

Molecular Characterization of Barley Methionine γ -Lyase and Gene Expression by Abiotic Stress and Aspartate Family Amino Acids

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Abstract

The concentrations of free proline, leucine, and isoleucine increase significantly in plant cells under osmotic stress. Methionine γ -lyase (MGL) catalyzes methionine to produce α -ketobutyrate, which is a precursor for isoleucine biosynthesis. Arabidopsis MGL gene is induced by drought and salt stresses, suggesting that plant MGL plays a role in isoleucine biosynthesis for abiotic stress tolerance in plants. However, enzymatic characterization and gene expression of plant MGL are described mostly based on results of Arabidopsis MGL. This study identified putative MGL genes from barley and some monocot plants that encode amino acid sequences showing homology with that of Arabidopsis MGL. Plant MGLs were distinguishable between monocots and dicots from their gene and amino acid sequences. Barley MGL catalyzed α , γ -elimination reaction more than α , β -elimination reaction, as did Arabidopsis MGL. Barley MGL gene was up-regulated considerably by drought stress, but down-regulated by the aspartate family amino acids, cystathionine, homocysteine, methionine, threonine, and isoleucine, with especially considerable reduction by methionine. These results suggest that barley MGL gene should be induced by drought stress specifically and be reduced by methionine to reserve methionine for the production of *S*-adenosylmethionine and the following metabolites, which are precursors for ethylene and polyamines playing a role in abiotic stress tolerance.

Keywords: Amino acid; Barley; Drought stress; Methionine γ -lyase; PLP-dependent enzyme

Introduction

Methionine in plants is not only an essential amino acid; it is also a precursor of *S*-adenosylmethionine (SAM), which functions as a primary methyl group donor for synthesis of ethylene, polyamines and vitamin B1, as well as *S*-methylmethionine (SMM), which is used as a transport molecule for reduced sulfur [1-4]. Methionine *de novo* synthesis is catalyzed by cystathionine γ -synthase (CGS) followed by cystathionine β -lyase (GBL) and methionine synthase (MS) [5,6]. Expression of CGS gene is regulated by the methionine concentration in Arabidopsis, but not in potato [7,8]. Methionine is catabolized to α -ketobutyrate and methanethiol, which are respectively a precursor for isoleucine biosynthesis and a pathogen of periodontal bacteria in plants, and ammonia by methionine γ -lyase (MGL; EC 4.4.1.11) [9,10]. MGL is a pyridoxal 5'-phosphate (PLP)-dependent enzyme distributed among bacteria, protozoans, fungi, archaea and plants, and is regarded as a key enzyme in the methionine metabolism [11,12]. Especially, MGL from *Pseudomonas putida* (PpMGL) is well characterized to define the relation between structure and function [13-16].

In plants, MGL gene from Arabidopsis (*AtMGL*) was isolated to express active enzymes, of which the amino acid sequence had 33% identity with PpMGL [17]. *AtMGL* was induced by drought and salt stresses, suggesting that MGL plays an important role in plant tolerance against osmotic stress by promoting isoleucine biosynthesis as an osmolyte [18-21]. The silencing of potato MGL gene (*StMGL1*) caused methionine accumulation in transgenic potato tubers [22,23], showing that *StMGL1* encodes a functional MGL. These results suggest that plant MGL might regulate methionine concentration. However, most studies of enzymatic and gene characterization of plant MGL are limited to Arabidopsis and potato, which are dicot plants. Gene regulation by amino acids constituting methionine biosynthesis is obscure.

This study identified putative MGL genes from barley (*HvMGL*)

and some monocot plants that encode homologues of *AtMGL*. *HvMGL* gene has about 70% GC contents as well as other monocot MGL genes, and encodes an active enzyme, of which amino acid residues of PLP binding and active site are conserved as with *AtMGL*. *HvMGL* was up-regulated by drought stress specifically, but was down-regulated by methionine to reserve methionine for SAM and subsequent metabolite production, which plays a role in tolerance against abiotic and biotic stresses.

Materials and Methods

Plant cultivation and stress treatment

Seeds of barley, *Hordeum vulgare L.*, Haruna Nijo, were soaked in water at 15°C for 5 h and were incubated on a filter paper filled with water at 23°C for 3 days in the dark. The seedlings with filter paper were set on an aquarium filter (2 cm thickness) filled with water and were cultivated under 20 h of metal halide light (350 $\mu\text{mol}/\text{m}^2/\text{s}$) and a 4 h dark cycle. After 3 days of cultivation, plants were treated as follows: soaked in 150 mM NaCl solution for 24 h; wounded by knife and incubated in the dark for 6 h; dehydrated in the incubator at 23°C in the dark for 6 h; soaked in 1 mM amino acid solution for 24 h; or cultivated in the dark as a control. After exposure to stress conditions, the shoots were harvested, frozen in liquid nitrogen, and stored at -80°C.

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Expression and purification of barley MGL

Total RNA was isolated from control shoots using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using a PrimerScript 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan) with oligo dT primer. The open reading frame of HvMGL gene was amplified with first-strand cDNA, Tks Gflex DNA polymerase (Takara Bio), and two primers designed from the putative open reading frame of the barley MGL gene (accession no. AK371929) as follows: sense primer, 5'-CCATATGCCCCACGTCCTCGCCGCCGCG-3', which creates a *NdeI* site (denoted as underlined), and antisense primer, 5'-CCTCGAGGACGGCCTTGCCGTA^TCTGG-3', which creates an *XhoI* site (denoted as underlined). The PCR product of 1,398 bp was cloned into the pGEM-T vector. Then the fragment of the plasmid digested by *NdeI* and *XhoI* were subcloned into a pET-20b (+) vector in which a polyhistidine tag gene is fused instead of the termination codon. The resulting plasmid, pHvMGL, was transformed into *Escherichia coli* BL21 (DE3) pLysS cells. *E. coli* cells harboring pHvMGL were grown at 37°C in Luria-Bertani (LB) medium containing 50 μ g/ml ampicillin. When the absorbance at 600 nm became 0.5, isopropyl- β -D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. After cultivation at 25°C for 18 h, the cells were harvested by centrifugation and were frozen at -80°C for at least 2 h. After the frozen cell pellets were suspended in extraction buffer consisting of 50 mM phosphate buffer (pH 7.0), 50 mM PLP, 10 mM GSH, and 5% trehalose, they were sonicated and centrifuged at 15,000 \times g for 20 min. The supernatant including the resulting recombinant protein HvMGL was purified using an Ni-NTA agarose column (Qiagen) initially equilibrated in 20 mM Tris-HCl buffer (pH 7.9) containing 0.3 M NaCl and 5 mM imidazole (Buffer A). The column was washed with Buffer A, followed by 60 mM imidazole in Buffer A, with the absorbed protein finally eluted with 0.3 M imidazole in Buffer A. After the protein solution of HvMGL was dialyzed against extraction buffer, the dialyzed solution was concentrated (Ultracent-30; Toso, Tokyo, Japan).

Cultivation of *E. coli* cells in nitrogen-restricted synthetic medium

E. coli cells harboring pHvMGL and pET20b (+) were cultivated overnight at 37°C in LB medium containing 50 μ g/ml ampicillin and 0.5 mM IPTG. Cells were collected by centrifugation at 8,000 \times g for 10 min and were suspended with saline to wash the cells. This washing was repeated three times. Then the aliquot of the washed cells was added to 20 ml of M9 minimal medium containing 0.4% glucose, trace elements (Mo, B, Co, Cu, Mn, Zn) [24], 50 μ g/ml ampicillin, and 0.5 mM IPTG without NH_4Cl to make the absorbance at 600 nm 0.2. Cells were incubated at 25°C on an orbital shaker (120 rpm) for 2 days. Absorbance at 600 nm was measured.

Enzyme and protein assays

The assay mixture containing 1 ml of 100 mM potassium phosphate buffer (pH 8.0), 25 mM L-methionine or other substrates, and 0.01 mM PLP was mixed with 0.01 ml of enzyme solution and was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.125 ml of 50% trichloroacetic acid. After centrifugation at 15,000 \times g for 5 min, 0.5 ml of the supernatant was mixed with 1 ml of 1 M sodium acetate buffer (pH 5.0) and 0.5 ml of 0.1% of 3-methyl-2-benzothiazolinone hydrazine (MBTH), incubated at 50°C for exactly 30 min and followed by 25°C for 30 min. The absorbance at 320 nm was measured. Then the amount of α -ketobutyrate or α -ketoacid products was estimated from the standard curve made with α -ketobutyrate. One unit is defined as

producing 1 μ mol of α -ketobutyrate per minute [13,25]. The protein concentration was quantified according to Bradford with bovine serum albumin as the standard [26].

Quantitative RT-PCR analysis

Total RNA was isolated from shoot samples using the RNeasy Plant mini kit (Qiagen) following the manufacturer's instructions. Poly(A)⁺ RNA was purified from total RNA with the Poly (A) Purist MAG (Ambion, Austin, Texas). Then the purified poly(A)⁺ RNA was dissolved in the RNA storage solution. First-strand cDNA for quantitative and semi-quantitative RT-PCR were synthesized, respectively, from total RNA and poly(A)⁺ RNA using a PrimeScript RT Master Mix (Takara Bio). Quantitative RT-PCR was performed in a mixture of 20 μ l containing first-strand cDNA, SYBR Premix Ex Taq (Takara Bio) and 0.2 μ mol forward and reverse primers (5'-CTACGACACGCTCATGTCTCTGCTCCGG-3', 5'-TCGAACTGCGCCAGCGCTGCTCCAGG-3') using LightCycler 2.0 (Roche Applied Science, Mannheim, Germany). The thermal cycle profile was 1 cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. The cDNA quantities of each gene were calculated using software (LightCycler 4.0; Roche Applied Science) and were normalized with that of the α -tubulin gene using forward and reverse primers (5'-CTCCATGATGGCCAAGTGTGA-3', 5'-AGTGCCTTGGTCTTGATGGT-3').

Results

Identification of barley MGL gene

The gene, which encodes amino acid sequence showing homology with that of AtMGL, was searched using the BLAST program [27,28]. The full-length cDNA of the barley gene (accession no. AK371929, 1,750 bp), which showed 65% identity with AtMGL, was identified. The open reading frame (ORF) of the gene from the database was amplified. The obtained 1,386 bp fragment was sequenced. The nucleotide sequence of the amplified fragment was matched to that of the database except for the substitution of A740 for G740, which translated into His247 instead of Arg247, encoding 461 amino acid residues. The genes which encode amino acid sequence showing homology with that of HvMGL were searched and were identified from rice (*Oryza sativa*), Brachypodium (*Brachypodium distachyon*), maize (*Zea mays*), and potato (*Solanum tuberosum*). The nucleotide sequences of putative ORF from monocot plants, barley, *O. sativa* (AK100465; OsMGL), *B. distachyon* (Bradi3g30860; BdMGL) and *Z. mays* (EU974363; ZmMGL) showed 72.5, 72.2, 70.2, and 72.9% of GC contents, respectively, whereas those from dicot plants, *A. thaliana* (At1g64660) and *S. tuberosum* (PGS0003DMT400031901; StMGL1, PGS0003DMT400015024; StMGL2) respectively showed 54.8, 48.5, and 46.6%. The putative amino acid sequences of HvMGL showed 85, 86, 85, 65, 63, and 63% identities, respectively, with those of OsMGL, BdMGL, ZmMGL, AtMGL, StMGL1, and StMGL2, and 34% with PsMGL. Alignment analysis of amino acid sequences with the Clustal W algorithm [29,30] showed that the amino acid residues, Met90, Asp187, His207, and Gly215 for PLP binding and Tyr59, Arg61, and Tyr114 for active site of PpMGL (amino acid numbers are based on PpMGL) [16] were conserved in plant MGLs, whereas Cys116, Lys240, and Asp241 for active site of PpMGL [16] were replaced with Gly, Leu, and Met in HvMGL as were other plant MGLs (Figure 1). The phylogenetic tree of these MGLs shows that plant MGLs are distinguishable between the monocot and dicot (Figure 2).

Pp	-----MHGSKLPGFATRAIHHGYDPQDHGGALVPPVYQTATFTTPTVEYGA	47
Hv	MAHVLAAAEPLTLLKRPTQNDGF--GDDNGSTEEKALKARRR-ESDPAAALAAARHEFGHEGGVNMS IEASATFTVMEPDTMR	81
Os	MAHVMAASELVTATIKRPFQNDNDSNNGNADG-KPKARRR-EADPAAALAAARHEFGHEGGVNMS IEASATFTVMEPDTMR	82
Bd	MAHVLAAAEPLISMLKRPPPPND-----DEKAPPVKNKAARRRDPSSDPAAALAAARHEFGHEGGVNMS IEASATFTVMEPDTMR	79
Zm	MAQTLASAASEL-ATLKRPFQNDGFGDGSNNGSATGEKPKARRR-EADPAAAMAAARHEFGHEGGVNMS IEASATFTVMEPDTMR	82
At	MAHFLETQEPVLFSGKKRRDRDDE----DGDALVAKKSALAVCDADPAAAIANI RHEFGHEGGVNMS IEASATFTVMEPDTMR	79
St1	MADTLNQNTNIVASNKRRSSGSDC-DHHDDSFVFKKQSKSLVWEDPAAALANARHEFGHEGGVNMS IEASATFTVMEPDTMR	83
St2	MAGKSEQ--NAVVLDKQRPAN-----VAQKSMKHLILEDPAAALANVRHEFGHEGGVNMS IEASITFTVMEPENLS	70
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Pp	ACFAGEQAGHF----YSRISNPTLNLEARMASLEGGEGALALASGGAITSTLWTLRLPGDEVLLGNTLYGCTFAFLHHGIG	126
Hv	RLFTGELGPERGDLIYISRFHNPVTLALGRQMAALEGTEAAYCTASGMSAISSVLMQLVAGGHVVASRCLYGGTHALLSRFLP	165
Os	RLFSGELGPERGDLIYISRFHNPVTLALGRQMAALEGTEAAYCTASGMSAISSVLMQLVAGGHVVASRCLYGGTHALLSRFLP	166
Bd	KLFAGELGPDGRDLYIYSRHFNPVTLALGRQMAALEGTEAAYCTASGMSAISSVLMQLVAGGHVVASRCLYGGTHALLSRFLP	163
Zm	RLFAGELGPDGRDLYIYSRHFNPVTLALGRQMAALEGTEAAYCTASGMSAISSVLMQLVAGGHVVASRCLYGGTHALLSRFLP	166
At	RMFTGELGPDN-DFYVYSRHFNPVTLNLSRQMAALEGTEAAYCTSSGMSAISSVMLQLCSSGGHVAASTLYGGTHALLSHFLP	162
St1	RMFAGELGDDR-DFFIYSRHFNPVTLNLSRQMAALEGTEAAYCTASGMSAISSVMLQLCSSGGHVAASQTYGGTHALLTHFLP	166
St2	RMFAGELGDDR-DFFIYSRHLNPTVTLNLSRQMAALEGTEAAYCTSSGMSAISSVMLQLCSSGDHIVASRSIYGGTYALLTHFLP	153
<div style="display: flex; justify-content: space-around; margin-top: 10px;"> ▼ ▼ </div>		
Pp	-EFGVKLRHVDMDLQALEAAMTP-ATRVLYFESPANPNMHMADIAGVAKIARKHGATVVVDNTYCTPYLQRPLELGADLVVHS	208
Hv	RTSGVKATFVDADDEAAVRAAMRPGETRVVYVETMSNPTLAVADIPMLARVAHEAGAKLVVDNTFT-PMVVS PARLGADV VVHS	248
Os	RASGVRATFVDADDEAAVRAAVRPGETRVVYVETMSNPTLAVADIPMLARVAHDAGAKLVVDNTFT-PMVVS PARLGADV VVHS	249
Bd	RTSGVTATFVDADDEAVRAAVRPGETKVVYVETMSNPTLAVADIPMLARVAHEAGAKLVVDNTFT-PMVVS PARLGADV VVHS	246
Zm	RASGVRATFVDADDEAAVRAAVRPGETRVVYVETMSNPTLAVADIPMLARVAHEAGAKLVVDNTFT-PMVVS PARLGADV VVHS	249
At	RTCNIITTSFVDITDHGAVANAIVEGRTQVLYFESVANPTLTVADIPELSRMAHEKGVTVVVDNTFA-PMVLSPAKLGADV VVHS	245
St1	RACNIITTSFVDVRDLEMVKEAIVEGRTNVL YFESVSNPTLTVANIPELSRMAHEKGVTVVVDNTFA-PMVLSPVKMGADV VVHS	249
St2	KACNIITTSFVDIRDLMVVEEAIVEGRTKVL YFESI SNPTLMVTDIPALCRIAHSKGVFVVVDNTFA-PMVLS PARLGADV VVHS	236
<div style="display: flex; justify-content: space-around; margin-top: 10px;"> ▼ ▼ </div>		
Pp	ATKYLSCGHGITAGIVVGSQALVDRIR--LQGLKDMTGAVLSPHDAALLMRGKTLNLRMDRHCANAQVLAEBFLARQPVELIH	290
Hv	VSKFISGGADIIAGAICGPASLVNAMMDLQDQALMLLGPMTNAKVASELAGRPLPRLMQEHSRRAAEFAARMRRQG--LRVT	330
Os	VSKFISGGADIIAGAICGPASLVNAMMDLQDQALMLLGPMTNAKVAFELSERLPHLPLRMQEHSRRAAEYASRMRLG--LRVA	331
Bd	VSKFISGGADIIAGAICGPASLVNSMMDLQDQALMLLGPMTNAKVASELAARLPHLPLRMQEHSRRAGEFAARMQRMG--LRVV	328
Zm	VSKFISGGADIIAGAICGPASLVNAMMDLQDQALMLLGPMTNAKVAFELSERLPHLPLRMQEHSRRALAFARMQGLG--LRVL	331
At	ISKFISGGADIIAGAVCGSENLVKEMMDLQDQALMLLGPMTNAKVAFELSERIPHLGLRMREHSRRAQVYAEARMRDG--MKVI	327
St1	ISKYISGAADIIAGAVCGPASLVNSMMDLHQGSLMLLGPMTNPKVAFELARLPHLGLRMKEHCKRALEYASRMTKLG--LKVI	331
St2	ISKYISGAADVIAVAVCGPASLINSMMDLREGLMLLGPMTNAKIAFELSERLPHLGLRMKEHSNRALVFATRITKLG--LKVI	318
<div style="display: flex; justify-content: space-around; margin-top: 10px;"> ▼ ▼ </div>		
Pp	YPGLASFPQYTLARQQMSQ--PGGMIAFELKGGI GAG---RRFMNALQLFSRAVSLGDAESLAQHPASMTSS YTPPEERAHYG	368
Hv	YPGLPEHPHARLARAGNPGYAGGMLCLDMGTTEERANRLMHLQNTTQFGLMAVSLGYYETLMSCSGNSTSSEMPEDRARAG	414
Os	YPGLPDHPHARLAIANPGYAGGMLCVDMGTEEDRANRLMHLQNTTQFGLMAVSLGYYETLMSCSGNSTSSEMPEDRARAG	415
Bd	YPGLQDHTHHGRLARMANPGYGFGLMCLVDMGTTEERARLMMHLQNTTQFGLMAVSLGYYETLMSCSGNSTSSEMPEDRALAG	412
Zm	YPGLPGHPHARLAAMGNPGYCGGMLCLDMGTTEERANRLMHLQNTTQFGLMAVSLGYYETLMSCSGNSTSSEMPEDRARAG	415
At	YPGLETHPQHKLKFGMVNRDYGGLLSIDMETEEKANKLMAYLQNTQFGLMAVSLGYYETLMSCSGNSTSSELDPQKEAAG	409
St1	YPGLEDPHDLALIKSMANKDYGYGILCVDMETTEERANRLMNLQNTQFGLMAVSLGYYETLMSCSGNSTSSELNNEKELAG	413
St2	YPGLENHDPHGLKSLANEDYGYGILCVDMETTEEKANCLMNLQNTQFGLIAVSLGYYETLMSCSGNSTSSEMNQEKELAG	400
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Pp	ISEGLVRLSVG-LEDIDDLADVQQALKASA-----	398
Hv	ISPLIRMSVGYNGTLEQRWAQFERALALMQQETPAAA-----ATAKYGKAV	461
Os	ISPLVIRMSVGYNGTLEQRWAQFERALSMLQQQQQH-----PDRD--AAKYCKV	464
Bd	ISPLIRMSVGYNGTLEQRWAQFERAFALMQQD-APS-----AAKYCKAI	458
Zm	ISPLVIRMSVGYNGTLEQRWAQFERALALMQTPPTPHHLKAAAARDGPEAANNHRKH-	473
At	ISPLVIRMSVGYVGTLEQKWTQFEKAFIRM-----	441
St1	ISPLVIRMSIGYNGSLEQKWSQLDKALSKMQEKMPF-----	451
St2	ISPLVIRMSIGYNGTLEQKWSQLEKALSOMQ-----	433

Figure 1: Alignment of the deduced amino acid sequences of barley MGL with those of monocot and dicot MGLs as well as *P. putida* MGL. Barley, rice, Brachypodium, maize, Arabidopsis, and two kinds of potato MGLs (Hv, Os, Bd, Zm, At, St1, St2) respectively correspond to gene nos. AK371929, AK100465, Bradi3g30860, EU974363, At1g64660, PGS0003DMT400031901, and PGS0003DMT400015024. Gaps, denoted by dashes, are introduced into the sequences to maximize the homology. The open and closed arrowheads respectively indicate the PLP binding and active site amino acid residues. Identical amino acid residues of PLP binding and active site among either plant MGLs or plant and *P. putida* MGLs are represented by black boxes.

Purification and enzymatic characterization of recombinant barley MGL

E. coli cells harboring pHvMGL produced an extra protein. Purification of the recombinant HvMGL with Ni-NTA column chromatography yielded a 95% pure preparation (Figure 3). The molecular mass of HvMGL estimated by SDS-PAGE was ca. 47 kDa, which matches to 49 kDa calculated from the amino acid sequence, and ca. 180 kDa by gel filtration, suggesting that HvMGL is a homotetramer protein. The purified HvMGL incubated with methionine showed the production of α -ketobutyrate, of which the specific activity was 0.36 U/mg. Results showed that MGL activity was inhibited completely by 1 mM hydroxylamine, the PLP antagonist, indicating that HvMGL depends on PLP. Actually, the HvMGL had maximum activity at pH 7.5 and high activities toward L-ethionine, L-homocysteine, and seleno-L-methionine in addition to L-methionine (Table 1).

E. coli harboring pHvMGL or pET20b (+) was cultivated in the synthetic medium with or without L-methionine as the solo nitrogen source (Figure 4). *E. coli* harboring pHvMGL did not grow in the medium without L-methionine as same as *E. coli* harboring pET20b (+), but it did grow in the L-methionine containing medium faster than *E. coli* harboring pET20b (+), and reached absorption of 600 nm to 1.55 after 48 h, whereas absorption was 1.10 from *E. coli* harboring pET20b (+).

Induction of barley MGL gene

Response of HvMGL expression to environmental stress and amino acids was evaluated using quantitative real-time PCR (Figure 5). HvMGL was up-regulated considerably, 4.7-fold, by drought stress, but it was unchanged by wounding and salt stresses, each of which had a level that was 1.2-fold higher. In response to amino acids constituting of the aspartate family including methionine and isoleucine biosynthesis in plants [5,6], MGL levels were decreased 1.9, 1.4, and 2.9-fold, respectively, by cystathionine, homocysteine, and methionine, which constitute methionine biosynthesis, and 1.1 and 1.4-fold, respectively, by threonine and isoleucine, which constitute isoleucine biosynthesis from O-phosphohomoserine through threonine.

Discussion

MGL gene encoding 65% homologue to AtMGL was identified from barley. The putative MGL from rice, Brachypodium, and maize showed 85-86% identities with HvMGL. These monocot MGLs had 63-65% identities with Arabidopsis and potato. The interesting feature of monocot MGL genes was a GC-rich sequence, which failed to amplify the gene by PCR using standard DNA polymerase and condition. These results indicate that plant MGL is subdivided into monocot and dicot groups. The deduced amino acid sequences of four monocot MGLs conserved four amino acid residues for PLP binding and six amino acid residues for the active site as well as three dicot MGLs. In these plant MGLs, the three amino acid residues for active sites corresponding to Cys116, Lys240, and Asp241 differed from those of PpMGL. The purified HvMGL was the PLP-dependent enzyme consisting of a homotetramer as well as PpMGL [14]. HvMGL had a specific activity of 0.36 U/mg with methionine, which was almost identical to AtMGL of 0.4 U/mg [17], but lower by 56 times than PpMGL of 20.4 U/mg [14]. HvMGL had the highest activity toward L-homocysteine, whereas L-ethionine and seleno-L-methionine are, respectively, the best substrates for AtMGL and PpMGL [14,17]. L-Methionine, L-ethionine, L-homocysteine, and seleno-L-methionine are catalyzed much more than L-cysteine, L-cystathionine, and S-methyl-L-cysteine by HvMGL,

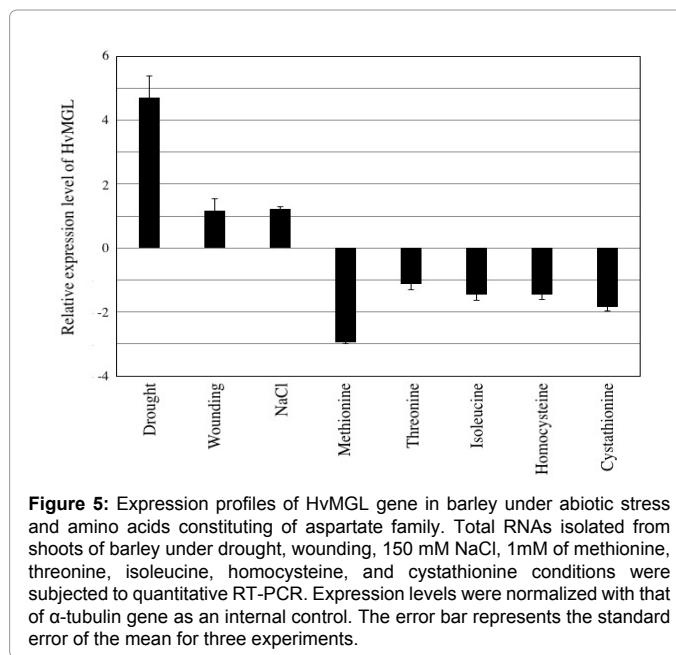
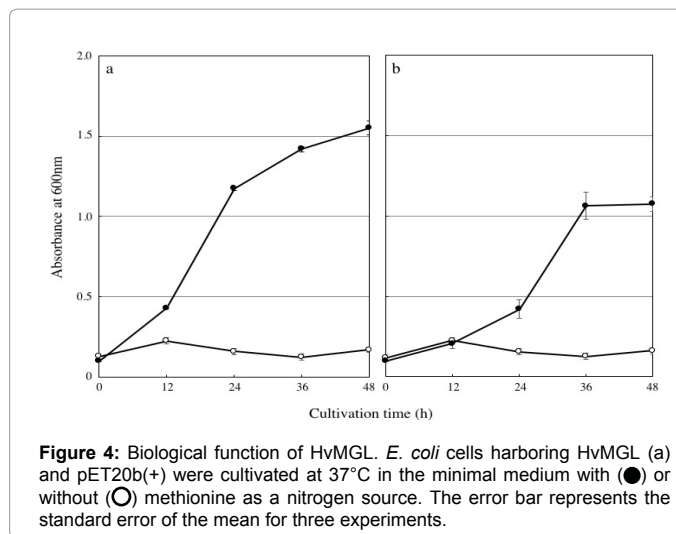
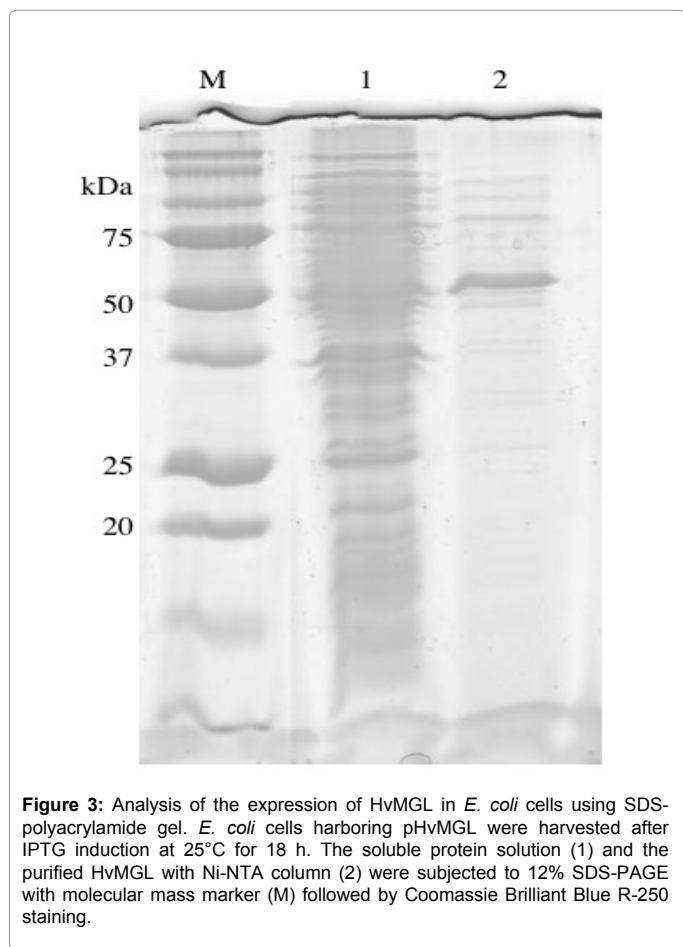
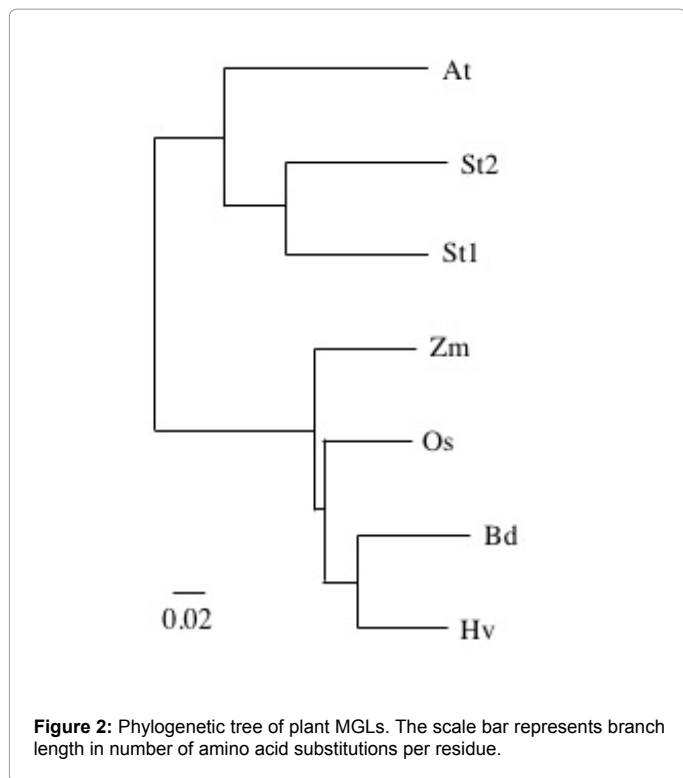
revealing that HvMGL prefers an α , γ -elimination reaction to an α , β -elimination reaction as do AtMGL and PpMGL. Reportedly, Lys240, Asp241, Arg61, Tyr114, and Cys116 of PpMGL form a hydrogen-bond network in the active site and PpMGL mutants, of which Cys116 was replaced by Ser or Thr, reduced respectively to 9 and 40% relative activity toward L-methionine [15]. Taken together, substitution of Gly, Leu, and Met of HvMGLs and other plant MGLs, respectively, for Cys116, Lys240, and Asp241 is expected to reduce the activity and change the substrate specificity.

It has been reported that MGL plays a role in protecting plant cells from abiotic stress because of production of α -ketobutyrate, a precursor of isoleucine accumulating in dehydrated plant cells as well as leucine, valine, and proline as osmolytes [31], and because of up-regulation of the gene expression by drought, osmotic, and salt stresses in Arabidopsis [18-21]. Our results demonstrated that HvMGL was up-regulated 4.7-fold, especially by drought stress. Although specific activity of HvMGL was lower than that of PpMGL, biological activity was revealed in *E. coli* cells expressing HvMGL, supporting that HvMGL functions increase the α -ketobutyrate concentration in barley under drought stress.

In mammals, amino acids have been shown to modify the expression of target genes at the levels of transcription and mRNA stability [32]. Methionine, which constitutes the aspartate family as do threonine and isoleucine in plants, is synthesized in three reactions as follows: from O-phosphohomoserine to cystathionine by CGS, from cystathionine to homocysteine by CBL, and from homocysteine to methionine by MS [5,6]. Methionine is catabolized by MGL to α -ketobutyrate, a precursor for isoleucine biosynthesis. On the other hand, α -ketobutyrate is also synthesized from O-phosphohomoserine through threonine [5,6]. In Arabidopsis, expression levels of CGS and MS were reduced by excess methionine in flowers, but those were increased in siliques [33,34]. Excess methionine was unable to regulate potato CGS transcript [8]. These results demonstrate that methionine concentration regulates gene expression associated with the methionine biosynthesis pathway. However, the effects of methionine and other amino acids constituting the aspartate family on MGL expression are not clear. Interestingly, our results showed that HvMGL was down-regulated by cystathionine, homocysteine, and methionine, especially 2.9-fold reduction by methionine. Most methionine is converted to SAM by SAM synthase in plants [35]. SAM reduces the expression level of GCS in Arabidopsis [36]. SAM acts as a precursor for ethylene and polyamines biosynthesis, which supports tolerance against abiotic and biotic stresses [2,37,38]. Taken together, HvMGL is expected to contribute specifically to the acquisition of tolerance against drought stress and to be reduced by methionine accumulation to reserve the methionine for producing SAM and following metabolites, contributing to the acquisition of tolerance under other abiotic stress.

Conclusion

Results of this study demonstrated that barley MGL conserved amino acid residues for PLP binding and active site as with other plant MGLs and plant MGLs were distinguishable between monocots and dicots. The expression level of HvMGL was increased by drought stress specifically and reduced considerably by methionine. These results suggest that HvMGL can contribute specifically to acquisition of drought tolerance and can reserve the methionine under other abiotic stresses.



Substrate	Relative activity (%)		
	HvMGL	AtMGL	PpMGL
L-Methionine	100	100	100
L-Ethionine	136	313	55
Seleno-L-methionine	119	107	282
L-Methionine sulfone	22	19	55
L-Methionine sulfoxide	29	14	21
L-Methionine sulfoximine	6	<3	1
L-Homocysteine	296	209	180
L-Cysteine	3	33	10
L-Cystathionine	3	11	0
S-Methyl-L-cysteine	2	<3	9

Table 1: Relative activities of HvMGL, AtMGL [17] and PpMGL [14].

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