I), flowers (F), and green siliques (GS). Individual tissue was collected from 4-week-old *Arabidopsis thaliana* plants (ecotype Col0). An ethidium bromide-stained gel is shown below.

(D) Northern blot of 10 µg total RNA from 5-day-old seedlings from Col0 and *hol-1* mutant plants. The probe was specific to 453 nucleotides of the mRNA downstream of the T-DNA insertion site. An ethidium bromide-stained gel is shown below.

plants to halide levels that could be found under natural conditions.

Wild-type *Arabidopsis* juvenile plants and adult plants produce and emit all three methyl halides with fluxes that are influenced by the concentrations of halides in the substrate (Figure 2A, black bars). Juvenile plants have large emissions even at the low halide concentrations found in unamended agar. Overall production rates showed relative emissions of $\text{CH}_3\text{I} \gg \text{CH}_3\text{Cl} > \text{CH}_3\text{Br}$. For the experiments on adult plants, in which bromide was enhanced and iodide was reduced relative to the agar in the juvenile experiments, CH_3Br emissions were greater than both CH_3I and CH_3Cl . Average production rates were linear with respect to the available halide concentration. CH_3Cl showed production even when incubated in deionized water for over an hour, presumably due to residual chloride in the plant.

In contrast to wild-type plants, *hol-1* mutants have dramatically lower methyl halide emissions (Figure 2A, white bars). For the experiments with juvenile plants grown at the same time and under the same conditions, *hol-1* mutants showed average emissions of only 15% CH_3Cl , 4% CH_3Br , and 1% CH_3I compared to wild-type. The adult *hol-1* plants showed an even more striking loss of activity, with emissions less than 1% of wild-type for all three methyl halides. A second mutant allele with a transposable Ds element in intron 1 (*hol-2*) showed a similar loss of function when it was compared to its *Landsberg* ecotype background (data not shown). Crude protein extracts from *hol-1* and Col0 juvenile plants, incubated with SAM and iodide or bromide in buffer, also show a similar magnitude of loss of activity (R.C.R. and S. Manley, data not shown). These results demonstrate that the production of methyl halides in *Arabidopsis* is primarily controlled by the *HOL* gene. However, the small emissions observed by the mutant plants provide some evidence for an alternative, although minor, means of producing methyl halides. One possibility is that this contribution is due to redundant

activity of the close homolog (At2g43920) described above. Alternatively, it may be the result of the abiotic conversion of halides to methyl halides by using the plant component pectin as a methyl donor [13]. However, the results demonstrate that such an abiotic mechanism, if present, would be minor compared to the enzymatic production of methyl halides in *Arabidopsis*.

The order of preference for halide methylation may be assessed by normalizing the observed emission rates of methyl halides (CH_3X) to the concentrations of the corresponding halide (X^-) in the substrate for each incubation experiment. All wild-type plants strongly favor the methylation of I^- to Br^- to Cl^- (Figure 2B). Adult plants showed a relative methylation preference ratio for I:Br:Cl of roughly 10,000:50:1. Juvenile plants showed a ratio of roughly 40,000:9:1.

This order of halide preference is also exhibited in juvenile plants of another ecotype of *Arabidopsis thaliana* (*Landsberg erecta*) and is also evident when normalizing fluxes to the halide concentrations within adult plant tissue itself (see Figure S2 in the Supplemental Data). This preferential methylation order of $\text{I}^- \gg \text{Br}^- > \text{Cl}^-$ is similar to the analogous SAM-dependent methyltransferases in the wood-rot fungi *Phellinus pomaceus* [10], the alga *Endocladia muricata* [8], the salt marsh plant *Batis maritima* [14], as well as cabbage (*Brassica oleracea*) [9, 11].

The *HOL* protein and the thiol methyltransferase (TMT) identified in *Brassica oleracea* [15, 16] share a high sequence similarity, and both are most highly expressed at early stages of development (Figure 1C), suggesting that they serve the same biological function. TMT putatively plays a role in the metabolism of glucosinolates, sulfur-containing secondary metabolites found in the tissues of all members of Brassicaceae. Upon tissue damage, glucosinolates hydrolyze in the presence of myrosinases to produce reactive thiols that act as deterrents to herbivory [17]. TMT methylates the products of glucosinolate degradation, creating volatile sulfur deriv-

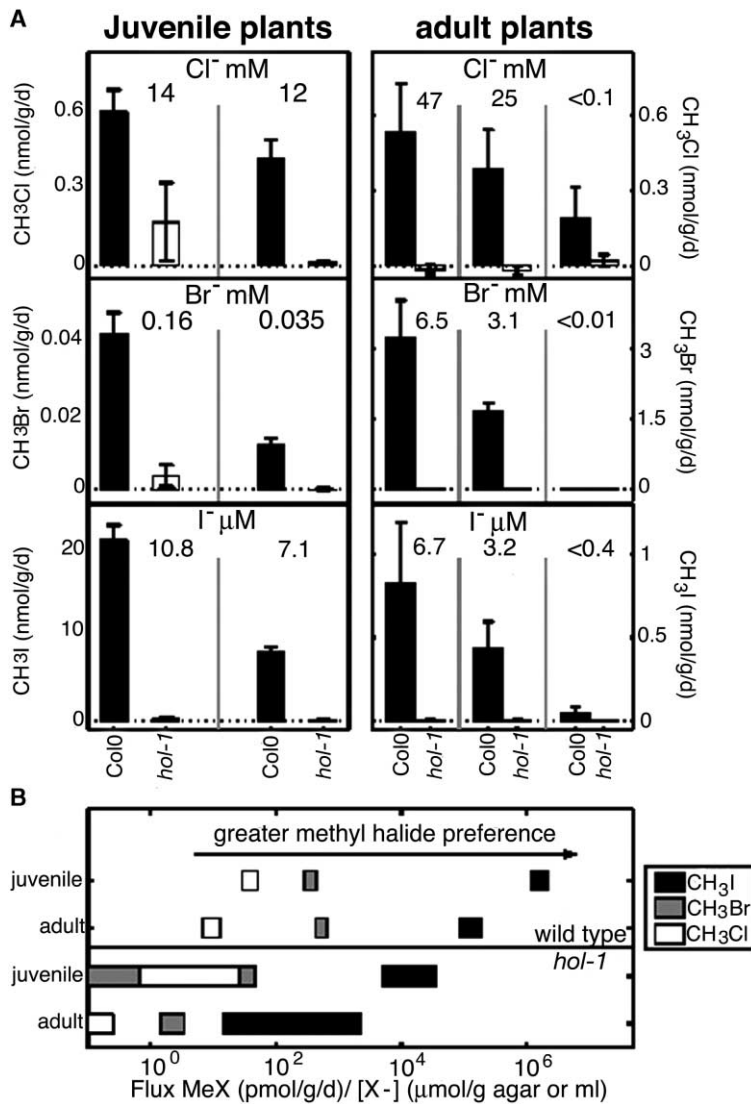


Figure 2. Effect of *hol-1* Mutation, Plant Life Stage, and Substrate Halide Concentrations on Methyl Halide Emissions

(A) Molar fluxes of CH₃Cl, CH₃Br, and CH₃I from wild-type Col0 and *hol-1* mutant plants on a fresh weight basis. Emissions of the methyl halides are dramatically decreased between the Col0 wild-type (black bars) and the mutant (white bars) grown and incubated under the same conditions. (Left panels) Each bar represents four flux measurements from two independent sets of juvenile plants. The numbers represent the mean measured halide concentration of the substrate, reported in μmol/g agar for Cl⁻ and Br⁻ and in nmol/g agar for I⁻ (SD = 14%, 40%, and 34%, respectively). (Right panels) Each bar represents flux measurements of 3–5 separate sets of adult plants. The numbers represent the mean measured halide concentration of the incubation solution (SD = 26%, 10%, and 10% for Cl⁻, Br⁻, and I⁻, respectively). (B) Halide-normalized fluxes demonstrate the preferential uptake of iodide >> bromide > chloride for the wild-type juvenile (n = 8) and adult (n = 7) plants. This trend is also generally followed in the *hol-1* juvenile (n = 8) and adult (n = 8) plants, although overall emission rates are much lower. The full range, down to the detection limit (note logarithmic scale), is shown.

atives such as methylthiocyanate (CH₃SCN), methylisothiocyanate (CH₃NCS), and methanethiol (CH₃SH), compounds that may act as allelochemicals or plant-insect signals [16, 18]. The most preferred substrate for TMT is thiocyanate (SCN⁻), based on its low *K_m* value and its ability to inhibit the methylation of iodide [18].

To test the activity of HOL to potential methyl group acceptors, we conducted a competitive inhibition study by adding Br⁻ and thiocyanate (SCN⁻) to groups of plants and monitoring the resultant change in methyl halide emissions (Figure 3). Two jars of juvenile wild-type (Col0) plants and two jars of juvenile *hol-1* mutant plants were grown in agar. Over the course of 5 days, substrates were added consecutively to the agar of one set, while the agar of the other set was unamended. Addition of 100 μmol NaBr to a set of wild-type juvenile plants caused more than a thousand-fold increase in CH₃Br emissions on the following day, but only a 20%–25% decrease in CH₃Cl and CH₃I. The subsequent addition of 100 μmol NaSCN caused a >95% decrease in all methyl halide emissions. This decrease suggests that

the enzyme preferentially methylated thiocyanate over the halides, and this finding is consistent with results from *B. oleracea* [18]. In contrast, the unamended jars of wild-type juvenile plants showed stable rates of methyl halide emissions over the course of the week.

Methyl halide emissions from the *hol-1* mutant juvenile plants were initially less than 5% of those from wild-type (Figure 3). CH₃Br emissions clearly increased following the addition of NaBr. This marked increase is similar to the wild-type experiment, although the *hol-1* emission rates were still significantly lower than the wild-type emission rates. However, the addition of NaSCN did not dramatically diminish the emission rates of the methyl halides, suggesting that it was not an effective inhibitor in the mutant plants.

The identification of the *HOL* gene and its integration into a phylogenetic map by using its encoded amino acid sequence can provide insight into the taxonomic diversity of methyl halide production by terrestrial plants. By searching the TIGR database (<http://www.tigr.org>), we identified a total of eight homologs of the

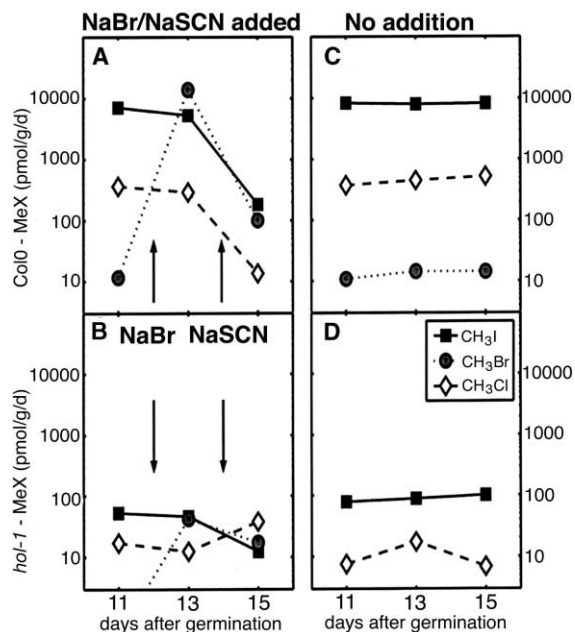


Figure 3. Activity of *HOL* in Response to Potential Methyl Group Acceptors

(A) Wild-type plants show markedly higher CH_3Br emissions and 20%–25% reduced CH_3Cl and CH_3I emissions following the addition of 100 μmol NaBr in solution. Emissions of all methyl halides are reduced >95% following the addition of NaSCN (note logarithmic scale).

(B) *hol-1* plants also show markedly higher CH_3Br emissions following NaBr addition, although emissions are still 330 times smaller than the wild-type plants. NaSCN addition has relatively little effect on *hol-1* fluxes.

(C) Wild-type plants without substrate additions.

(D) *hol-1* mutant plants without substrate additions. CH_3Br fluxes are not significantly different than zero.

HOL protein from seven different plant species: four dicotyledonous (*Arabidopsis thaliana*, *Brassica oleracea*, *Batis maritima*, and *Gossypium hirsutum*) and three monocotyledonous (*Hordeum vulgare*, *Oryza sativa*, and *Zea mays*) plants. In the phylogenetic tree, the *HOL* homologs divide into two main clades, with the five dicot sequences forming one, and the three monocot sequences forming the other. This division indicates that the common ancestor of *HOL* existed before plants diverged into monocots and dicots (Figure 4).

The phylogenetic tree shows that the gene most similar to *HOL* is At2g43920 (83% identity), which is also from *Arabidopsis* and located tandemly to the *HOL* gene. We propose that this second enzyme may be responsible for the small amount of remaining methyl halide production in the *hol-1* mutant. *Brassica oleracea* also has two closely related *HOL*-like genes [16] (Figure 4). The *Batis maritima* MCT, while showing a high degree of identity to *HOL*, is on a separate branch from all the Brassicaceae proteins; this finding is consistent with a different functionality. BmMCT does not effectively catalyze the methylation of thiol and consequently does not appear to participate in the metabolism of sulfur compounds [19]. Instead, its purported function is to maintain homeostatic levels of cytoplasmic chloride ion concentration [14].

Based on homology to *HOL*, we have also identified a putative ortholog in *Oryza sativa* (rice), a monocotyledonous plant, which emits CH_3Br and CH_3Cl and copious amounts of CH_3I [20]. In addition, homologs have been discovered in corn (*Zea mays*) and barley (*Hordeum vulgare*). The presence of *HOL*-like genes in both dicots and monocots suggests that this particular methyltransferase gene is widespread, if not ubiquitous, among vascular plants.

The expression of the gene and the physiological role of the encoded protein may vary widely among taxonomic lines, but available evidence suggests that additional sources of methyl halides will be discovered by following taxonomic branches of known producers. Currently, the largest emissions of CH_3Br and CH_3Cl were observed in plants from the families Brassicaceae, Bataceae, Frankeniaceae, and Dipterocarpaceae [12, 21, 22], and all of these families belong to the same subclass, Dilleniidae. Cotton (*Gossypium hirsutum*), a member of the Malvaceae family, is also part of the same subclass, and the open reading frame sequence of a *HOL*-like SAM:halide methyltransferase gene from cotton was identified (GenBank Number AI731595). Cotton is a salt-tolerant crop that has been planted widely in saline soils and may consequently be a previously unidentified source of methyl halide emissions. Our work further suggests that while overexpression of the *TMT* gene in *Brassica oleracea* plants may increase the detoxification of thiocyanate compounds and subsequently enhance food quality [16], it may also result in increased methyl halide emissions with unknown implications for the ozone chemistry of the atmosphere.

Supplemental Data

Supplemental Data including the Experimental Procedures, three figures, and associated references are available at <http://www.current-biology.com/cgi/content/full/13/20/1809/DC1/>.

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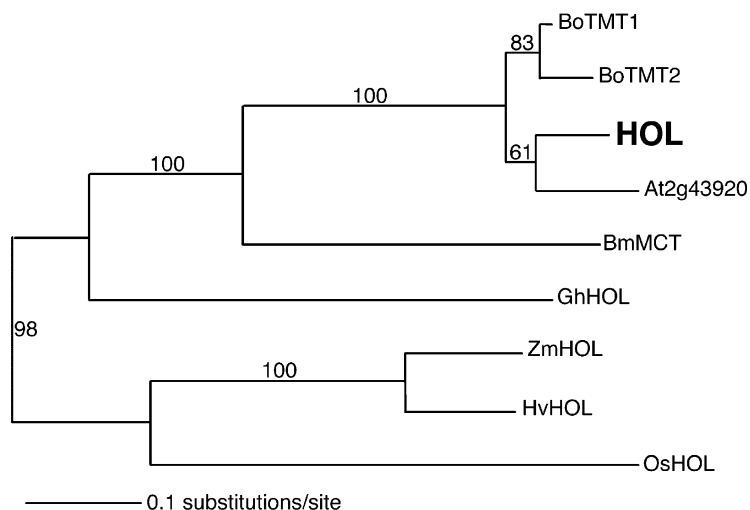


Figure 4. Phylogenetic Analysis of HOL/TMT Genes

Bootstrap values from 100 replicates are shown above each branch of the neighbor-joining tree. Sequences in the tree are from *Arabidopsis thaliana* (At2g43920), *Batis maritima* (BmMCT; GenBank Accession Number AF109128), *Brassica oleracea* (BoTMT1 and BoTMT2; Accession Numbers AF387791 and AF387792, respectively), *Gossypium hirsutum* (GhHOL; Accession Number AI731595), *Hordeum vulgare* (HvHOL; Accession Number TC102403), *Oryza sativa* (OsHOL; Accession Number AC092852), and *Zea mays* (ZmHOL; Accession Number BQ667926).

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