

Characterization of a Selenium-resistance-enhancing Homocysteine S-methyltransferase from *Aegilops tauschii*

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In this study, the cDNA of homocysteine S-methyltransferase was isolated from *Aegilops tauschii* Coss., with the gene accordingly designated as *AetHMT1*. Similar to other methyltransferases, *AetHMT1* contains a GGCCR consensus sequence for a possible zinc-binding motif near the C-terminal and a conserved cysteine residue upstream of the zinc-binding motif. Analysis of *AetHMT1* uncovered no obvious chloroplast or mitochondrial targeting sequences. We functionally expressed *AetHMT1* in *Escherichia coli* and confirmed its biological activity, as evidenced by a positive HMT enzyme activity of 164.516 ± 17.378 nmol min⁻¹ mg⁻¹ protein when catalyzing the transformation of L-homocysteine. Compared with the bacterium containing the empty vector, *E. coli* harboring the recombinant *AetHMT1* plasmid showed much higher tolerance to selenate and selenite. *AetHMT1* transcript amounts in different organs were increased by Na₂SeO₄ treatment, with roots accumulating higher amounts than stems, old leaves and new leaves. We have therefore successfully isolated *HMT1* from *Ae. tauschii* and characterized the biochemical and physiological functions of the corresponding protein.

Keywords: homocysteine S-methyltransferase, *Aegilops tauschii*, selenium

Abbreviations: HMT: homocysteine S-methyltransferase, Bp: base pair, RT-PCR: reverse transcription polymerase chain reaction, SMT: selenocysteine methyltransferase

Introduction

Selenium (Se) is an essential micronutrient in both animals and humans, with several specific proteins requiring Se-cysteine in their active positions (White and Brown 2010; Hu et al. 2016). Se deficiency-related diseases caused by local low availability in food are well documented (Chen et al. 1980; Peng and Yang 1991). A daily dietary supplement of Se is one of the most effective Se-enrichment strategies. The main food sources of Se are wheat (Guo et al. 2013; Szira et al. 2014), red meat and seafood (Ebert and Jakob 2007),

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while plants can serve as an effective buffer to prevent accidental intake of excessive Se by humans (Schiavon and Pilon-Smits 2017), which is sometimes caused by direct supplementation (Hartikainen 2005). The breeding or selection of crops with greater Se concentrations in their edible tissues is an effective approach to increase the dietary Se intake of animals and humans (White 2016).

A precondition of higher Se accumulation ability in plants is toleration of high Se concentrations in large tissues (Brown and Shrift 1982). Se tolerance is primarily related to the ability to divert Se away from the production of selenocysteine and selenomethionine, which might be incorporated into non-functional proteins, through the synthesis of less toxic Se metabolites (White 2016). Selenocysteine methyltransferase (SMT) is thought to be the main enzyme specifically catalyzing methylate selenocysteine (SeCys) to produce the non-protein amino acid methylselenocysteine, thereby reducing intracellular concentrations of SeCys and selenomethionine (SeMet) (Neuhierl and Böck 1996). The SMT protein has been characterized as having over 40% identity to yagD from *Escherichia coli*, with the latter protein having been shown to catalyze the methylation of homocysteine, selenohomocysteine and SeCys (Neuhierl et al. 1999). Similarly, comparison of the SMT amino acid sequence from *Astragalus bisulcatus* against various databases has revealed that this sequence is 70% and 65% identical to homocysteine S-methyltransferase (HMT, EC 2.1.1.10) proteins from *Arabidopsis thaliana* and *Oryza sativa*, respectively. In fact, the cDNAs encoding HMT from *Arabidopsis thaliana* were cloned through functional complementation of yagD *E. coli* mutations (Ranocha et al. 2000). The HMT enzyme catalyzes the synthesis of methionine from homocysteine using SMM as a methyl donor. These results collectively suggest that SMT and HMT have similar structures and functions, with both possessing the ability to methylate cysteine and homocysteine, as well as their Se isologs, with varying specificities (Sors et al. 2005).

Se in wheat grains has high bioavailability (Thomson 2004). Bread wheat (*Triticum aestivum* L., genome AABBDD), a major staple crop worldwide, is humanity's main food source. Bread wheat is an allohexaploid that originated from hybridization between cultivated tetraploid wheat (*T. turgidum*, BBAA) and the diploid wheat relative *Aegilops tauschii* (DD). A previous study has indicated that *Ae. tauschii* has a higher Se concentration than bread wheat (Lyons et al. 2005).

In the present study, we isolated and characterized a novel gene that encodes the HMT enzyme from *Ae. tauschii* (AetHMT1). To functionally analyze *AetHMT1*, we expressed this gene in *E. coli* exposed to high Se concentrations. We investigated the potential activity of the enzyme encoded by *AetHMT1* and also analyzed temporal and spatial patterns of *AetHMT1* expression in response to Se treatments.

Materials and Methods

Plant materials and growth conditions

Aegilops tauschii, ssp. *strangulata* accession AS2407 was obtained from Sichuan Agricultural University and used in this study. One-week-old seedlings germinated in dishes

were transferred to pots containing a 1:3 (v/v) mixture of natural soil and artificial substrate (Klasmann, Germany) and were grown in a greenhouse at 24 °C under 16-h day-length conditions for 8 weeks. Young leaf samples from at least three individual plants were then combined, frozen in liquid nitrogen, and stored at -80 °C for RNA extraction.

For RT-PCR, AS2407 seedlings with consistent growth were selected, transplanted into a hydroponic growth system, and aerated hourly with Hoagland's solution for 10 min. When plants were at the two-leaf and one-center stage, sodium selenate (Na_2SeO_4) at a concentration of 10 μM was added to half of the plants, with the non-treated plants serving as a control. Various organs, including new leaves, old leaves, stems and roots, were harvested after 72 h of treatment, frozen in liquid nitrogen, and stored at -80 °C for RNA extraction.

Isolation of HMT1 from Ae. tauschii

Total mRNA was extracted from young leaf samples using an RNAprep Pure kit (Tiangen) and stored at -80 °C. The extracted mRNA was reverse transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) according to the manufacturer's instructions, detected by electrophoresis, and stored at -20 °C. The homocysteine S-methyltransferase 1 gene from *Aegilops tauschii* (*AetHMT1*) was cloned using *HMT1*-specific primers HMT1F and HMT1R (Table S1*) designed according to <http://www.ncbi.nlm.nih.gov/protein/EMT27714.1>. PCR amplifications were performed with Phusion High-Fidelity DNA polymerase (Thermo). The resulting products were purified using an EasyPure Quick Gel Extraction kit (Tran), sub-cloned into a pGEM-T Easy vector, transformed into *E. coli* strain DH5 α , and sequenced (Ausubel et al. 1993).

Construction of a prokaryotic expression vector

The *AetHMT1* coding sequence was amplified from the original cDNA clones with Phusion High-Fidelity DNA polymerase using primers BH1F and BH1R (Table S1). RT-PCR products of the *AetHMT1* gene were gel-purified and cloned into a pGEM-T Easy vector to yield pGEM-*AetHMT1*. The sequenced recombinant pGEM-*AetHMT1* plasmid was digested with BamHI and ligated into a pET-30a-c (+) expression vector. The recombinant plasmid was verified by PCR and restriction enzyme digestion. The correct recombinant plasmid was designated as pET-*AetHMT1* and transformed into competent cells of *E. coli* strain BL21 (DE3).

Overproduction of the AetHMT1 gene in E. coli and preparation of a crude extract

Escherichia coli strain BL21 (DE3) containing the pET-*AetHMT1* plasmid was cultured in liquid Luria-Bertani medium containing kanamycin (50 U ml⁻¹) at 37 °C with rotation at 250 rpm until an OD₆₀₀ of 0.6 was obtained. IPTG was then added to a final concentra-

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

tion of 0.01 mM. After incubating the culture at 17 °C for 20 h, 1-ml aliquots were collected. All collected samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Bacterial cells (50 ml) cultured above were collected by centrifugation at 12,000 rpm for 5 min. The supernatant was discarded, and the cell pellets were resuspended in 10 ml PBS buffer containing 1 mM phenylmethanesulfonyl fluoride and 1 mM DL-dithiothreitol. The prepared samples were then sonicated in a JY96-II sonifier cell disrupter (Scientec) on ice. The sonicated cells were collected by centrifugation at 4 °C and 12,000 rpm for 15 min. To check for *AetHMT1* expression, the supernatant and precipitate were analyzed by SDS-PAGE. The supernatant containing AetHMT1 protein was sterilized by filtration with a 0.22- μ m filter unit (Millex GP). The expressed protein was isolated with a His GravTrap and His Buffer kit (GE Healthcare) according to the manufacturer's instructions and stored in a -80 °C freezer.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970). All samples were denatured in boiling water for 10 min after adding protein denaturation liquid and then centrifuged at 12,000 rpm for 1 min. Following SDS-PAGE, gels were stained overnight with Coomassie brilliant blue, decolorized with glacial acetic acid:alcohol:water (1:3:6, v/v/v), and visualized.

Enzyme separation and activity assay

AetHMT1 enzyme activity was assayed by measuring the reduction of the substrate L-homocysteine according to previous methods (Liu et al. 2014; Lozada-Ramirez et al. 2008) with slight modification. A 2-ml centrifuge tube containing 20 mM HEPES-KOH buffer (pH 7.2), 200 μ l enzyme extract, 0.2 mM L-homocysteine and 200 μ M S-adenosyl-L-methionine was incubated at 37 °C for 25 min. A standard curve was prepared for L-homocysteine (0.00, 0.05, 0.10, 0.15, 0.20 and 0.25 mM). After the addition of 200 μ l of 100 mM 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) for visualization, samples were incubated for 10 min in 96-well plates (EIA/RIA 1 \times 8 Stripwell Plant, Costar). Following the reaction, 100 μ l of solution remaining on the 96-well plates was incubated with an equal volume of DTNB for 10 min. The concentration of L-homocysteine was then measured by reading the absorbance value at 412 nm on a Multimode Plate Reader (PerkinElmer EnSpire). The concentration of AetHMT1 protein was determined according to the Bradford method (Bradford 1976).

Bacterial Se tolerance analysis

To test clone functionality in the BL21 (DE3) cells, an individual colony from each construct was grown overnight in M9 medium (Sambrook et al. 1989) containing 0.8% glycine, 100 mM L-methionine and 100 μ g ml⁻¹ kanamycin (Ranocha et al. 2000). The

overnight culture was reinoculated in 3 ml of the same medium, collected at an OD₆₀₀ of approximately 0.4, and washed twice with 1 ml of 0.9% NaCl. The pretreated cells were adjusted to an OD₆₀₀ of 0.05 and grown in the presence or absence of 100 µM of selenate or selenite at 37 °C for 16 h on a shaker (Shaker). Cell density (OD₆₀₀) was then measured (Lyi et al. 2005).

AetHMT1 gene expression pattern after Se treatment

To analyze *AetHMT1* gene expression patterns, different organs (new leaves, old leaves, stems and roots) of AS2407 were harvested after Se treatment. HMT1F and HMT1R primers (Table S1) were used for semi-quantitative RT-PCR, with the *tubulin* gene chosen as an internal control.

Results

Isolation and characterization of a cDNA encoding AetHMT1 from Ae. tauschii

A full-length sequence with close homology to HMT was obtained by a search against the NCBI GenBank database. A primer pair based on this sequence was then designed for RT-PCR amplification of the open reading frame of the *HMT1* gene of *Ae. tauschii* ssp. *strangulata* AS2407. Sequencing of the PCR product indicated a size of 975 bp, exactly the same as the sequence identified in GenBank (accession number KD510783.1). The deduced AetHMT1 sequence was predicted to encode 324 amino acid residues, with a calculated molecular mass of approximately 35.15 kDa and an isoelectric point of 5.74. Blast searches revealed that AetHMT1 shared high sequence similarity with several methyltransferases. Some *HMT* or *SMT* genes have been cloned: for example, *SMT* from *Astragalus bisulcatus* (*AbSMT*) (Ari et al. 2010), *HMT-1* from *Arabidopsis thaliana* (*AtHMT-1*) (Ranocha et al. 2000), *SMT* from broccoli (*Brassica oleracea* var. *italica*; *BoSMT*) (Lyi et al. 2007), and *SMT* from *Camellia sinensis* (*CsSMT*) (Zhu et al. 2007). Like other related methyltransferases (Ranocha et al. 2000; Lyi et al. 2005), AetHMT1 contained a GGCCR consensus sequence near the C-terminal and three conserved cysteine residues, Cys236, Cys303 and Cys304, considered to play a crucial role in creating a ternary structure for the zinc-binding motif (Millian and Garrow 1998; Peariso et al. 1998; Koutmos et al. 2008). The deduced amino acid sequence of AetHMT1 was found to share the following identities: 51.92% with AbSMT, 52.02% with BoSMT, 91.09% with TuHMT1 and 65.96% with GaHMT (Fig. S1). AetHMT1 was found to share 65.96% identity with AtHMT-1 and 86.73% with ZmHMT1, and contained no obvious chloroplast or mitochondrial targeting sequences.

The high sequence similarity between SMT and HMT is responsible for their common function of catalyzing cysteine into methyleysteine, but with different affinity capabilities (Sors et al. 2009). This similarity suggests that SMT and HMT are derived from a common ancestor and are functionally related. Different conserved amino acids may be responsible for the different enzyme catalytic capabilities between HMT and SMT (Zhao et al. 2015).

Phylogenetic analysis placed AetHMT1 in a clade together with HMT-1 from *Arabidopsis thaliana* and HMTs from *T. urartu* and *Zea mays*, which was separate from SMTs of *Astragalus bisulcatus* and *C. sinensis* (Fig. S2).

Expression and purification of recombinant protein

To confirm the identity of the protein encoded by *AetHMT1*, the gene was cloned into plasmid pET-30a-c (+) to be expressed in *E. coli* strain BL21 (DE3). The pET-30a-c (+) vector is designed for cloning and high-level expression of sequences fused with a His tag. Cloning sites (BamHI) are available for producing proteins. Overexpression in *E. coli* BL21 (DE3) containing *AetHMT1* recombinant plasmid generated a gene product (Fig. 1). The molecular mass of AetHMT1 was approximately 40 kDa according to SDS-PAGE (Fig. 1), which is basically in agreement with the expected size of 40.58 kDa (35.15 kDa derived from the gene sequence and 5.44 kDa from the pET-30a-c (+) expression vector) as calculated using the tool at http://web.expasy.org/peptide_mass/. In addition, the empty pET-30a-c (+) vector and non-IPTG-induced pET-*AetHMT1* did not produce AetHMT1 protein in *E. coli* BL21 (DE3).

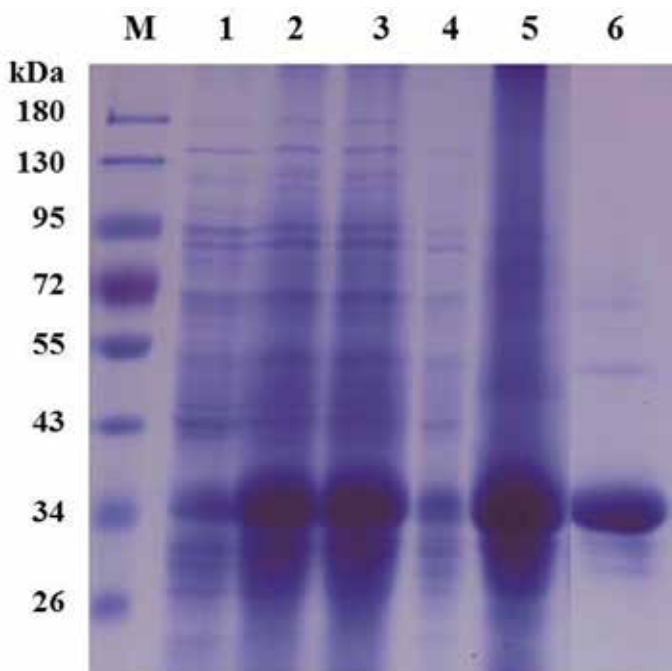


Figure 1. SDS-PAGE analysis of recombinant AetHMT1 protein expressed in *E. coli*. The AetHMT1 enzyme was purified by using 20 mM imidazole to elute other proteins. **M**, standard protein molecule weight marker; **1**, crude cell extracts before induction; **2 and 3**, crude cell extracts after induction; **4**, supernatants from induced *E. coli*; **5**, in precipitation; **6**, affinity-purified recombinant AetHMT1 eluted by 20 mM imidazole

AetHMT1 enzyme activity assays

Enzyme extracts from *E. coli* cells expressing *AetHMT1* and the empty vector control were prepared and assayed for HMT activity, with S-adenosyl-L-methionine (SAM) used as the methyl donor and L-homocysteine as the methyl acceptor at saturating concentrations (Ranocha et al. 2000). Extracts from the *E. coli* cells possessing *AetHMT1* clones were found to catalyze methyl transfer from SAM to homocysteine, thus demonstrating HMT activity, with testing to reduce the amount of homocysteine also conducted (Lozada-Ramirez et al. 2008). Homocysteine and SAM concentrations for optimal *AetHMT1* activity were determined to be 200 μM and 200 μM , respectively. The apparent HMT activity of the *AetHMT1* extracts was $164.516 \pm 17.378 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. No enzyme activity was detected in the empty vector control.

Increased Se tolerance of AetHMT1-expressing E. coli

To examine if *AetHMT1* improves the Se tolerance of *E. coli*, the growth of BL21 (DE3) cells expressing *AetHMT1* and the empty vector were examined in the presence or absence of 100 μM selenate or selenite. After 16 h of incubation with 100 μM of selenate, the growth of empty vector control cells was significantly reduced by 69.96%. In contrast, cells containing *AetHMT1* showed Se tolerance, with only a 26.76% reduction in growth (Fig. 2). Similarly, enhanced Se tolerance was also observed when cells expressing *AetHMT1* were exposed to 100 μM selenite. Compared with selenate, selenite appeared to be more toxic to bacterial cell growth (Fig. 2; pET-30a-c (+) control). In contrast to cells containing *AetHMT1*, which showed Se tolerance with only a 75.56% reduction in growth, the growth of empty vector control cells was significantly reduced (by 83.91%).

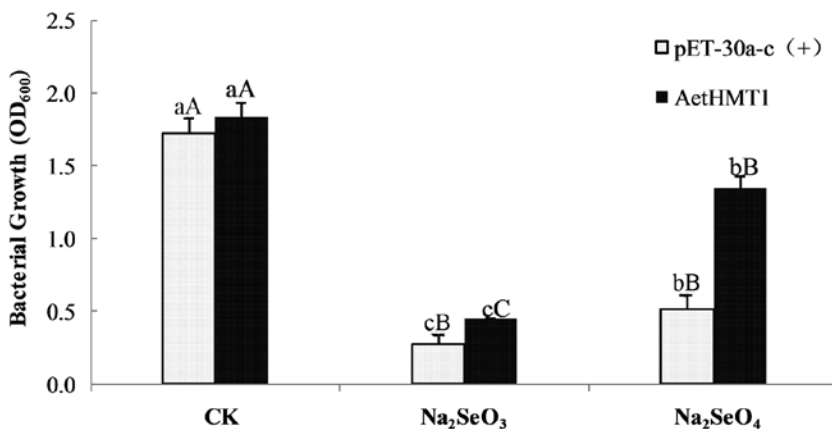


Figure 2. The effects of *AetHMT1* expression on bacterial tolerance to selenate and selenite. Bacterial strain BL21 (DE3) transformed with empty pET-30a-c (+) vector and *AetHMT1* were grown overnight and the cell density in the absence and presence of 100 μM Na₂SeO₄ or 100 μM Na₂SeO₃, then samples were measured as detailed in “Materials and Methods”. The experiments were repeated with three replications

Transcriptional expression analysis of *AetHMT1* in *Ae. tauschii*

The expression of *AetHMT1* in new leaves, old leaves, stems and roots was examined by semi-quantitative RT-PCR using gene-specific HMT1F and HMT1R primers, with the constitutively expressed *tubulin* gene as a reference (Fig. 3). *AetHMT1* transcripts were relatively abundant in all tissues. *AetHMT1* expression was dramatically upregulated in all tissues after 72-h treatment with 10 mM Na_2SeO_4 . Interestingly, roots accumulated higher transcript amounts than stems or leaves, which implies that *AetHMT1* may play a crucial role in Se uptake in *Ae. tauschii*.

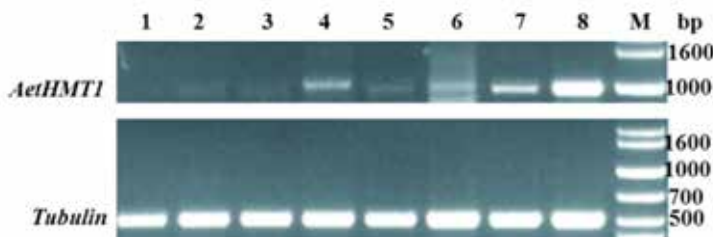


Figure 3. Semi-quantitative RT-PCR analysis of *AetHMT1* gene expression in new leaves, old leaves, stems and roots after treated by selenium for 72 hours. The As2407 seedlings were cultivated under 0 mM Na_2SeO_4 conditions and 10 mM Na_2SeO_4 conditions as described in the Materials and methods. Levels of mRNA were analyzed by semi-quantitative RT-PCR using gene specific primers for *AetHMT1*. And *Tubulin* was used as internal control. 1, new leaves treated by 0 mM Na_2SeO_4 ; 2, new leaves treated by 10 mM Na_2SeO_4 ; 3, old leaves treated by 0 mM Na_2SeO_4 ; 4, old leaves treated by 10 mM Na_2SeO_4 ; 5, stems treated by 0 mM Na_2SeO_4 ; 6, stems treated by 10 mM Na_2SeO_4 ; 7, roots treated by 0 mM Na_2SeO_4 ; 8, roots treated by 10 mM Na_2SeO_4 ; M, Marker

Discussion

Plants are known for their unique ability to synthesize methionine from homocysteine using HMT (Lyi et al. 2007). Three *HMT* cDNAs have been identified from *Arabidopsis thaliana* on the basis of homology to *E. coli YagD* (Ranocha et al. 2000; Ranocha et al. 2001). The *HMT* gene encodes cytosolic enzymes that have zinc-binding motifs and share significant protein sequence similarity with SMT from *Astragalus bisulcatus*, a Se hyper-accumulator (Neuhierl et al. 1999). Although HMT and SMT enzymes may play different roles in plants (Ranocha et al. 2001), both *HMT* and *SMT* genes are thought to confer Se tolerance. Because *Ae. tauschii* is an important genetic resource and the D-genome donor for common wheat, the characterization of the HMT1 protein from *Ae. tauschii* can indirectly contribute to the development of Se-rich wheat. In this study, the complete cDNA of *AetHMT1* was isolated from *Ae. tauschii* for the first time. The cDNA was cloned into a prokaryotic expression vector, pET-30a (+), and expressed in *E. coli* BL21 (DE3) after induction with IPTG. According to SDS-PAGE, *AetHMT1* was successfully expressed in the pET prokaryotic expression system.

Sequence alignment and phylogenetic analysis of the deduced AetHMT1 protein with other HMT and SMT proteins grouped AetHMT1 in the same clade with HMTs, which is separate from SMTs (Fig. S2). The deduced amino acid sequence of AetHMT1 shares high sequence identity with TuHMT1 from *T. urartu* (Ling et al. 2013), ZmHMT1 from *Z. mays* (Ranocha et al. 2001) and BoSMT from *B. oleracea* var. *italica* (Lyi et al. 2005). AetHMT1 contains a zinc-binding domain as well as an upstream conserved cysteine residue, characteristics typical of Zn^{2+} dependent thiol and selenol methyltransferases (Zhu et al. 2007; Lyi et al. 2007; Lyi et al. 2005). The presence of these features suggests that this enzyme requires a zinc cofactor for binding and/or activating the thiol group of homocysteine (Millian and Garrow 1998; Ranocha et al. 2000), as the GGCCR binding motif may be important for methyltransferase activity (Lyi et al. 2005). AetHMT1 has no obvious chloroplast or mitochondrial targeting sequences and appears to be a cytosolic enzyme, which is consistent with previous results (Lyi et al. 2005; Zhu et al. 2007; Lyi et al. 2007). Methylation of selenocysteine or selenomethionine, as well as metabolism involving the synthesis of S-methylmethionine, most likely takes place in the cytosol (Bourgis et al. 1999). According to our SDS-PAGE results, the AetHMT1 protein had the same mass as conjectured (Jia et al. 2013). The biochemical identity of purified AetHMT1 protein was confirmed, with its positive HMT enzyme activity in catalyzing the transformation of L-homocysteine measured as $164.516 \pm 17.378 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$.

The Se tolerance of *E. coli* was enhanced by the expression of recombinant AetHMT1. AetHMT1 conferred better protection against selenite than selenate, as the *AetHMT1*-transformed cells had a higher ratio of growth on selenite vs. selenate compared with the empty vector. This result is consistent with observations of previous studies (Lyi et al. 2005; Zhu et al. 2007). A precondition of higher Se accumulation ability is tolerance to high Se concentrations in large tissues (Brown and Shrift 1982). Our results demonstrated that AetHMT1 can improve bacterial Se endurance.

AetHMT1 is inferred to play an active role in Se absorption, as *AetHMT1* expression was dramatically upregulated in all tissues, especially roots, after treatment with 10 mM Na_2SeO_4 . The expression pattern of *AetHMT1* after exposure to selenate is similar to that of *BoSMT* (Lyi et al. 2007). *AetHMT1* expression amount was highest in roots, followed by stems, old leaves and then new leaves. This order may be similar to the pattern of Se delivery in plants. This observation indicates that the effect of Na_2SeO_4 on *AetHMT1* is likely a consequence of the role of this gene in Se metabolism.

In summary, cDNA of the *HMT* gene from *Ae. tauschii*, designated as *AetHMT1*, was isolated for the first time. The AetHMT1 protein was successfully expressed using the pET prokaryotic expression system. The biochemical activity of AetHMT1 was confirmed, with a positive HMT enzyme activity in catalyzing the transformation of L-homocysteine measured as $164.516 \pm 17.378 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$. AetHMT1 can improve bacterial Se endurance. Our research suggests the possibility of developing Se-rich bread wheat by selecting new *Ae. tauschii* AetHMT1 haplotypes with strong Se endurance and Se accumulation ability.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at <http://www.akademai.com/content/120427/>

Electronic Supplementary *Table S1*. DNA primers used for amplification of cloning, expressing protein in *E. coli* and semi-quantitative RT-PCR for *AetHMT1*

Electronic Supplementary *Figure S1*. Sequence alignment of the deduced amino acids for AetHMT1 (EMT27714.1) with those of related proteins. Conserved amino acids residues are shaded in black. Dash depict gaps were added for optimum alignment. The arrowhead indicated the third conserved Cys residue. The left-right arrow shows the possible zinc-binding motif that is typical for the structural family of Zn²⁺-dependent thiol and selenol methyltransferases is underlined. TuHMT1, *Triticum urartu* HMT1(EMS47619.1); ZmHMT1, *Zea mays* HMT1(AAG22537.1); AtHMT-1, *Arabidopsis thaliana* HMT1(NP_189219.1); GaHMT1, *Gossypium arboreum* HMT1(KHG14282.1); BoSMT, *Brassica oleracea* var. *italica* SMT(AAX20123.1); AbSMT, *A. bisulcatus* SMT (CAA10368); AtHMT-1, *Arabidopsis thaliana* HMT-1(AAF23821.1); CsSMT, *Camellia sinensis* SMT(ABF47292.1)

Electronic Supplementary *Figure S2*. Phylogenetic analysis of AetHMT1 and the aligned protein sequences. The analysis was conducted using the maximum likelihood method and a single unrooted phylogram tree was identified. Numbers presented at each branch point represent bootstrap values from 1000 replicates. Branch lengths on the tree are proportional to the amount of change as shown by the bar