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Citation
Le, Dung Tien, Lionel Tarrago, Yasuko Watanabe, Alaattin Kaya, Byung Cheon Lee, Uyen Tran, Rie Nishiyama, Dmitri E. Fomenko, Vadim N. Gladyshev, and Lam-Son Phan Tran. 2013. “Diversity of Plant Methionine Sulfoxide Reductases B and Evolution of a Form Specific for Free Methionine Sulfoxide.” PLoS ONE 8 (6): e65637. doi:10.1371/journal.pone.0065637. http://dx.doi.org/10.1371/journal.pone.0065637.

Published Version
doi:10.1371/journal.pone.0065637

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Diversity of Plant Methionine Sulfoxide Reductases B and Evolution of a Form Specific for Free Methionine Sulfoxide

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Abstract

Methionine can be reversibly oxidized to methionine sulfoxide (MetO) under physiological conditions. Organisms evolved two distinct methionine sulfoxide reductase families (MSRA & MSRB) to repair oxidized methionine residues. We found that 5 MSRB genes exist in the soybean genome, including GmMSRB1 and two segmentally duplicated gene pairs (GmMSRB2 and GmMSRB5, GmMSRB3 and GmMSRB4). GmMSRB2 and GmMSRB4 proteins showed MSRB activity toward protein-based MetO with either DTT or thioredoxin (TRX) as reductants, whereas GmMSRB1 was active only with DTT. GmMSRB2 had a typical MSRB mechanism with Cys121 and Cys68 as catalytic and resolving residues, respectively. Surprisingly, this enzyme also possessed the MSRB activity toward free Met-R-O with kinetic parameters similar to those reported for fRMSR from Escherichia coli, an enzyme specific for free Met-R-O. Overexpression of GmMSRB2 or GmMSRB4 in the yeast cytosol supported the growth of the triple MSRA/MSRB/fRMSR (A3MSRs) mutant on MetO and protected cells against H2O2-induced stress. Taken together, our data reveal an unexpected diversity of MSRBs in plants and indicate that, in contrast to mammals that cannot reduce free Met-R-O and microorganisms that use fRMSR for this purpose, plants evolved MSRBs for the reduction of both free and protein-based MetO.

Introduction

Among the 20 common amino acids, methionine (Met) is among the most susceptible to oxidation by reactive oxygen species (ROS). Under elevated ROS levels, free and protein-based Met are converted to methionine sulfoxide (MetO), which occurs in a diastereomeric mixture of methionine-S-sulfoxide (Met-S-O) and methionine-R-sulfoxide (Met-R-O) [1]. Several proteins have been reported, in which oxidation of Met residues is linked to protein dysfunction or aggregation (reviewed in [2,3]). Oxidation of Met was also described in signaling proteins, modulating their functions [1,4]. To repair oxidized Met in proteins, organisms evolved two enzyme families, methionine sulfoxide reductase A (MSRA) that reduces Met-S-O and methionine sulfoxide reductase B (MSRB) that reduces Met-R-O. It was reported that MSRAs can act on both protein-based and free Met-S-O, whereas MSRBs are inefficient against free Met-R-O because of extremely low affinity for this substrate [5–8]. Lee et al. showed that mammalian cells are unable to use Met-R-O as a source of Met to support growth [9] and it was found also that yeast cells carrying only MSRB gene (with other MSRB deleted) failed to grow in both liquid and solid media containing Met-R-O as the sole source of Met [10]. Recently, a new enzyme family unique to some unicellular organisms capable of reducing free Met-R-O (fRMSRs) was characterized [10,11]. As reported by Lin et al., the fRMSR from E. coli, which contains a GAF domain, reduced Met-R-O with the Km of 3,900 μM [11]. Following this study, a yeast homolog of E. coli fRMSR was characterized with the Km of 230 μM. It should be noted that due to the nature of the discontinuous assay (where NADPH may become limited at high substrate concentrations), this Km might not represent a true value [10].

The catalytic mechanisms of 2-Cys MSRBs and fRMSRs are similar to that of MSRAs and involve transient formation of a sulfenic or selenic acid intermediates on the catalytic Cys or selenocysteine [12,13], which subsequently condenses with a resolving Cys to form a disulfide or selenosulfide bond [10,11,14]. 1-Cys MSRBs, such as human MSRB2 and MSRB3 and A. thaliana plastidic MSRB1, which do not possess resolving Cys residues, can be reduced in vitro by thioredoxin (TRX) by the direct reduction of the sulfenic acid intermediate [15–17]. Glutaredoxin was shown to serve as a possible alternative reducing system [18–20].
In vivo modulation of MSR activities has been reported in yeast [10,21], fruit fly [22] and mammals [23], which in turn affected resistance to oxidative stress and lifespan. In plants, MSR activities were identified many years ago [24], but their functional characterization has not been carried out until recently [8,25,26]. In an attempt to understand the importance of Met oxidation and MetO reduction in soybean’s defense against biotic and abiotic stresses, we carried out a comprehensive characterization of its MSRBs. An exhaustive search of the genome identified 5 members of the \textit{GmMSRB} family. We analyzed their expression profiles in various tissues under normal and drought stress conditions, and characterized their enzymatic properties as well as their roles in protecting against oxidative stress using yeast.

Interestingly, characterization of their enzymatic properties revealed that \textit{GmMSRB2} could reduce free Met-O as efficiently as yeast fMSR. Expression of some of the \textit{GmMSRB} genes in the \textit{DMSR} mutant yeast restored the ability to use free-Met-O as a source of Met to support growth, indicating that soybean MSRBs function in the reduction of both free and protein-based Met-O.

### Materials and Methods

In Silico Analysis of \textit{GmMSRBs}

Using \textit{Arabidopsis} MSRBs as seed sequences, \textit{GmMSRBs} were identified by reciprocal BLAST, and genes were further examined by manual inspection. Full-length sequences containing natural stop codons were used for further analyses. Multiple sequence analyses were done with MEGA4 [27]. Synteny analysis was performed using the online locus search (http://chibba.agtec.uga.edu/duplication/index/locus).

Soybean Growth, Stress Treatment and Sample Collection

Stress treatment and sample collection of young soybean seedlings were performed as previously described [28]. Drought treatment of V6 vegetative soybean plants (28 days after sowing, containing 6 fully developed trifoliate leaves) was carried out by withholding plants from watering, and sample collection was performed exactly as described previously [29,30].

### Table 1. Domain feature and signal peptide prediction of soybean MSRB proteins.

| Names | Gene ID | E values* | Position of catalytic Cys | cTPb | mTP | SP | Other Locationc | Reliabilityd | TPlength |
|-------|---------|-----------|---------------------------|------|-----|---|-----------------|-------------|----------|
| GmMSRB1 | Glyma08g25610 | 1.0E-48 [190] | 0.869 0.061 0.02 0.195 | C | 2 | 45 |
| GmMSRB2 | Glyma13g28320 | 6.0E-52 [121] | 0.133 0.083 0.11 0.854 | – | 2 | – |
| GmMSRB3 | Glyma13g32680 | 1.1E-51 | 0.971 0.088 0.01 0.038 | C | 1 | 67 |
| GmMSRB4 | Glyma15g06650 | 1.5E-51 | 0.956 0.102 0.01 0.056 | C | 1 | 67 |
| GmMSRB5 | Glyma15g10750 | 1.2E-52 [121] | 0.132 0.071 0.1 0.871 | – | 2 | – |

*E values for SelR domain prediction by PFAM.

*Target prediction by TargetP.

*Locations: C, Chloroplast; -, not known.

*Reliability score scale from 1 to 5, lower values have higher probability.

doi:10.1371/journal.pone.0065637.t001

Figure 1. Multiple sequence alignment of \textit{GmMSRB} proteins. Arrows indicate predicted resolving and catalytic Cys residues, respectively. Black bars indicate two CxxC motifs coordinating a zinc atom. Residues identical in all the 5 sequences were colored red and shaded in yellow, whereas similar residues were shaded in blue.

doi:10.1371/journal.pone.0065637.g001
samples were quickly frozen in liquid nitrogen and stored at −80°C until use.

RNA Extraction, cDNA Synthesis and Transcript Analyses by Quantitative PCR (qPCR)

Tissue samples were ground into fine powder using pestle and mortar, and TRIZOL reagent (Invitrogen) was used to isolate total RNA. Total RNA was then treated with Turbo DNA-free DNase I (Ambion) and subsequently used for first stranded cDNA synthesis. All steps were performed as described [28,29]. For transcription profiling of GmMSRB genes in soybean, primers were designed using Primer3 [31]. Primer specificity was confirmed by BLAST against the soybean genome. For normalization, primers specific for genes encoding F-box and 60S were used as described previously [32]. qPCR was performed as previously described, including data calculation [28].

Statistical Analysis of Data

qPCR was performed on 3 biological replicates for each treatment, and mean values and standard errors were used for data presentation. For comparison of two mean values, a Student’s t-test was applied. All differences with p-values less than 0.05 were considered statistically significant. To compare expression of genes with alternative splicing, the sums of primary and secondary transcripts were used.

Figure 2. Steady-state transcript abundance (in arbitrary units) of GmMSRB genes under normal and drought conditions. (A) Transcript levels in V6-vegetative-stage leaves, (B) transcript levels in roots and shoots of young seedlings; R0, R2, R10 and S0, S2, S10 represented roots (R) or shoots (S) at 0, 2 or 10 h, respectively, under dehydration stress treatment.
doi:10.1371/journal.pone.0065637.g002

Figure 3. In vitro and in vivo activities of GmMSRBs. MSR activities of GmMSRBs using DTT as electron donor toward free Met-R-O (A) or dabsyl-Met-R-O (B). Data presented are the means ± SE of 3 replicates. (C) In vivo complementation assay of GmMSRBs. Yeast strain lacking MSRA, MSRB and fRMSR was transformed with indicated plasmids and grown under selective media (right panel) or selective media plus MetO as the sole source of Met (left panel). Experiment was performed in triplicate and representative data are shown. pGmMSRB1 and pGmMSRB4 did not include sequences encoding signal peptides.
doi:10.1371/journal.pone.0065637.g003
Gene Cloning and Site-directed Mutagenesis

Coding sequences of GmMSRBs were cloned from the soybean cDNA pool extracted from various tissues and under various treatments using primers listed in Table S1. For construction of expression vectors in yeast, blunt-ended PCR products were first ligated into the pKS vector and sequenced. Correct inserts were excised using SpeI and SalI restriction enzymes (see Table S1) and ligated into the p425-GPD vector. To create yeast expression vectors carrying GmMSRB1 and GmMSRB4 that do not encode signal peptides, the coding sequences were PCR-amplified from pDEST17 plasmids using primers shown in Table S1, digested with BamHI/XhoI (for GmMSRB1) or NdeI/XhoI (for GmMSRB4) and ligated into appropriately cut p425-GPD vector.

For production of recombinant proteins in E. coli, sequences coding for full-length proteins were PCR-amplified from pKS plasmids and ligated into pENTR D/TOPO. The pENTR plasmids carrying correct sequences were recombined into pDEST17 vectors using Gateway® technology. Initial expression analysis showed that full-length GmMSRB1 and GmMSRB4 proteins were not soluble; therefore, constructs that overexpressed proteins lacking the predicted signal peptides were prepared. For this purpose, coding sequences of N-terminal truncated GmMSRB1 & GmMSRB4 (without signal peptides) were PCR-amplified from respective pDEST17 plasmids and inserted into pET21b. For purification of yeast TRX2 (YGR209C) and GRX4 (YER174C) recombinant proteins, TRX2 and GRX4 were PCR-amplified using primers listed in Table S1 and inserted into pET15b and pET21b, respectively. Site-directed mutagenesis was performed following the Quickchange® protocol using primers listed in Table S1.

Protein Expression and Purification

pET21b, pDEST17 and pET15b carrying appropriate coding sequences were transformed into E. coli BL21 (DE3) T7 Express® (New England Biolabs) and cultured in media containing recommended concentrations of antibiotics. Protein expression

| Table 2. Kinetic properties of soybean methionine sulfoxide reductases B. |
|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                | N-acetyl-MetO |                | Free l-MetO   |                |                |                |
|                | kcat (s⁻¹)     | K_m (µM)       | kcat/K_m (M⁻¹.s⁻¹) | kcat (s⁻¹)     | K_m (µM)       | kcat/K_m (M⁻¹.s⁻¹) |
| GmMSRB1        |                |                |                | 2.04±0.06      | 49±5           | 42×10²          |
| GmMSRB2        | 2.04±0.06      | 49±5           | 42×10²         | 2.86±0.16      | 2,093±251      | 1,400           |
| C68S GmMSRB2   | No activity    |                |                |                |                |                |
| C121S GmMSRB2  | No activity    |                |                |                |                |                |
| GmMSRB4       | 0.24±0.01      | 49±6           | 5×10⁴          | 0.13±0.01      | 1,451±239      | 90              |
| E. coli frMSR  | –              | –              | –              | 6.90±0.40      | 3,900±400      | 1,700           |
| Data presented are means ± SE of 3 replicates; a,b: the GmMSRB1 and GmMSRB4 proteins were without the N-terminal signal peptides. c: because the substrate used was a mixture of S- and R- forms, the K_m shown is half of the measured values. d: from reference [11].

doi:10.1371/journal.pone.0065637.t002

Figure 4. Overexpression of GmMSRBs in the MSR triple-mutant yeast strain. Yeast cells harboring indicated plasmids (at the OD₆₀₀ of 0.6) were treated with 2 mM H₂O₂ for 60 minutes (left), washed to remove excess peroxide and plated. Mock-treated cells were also spotted as a control (right). pGmMSRB1 and pGmMSRB4 did not include sequences encoding signal peptides. Experiment was repeated twice and a typical result is shown.

doi:10.1371/journal.pone.0065637.g004
was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to achieve a concentration of 100 μM. Induction of protein synthesis was conducted at 30°C for 4 hours, and the cells were harvested by centrifugation [33]. Purification of His-tag recombinant proteins was performed essentially as described [7,34].

Measurements of Methionine Sulfoxide Reductase Activities
TRX-dependent MSR activities were measured by monitoring NADPH consumption as described by Tarrago et al. [7]. Briefly, the reaction was initiated by the addition of 200 μM NADPH to the reaction mixture containing 2 μM TRX reductase, 25 μM yeast TRX2, 1–5 μM GmMSRB proteins and variable amounts of MetO or N-acetyl-MetO. For the GRX-reducing system, the reaction mixture contained 400 μM NADPH, 0.5 unit yeast glutathione reductase (Sigma), 10 mM GSH, 5 μM yeast GRX4 and 1 mM MetO or N-acetyl-MetO. DTT-dependent MSR activities toward dabsyl-MetO or free MetO were also determined using published procedures [10,35].

Yeast Complementation and Oxidative Stress Tolerance Assays
A triple yeast mutant strain, whose all 3 MSRs (MSRA/MSRB/FRMSR) were knocked out, was transformed with p425-GPD plasmids expressing soybean MSRBs or yeast MSRBs//FRMSR genes under the control of a strong promoter [36]. For complementation assays, the recombinant strains were grown in synthetic media without L-Leu, L-Met and with the addition of L-MetO (20 mg·L⁻¹). For oxidative stress protection assay, strains were grown in selective liquid media until the OD₆₀₀ reached 0.6. Subsequently, H₂O₂ was added to achieve a final concentration of 2 mM and the treatment was continued for 60 minutes. Cells were washed, diluted and spotted on agar plates.

Results and Discussion
Identification and in Silico Analysis of Soybean GmMSRB Genes
Although soybean is a palaeopolyploid, its genome possesses only 5 genes encoding MSRBs (GmMSRBs), the same number as in rice, poplar and grapevine, but fewer than in Arabidopsis (9 MSRB genes) [8,37]. The GmMSRB proteins contain a SelR domain with catalytic and resolving Cys residues predicted to be at positions 121 and 68, respectively (numbering follows the published sequences) using qPCR in V6 vegetative-stage trifoliate leaves, young seedling roots and shoots under normal and dehydration conditions. Results shown in Fig. 2 confirmed the occurrence of alternative splicing in GmMSRB2 but the data were ambiguous for GmMSRB4. Because GmMSRB4.2 is predicted to be of very low abundance and encodes a predicted protein lacking its catalytic residue, we consider that the presence of this transcript was due to splicing error. The data shown in Fig. 2 also indicated that under drought conditions, the expression of all GmMSRBs was induced in the V6-stage leaves, and this effect was more pronounced in younger trifoliate leaves (Fig. 2A). In young seedling roots and shoots, GmMSRBs were less responsive to dehydration stress with the exception of GmMSRB2, whose expression was upregulated in both roots and shoots, and GmMSRB5 whose expression was induced only in the shoots (Fig. 2B).

Expression of GmMSRBs in Various Tissues Under Normal and Stress Conditions
To obtain insights into biological functions of GmMSRBs under normal and abiotic stress conditions, we analyzed their expression profiles. GmMSRB1 and GmMSRB3 were highly expressed in various tissues, especially in the aerial parts, reaching highest levels in leaf (Fig. S3) [39]. Although GmMSRB3 and GmMSRB4 formed a duplicated pair, their expression levels were significantly different as judged by steady-state transcript abundance. To gain insight into how these genes function under abiotic stresses, we quantified the steady-state levels of their transcripts (including their spliced forms) using qPCR in V6 vegetative-stage trifoliate leaves, young seedling roots and shoots under normal and dehydration conditions. Results shown in Fig. 2 confirmed the occurrence of alternative splicing in GmMSRB2 but the data were ambiguous for GmMSRB4. Because GmMSRB4.2 is predicted to be of very low abundance and encodes a predicted protein lacking its catalytic residue, we consider that the presence of this transcript was due to splicing error. The data shown in Fig. 2 also indicated that under drought conditions, the expression of all GmMSRBs was induced in the V6-stage leaves, and this effect was more pronounced in younger trifoliate leaves (Fig. 2A). In young seedling roots and shoots, GmMSRBs were less responsive to dehydration stress with the exception of GmMSRB2, whose expression was upregulated in both roots and shoots, and GmMSRB5 whose expression was induced only in the shoots (Fig. 2B).

In vitro and in vivo Activities of GmMSRB Proteins
To characterize the function of GmMSRBs, we selected GmMSRB1, GmMSRB2 and GmMSRB4 as representatives. Since GmMSRB3 and GmMSRB5 were duplicated members of GmMSRB4 and GmMSRB2 with very high homology (90 and 95% identity in amino acid sequence, respectively, Fig. 1 and Fig. S1), they likely function very similarly to the corresponding paralogs. The purified GmMSRB proteins were assayed for MSR activities with either DTT or yeast TRX system as reductants. As shown in Fig. 3, in the reaction mixtures containing equivalent amounts of enzymes, GmMSRB1 was two-fold more efficient than GmMSRB2 or GmMSRB4 in reducing dabsylated Met-R-O (Fig. 3A). Although MSRBs are known to have activity only for protein-based MetO, several reports suggested that the enzymes may have very low activity with free MetO [5,7,8]. This possibility prompted us to assay GmMSRB proteins for their activities toward free MetO. Surprisingly, we found that GmMSRB2 exhibited a high MSR activity with free Met-R-O, as this protein released 7621 pmole of Met per minute per milligram protein (Fig. 3B). This activity was 7- and 10-fold higher than those of GmMSRB1 and GmMSRB4, respectively. As oxidized MSRs can be regenerated by TRX or GRX [18,19,40], we also assayed the soybean enzymes using yeast TRX and GRX as reduction systems. The data presented in Table 2 show that GmMSRB1 did not exhibit MSR activity with yeast TRX2 or GRX4, whereas GmMSRB2 and GmMSRB4 were active with the TRX system. Although GmMSRB4 exhibited much lower kₐ values than GmMSRB2 with either N-acetyl-MetO or free MetO, the apparent kₐ values for both substrates were the same, and 15- to 20-fold lower than the reported values for Arabidopsis MSRB2 [8]. In addition, we observed that the kₐ values of GmMSRB2 and GmMSRB4 for free Met-R-O were actually lower than that reported for fMRSR from E. coli [11]. The catalytic efficiency (kₐ/Kₐ) of GmMSRB2 was 15- to 150-fold higher than that of any other characterized MSRBs [as reviewed in [41]]. In the case of GmMSRB4, the calculated Kₐ was low, showing a strong affinity for the substrate, but the low kₐ led to the question...
whether or not the yeast TRX regenerated its activity as efficient as it did to GmMSRB2. To clarify this question, we measured the $k_a$ values of yeast TRX2 for GmMSRB2 and GmMSRB4 using N-acetyl MetO as substrate. We found the values for GmMSRB2 and GmMSRB4 to be 4.54±0.46 and 2.27±0.51 μM, respectively, suggesting both enzymes can be regenerated by TRX at similar efficiencies.

To confirm the functions of catalytic and resolving Cys, we performed multiple sequence analysis and identified additional conserved Cys at residue 68 (Fig. 1). Site-directed mutagenesis was then performed and two mutants were obtained (C68S GmMSRB2 and C121S GmMSRB2). As shown in Fig. 3, with DTT as the reducing agent, the C68S GmMSRB2 mutant was active towards both protein-based and free MetO with the catalytic parameters similar to those of the wild type enzyme. However, when TRX was used as a reductant, the mutant showed no activity (Table 2), clearly demonstrating a role of Cys68 as a resolving residue. The Cys-to-Ser mutation at residue 121 rendered GmMSRB2 completely inactive toward both substrates using either DTT or TRX as reductants (Fig. 3A,B and Table 2), confirming its role as the catalytic residue.

To test whether the observed activity towards free MetO was taking place in vivo, we performed a complementation assay using an yeast strain whose all three MSR genes were knocked out [10]. As shown in Fig. 3C, GmMSRB2 and GmMSRB4, expressed under the control of a strong GPD promoter, supported the growth of yeast mutant cells on free MetO as the only source of Met. The level of complementation was similar to that of yeast JRMRS, and much stronger than the yeast MSR2 under the control of the same promoter. It should be noted that, under the control of its own promoter, yeast MSR2 did not complement at all (Fig. S4). Despite the low calculated $k_a$ observed with the recombinant GmMSRB4, overexpression of GmMSRB4 could also provide complementation, suggesting that yeast cells require only a trace amount of free Met to maintain growth. The fact that GmMSRB1 cannot complement the growth of the yeast triple mutant (Fig. 3C) and that yeast TRX2 and GRX4 were unable to regenerate this enzyme in vitro (Table 2) implied that GmMSRB1 may require a plant-specific regeneration system.

Overexpression of GmMSRBs Confers Oxidative Stress Tolerance in Yeast

We previously showed that overexpression of MSR could protect the yeast strain lacking all three MSR against oxidative stress [10]. Thus, mutant yeast strains overexpressing GmMSRBs were tested for their viability in the presence of hydrogen peroxide. As shown in Fig. 4, overexpression of either soybean or yeast MSRs protected cells from H2O2-induced stress, and the protection was higher in cells overexpressing GmMSRB2 or GmMSRB4, while overexpressing yeast JRMRS alone did not confer significant protection under conditions of our study. Overall, these data suggest that the presence of a GmMSRB possessing a novel activity for free MetO could provide better protection against oxidative stress than MSRBs lacking such activity or JRMRS.

Overall, this work reports the discovery of a unique MSRB from soybean that acquired activity for free MetO. This enzyme is as efficient as JRMRS both in vitro and in vivo. We also demonstrate that this enzyme conferred better protection against oxidative stress to yeast cells than either other MSRBs or JRMRS. Our work uncovered an unexpected function of MSRBs in plants, which should facilitate research into the roles of MSRBs under physiological and pathophysiological conditions as well as potential application in agriculture.

Supporting Information

Figure S1 Synteny analysis of soybean GmMSRB genes revealed two segmental duplicated pairs. Both pairs shared a large block with 397 anchors. Locus search and image acquisition were done via the web service at http://chibba.agtec.uga.edu/duplication/index/locus. (PDF)

Figure S2 Sequence alignments of proteins encoded by different alternate transcripts of GmMSRB2 (upper panel) and GmMSRB4 (lower panel). Black arrows indicate catalytic Cys, and gray arrows indicate resolving Cys. (PDF)

Figure S3 Relative expression of soybean MSRB transcripts in various tissues. Data (normalized reads per million) taken from the cDNA sequencing study by Libault et al. (Plant J., 2010, 68:86-99) [39]. (PDF)

Figure S4 Complementation assay of the Δ3MSR yeast cells transformed with p425-GPD plasmids harboring indicated yeast MSRs. The triple mutant was transformed with plasmids and grown on selective media (right panel) or selective media minus L-Met and plus L-MetO (left panel). In the p&3P::MSRA and p&3P::MSRB plasmids, the GPD promoter was replaced with natural yeast promoters for the respective genes. (PDF)

Table S1 Primers used in this study. (DOC)

Acknowledgments

The authors wish to thank N. Doi and T. Kawada for technical assistance and N. Etohara for help in preparing this manuscript.

Author Contributions

Conceived and designed the experiments: DTL, VNG, L-SPT. Performed the experiments: DTL, YW AK LT DEF UT RN. Analyzed the data: DTL, YW AK LT. Contributed reagents/materials/analysis tools: BCL, LT DEF. Wrote the paper: DTL, VNG L-SPT LT.

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