Short title: Met γ-lyase suppression leads to high S-methyl Met

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Suppressed Methionine γ-Lyase Expression Causes Hyperaccumulation of S-Methylmethionine in Soybean Seeds

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One-sentence Summary:
Suppression of the methionine catabolism in soybean seeds causes hyperaccumulation of S-methylmethionine.

Author Contributions:
K.M. conceived this project, designed experiments, and wrote the article. T.T. analyzed metabolites, characterized recombinant enzyme, N.Y. performed crossbreeding and harvested seeds, M.I., T. S., and Y. Y. performed genotyping and positional cloning, K.I. provided enzymes, T.K. mined database and constructed phylogenetic trees, M.U. performed statistical analyses.

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Abstract

Several soybean (*Glycine max* L.) germplasms, such as Nishiyamahitashi 98-5 (NH), have an intense seaweed-like flavor after cooking because of their high seed S-methylmethionine (SMM) content. In this study, we compared the amounts of amino acids in the phloem sap, leaves, pods, and seeds between NH and the common soybean cultivar Fukuyutaka (FY). This revealed a comparably higher SMM content alongside a higher free methionine (Met) content in NH seeds, suggesting that the SMM-hyperaccumulation phenotype of NH soybean was related to Met metabolism in seeds. To investigate the molecular mechanism behind SMM hyperaccumulation, we examined the phenotype-associated gene locus in NH plants. Analyses of the quantitative trait loci in segregated offspring of the cross between NH and the common soybean cultivar Williams82 indicated that one locus on chromosome 10 explains 71.4% of SMM hyperaccumulation. Subsequent fine-mapping revealed that a transposon insertion into the intron of a gene, *Glyma.10g172700*, is associated with the SMM-hyperaccumulation phenotype. The *Glyma.10g172700*-encoded recombinant protein showed Met-γ-lyase (MGL) activity in vitro, and the transposon-insertion mutation in NH efficiently suppressed *Glyma.10g172700* expression in developing seeds. Exogenous administration of Met to sections of developing soybean seeds resulted in transient increases in Met levels, followed by continuous increases in SMM concentrations, which was likely caused by Met methyltransferase activity in the seeds. Accordingly, we propose that the SMM-hyperaccumulation phenotype is caused by suppressed MGL expression in developing soybean seeds resulting in transient accumulation of Met, which is converted into SMM to avoid the harmful effects caused by excess free Met.

Introduction

In the 2017/2018 market year, 346.2 million tons of soybean (*Glycine max* L. Merr.) were produced (FAOSTAT; http://www.fao.org/faostat/en/#home). This large amount of soybean production was driven by the high oil and protein contents of the plant. Soybean meal is widely used as animal feed and has high protein contents and a well-balanced amino acid profile. However, its nutritional value for monogastric animals could be improved by increasing its cysteine (Cys) and methionine (Met)
Accordingly, the regulatory mechanisms relating to Met and Cys biosynthesis in soybean have been investigated extensively (Hesse & Hoefgen, 2003, Galili et al., 2016, Krishnan & Jez, 2018, Amir et al., 2019). In the central region of Japan, specific soybean seeds have been cultivated for their seaweed odor, which is strongly related to dimethyl sulfide. Dimethyl sulfide is formed spontaneously from S-methylmethionine (SMM) during heating of the seeds for cooking (Morisaki et al., 2014). In a representative cultivar, Nishiyamahitashi 98-5 (NH), the SMM level in the seeds is more than 100-fold higher than in common soybean cultivars, such as Fukuyutaka (FY) (Morisaki et al., 2014). Because SMM is a direct product of Met metabolism by Met methyltransferase (MMT), it is assumed that the cultivars with higher SMM content are likely to metabolize Met and its derivatives via mechanisms that are distinctive from that in ordinary soybean cultivars.

Met is a biosynthetic member of the aspartate (Asp) family (Fig. 1) (Galili et al., 2016, Amir et al., 2019). Cystathionine γ-synthase (CGS) performs the crucial regulatory step in Met biosynthesis and determines the rate of Met production from O-phosphohomoserine (Galili et al., 2016). Free Met is used to form proteins, but a portion of Met is converted to SAM by a SAM synthetase. Another portion of free Met accepts a methyl group from SAM through MMT and is converted into SMM. Homocysteine methyltransferase (HMT) converts SMM back to Met. MMT and HMT constitute the SMM cycle, which seems to operate throughout plant tissues, including reproductive tissues of various plant species (Ranocha et al., 2001). In several flowering plants, SMM is produced in the leaves by MMT and is transported through the phloem toward the reproductive organs, where it is reconverted to Met by HMT (Bourgis et al., 1999, Cohen et al., 2017a). With developing seeds of Medicago truncatula, SMM is converted back to Met via HMT in seed coats, and Met released into the seed apoplast is taken up by seeds (Gallardo et al., 2007). Both the in situ formation of Met through Asp family enzymes and the biosynthesis of SMM in the leaves following phloem transport likely regulate Met contents simultaneously in the seeds (Cohen et al., 2017a, Amir et al., 2019). Met catabolism also controls Met levels in plant tissues. Met γ-lyase (MGL) is a pyridoxal phosphate (PLP)-dependent enzyme that metabolizes Met into 2-ketobutyric acid, methanethiol, and ammonia (Sato & Nozaki, 2009). 2-Ketobutyric acid can be metabolized to form isoleucine (Ile) even though this pathway is auxiliary to the major Ile biosynthetic pathway through threonine (Thr) deaminase (Joshi & Jander,
Based on accumulating studies of Met regulation in plant tissues, numerous attempts to improve Met levels in crops have been made through genetic engineering particularly of the Asp family pathway (Hacham et al., 2008, Hanafy et al., 2013, Song et al., 2013, Cohen et al., 2014, Kumar & Jander, 2017, Amir et al., 2019). Acceleration of SMM transport from non-seed tissues to seeds was also attempted to increase Met levels in seeds (Lee et al., 2008, Cohen et al., 2017a). As such, attempts to increase seed Met levels are becoming more successful but are sometimes disturbed by abnormal phenotypes of the plants (Krishnan & Jez, 2018, Amir et al., 2019). Severe growth retardation was observed in potato (*Solanum tuberosum*) plants overexpressing the feedback-insensitive CGS to form more Met and β-zein to store Met (Dancs et al., 2008). Tobacco (*Nicotiana tabacum*) plants overexpressing CGS and with elevated free Met levels also had increased sensitivity to oxidative stress (Hacham et al., 2017). In addition, *Arabidopsis* seeds overexpressing a mutant CGS accumulated Met to 2.5-fold higher levels, and these conditions were associated with increased expression of stress-related transcripts (Cohen et al., 2014, Cohen & Amir, 2017). These studies suggest that Met levels are tightly controlled in plant tissues and that excessive free Met is deleterious to plant health. Yet, the mechanisms by which Met levels are regulated in some tissues remain poorly understood.

The soybean SMM-hyperaccumulation phenotype, like that of NH, was assumed to be attributable to a genotype related to Met metabolism in seeds. We investigated the genotype of SMM-hyperaccumulating soybean plants and identified the gene that is responsible for SMM accumulation in soybean. Through analyses of gene function and NH phenotypes, we propose a mechanism underlying SMM hyperaccumulation.
RESULTS

S-Methylmethionine and Free Methionine Levels

The quantities of SMM and free Met were determined in the leaves, pods, and seeds of two soybean cultivars, Fukuyutaka (FY) and Nishiyamahitashi 98-5 (NH) at the flowering stage, immature green seed stage, mature green seed stage, and in dry seeds (Fig. 2). The seed coats were removed from the other parts of seeds (i.e., cotyledons, plumules, and radicles) before analyses to avoid mixing tissues of maternal genotype (i.e., seed coats) and those of offspring genotypes (cotyledons, plumules, and radicles). The contents of SMM and free Met were low in the leaves, and no significant differences were identified between FY and NH. The SMM level in the pods seemed to be a little higher than that found in the leaves, but a statistically significant difference in its level between FY and NH was hardly detected. The free Met level in the NH pods at the immature green stage was significantly lower than that of FY, but the level in the FY pods lowered at the mature green stage to the level found in the NH pods. The levels of SMM in the NH seeds were 8.0- and 15.6-fold higher at the immature and mature green stage, respectively, than those found in the FY seeds. A substantial amount of SMM was detected in dry NH seeds, whereas it was under the detection limit in the dry FY seeds. The levels of free Met in the NH seeds were significantly higher at the mature green stage and in dry seeds than those found in the FY seeds.

The SMM level in the phloem exudate collected through a cut petiole of NH at the seed-developing stage was significantly higher than that found for FY, whereas the level of free Met was lower than that found for FY (Table 1). The levels of SMM and free Met in phloem exudate collected using the same procedure through Arabidopsis petioles at the seed-filling stage were more than 93-fold higher than those found for NH and FY.

The free amino acid contents of mature seeds were mostly similar between FY and NH, yet the histidine (His), Met, phenylalanine (Phe), Thr, and homoserine contents were significantly higher in NH than FY seeds (Fig. 3). Homocysteine was under the detection level (4.2 µg g⁻¹) in both NH and FY. The total protein contents, the protein profiles examined using Coomassie Brilliant Blue staining after SDS-PAGE (Supplemental Fig. S1), and total amino acid contents showed no significant differences.
between the FY and NH seeds (Supplemental Table S1).

**Positional Cloning of the Gene Responsible for Hyperaccumulation of S-Methylmethionine**

The contents of SMM and free Met were determined with four F\(_1\) seeds after reciprocal crossing of the FY and NH cultivars. The hyperaccumulation of SMM was only evident in self-pollinated NH seeds, and the maternal and paternal genotypes did not play a significant role in the accumulation of SMM in seeds (Fig. 4). Moreover, F\(_2\) seeds of the FY × NH cross were segregated into high SMM/low SMM at a ratio of 3/17 with a consistent segregation ratio of 1:3 (Chi-squared test, \(P = 0.30\); Supplemental Fig. S2A).

These data indicate that hyperaccumulation of SMM is essentially regulated by a single recessive allele. In order to identify the gene responsible for hyperaccumulation of SMM, we crossbred NH to Williams 82 (WI) cultivar. WI was used because the SMM contents in WI seeds were as low as those in FY seeds (see below) and also because the reference genome sequence was produced with WI (Schmutz et al., 2010). A total of 156 F\(_5\) recombinant inbred lines (RILs) were generated from the cross between NH and WI, and SMM levels in their mature seeds were determined (Supplemental Fig. S2B).

Quantitative trait loci (QTL) analyses with molecular markers indicated that an allele near a simple sequence repeat marker (Satt477) on chromosome 10 explained 71.4% of the phenotypic variation (Fig. 5A). No other QTL with a significant influence on the hyperaccumulation of SMM was detected. Fine-mapping of the allele responsible for the hyperaccumulation of SMM in F\(_6\) and F\(_7\) residual heterozygous lines narrowed down this region to a 12-kb sequence on chromosome 10 (Fig. 5B). Furthermore, examination of the Phytozome soybean genome sequence database (https://phytozome.jgi.doe.gov/pz/portal.html) revealed only one open reading frame (ORF) of Glyma.10g172700 in this region. Glyma.10g172700 comprises two exons flanking a single intron. Finally, sequence analyses of Glyma.10g172700 in NH (GenBank acc. no. MK887190) indicated that a *copia*-type retrotransposon (AB370254, Liu et al. 2008) was inserted into the intron.

**Hyperaccumulation of SMM Correlates Well with Transposon Insertion in the Glyma.10g172700 Gene**
We collected local soybean cultivars in Nagano Prefecture, Japan (Supplemental Fig. S3). These were previously shown to have differing SMM levels (Morisaki et al. 2014). We extracted genomic DNA and then amplified the Glyma.10g172700 gene using primers for 5′- and 3′-termini of its deduced ORF. In normal soybean cultivars, such as FY and WI, the resulting DNA fragment was 2885 bp, as expected from genome sequences in the Phytozome. The Glyma.10g172700 gene length was the same in five of the ten local cultivars as that detected in normal cultivars, but was longer in the other five cultivars and was similar in length to that amplified from the NH cultivar (ca. 9.0 kb; Fig. 6A). Therefore, it was expected that these latter five soybean cultivars carry an inserted transposon in the Glyma.10g172700 gene of the same size as the inserted transposon in NH. Determinations of SMM levels in seeds of these cultivars showed higher SMM levels in cultivars harboring the Glyma.10g172700 gene with the transposon insertion than in those without the transposon-insertion (Fig. 6B). Free Met levels were higher in some seeds having the transposon insertion in Glyma.10g172700 gene than those without the insertion, but the correlation was not always evident. Reverse transcription quantitative PCR (RT-qPCR) analyses on RNA extracted from maturing seeds showed that the lower levels of transcript derived from Glyma.10g172700 were detected with cultivars that had the transposon insertion in Glyma.10g172700 (Fig. 6C). The product sizes obtained with RT-PCR with NH and FY seeds with excess amplification cycles using primers for 5′- and 3′-termini of GmMGL1 ORF were same (Supplemental Fig. S4), and no sign of alternative splicing was detected.

**Glyma.10g172700 Encodes a Functional Methionine γ-Lyase**

The ORF of Glyma.10g172700 encodes a protein of 48,069 Da, yet the deduced protein sequence had no predictable targeting signal in TargetP analyses (http://www.cbs.dtu.dk/services/TargetP/), suggesting a cytosolic location of the protein. The Glyma.10g172700 gene was tentatively assigned as a gene encoding an MGL, and the deduced protein sequence had 77.2% and 78.6% identities with Arabidopsis MGL (Q9SGU9, AEE34271.1) and melon (Cucumis melo) MGL (M1NFB7, NP_001315378.1), respectively (Rébeillé et al. 2006, Gonda et al. 2013) (Supplemental
Moreover, the protein sequence has a motif (Ser237-Xaa-Xaa-Lys240) that is conserved in pyridoxal-5′-phosphate (PLP) enzymes of the γ-subfamily and associates with the cofactor PLP (Martel et al. 1987, Sato & Nozaki, 2009) (Supplemental Fig. S5). Tyr142, Asp216, and Arg410 residues are also involved in substrate binding and catalysis at appropriate positions (Goyer et al. 2007), and Gly144 is conserved as in Arabidopsis and melon MGLs that retain restricted substrate specificity for L-Met (Gonda et al. 2013) (Supplemental Fig. S5).

Glyma.10g172700 cDNA was cloned using RNA that was extracted from developing WI seeds. We expressed the recombinant protein as an N-terminal His-tagged protein and purified it using Ni²⁺-affinity chromatography (Fig. 7A). Subsequently, L-Met reacted with the recombinant protein in the presence of PLP, and the products were converted into their 3-methyl-2-benzothiazoline hydrazone derivatives. This derivatization resulted in increased absorption at 320 nm (Fig. 7B), suggesting the formation of an aliphatic carbonyl compound (Esaki & Soda, 1987, Inoue et al., 1995). To confirm its structure, the reaction product that was extracted using ethyl acetate was reacted with N,O-bis(trimethylsilyl)trifluoroacetamide and was then analyzed using GC-MS. A peak at the retention time of 9.5 min was assigned as trimethylsilylated 2-ketobutyric acid by comparing its MS profile and retention time with that prepared from a standard compound (Fig. 7C and 7D). Accordingly, we concluded that Glyma.10g172700 encodes MGL that catalyzes γ-elimination of L-Met. We denoted the gene GmMGL1. This reaction had optimal activity at pH 7.0 and followed Michaelis–Menten kinetics, with $K_m$ and $V_{max}$ values of 7.72 mM and 0.55 µmol mg⁻¹ min⁻¹, respectively (Supplemental Fig. S6).

Comparison of Methionine Metabolism Genes in Fukuyutaka and Nishiyamahitashi 98-5

BLAST searches for GmMGL1 indicated that the soybean genome encodes the MGL-like genes Glyma.02g087900 and Glyma.13g001200 (hereafter referred to as GmMGL2 and GmMGL3, respectively). These genes encode proteins with the amino acid signatures that are conserved among the MGLs described above (Supplemental Fig. S5). Among the three GmMGLs, GmMGL2 and -3 showed higher sequence similarity than the other combinations. The phylogenetic analyses with MGL and MGL-like
sequences found in several plant species indicated that GmMGL1 is located in a clade
different from the one GmMGL2 and GmMGL3 belong to (Supplemental Fig. S7).

The RT-qPCR analyses of the FY seeds showed that GmMGL1 mRNA
expression was enhanced at the early stage of seed maturation (from stages one to two)
and remained constant thereafter until the matured green stage (stage five; Fig. 8A).
However, GmMGL1 expression was considerably lower in NH seeds than in FY seeds
throughout seed development and differed little between developmental stages.
GmMGL2 and GmMGL3 expression levels were transiently induced during stage three,
but only in NH seeds, and they were not significantly different between FY and NH
cultivars at the other stages. The MGL activity in crude protein extracts prepared from
developing seeds (at stage four) of NH (5.86 ± 0.81 nmol h⁻¹ g⁻¹) was significantly
lower than that detected in FY seeds (12.1 ± 2.28 nmol h⁻¹ g⁻¹) (P < 0.05, Student’s t-test,
n=4). GmMGL1 expression was significantly lower in the leaves, stems, and roots of
NH plants than in the leaves, stems, and roots of FY plantlets at the leaf-expansion stage
before flowering (Fig. 8B). Among these, the transcript levels of GmMGL2 and
GmMGL3 were highest in the leaves and did not differ significantly between the
soybean cultivars.

Because the genes of Met metabolism are regulated coordinately (Liao et al.
2012), we examined the effects of GmMGL1 suppression on the expression of
cystathionine γ-synthase (CGS), which catalyzes a key regulatory step of the Met
biosynthetic pathway (Hesse & Hoeftgen, 2003), and of Met methyltransferase (MMT)
and homocysteine methyltransferase (HMT), which are directly involved in the
formation and decomposition of SMM (Fig. 1; Cohen et al., 2017a). In SoyBase
BLAST searches using AtCGS (At3g01120), AtMMT (At5g49810), and AtHMT1
(At3g25900) as queries, two CGS homologs (Glyma.18g261600 and Glyma.09g235400;
referred to as GmCGS1 and GmCGS2, respectively), two MMT homologs
(Glyma.12g163700 and Glyma.16g000200; GmMMT1 and GmMMT2, respectively),
and three HMT homologs (Glyma.08g261200, Glyma.19g158800, and
Glyma.20g148900; GmHMT1, GmHMT2, and GmHMT3, respectively) were identified.
RT-qPCR analyses of mRNA expression from soybean seeds at different developing
stages revealed no significant differences between FY and NH, except for GmCGS1/2,
GmHMT2, and GmHMT3 at stage one (Supplemental Fig. S7).
Administration of Methionine Causes Accumulation of $S$-Methylmethionine in Developing Seeds

Suppression of *GmMGL1* expression in developing soybean seeds might lead to the accumulation of Met, which would otherwise be catabolized to ammonia, methanethiol, and 2-ketobutyric acid. One of the alternative fates of free Met is the formation of SMM via the activity of MMT (Fig. 1), which is likely to occur in developing soybean seeds because of the substantial expression levels of *GmMMT1* and -2 (Supplemental Fig. S8). To examine whether MMTs are active in developing soybean seeds, we conducted a Met-feeding experiment. We fed free Met solution onto slices of immature green soybean seeds of the FY and NH cultivars and determined SMM and Met contents using LC-MS/MS (Fig. 9). Inclusion of 1 or 5 mM Met in the solution covering the cut surfaces of the FY seeds yielded incremental increases in the SMM contents, and after 24 h of treatment, the SMM levels increased up to 37.9 and 135 µg g$^{-1}$ for the 1 and 5 mM Met solutions, respectively. The SMM levels of the NH seeds also showed similar incremental increases, but in a more prominent manner, and after 24 h, the SMM levels increased up to 214 and 316 µg g$^{-1}$ for the 1 and 5 mM Met solutions, respectively. The SMM level in the NH seeds treated only with water also significantly increased to 80.0 µg g$^{-1}$ after 24 h. No significant difference in the Met levels was observed for FY and NH seeds treated with 1 mM Met in comparison with the levels in seeds treated with water except those after 24 h with NH seeds; however, following feeding with 5 mM Met solution, the Met levels increased significantly in both FY and NH, with more prominent increases in NH. The highest Met level for FY was 9.71 µg g$^{-1}$ at 8 h and for NH was 27.2 µg g$^{-1}$ at 24 h.

To confirm substrate–product relationships, we performed feeding experiments using $^{13}$C-Met (C5, 99 atom %) with NH seeds. Subsequent LC-MS/MS analyses of SMM in the extract confirmed that it was predominantly formed from $^{13}$C-Met, as indicated by $m/z$ 169.1 and 106.1 that were generated from $^{13}$C5-labeled SMM (Fig. 9B).
Using a molecular genetic approach to locate the allele responsible for hyperaccumulation of SMM, we found that a transposon insertion into the intron of \textit{GmMGL1} is strongly associated with SMM hyperaccumulation in soybean seeds. Expression of the \textit{GmMGL1} gene and, accordingly, MGL activity in seeds were suppressed due to the transposon insertion. Under these conditions, Met catabolism would be low in seeds, leading to Met accumulation. Because excess Met levels have been associated with various adverse effects in plant tissues, we hypothesized that surplus Met in soybean seeds with MGL deficiencies was converted to the better-tolerated compound SMM by MMT activity (Fig. 10). In line with this hypothesis, Met-feeding experiments showed that surplus Met was efficiently converted into SMM in green mature soybean seeds and that the conversion was more prominent with MGL-deficiency.

\section*{The Transposon Insertion Suppresses Expression of \textit{GmMGL1}}

The insertion of a transposon into the intron of \textit{GmMGL1} strongly suppressed its mRNA expression, whereas processing of the corresponding precursor mRNA through splicing at the inherent positions was little affected by the intronic insertion. Intronic insertion of transposons generally have minimal impacts on gene expression levels or splicing events (Hirsch & Springer, 2017). For example, insertion of the retrotransposon Ty1-\textit{copia}, which is approximately 5000 bp in length, had little impact on transcription with flax (\textit{Linum usitatissimum}) (Galindo-González et al. 2016). Hence, the present marked repressive effects of transposon insertion into the intron of \textit{GmMGL1} are unique. Alternatively, in a previous study of soybeans, transposons in or near a gene were related to increased CHG/CHH methylation, and consequently, lower expression levels (Kim et al., 2015). Hence, epigenetic regulatory mechanisms likely play roles in the present repression of \textit{GmMGL1}. As such, analyses of DNA methylation should be one of the next priority research areas to reveal more details about the mechanisms of this type of gene suppression.

\section*{Suppression of \textit{GmMGL1} Accounts for SMM Hyperaccumulation
In plant tissues, Met levels are tightly regulated through biosynthesis and catabolism (Fig. 1). Higher free Met contents (in addition to SMM contents) in NH seeds than in FY seeds prompted us to assume that GmMGL1 participated in controlling free Met levels in seeds. If this is the case, in the absence of substantial MGL activity as found with NH, free Met levels should increase, and surplus Met could be converted into SMM by the MMT activity in seeds. This scenario showed no fundamental inconsistency in the results obtained in this study about the Met metabolism of NH. The function of MGL to adjust free Met levels has been demonstrated with Arabidopsis, in which knock-out of the AtMGL gene increased free Met contents in leaves, flowers, and seeds (Goyer et al., 2007, Joshi & Jander, 2009). Notably, the Arabidopsis knock-out mutant contained 4.5-fold higher SMM contents in leaves than its parental wild type; therefore, it is presumed that conversion of surplus Met to SMM is common among plants. In support of this hypothesis, SMM accumulation has been reported in multiple transgenic plants with high free Met levels (Kim et al., 2002, Hacham et al., 2008, Hacham et al., 2017). Taken together, it is suggested that GmMGL1 was involved in controlling free Met levels in developing soybean seeds. SMM-hyperaccumulation is likely to be a consequence of suppressed GmMGL1, and a subsequent “fail-safe” system employing MMT activity to avoid the adverse effect of excess Met.

One of the MGL products, 2-ketobutyric acid, is partly converted to Ile in Arabidopsis, especially under drought stress (Rébeillé et al., 2006, Joshi & Jander, 2009). However, we found no significant difference in either free or total Ile content between NH and FY seeds, suggesting that GmMGL1 accounted little for Ile formation in developing seeds. On the contrary, dry NH seeds had higher free Thr, Phe, His, and homoserine levels in addition to increased free Met and SMM levels. Therefore, the MGL deficiency is likely to cause pleiotropic effects on the metabolism of other amino acids. Accumulation of free amino acids was often observed in several transgenic plants generated to enhance Met levels (Hanafy et al., 2013, Cohen et al., 2014, Hacham et al., 2017, Huang et al., 2014). Accordingly, it is suggested that the adverse effect of surplus Met in NH induced a stress response that led to higher Thr, Phe, His, and homoserine levels.

Limited Significance of Phloem Transport of SMM for SMM Hyperaccumulation
It has been reported for several plant species, including *Arabidopsis* and wheat (*Triticum aestivum*), that SMM formed in vegetative tissues is transported to seeds through the phloem (Bourgis et al., 1999, Lee et al., 2008, Frank et al., 2015, Cohen et al., 2017b). Therefore, it was assumed that the phloem transportation of SMM formed in vegetative tissues to seeds could also be accountable for hyperaccumulation of SMM in NH seeds. The level of SMM in the phloem exudate collected from leaves of NH was higher than that found for FY plants. Therefore, transportation of SMM through the phloem toward the seeds is likely to be at least partly accountable for the hyperaccumulation of SMM in NH seeds. The SMM levels in the pods of both NH and FY showed a tendency to decrease during maturation; thus, the transportation of SMM from the pods to the seeds should also be taken into consideration. However, it was remarkable that the SMM levels found in the phloem exudate of NH plants were 291-fold lower than that in *Arabidopsis* phloem exudate. Concordant with the fact that amino acid levels in soybean phloem exudate were 8-fold lower than those in *Arabidopsis* and wheat (Bourgis et al., 1999), our observation of low levels of SMM in soybean phloem exudate prompted us to consider that the contribution of phloem transport of SMM toward seeds for SMM hyperaccumulation is not negligible, but is limited. Furthermore, the results of the reciprocal crossing of NH and FY indicated that maternal as well as paternal genotypes did not play a substantial role in determining the seed phenotype of SMM hyperaccumulation, which was caused only when the genotype of the seeds was homozygous for *mgl1*, and thus, the involvement of vegetative organs in SMM hyperaccumulation in the seeds of NH is likely limited.

The extensively lower levels of SMM and Met in the soybean phloem, compared to levels in the *Arabidopsis* phloem, are noteworthy because the levels of free and total amino acids including Met are more than 10-fold higher in soybean seeds than in *Arabidopsis* seeds (Cohen et al., 2017c). Source-sink transport of amino acids from vegetative organs to seeds, and *in situ* synthesis of amino acids in seeds, might be accountable in different ways for accumulation of amino acids in seeds in these two plant species.

**Exogenous Methionine is Converted into S-Methylmethionine**
The addition of Met at 1 mM onto developing seeds of FY had only a slight effect on Met or SMM concentrations, probably because Met, supplied exogenously, was appropriately catabolized in part by intrinsic GmMGL1 activity in FY seeds. This Met-catabolizing system was, however, overwhelmed by treatments with 5 mM Met solution, as indicated by transient increases in free Met in developing FY seeds, followed by a significant increase in the amount of SMM. The accumulation of Met and SMM seemed to be further emphasized for NH. This result indicated that both NH and FY seeds exhibited enough MMT to convert Met supplied exogenously into SMM. The exaggerated responses of accumulation of Met and SMM in NH seeds could be explained by the lower activity of GmMGL1 in the seeds, and the surplus Met left behind in the seed tissues being converted into SMM by MMT. SMM has been considered to be a tentative storage form of Met to avoid excessive Met concentrations (Mudd & Datko, 1990). In agreement, the SMM concentrations in soybean seeds treated with 5 mM Met solution were much higher than the Met concentrations, suggesting that SMM is a safer storage form of Met. In summary, SMM-hyperaccumulation was caused exclusively by suppression of GmMGL1 that regulates free Met levels in developing soybean seeds. MMT activity in developing soybean seeds should be sufficient to convert surplus Met into SMM, irrespective of MGL activity (Fig. 10).

The present data indicate that the genetic suppression of MGL in soybean seeds affects Met metabolism, favors hyperaccumulation of SMM, and provides further insights into the regulatory mechanisms of Met metabolism. This knowledge should be taken into consideration when attempting to modify Met metabolism in soybean seeds.

MATERIALS AND METHODS

Plant Materials. Seeds of the soybean cultivars NH, FY, and WI were grown and harvested during 2016 in an experimental field at the Nagano Vegetable and Ornamental Crops Experiment Station, Shiojiri City (E 137°57', N 36°06'; annual mean temperature, 11°C). For the quantification of SMM levels in leaves, pods, and seeds, NH and FY plants were grown and harvested in 2019 in an experimental field at the Yoshida campus of Yamaguchi University, Yamaguchi City (E 131°47', N 34°15'; annual mean
temperature, 15°C). To prepare samples at a similar developmental stage, each organ was collected at slightly different dates because FY showed a little early growth phenotype when compared with NH. For collecting seeds for the RT-qPCR analyses and Met-feeding experiments, plants were grown and harvested in 2017 and 2019 in an experimental field at the Yoshida campus of Yamaguchi University. For extraction of RNA from leaves, stems, and roots, plantlets (NH and FY) were germinated with vermiculite and were transplanted to the hydroponic culture system (Kuroda & Ikenaga, 2015) with a 12-h light (at 27°C)/12-h dark (22°C) cycle. NH and FY plants were cultured under these conditions for 40 and 34 days, respectively. Seeds of the cultivars Shinano-Kurakake, Nishiyamahitashi 94-5, Sinanomachi Arasebara Kurakake, Togakushi Morozawa Ganimame, Usuda Zairai, Shinano Midori, Nishiyamahitashi 94-1, Tousanhitashi 94-1, Ogawa Zairai 5, and Chino Zairai 4 were harvested in 2012 and 2015 from the experimental field at the Nagano Vegetable and Ornamental Crops Experiment Station, or in 2019 from the experimental field at the Yoshida campus of Yamaguchi University.

**Determination of SMM contents**

Seed coats were carefully removed and soybean seeds containing hypocotyls were then powdered using a multi-beads shocker (PM2000, Yasui Kikai, Osaka, Japan) equipped with stainless metal cones (MC-0316S, Yasui Kikai) and operated at 2500 rpm for two 30-s periods with a 10-s interval. Powder samples of 20 mg were then mixed with 1 mL of distilled water containing 50 µg mL\(^{-1}\) L-methionine-S-methyl d6 sulfonium chloride (d6-SMM; Toronto Research Chemicals Inc. Toronto, Ontario, Canada) and were then placed in a water bath sonicator (US-2, SND Co. Ltd., Suwa City, Nagano, Japan) for 10 min. The resulting suspensions were centrifuged at 15,000 rpm (20,000 \(\times\) g) for 10 min at 4°C. Subsequently, 100-µL aliquots were added to Strata C18-E (100 mg mL\(^{-1}\); Phenomenex Inc. Torrance, CA, USA), and SMM was eluted twice with 0.5-mL aliquots of distilled water. Eluates were cleared using an Ekicordisc 3 (0.45 µm, 3 mm, Pall Co., Tokyo, Japan).

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) analyses were performed using an AB Sciex (Framingham, MA, USA) 3200 Q-TRAP LC-MS/MS system equipped with a Prominence UFLC (Shimadzu, Kyoto, Japan) in multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI).
Chromatography was conducted using a Discovery HS F5 column (15 cm × 2.1 mm, 3 µm; Supelco, Bellefonte, PA, USA), and HPLC and MS analyses were performed using previously described conditions (Morisaki et al., 2014). To quantify $^{13}$C-labeled SMM formation from $^{13}$C-Met (Cambridge Isotope Laboratories, Tewksbury, MA, USA), MS analyses were performed in the enhanced production mode with $m/z$ 169.2 (for labeled SMM) or 164.2 (for non-labeled SMM) using positive electron spray ionization (ESI) with a capillary voltage of 4500 V, an arbitrary source temperature, a curtain gas of 10 (arbitrary units), ion source gases 1 and 2 of 16 and 0 (arbitrary units), respectively, a declustering potential of 26 V, and an entrance potential of 2.5 V. The level of free Met was also analyzed using the same LC-MS/MS condition but with different MRM transitions. The detailed parameters for LC-MS/MS analysis are shown in Supplemental Table S2.

To collect phloem exudate, fully expanded leaves of NH and FY plants at their seed-filling stage were detached with the base of the petiole under a solution of 20 mM EDTA (pH 7.0) and immersed into 0.2 mL of the same solution in 0.5-mL microtubes, and placed in humid chambers in the dark at 25°C (Urquhart & Joy, 1981). After 5 h, the EDTA solution was collected and used for the LC-MS/MS analyses as described above to estimate the concentration of SMM and free Met. As a comparison, fully expanded rosette leaves of *Arabidopsis* (ecotype Ws-0) were used at its seed-filling stage.

**Determinations of amino acid and protein contents**

Soluble amino acids were extracted from dry seed flour (20 mg) as described by Hanafy *et al.* (2013). Flour samples were suspended in 240 µL of 3% (w/v) sulfosalicylic acid and were suspended with vigorous shaking for 30 min. After centrifugation at 12,000 × g for 10 min at 25°C, precipitates were extracted two more times as described above. Combined supernatants were then filtered and analyzed using LC-MS/MS with an ESI interface (Tomita et al., 2016) as detailed above. An Intrada amino acid column (100 × 3 mm i.d., 3 µm; Imtakt, Kyoto, Japan) was used with a column temperature of 40°C. Mobile phases were applied at 0.4 mL min$^{-1}$ and comprised solvents A (acetonitrile/formic acid at 100/0.3, v/v) and B (0.1 M acetonitrile/ammonium formate at 20/80, v/v) at 15% B for 10 min, followed by a linear increase from 15% B to 60% B over 15 min, then from 60% B to 100% B over 5 min, and then 100% B for 10 min. The
injection volume was 4 µL. The MS system was operated in MRM mode using positive ESI with a capillary voltage of 3000 V, a source temperature at 550°C, a curtain gas of 35 (arbitrary units), ion source gases 1 and 2 of 80 and 60 (arbitrary units), respectively, a declustering potential of 16 V, and an entrance potential of 5 V. MRM transitions of the precursor to product ions used for the quantification and collision energy are summarized in Supplementary Table S2. Quantification was done using calibration curves constructed using amino acid mixture standard solution (Wako Pure Chemicals, Osaka, Japan) supplemented with homoserine and homocysteine (Wako Pure Chemicals).

Proteins were extracted from 20-mg samples of soybean flour using 500-µL aliquots of 10 mM Tris HCl (pH 8.0) containing 2.5% (w/v) sodium dodecyl sulfate and 10 mM 2-mercaptoethanol. Proteins were extracted for 10 min with vigorous vortexing at the highest speed using a Micro Tube Mixer (MT-360, Tomy Seiko, Tokyo, Japan) and subsequent treatment with a water bath sonicator (120 W, 38 kHz, US-2, SND Co. Nagano, Japan) for 10 min at 25°C. A clear protein solution was obtained after centrifugation at 20,000 × g for 20 min at 4°C. Protein contents were determined using Protein Assay (Bio-Rad, Hercules, CA, USA). After the separation of proteins with SDS-PAGE, each protein band was quantified using ImageJ 1.48v (http://imagej.nih.gov/ij).

Total amino acids were determined according to the method employed by Ishimoto et al. (2019). In brief, 10 mg of soybean seed flour was hydrolyzed with 1 mL of 6N HCl at 100°C for 22 h under argon. The dried hydrolysate was dissolved in 0.02N HCl and served to LC-MS/MS analysis, as described above. To analyze total Cys and Met, the flour was oxidized with 1 mL of performic acid at 0°C for 16 h to give cysteic acid and methionine sulfone prior to acid hydrolysis.

Analyses of quantitative trait loci for S-Methylmethionine contents

RILs, including 155 F₅ lines were developed from a single seed descendant of the cross between NH and WI. Total DNA extraction and linkage map construction by simple sequence repeat markers (WSGP ver. 2) were performed as described previously (Fujii et al., 2018). SMM contents in each RIL were quantified using bulked F₆ seeds that were derived from the F₅ individual. Because SMM contents varied widely among RILs, QTL analysis was conducted with the common logarithm (log) value for contents.
QTL analyses were performed using composite interval mapping, as implemented in QTL Cartographer 2.5 software (Wang et al., 2005). The genome was scanned at 1-cM intervals. One thousand permutation tests were conducted to determine the threshold value of the limit of detection score.

**Mapping of responsible genes**

F6 and F7 progenies of parent individuals with hetero genotypes in the chromosomal region that corresponded with QTL were used for fine genetic mapping of responsible genes. Initial QTL analyses indicated a region of around 486 kb ranging from Satt477 and Satt592 on chromosome 10. A population of F7 progeny was then used to delimit the locus using marker-genotyping and SMM quantification. Gene mapping was performed using the BARCSOYSSR markers (BSSR) described by Song et al., 2010. The tail sequence CACGACGTTGTAAAACGAC was added to the 5’ end of the reverse primer and oligonucleotides that were complementary to tail sequences were fluorescently labeled with 6-FAM, VIC, NED, and PET (Thermo Fisher Scientific, Waltham, MA, USA) before addition to PCR reaction solutions. PCRs and PCR fragment length analyses were conducted following Fujii et al. (2018). Only one ORF (i.e., Glyma.10g172700) was identified in the region after the second round of fine-mapping.

**cDNA cloning and expression of recombinant proteins**

Total RNA was extracted from developing seeds of WI using Qiagen RNaseat Plant Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was degraded using DNA-free Kits (Ambion, Thermo Fischer Scientific, Waltham, MA, USA), and cDNA was synthesized using SuperScript VILO cDNA Synthesis Kits (Invitrogen). Subsequently, Glyma.10g172700 (GmMGL1) cDNA was PCR amplified using primers for 5’ and 3’ ends of the translation initiation site (Supplementary Table S3). The resulting PCR products were cloned into pGEM T-easy vectors (Promega, Madison, WI, USA) for sequencing. PCR products were then sub-cloned into the EcoRI-XhoI site of the pET24a vector (Merck) and the resulting plasmid was transfected into Escherichia coli Rosetta2 (DE3) pLysS cells (Merck). Cells were subsequently grown in Luria broth supplemented with kanamycin (50 μg mL−1) and
chloramphenicol (30 µg mL\(^{-1}\)) at 37°C to an optical density of 0.6–0.8 at 600 nm. After chilling the cultures on ice for 15 min, isopropyl β-D-1-thiogalactosylpyranoside was added to a concentration of 1 mM, and cells were then cultured at 30°C for 16 h.

Cells from 50-mL cultures were recovered by centrifugation at 4,000 \(\times\) \(g\) for 20 min at 4°C and were resuspended in 5 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 0.01% (w/v) dithiothreitol and 1.3 mM pyridoxal phosphate (PLP).

After the addition of 5-µL aliquots of 100 mM phenylmethane sulfonyl fluoride and 50 mg mL\(^{-1}\) lysozyme, suspensions were kept on ice for 15 min, and cells were then disrupted using a tip-type ultrasonic disruptor (UD-211, Tomy Seiko, Tokyo, Japan).

After centrifugation at 12,000 \(\times\) \(g\) for 10 min, supernatants were directly applied to a column (2 mL) of Ni-NTA agarose (Nacalai Tesque, Kyoto, Japan) that had been equilibrated with 100 mM potassium phosphate buffer (pH 7.5) containing 0.01% (w/v) dithiothreitol and 10 µM pyridoxal phosphate (PLP). The column was then washed with 10 mL of the same buffer containing 10 mM imidazole, and His-tagged recombinant proteins were eluted with 10 mL of the same buffer containing 250 mM imidazole.

Active fractions were finally combined and desalted using a PD-10 column (GE Healthcare, Chicago, IL, USA).

**Enzyme assays**

Methionine-γ-lyase enzyme assays were performed according to previous reports (Esaki and Soda, 1987, Takakura et al., 2004). Given volumes of purified enzyme solution were mixed with 100 mM K-phosphate buffer (pH 7.5) containing 10 µM PLP and 40 mM L-Met and were incubated at 30°C with gentle shaking. Aliquots of 2 mL were taken every 2 min and were added to 100 µL of 50% (w/v) trichloroacetic acid. After centrifugation at 20,000 \(\times\) \(g\) for 8 min at 25°C, 0.8-mL supernatants were mixed with 1.6-mL aliquots of 1 M sodium acetate buffer (pH 5.0) and 0.6-mL aliquots of 0.1 % (w/v) 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH). Reaction tubes were then tightly closed and incubated at 50°C for 40 min. The MGL product 2-ketobutyric acid was quantified according to absorbance at 278 nm, which is derived from the MBTH derivative of 2-ketobutyric acid. The absorbance at 0 min was subtracted from later measurements, and a calibration curve was generated using authentic 2-ketobutyric acid (Sigma-Aldrich). The structure of 2-ketobutyric acid was confirmed using GC-MS after converting the acid into a trimethylsilylated product.
(Gonda et al. 2013). After incubating the recombinant enzyme with L-Met and PLP overnight in a total volume of 3 mL, reactions were terminated by adding 40-µL aliquots of 6N HCl. Products were then extracted in 2 mL of ethyl acetate and extracts were washed once with 1 mL of water prior to removing the solvent under a stream of nitrogen-gas. After confirming complete dryness, extracts were incubated with 100-µL aliquots of anhydrous pyridine at 25°C for 90 min. Thereafter, 100-µL aliquots of O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Tokyo Chemical Industry, Tokyo, Japan) were added and incubated for 90 min. GC-MS was performed using a QP-5050 (Shimadzu, Kyoto, Japan) instrument equipped with a 0.25 mm × 30 m DB-5MS column (film thickness; 0.25 µm, Restek, Bellefonte, PA, USA). The column temperature was programmed as follows: 50°C for 1 min, increasing by 5°C min⁻¹ to 120°C, then by 20°C min⁻¹ to 280°C, and then maintenance at 280°C for 1 min. The carrier gas (He) was delivered at a flow rate of 30 kPa. Injector and interface temperatures were 240 °C and 300 °C, respectively. The mass detector was operated in electron impact mode with an ionization energy of 70 eV. Compounds were assigned by comparing MS profiles and retention times with those of TMS-derivatized 2-ketobutyric acid that was prepared separately.

To determine MGL activity in developing soybean seeds, the seeds of NH and FY at seed developmental stage four (cf. the photo in Fig. 8) were homogenized with four volumes of 50 mM sodium phosphate buffer (pH 7.5) containing 5% (w/v) sorbitol, 10 mM dithiothreitol, 5 mM sodium metabisulfite, and 2.5 µM PLP. After centrifugation at 20,000 × g for 20 min at 4°C, the cleared supernatant (0.5 mL) was mixed with 0.25 mL of 0.2 M Met in a buffer (50 mM sodium phosphate, pH 7.5 containing 2.5 µM PLP) in a total volume of 4.5 mL, and incubated at 30°C with a shaking water bath for 17 h. After the reaction, the reaction mixture was acidified by adding 66.6 µL of 6N HCl, and then, the products were extracted with 4 mL of ethylacetate. After washing the ethylacetate extract with 1 mL of saturated NaCl solution, the extract was used for derivatization with BSTFA as described above. The amount of 2-ketobutyric acid was determined using GC-MS analysis and a calibration curve constructed with authentic 2-ketobutyric acid. Molecular ion chromatograms with m/z 73 and 115 were used for quantification.

Genomic PCR and Reverse Transcription Quantitative PCR Analysis
Genomic DNA was isolated according to Hanafy et al. (2013). Total RNA was isolated using the Qiagen RNeasy Plant Mini Kit according to the manufacturer’s instruction. Total RNA (0.25 µg) was then reverse transcribed with 2.5-µM aliquots of oligo(dT)$_{15}$ primer (Invitrogen) and ReverTra Ace (derived from moloney murine leukemia virus reverse transcriptase; Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Reverse transcription quantitative PCR (RT-qPCR) was performed with an Eco Real-Time PCR System (Illumina). Ct values for the genes of interest were normalized to means of the reference gene for the 20S proteasome subunit beta (Glyma.06g078500; Pereira Lima et al. 2017). Expression levels were calculated as relative amounts using $\Delta\Delta$Ct values. The lowest $\Delta\Delta$Ct value in each experiment was set at 1.

Homologs of MGL, CGS, MMT, and HMT were searched using BLASTP analysis on SoyBase (https://soybase.org/) with GmMGL1 (Glyma.10g17200), AtCGS (At3g01120), AtMMT (At5g49810), and AtHMT1 (At3g25900) as queries, respectively. Primers for genomic PCR and RT-qPCR are shown in Supplemental Table S3.

**Met-feeding**

Pods harboring the seeds of developmental stage two (cf. the photo in Fig. 8) were collected and were gently removed with their seed coats. Thin sections of 1-mm thickness were excised at the short axis using a razor blade, and they were immediately placed on a sheet of Parafilm (Bemis Flexible Packaging, Chicago, IL, USA) in a glass Petri dish. The inner surface of the Petri dish was covered with a moistened paper towel. Fifty-microliter aliquots of 0, 1, or 5 mM Met or $^{13}$C-Met in water were then placed on the surfaces of seed sections at 11:00 am, and the Petri dish was immediately closed and incubated at 25°C for 0, 4, 8, and 24 h under light/dark conditions of 14-h light (8:00 am–10:00 pm)/10-h dark (10:00 pm–8:00 am). To determine Met and SMM concentrations, sections were carefully washed with water and were mixed with 1-mL aliquots of distilled water containing 1-μg mL$^{-1}$ d6-SMM. Sections were then homogenized in a mortar, and homogenates were placed in a water bath sonicator (US-2, SND Co. Ltd., Suwa City, Nagano, Japan) for 10 min to facilitate extraction of Met and SMM. Suspensions were centrifuged at 15,000 rpm (20,000 $\times$ g) for 10 min at 4°C, and 100-μL aliquots were applied to a Strata C18-E (100 mg mL$^{-1}$) cartridge (Phenomenex Inc. Torrance, CA, USA). Met and SMM were eluted twice with 0.5 mL of distilled
water and eluted solutions were cleared with Ekicrodisc 3 (0.45 µm, 3 mm, Pall Co.,
Tokyo, Japan) prior to LC-MS/MS analyses as described above.

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under
accession number MK887190 (*Glyma.10g172700* in NH).

**SUPPLEMENTAL DATA**

The following supplemental materials are available.

**Supplemental Figure S1.** Protein contents and profile of soybean cultivars used in this study.

**Supplemental Figure S2.** The inheritance pattern of *S*-methylmethionine (SMM) contents.

**Supplemental Figure S3.** Appearance of local soybean cultivars collected in Nagano Prefecture, Japan. Bar = 3 cm.

**Supplemental Figure S4.** Polymerase chain reactions with complementary and genomic DNA.

**Supplemental Figure S5.** Amino acid sequence alignments of methionine γ-lyases (MGLs) from soybean, melon, *Arabidopsis*, potato, *Pseudomonas putida*, and *Streptomyces avermitilis*.

**Supplemental Figure S6.** Properties of recombinant *GmMGL1*.

**Supplemental Figure S7.** Phylogenetic analysis of *GmMGL1* and its related MGL-like proteins.

**Supplemental Figure S8.** Expression levels of *GmCGS1/2, GmMMT1, GmMMT2,*
Supplemental Table S1. Total amino acid contents in the dry seeds of the Fukuyutaka (FY) and Nishiyamahitashi 98-5 (NH) soybean cultivars.

Supplemental Table S2. Multiple reaction monitoring (MRM) transitions and MS parameters used to detect amino acids.

Supplemental Table S3. Primers used in this study.

Supplemental Table S4. Protein sequences used to construct the phylogenetic tree.

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TABLES

Table 1. Level of SMM and Met in soybean and Arabidopsis phloem exudates.

| Plant    | Cultivar/Ecotype | SMM (nmol g⁻¹ leaf DW) | Met (nmol g⁻¹ leaf DW) |
|----------|------------------|------------------------|------------------------|
| Soybean  | NH               | 3.40 ± 1.06*           | 0.063 ± 0.032†         |
| Soybean  | FY               | 0.63 ± 0.23            | 0.203 ± 0.033          |
| Arabidopsis | Ws-0        | 990 ± 150              | 18.8± 5.2              |

*: t-test after Box-Cox transformation (between soybean NH and FY) \( P = 0.0163 \), †: t-test after Box-Cox transformation (between soybean NH and FY) \( P = 0.0292 \). To all the values of Met 1 was added before Box-Cox transformation to avoid the values of 0.

FIGURE LEGENDS

Figure 1. Schematic representation of Met metabolism in plants. The pathways mentioned in this study are highlighted. Solid arrows represent one metabolic step,
whereas dashed arrows represent several metabolic steps. The enzyme names are underlined. The canonical Asp family pathway is shown with gray background. The S-methylmethionine cycle is shown with striped background. Asp, aspartic acid; CGS, cystathionine \( \gamma \)-synthase; Cys, cysteine; HMT, homocysteine methyltransferase; HomoCys, homocysteine; Ile, isoleucine; 2KB, 2-ketobutyric acid; Met, methionine; MGL, methionine \( \gamma \)-lyase; MMT, methionine methyltransferase; MS, methionine synthase; 5MeTHF, 5-methyltetrahydrofolate; O-PhosphohomoSer, O-phosphohomoserine; SAM, S-adenosylmethionine; SMM, S-methylmethionine; Thr, threonine.

**Figure 2.** S-Methylmethionine and methionine contents in soybean plants. S-Methylmethionine (SMM) (upper panels) and methionine (Met) (lower panels) contents in the leaves (A), pods (B), and seeds (without seed coats) (C) harvested at the flowering stage (F), the immature green seed stage (IG; corresponding to stage two in Fig. 8), and the mature green seed stage (MG; corresponding to stage five in Fig. 8) are shown as means ± standard errors (SE) of four replicates. Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation and Fisher’s least significant difference test (LSD; \( P < 0.05 \)). Different lowercase letters indicate significant differences between the developing stages (\( P < 0.05 \), Tukey’s HSD test after two-way ANOVA). Different capital letters indicate significant differences between all treatments (\( P < 0.05 \), Tukey’s HSD test after two-way ANOVA). ***: \( P < 0.001 \), **: 0.001 < \( P < 0.01 \), NS: 0.05 < \( P \) (simple main effect test after two-way ANOVA).

**Figure 3.** Free amino acids in seeds. Quantities of free amino acids in dry matured seeds of Fukuyutaka (FY; white bar) and Nishiyamahitashi 98-5 (NH; gray bar) are shown as \( \mu g \) per g of mature dry seeds. The data are shown as means ± SE of three replicates. Significant differences were identified using Student’s \( t \)-test after Box-Cox transformation (**: \( P < 0.01 \), *: 0.01 < \( P < 0.05 \), n.s.: 0.05 < \( P \)). HomoSer: homoserine, HomoCys: homocysteine, n.d.: not detected.

**Figure 4.** Inheritance of hyperaccumulation of S-methylmethionine. Concentrations of S-methylmethionine (SMM) in F1 progenies that were generated by reciprocal crossing
of Fukuyutaka (FY) (MGL1) and Nishiyamahitashi 98-5 (NH) (mgl1) soybean strains; data are shown as means ± SE of three replicates. Significant differences between plant lines in SMM were identified using Tukey’s HSD tests after Box-Cox transformation ($P < 0.05$).

**Figure 5.** Map-based cloning of the allele responsible for the hyperaccumulation of S-methylmethionine in soybean. A. QTL-regions detected in chromosome 10 of soybean. B. Graphical genotypes of 26 F$_6$ and F$_7$ residual heterozygous lines determined with markers are shown (WI: Williams 82, NH: Nishiyamahitashi 98-5). The amounts of S-methylmethionine (SMM) in the respective lines are shown in the bar graph on the right. After delimiting the region, only one open reading frame (Glyma.10g172700) was identified. Glyma.10g172700 was tentatively assigned as the gene encoding Met γ-lyase and comprises two exons flanking one intron. The DNA sequencing of genes from NH and WI strains showed a copia-type retrotransposon inserted into the intron of the gene in the NH strain only.

**Figure 6.** Hyperaccumulation of S-methylmethionine correlates with the insertion of a transposon into the GmMGL1 gene. A. Sizes of DNA fragments that were amplified with primers for the full-length coding sequence of Glyma.10g172700; M: molecular weight marker (l/Hind III digests), 1: Shinano-Kurakake, 2: Nishiyamahitashi 94-5, 3: Sinanomachi Arasehara Kurakake, 4: Togakushi Morozawa Ganimame, 5: Usuda Zairai, 6: Shinano Midori, 7: Nishiyamahitashi 94-1, 8: Tousanhitashi 106, 9: Ogawa Zairai 5, 10: Chino Zairai 4. NH: Nishiyamahitashi 98-5, WI: Williams 82, FY: Fukuyutaka. B. Quantities of S-methylmethionine (SMM; upper panel: black bars) and Met (lower panel: white bars) in dry matured seeds of each soybean line. Data are shown as means ± SE of three replicates. C. Expression levels of Glyma.10g172700 in seeds at the green mature stage (stage five in Fig. 8). The Glycine max 20S proteasome subunit (Glyma.06g078500) was used as an internal control in RT-qPCR analyses. Transcript levels relative to the internal control are shown as multiples of the lowest value of 1. Data are presented as means ± SE ($n = 3$). Significant differences indicated with different lowercase letters were identified using Tukey’s HSD tests after Box-Cox transformation ($P < 0.05$).

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Figure 7. *Glyma.10g172700* encodes a functional MGL. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant soybean MGL1 protein with an N-terminal His-tag; lanes 1 and 4, molecular weight marker; lane 2, *E. coli* lysate expressing recombinant GmMGL1 protein; lane 3, *E. coli* lysate with the empty vector (a negative control); lane 5, purified recombinant GmMGL1. B, Absorption spectra of 3-methyl-2-benzothiazolinone hydrazone (MBTH) derivatives of the product formed during metabolism of Met by recombinant GmMGL1; C, Chromatograms of trimethylsilylated products of recombinant GmMGL1 and Met from retention times of 8.25–10.75 min; the solid line represents chromatograms from experiments with the recombinant enzyme, and the broken line shows those without enzyme (negative control). The peak specifically found with the product formed with recombinant enzyme is shown with arrow (peak A). D, Mass spectra obtained with authentic trimethylsilylated 2-ketobutyric acid (upper panel) and with peak A (lower panel).

Figure 8. Expression of *GmMGL1*, *GmMGL2*, and *GmMGL3* in soybean plants. Expression of *GmMGL1*, *GmMGL2*, and *GmMGL3* in developing seeds (A) and in the leaves, stems, and roots (B) of Fukuyutaka (FY) and Nishiyamahitashi 98-5 (NH) soybean cultivars. Sizes of NH and FY seeds collected for RNA extraction (stages one to five) are shown in the upper panel. The *Glycine max* 20S proteasome subunit (*Glyma.06g078500*) was used as an internal control in RT-qPCR analyses. Transcript levels relative to the internal control are shown as multiples of the lowest value of 1. Data are presented as means ± SE (*n* = 3). Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation. *** above the symbols in A indicate significant differences between plant lines in each developing stage (*P* < 0.001, simple main effect test after two-way ANOVA). ** indicate significant differences between plant lines in B (*MGL1*) (*P* < 0.05, simple main effect test after two-way ANOVA). Different lowercase letters above columns indicate significant differences between organs in B (*MGL2* and *MGL3*) (*P* < 0.05, Tukey’ HSD test after two-way ANOVA).

Figure 9. Absorption and conversion of exogenously supplied methionine to S-methylmethionine in a section of a developing soybean seed. A. *S*-methylmethionine
(SMM) and methionine (Met) contents after treating with 0 (circle and white)-, 1 (square and gray), and 5 (triangle and black) mM Met. Data are shown as means ± SE \((n = 3)\). Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation. Different letters above the symbols indicate significant differences between Met concentrations in each hour \((P < 0.05, \text{Tukey' HSD tests after simple main effect tests})\). B. Mass spectrum of SMM extracted from the seed sections treated with 5 mM \(^{13}\text{C}_5\)-Met for 24 h (upper panel). Reaction of MMT from \(^{13}\text{C}_5\)-Met is shown as an inset. The positions of \(^{13}\text{C}\) in Met and SMM are shown with asterisks. Mass spectrum of non-labelled SMM is shown (lower panel). Tentative assignments of molecular and fragment ions are also shown.

**Figure 10.** A proposed mechanism of hyperaccumulation of \(S\)-methylmethionine in soybean seeds with low MGL activity. (A) When the MGL activity is sufficient as in normal soybean seeds, the level of free Met is properly controlled. (B) When MGL activity is suppressed by transposon insertion as in NH soybean seeds, surplus Met left behind is converted into SMM, which seems to account for hyperaccumulation of SMM. Met, methionine; SMM, \(S\)-methylmethionine; MGL, methionine \(\gamma\)-lyase; MMT, methionine methyltransferase; HMT, homocysteine methyltransferase; SAM, \(S\)-adenosylmethionine; HomoCys, homocysteine; 2KB, 2-ketobutyric acid.
Figure 1. Schematic representation of Met metabolism in plants. The pathways mentioned in this study are highlighted. Solid arrows represent one metabolic step, whereas dashed arrows represent several metabolic steps. The enzyme names are underlined. The canonical Asp family pathway is shown with gray background. The S-methylmethionine cycle is shown with striped background. Asp, aspartic acid; CGS, cystathionine γ-synthase; Cys, cysteine; HMT, homocysteine methyltransferase; HomoCys, homocysteine; Ile, isoleucine; 2KB, 2-ketobutyric acid; Met, methionine; MGL, methionine γ-lyase; MMT, methionine methyltransferase; MS, methionine synthase; O-PhosphohomoSer, O-phosphohomoserine; SAM, S-adenosylmethionine; SMM, S-methylmethionine; Thr, threonine.
Figure 2. S-Methylmethionine and methionine contents in soybean plants. S-Methylmethionine (SMM) (upper panels) and methionine (Met) (lower panels) contents in the leaves (A), pods (B), and seeds (without seed coats) (C) harvested at the flowering stage (F), the immature green seed stage (IG; corresponding to stage two in Fig. 8), and the mature green seed stage (MG; corresponding to stage five in Fig. 8) are shown as means ± standard errors (SE) of four replicates. Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation and Fisher’s least significant difference test (LSD; P < 0.05). Different lowercase letters indicate significant differences between the developing stages (P < 0.05, Tukey’s HSD test after two-way ANOVA). ***: P < 0.001, **: 0.001 < P < 0.01, NS: 0.05 < P (simple main effect test after two-way ANOVA).
Figure 3. Free amino acids in seeds. Quantities of free amino acids in dry matured seeds of Fukuyutaka (FY; white bar) and Nishiyamahitashi 98-5 (NH; gray bar) are shown as µg per g of mature dry seeds. The data are shown as means ± SE of three replicates. Significant differences were identified using Student’s t-test after Box-Cox transformation (**: P < 0.01, *: 0.01 < P < 0.05, n.s.: 0.05 < P). HomoSer: homoserine, HomoCys: homocysteine, n.d.: not detected.
Figure 4. Inheritance of hyperaccumulation of S-methylmethionine. Concentrations of S-methylmethionine (SMM) in F₁ progenies that were generated by reciprocal crossing of Fukuyutaka (FY) (MGL1) and Nishiyamahitashi 98-5 (NH) (mgl1) soybean strains; data are shown as means ± SE of three replicates. Significant differences between plant lines in SMM were identified using Tukey’s HSD tests after Box-Cox transformation (P < 0.05).
Figure 5. Map-based cloning of the allele responsible for the hyperaccumulation of S-methylmethionine in soybean. A. QTL-regions detected in chromosome 10 of soybean. B. Graphical genotypes of 26 F$_6$ and F$_7$ residual heterozygous lines determined with markers are shown (WI: Williams 82, NH: Nishiyamahitashi 98-5). The amounts of S-methylmethionine (SMM) in the respective lines are shown in the bar graph on the right. After delimiting the region, only one open reading frame (Glyma.10g172700) was identified. Glyma.10g172700 was tentatively assigned as the gene encoding Met $\gamma$-lyase and comprises two exons flanking one intron. The DNA sequencing of genes from NH and WI strains showed a copia-type retrotransposon inserted into the intron of the gene in the NH strain only.
Figure 6. Hyperaccumulation of S-methylmethionine correlates with the insertion of a transposon into the *GmMGL1* gene. 

A. Sizes of DNA fragments that were amplified with primers for the full-length coding sequence of *Glyma.10g172700*; M: molecular weight marker (*lHind III* digests), 1: Shinano-Kurakake, 2: Nishiyamahitashi 94-5, 3: Sinanomachi Arasehara Kurakake, 4: Togakushi Morozawa Ganimame, 5: Usuda Zairai, 6: Shinano Midori, 7: Nishiyamahitashi 94-1, 8: Tousanhitashi 106, 9: Ogawa Zairai 5, 10: Chino Zairai 4. NH: Nishiyamahitashi 98-5, WI: Williams 82, FY: Fukuyutaka. 

B. Quantities of S-methylmethionine (SMM; upper panel: black bars) and Met (lower panel: white bars) in dry matured seeds of each soybean line. Data are shown as means ± SE of three replicates. 

C. Expression levels of *Glyma.10g172700* in seeds at the green mature stage (stage five in Fig. 8). The *Glycine max* 20S proteasome subunit (*Glyma.06g078500*) was used as an internal control in RT-qPCR analyses. Transcript levels relative to the internal control are shown as multiples of the lowest value of 1. Data are presented as means ± SE (n = 3). Significant differences indicated with different lowercase letters were identified using Tukey’s HSD tests after Box-Cox transformation (P < 0.05).
Figure 7. *Glyma.10g172700* encodes a functional MGL. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the recombinant soybean MGL1 protein with an N-terminal His-tag; lanes 1 and 4, molecular weight marker; lane 2, *E. coli* lysate expressing recombinant GmMGL1 protein; lane 3, *E. coli* lysate with the empty vector (a negative control); lane 5, purified recombinant GmMGL1.

B, Absorption spectra of 3-methyl-2-benzothiazolinone hydrazone (MBTH) derivatives of the product formed during metabolism of Met by recombinant GmMGL1.

C, Chromatograms of trimethylsilylated products of recombinant GmMGL1 and Met from retention times of 8.25–10.75 min; the solid line represents chromatograms from experiments with the recombinant enzyme, and the broken line shows those without enzyme (negative control). The peak specifically found with the product formed with recombinant enzyme is shown with arrow (peak A).

D, Mass spectra obtained with authentic trimethylsilylated 2-ketobutyric acid (upper panel) and with peak A (lower panel).
Figure 8. Expression of GmMGL1, GmMGL2, and GmMGL3 in soybean plants. Expression of GmMGL1, GmMGL2, and GmMGL3 in developing seeds (A) and in the leaves, stems, and roots (B) of Fukuyutaka (FY) and Nishiyamahitashi 98-5 (NH) soybean cultivars. Sizes of NH and FY seeds collected for RNA extraction (stages one to five) are shown in the upper panel. The Glycine max 20S proteasome subunit (Glyma.06g078500) was used as an internal control in RT-qPCR analyses. Transcript levels relative to the internal control are shown as multiples of the lowest value of 1. Data are presented as means ± SE (n = 3). Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation. *** above the symbols in A indicate significant differences between plant lines in each developing stage (P < 0.001, simple main effect test after two-way ANOVA). ** indicate significant differences between plant lines in B (MGL1) (P < 0.05, simple main effect test after two-way ANOVA). Different lowercase letters above columns indicate significant differences between organs in B (MGL2 and MGL3) (P < 0.05, Tukey’s HSD test after two-way ANOVA).
Figure 9. Absorption and conversion of exogenously supplied methionine to S-methylmethionine in a section of a developing soybean seed. A. S-methylmethionine (SMM) and methionine (Met) contents after treating with 0 (circle and white), 1 (square and gray), and 5 (triangle and black) mM Met. Data are shown as means ± SE (n = 3). Significant differences were identified using a two-way analysis of variance (ANOVA) after Box-Cox transformation. Different letters above the symbols indicate significant differences between Met concentrations in each hour (P < 0.05, Tukey' HSD tests after simple main effect tests). B. Mass spectrum of SMM extracted from the seed sections treated with 5 mM $^{13}$C5-Met for 24 h (upper panel). Reaction of MMT from $^{13}$C5-Met is shown as an inset. The positions of $^{13}$C in Met and SMM are shown with asterisks. Mass spectrum of non-labelled SMM is shown (lower panel). Tentative assignments of molecular and fragment ions are also shown.
Figure 10. A proposed mechanism of hyperaccumulation of S-methylmethionine in soybean seeds with low MGL activity. (A) When the MGL activity is sufficient as in normal soybean seeds, the level of free Met is properly controlled. (B) When MGL activity is suppressed by transposon insertion as in NH soybean seeds, surplus Met left behind is converted into SMM, which seems to account for hyperaccumulation of SMM. Met, methionine; SMM, S-methylmethionine; MGL, methionine γ-lyase; MMT, methionine methyltransferase; HMT, homocysteine methyltransferase; SAM, S-adenosylmethionine; HomoCys, homocysteine; 2KB, 2-ketobutyric acid.
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