Different B-Type Methionine Sulfoxide Reductases in *Chlamydomonas* May Protect the Alga against High-Light, Sulfur-Depletion, or Oxidative Stress

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Available online on 30 August 2013 at www.jipb.net and www.wileyonlinelibrary.com/journal/jipb
doi: 10.1111/jipb.12104

Abstract

The genome of unicellular green alga *Chlamydomonas reinhardtii* contains four genes encoding B-type methionine sulfoxide reductases, MSRB1.1, MSRB1.2, MSRB2.1, and MSRB2.2, with functions largely unknown. To understand the cell defense system mediated by the methionine sulfoxide reductases in *Chlamydomonas*, we analyzed expression and physiological roles of the MSRBs under different abiotic stress conditions using immunoblotting and quantitative polymerase chain reaction (PCR) analyses. We showed that the MSRB2.2 protein was accumulated in cells treated with high light (1,300 μE/m2 per s), whereas MSRB1.1 was accumulated in the cells under 1 mmol/L H₂O₂ treatment or sulfur depletion. We observed that the cells with the MSRB2.2 knockdown and overexpression displayed increased and decreased sensitivity to high light, respectively, based on *in situ* chlorophyll a fluorescence measures. We also observed that the cells with the MSRB1.1 knockdown and overexpression displayed decreased and increased tolerance to sulfur-depletion and oxidative stresses, respectively, based on growth and H₂-producing performance. The physiological implications revealed from the experimental data highlight the importance of MSRB2.2 and MSRB1.1 in protecting *Chlamydomonas* cells against adverse conditions such as high-light, sulfur-depletion, and oxidative stresses.

Keywords: *Chlamydomonas*; high light; MSRB; oxidative stress; sulfur deprivation.


Introduction

Oxidation of methionine residues represents one of the major oxidative modifications occurring in protein molecules. The modification is primarily mediated by various biological oxidants such as reactive oxygen species (ROS) as well as metal-catalyzed oxidation reactions (Moskovitz et al. 1997), resulting in the formation of diastereoisomers called S- and R-methionine sulfoxide (MetO) (Shechter et al. 1975; Stadtman 2006). Oxidized proteins may become non-functional due to catalytic malfunction and structural distortions leading to toxic aggregation of unfolded proteins (Berlett and Stadtman 1997; Davies 2005; Lee et al. 2009). Therefore, it is vital to reduce the MetO back to methionine efficiently in living cells.

Type A and B methionine sulfoxide reductase (MSRA and MSRB, respectively) are the two well-known types of methionine sulfoxide reductases (MSRs) catalyzing the reduction of oxidized S-methionine residues (S-MetO) and R-methionine residues (R-MetO) back to methionine, respectively. Genes encoding these MSRs are ubiquitous in all organisms from bacteria to
mammals (Delaye et al. 2007). Comparison of bacterial genomes revealed that these are among the minimal set of proteins required for cell life (Mushegian and Koonin 1996). Considerable research has been conducted in heterotrophic organisms during the past decades to determine the structure of MSR proteins (Nakajima et al. 1998; Aachmann et al. 2011), and to elucidate the catalytic mechanisms of MSR enzymes (Kim and Gladyshev 2005; Lee et al. 2008; Tarrago et al. 2012). Physiological roles of MSRs in heterotrophic organisms also have been the subject of intensive research. It was reported that these proteins were crucial in determination of cell viability or lifespan (Moskovitz et al. 2001; Lee et al. 2009) and in development of age-associated disorders (Gabbita et al. 1999), virtually through their role in protecting cellular proteins against oxidative stress (Lee et al. 2009).

Genome analysis revealed more gene copies encoding MSRs in eukaryotic photosynthetic organisms, such as higher plants and green algae, than other organisms (Tarrago et al. 2009a). Compared to the heterotrophic organisms, experimental data regarding functional significance of MSRs in photosynthetic organisms are yet limited. Results obtained from several model species of higher plants indicated more complex roles of MSRs in these organisms than in heterotrophs (Romero et al. 2004; Kwon et al. 2007; Vieira Dos Santos et al. 2007; Guo et al. 2009; Tarrago et al. 2009b; Oh et al. 2010; Tarrago et al. 2010). Recently, it has been suggested that in Arabidopsis the plastid-located MSRs participate in protection of photosynthetic antennae under adverse conditions (Laugier et al. 2010). These findings have advanced current understanding of the functional significance of MSRs in higher plants. With regard to MSRs in unicellular photosynthetic organisms such as green algae and cyanobacteria, however, experimental data are still scarce (Novoselov et al. 2002; Allen et al. 2007; Hsu and Lee 2010, 2012). It remains largely unknown in terms of the expression of multiple copies of MSR genes as well as the specific functions of MSR proteins in these evolutionally and ecologically important organisms.

The green alga, Chlamydomonas reinhardtii, is a unicellular photosynthetic organism that has been used as a model system for studying photosynthesis and cell adaptation to various stress conditions (Lemaire et al. 2004; Ma et al. 2007). The genome of Chlamydomonas encodes five MSRs and four MSRBs, respectively (Merchant et al. 2007). Although the first Chlamydomonas MSR gene, that is, MSRA1, was identified a decade ago (Novoselov et al. 2002), the functional significance of MSRA1 and other MSR proteins in the organism has not been established. More recent proteomic data shows that MSRA4 is among the thioredoxin targets in Chlamydomonas (Lemaire et al. 2004). Differential expression of MSRA2 was reported in Chlamydomonas under manganese deficiency (Allen et al. 2007). In contrast to the MSRAs, no experimental data has been so far reported for the MSRBs in Chlamydomonas. The discovery of the presence of four MSRB genes in the single cell of Chlamydomonas raises questions about their expression patterns as well as physiological roles.

In this work, we have carried out an initial investigation into expression and physiological roles of the MSRBs in Chlamydomonas under abiotic stress. Expression of MSRB proteins and genes were examined using immunoblotting and quantitative real-time PCR (qRT-PCR) analysis. We show that all of them are expressed in Chlamydomonas cells, but display distinct patterns in response to different stress signals. We found that the level of MSRB2.2 protein increased significantly in response to high light while MSRB1.1 was apparently accumulated under sulfur-depletion and oxidative stresses. The correlation between their variations in protein abundance and the stress responses above was further verified by distinction of phenotype between wild-type and transgenic cells generated using artificial miRNA (amiRNA) and overexpressing tools. The results highlight the importance of MSRB2.2 and MSRB1.1 in protecting Chlamydomonas cells against adverse conditions such as high-light, sulfur-depletion, and oxidative stresses.

### Results

#### Analysis of MSRB protein sequences and antibody production

The genome of Chlamydomonas contains four MSRB genes encoding B-type methionine sulfoxide reductases, that is, CrMSRB1.1, CrMSRB1.2, CrMSRB2.1, and CrMSRB2.2 (http://genome.jgi-psf.org/Chlre4). Sequence comparison of CrMSRB proteins using Clustal W revealed that CrMSRB1.1 and CrMSRB1.2 were only 33% identical to each other while CrMSRB2.1 and CrMSRB2.2 were significantly similar (72%) at the protein sequence level (Figure S1). The CrMSRB2 proteins exhibited 50%–60% identity to Arabidopsis MSRB2 whereas CrMSRB1 proteins show higher similarity to cyanobacterial MSRB1 (39%–44%) than those from higher plants (34%–36%). The higher similarity of CrMSRB1 to those in cyanobacteria may indicate a closer relationship of CrMSRB1 proteins with the MSRB1 from cyanobacteria than to those from higher plants (Tarrago et al. 2009a). By using online programs, such as WoLFPSORT, TargetP, MitoProt, and Predotar, these proteins were predicted to be present in chloroplast, mitochondria, or cytoplasm of the organism (Table S1).

Making use of the genomic information, we generated peptide antibodies against each of the MSRB proteins. Antigenic peptides corresponding to specific regions of the predicted proteins were synthesized by MBL (Nagoya, Japan). Heterologous expression of recombinant MSRB proteins and antibody specificity verification was performed as described (Zhou et al. 2008; Terauchi et al. 2009). The initial analysis of crude
protein extracts from induced *Escherichia coli* Transetta (DE3) cells shows that the bacteria containing the respective plasmids (pET-28a with a His-tag sequence) produced a substantial amount of expected recombinant proteins with an estimated molecular mass of 23, 27, 20, and 16 kDa, which correspond to the molecular masses calculated from their coding sequences (Figure S2A). Equal amounts of total proteins extracted from the *E. coli* Transetta (DE3) cells expressing corresponding recombinant MSRB protein were analyzed by immunoblot with each antibody. As demonstrated in Figure S2B, all of them showed high specificity to the respective antigens, and no cross-reaction between the MSRBs was detected. The antibodies were therefore used for subsequent analysis of MSRB proteins in *Chlamydomonas*.

Expression patterns of MSRBs in response to abiotic stress

To investigate the physiological roles of MSRBs in *Chlamydomonas*, we designed and carried out a number of stress experiments. The expression patterns of MSRB genes and proteins were monitored using immunoblot and qRT-PCR analyses. Because high light is one of the most common stress conditions encountered by photosynthetic organisms, and ROS can be generated under such stress, we first investigated the expression patterns of MSRBs in response to high light. As shown in Figure 1, the protein level of MSRB2.2 increased significantly during high-light treatment (Figure 1D). Remarkable accumulation of MSRB2.2 was detected upon exposure of...
Chlamydomonas cells to high light for 15 min. The protein level of MSRB2.2 increased approximately 62% compared to that at 0 h. Prolonged treatment retained a high level of the protein although photobleaching was visible after 60 min (data not shown). Protein levels of the other three MSRBs appeared reduced under high-light treatment (Figure 1A–C). Compared to that at 0 h, the protein levels of those declined by an average of 33.7% (MSRB1.1), 34.7% (MSRB1.2), and 18.2% (MSRB2.1), respectively. To examine the gene expression of MSRBs in response to high light, qRT-PCR was carried out using total mRNA isolated from Chlamydomonas collected at different time points of high-light treatment (Figure 1E). Our experimental data shows that, compared to other MSRBs, no significant changes in the transcript level of MSRB2.2 was detected during 2 h of high-light treatment. Incongruent expression pattern between mRNAs and protein has been reported in a variety of cells (Griffin et al. 2002; Deng et al. 2007; Chen et al. 2010). The remarkable accumulation of MSRB2.2 protein may indicate that degradation of the protein was inhibited under such stress conditions.

It is generally known that methionine oxidation is primarily mediated by ROS. To determine whether oxidative stress would affect the expression of Chlamydomonas MSRBs, we applied H$_2$O$_2$ treatment in this initial investigation of MSRBs. Growth profiles of Chlamydomonas were at first monitored in the presence of H$_2$O$_2$ (1 mmol/L). As expected, cell growth was significantly repressed by H$_2$O$_2$ throughout the measured period (Figure S3A). Because a remarkable reduction of cell growth was observed at 12 h of H$_2$O$_2$ treatment, we examined the changes in the protein level of MSRBs in the duration of 12 h of H$_2$O$_2$ treatment using immunoblotting (Figure 2A–D). The data shows that the level of MSRB1.1 increased continuously during 12 h of H$_2$O$_2$ treatment. Gene expression of MSRB1.1 shows a similar pattern, as revealed by qRT-PCR analysis (Figure 2E). Compared to 0 h, the protein and mRNA levels of MSRB1.1 elevated 3.5 and 8.8 fold, respectively.

In higher plants, ROS can be stimulated rapidly by nutrient deprivation (Schachtman and Shin 2007). Considering that sulfur is one of the essential nutrient elements as cofactor/component for numerous enzymes/proteins in redox reactions, as well as its close relationship with H$_2$ production in Chlamydomonas (Melis et al. 2000), we investigated the expression of MSRBs under sulfur deficiency. To determine the sampling time points for this purpose, growth profiles were first monitored within 30 h of sulfur-depletion treatment (Figure S3B). Cell growth was apparently repressed: only 54% of cell growth was achieved after 24 h of the treatment (Figure S3B). Interestingly, MSRB1.1 protein was also accumulated during 12 h of sulfur deprivation (Figure 3A). Taken together, these data demonstrate that MSRB1.1 protein was upregulated by H$_2$O$_2$ treatment and sulfur depletion (Figures 2, 3). Gene expression profiles of MSRB1.1 under oxidative stress were different from sulfur depletion. In the presence of H$_2$O$_2$, the level of MSRB1.1 transcript increased steadily (up to ninefold), which was closely correlated with the protein expression (Figure 2A, E). Under sulfur depletion, however, the highest level of MSRB1.1 transcript (sevenfold) was detected at 6 h, followed by a gradual decline (Figure 3E). Gene expression of the other three MSRBs in response to the sulfur deprivation shows more complex patterns than that of MSRB1.1 described above. Despite the decrease in their protein levels, a clear increase in the transcripts of MSRB1.2 and MSRB2.2 (eight and fourfold, respectively) was observed. On the other hand, no dramatic change in protein and mRNA levels of MSRB2.1 was observed under sulfur deficiency (Figure 3C, E).

Methionine oxidation also could be mediated by metal catalyzed oxidation reactions (Moskovitz et al. 1997). Because iron is one of the essential metal elements presented in heme- and/or iron-sulfur proteins that are required for energy-transducing pathways such as photosynthesis, we investigated the effect of iron deficiency on growth (Figure S3C), and the expression of Chlamydomonas MSRBs (Figure 4). Similar trends were found for all MSRBs under this stress condition: the protein levels reduced dramatically during 6 h of iron starvation, followed by a slight recovery. Increased expression of MSRB genes was evident after 6 h of iron deficiency. The highest expression level (~33 fold) was found for MSRB1.2 under such stress conditions (Figure 4E). Based on these observations, we postulate that overall turnover of MSRB proteins may be enhanced in Chlamydomonas in order to cope with iron starvation.

**Phenotypic analysis of MSRB2.2- and MSRB1.1-transgenic cell lines**

To confirm the functional significance of MSRB2.2 and MSRB1.1 involved in the stress responses described above, we generated transgenic cell lines of MSRB2.2 and MSRB1.1 using amiRNA and overexpressing approaches (Sizova et al. 2001; Molnar et al. 2009), respectively. Positive clones were selected by antibiotic resistance (paromomycin), followed by immunoblotting using the antibody specific to each of the proteins. Dozens of amiRNA and overexpressing transformants were isolated, in which the level of MSRB2.2 or MSRB1.1 protein was reduced or accumulated to a different extent in comparison with that in wild-type (data not shown). Three transgenic cell lines with significantly lower (KD) and higher (OE) levels of MSRB2.2 (Figure 5) or MSRB1.1 (Figures 6, 7) were subjected to further analysis.

Chlorophyll fluorescence is one of the sensitive indicators widely used for evaluating high-light stress response in photosynthetic organisms (Krause and Weis 1991; Peers et al. 2009). To determine whether the altered level of MSRB2.2 protein would affect high-light sensitivity of the organism, we measured chlorophyll a fluorescence parameters...
of wild-type and MSRB2.2-transgenic cells using Maxi-Imaging PAM chlorophyll fluorometry (Heinz Walz, Effeltrich, Germany). Figure 5C shows the light intensity dependence of relative electron transport rate (ETR) through PSII in wild-type and MSRB2.2-amiRNA-transgenic cells (KD). The ETR was not distinct in the knockdown cells (KD36, KD39, and KD47) at light intensity below 100 μE/m² per s. However, an overall 20% lower ETR in the knockdown cells were observed with further increase of light intensity (Figure 5C). High-light-sensitive phenotype of the knockdown cells was also verified by their greater decrease in maximum quantum yield (Fv/Fm) after high-light treatment. As summarized in Table 1, Fv/Fm was highly similar between wild-type and knockdown cells and constant at approximately 0.73 under normal condition (100 μE/m² per s). After 40 min of high-light treatment (1,300 μE/m² per s), however, a greater reduction of Fv/Fm was observed in the knockdown cells (KD36, 0.07; KD39, 0.05; KD47, 0.08) than in wild-type (WT, 0.22). The average reduction of Fv/Fm in the knockdown cell lines was 10.7 fold, which was threefold more reduction than in wild-type.

To further clarify the correlation between elevated level of MSRB2.2 and cell resistance to high light, Fv/Fm was also compared between wild-type and MSRB2.2-overexpressing cells under normal and high-light conditions (Table 1). As expected, no difference was observed between the two types of cells under normal conditions. Reduction of Fv/Fm was also

Figure 2. Expression profiles of B-type methionine sulfoxide reductases (MSRBs) in Chlamydomonas under oxidative stress.

(A–D) Immunoblot detection of each MSRB in total protein extracts. Proteins (20 μg, per lane) were separated by 12% sodium dodecylsulfate polyacrylamide gel electrophoresis.

(E) Real-time quantitative polymerase chain reaction analysis of each MSRB gene. All experiments were done three times, and similar results were obtained.
similar in the above cell lines subjected to high-light treatment for 30 min (data not shown). Variation of Fv/Fm was, however, pronounced after prolonged high-light treatment (40 min). The average decrease of Fv/Fm in overexpression cells (OE3, OE4, OE33) was 1.1 fold, which was significantly less than that in wild-type (3.4 fold). These observations strongly suggest that MSRB2.2 is involved in high-light response. To test whether this functional role is specific for MSRB2.2, a parallel experiment was carried out using MSRB1.1-transgenic cells. Comparison of Fv/Fm between wild-type and MSRB1.1-transgenic cells indicated that the reduction of Fv/Fm was similar under high-light conditions (Table S6). These results exclude a close relationship of MSRB1.1 with high light. Taken together, we propose that MSRB2.2, rather than MSRB1.1, plays an important role in Chlamydomonas coping with high-light stress.

To confirm functional significance of MSRB1.1 in oxidative- and sulfur-depletion stresses, we compared phenotype of wild-type and MSRB1.1-transgenic cells subjected to the respective stress conditions. Figure 6A shows that MSRB1.1-amirNA cells (KD20, KD33, and KD45) were more susceptible to H2O2, due to the lower level of MSRB1.1, than wild-type. On the other hand, elevated level of MSRB1.1 (OE5, OE8, and OE23) caused a slightly better growth than wild-type (Figure 6B). To examine whether the observed response of cells with increased/decreased MSRB1.1...
expression was specific to \( \text{H}_2\text{O}_2 \), growth of wild-type and \( \text{MSRB1.1} \)-transgenic cells were also monitored in the presence of methyl viologen (0.5 \( \mu \text{M} \)) and rose bengal (2.5 \( \mu \text{M} \)), which are the generators of singlet oxygen and superoxide (Baroli et al. 2004), respectively. In the presence of methyl viologen (0.5 \( \mu \text{M} \)), no difference in cell growth was observed between wild-type and \( \text{MSRB1.1} \)-transgenic cells (Figure S5). In the presence of rose bengal (2.5 \( \mu \text{M} \)), growth of both \( \text{MSRB1.1} \)-amiRNA cells (KD20, KD33, KD45) and overexpression cells (OE5, OE8, OE23) was more repressed than wild-type (Figure S6). These results indicate that the level of \( \text{MSRB-1.1} \) expression was not correlated to oxidative stress caused by singlet oxygen and superoxide. Taken together, we conclude that \( \text{MSRB1.1} \) plays a crucial role in protecting \( \text{Chlamydomonas} \) against oxidative stress specifically caused by \( \text{H}_2\text{O}_2 \).

The physiological role of \( \text{MSRB1.1} \) in cell acclimation to sulfur depletion was also determined based on growth profiles of wild-type and \( \text{MSRB1.1} \)-transgenic cells under aerobic sulfur-depletion conditions (Figure 7). Growth of \( \text{MSRB1.1} \)-amiRNA cells (KD20, KD33, KD45) was generally more repressed than wild-type (Figure 7A), whereas that of overexpression cells (OE5, OE8, OE23) was significantly enhanced (up to 30%) under such stress conditions (Figure 7B). The parallel experiment using wild-type and \( \text{MSRB2.2} \)-transgenic cells shows no
phenotypic differences under either oxidative stress (data not shown) or sulfur deprivation (Figure S4). These results address the specific roles of MSRB1.1, rather than MSRB2.2, involved in both stresses.

Under anaerobic sulfur-depletion conditions, *Chlamydomonas* can produce a substantial quantity of hydrogen (H₂) (Melis et al. 2000). Based on the proteomics data, we have previously suggested that H₂O₂ could be one of the major ROS produced in *Chlamydomonas* during a prolonged period of anaerobic sulfur deprivation (Chen et al. 2010). Considering that MSRB1.1 is involved in both oxidative- and sulfur-depletion stresses (Figures 3, 7), and H₂ production by green algae is of biotechnological importance (Melis et al. 2000), we further compared the capacity of H₂ production in wild-type and the MSRB1.1-overexpressing cells under anaerobic sulfur-depletion conditions. The experimental data shows that MSRB1.1-overexpressing cells exhibited better capability in H₂ production than wild-type, especially at the later stages of H₂ evolution (Figure 7C). At 126 h of sulfur deprivation, the yield of H₂ evolved from the transgenic cells was on average 60% higher than that from wild-type. Thus, modulation of MSRB1.1 expression could be one of the new perspectives toward improvement of H₂ production in *Chlamydomonas*.

**Discussion**

*Chlamydomonas* is a model organism for studying photosynthesis and cell adaptation to adverse conditions including oxidative stress. Compared to the genes involved in cell defense to oxidative stress such as superoxide dismutase, our knowledge of the surveillance system mediated by MSRs in *Chlamydomonas* against oxidative stress remains modest (Tarrago et al. 2009a). In this study, we have investigated the expression patterns of all MSRBs in the *Chlamydomonas* gene family. We have shown that each MSRB gene and protein was differentially expressed in response to the stress conditions (Figures 1-4). Protein level of MSRB1.1 was elevated remarkably under sulfur-depletion and oxidative stresses, whereas MSRB2.2 protein was apparently accumulated under high-light stress. We have also shown that MSRB2.2 transgenic cells displayed higher (KD) or lower (OE) sensitivity to high-light stress, whereas MSRB1.1 transgenic cells exhibited the phenotype that was more susceptible (KD) or tolerant (OE) to sulfur-depletion and
oxidative stresses (Figures 5–7). Therefore, we hypothesize that MSRB2.2 and MSRB1.1 are the major MSRBs for protecting *Chlamydomonas* cells against adverse conditions such as high-light, sulfur-depletion, and oxidative stresses.

High light can cause ROS production in photosynthetic organisms (Niyogi 1999). However, no experimental data has been reported so far regarding expression or functions of MSR proteins in *Chlamydomonas* under such stress. Recent results obtained from the green macroalga, *Ulva fasciata*, indicated that expression of both MSRA and MSRB was upregulated by high light (Hsu and Lee 2012). In this work, we found that the protein level of MSRB2.2 increased up to 62% upon exposure of *Chlamydomonas* to high light (Figure 1). These observations may indicate that protein damage by oxidation is stimulated by high-light stress. Because no significant enhancement of the other MSRBs (MSRB1.1, MSRB1.2, and MSRB2.1) was observed, we presume that MSRB2.2 is the major *Chlamydomonas* MSRB involved in high-light stress response. Our findings that *MSRB2.2 amiRNA* cells, rather than *MSRB1.1 amiRNA* cells, were more sensitive to high light strongly support this assumption.

Although the protein sequence of MSRB2.2 and MSRB2.1 is highly similar, the effect of high light on their expression is different (Figure 1). In contrast to MSRB2.2, the level of MSRB2.1 was not accumulated significantly. This may exclude its close relationship with high-light stress in *Chlamydomonas*. Based on the increased level of MSRB2.2 protein, the high-light-sensitive phenotype of *MSRB2.2 amiRNA* cells (rather than *MSRB1.1 amiRNA* cells) observed in this work, as well as the high similarity of protein sequences, we postulate that MSRB2.2 is most likely the homolog to the plastidial MSRB2 in *Arabidopsis* (Vieira Dos Santos et al. 2005; Tarrago et al. 2009b). Predicting chloroplast localization of proteins in *Chlamydomonas* using the current version of TargetP is not as reliable as in higher plants (Dr O. Emanuelsson, personal communication; Tarrago et al. 2009a), and the prediction results from other programs were also not clear-cut for this location (Table S1), therefore more experimental work is required to determine the subcellular location(s) of MSRB2.1 and MSRB2.2 proteins as well as their potential substrates in *Chlamydomonas* under high-light stress conditions.

Clearly, both protein and mRNA levels of *MSRB1.1* were significantly elevated under sulfur-depletion and oxidative stresses (Figures 2, 3). These findings suggest that MSRB1.1 is involved in cell response to both stresses. Functional significance of MSRB1.1 was further demonstrated by the distinction of phenotype between the transgenic cell lines of MSRB1.1 and wild-type observed under these stress conditions (Figures 6, 7). Based on the increased sensitivity/more repressed growth observed in *MSRB1.1 amiRNA* cell lines under H$_2$O$_2$ treatment/sulfur depletion, and the enhanced tolerance/growth displayed in *MSRB1.1 overexpressing* cell lines under these stress conditions, we propose that MSRB1.1...
plays important roles in *Chlamydomonas* coping with sulfur-depletion and oxidative stresses.

The kinetics of MSRB1.1 gene expression in response to oxidative stress was different from that to sulfur-depletion stress (Figures 2, 3). Under oxidative stress (1 mmol/L H2O2), increase in the MSRB1.1 transcript was closely correlated with elevated level of the protein, indicating the direct involvement of MSRB1.1 in antioxidant response. This is confirmed by the increased H2O2 sensitivity observed in MSRB1.1 amiRNA cell lines mentioned above. It remains unclear whether the protein functions as a key player in repair of damaged proteins by oxidation or as a ROS scavengers through cyclic oxidation and reduction of methionine and methionine sulfoxide residues (Kim and Gladyshev 2007) during this process. Under sulfur depletion, the transcript level of MSRB1.1 reached the highest at 6 h of sulfur depletion, and then declined gradually. This may suggest that MSRB1.1 is one of the genes responsive to the sulfur-starvation stress. Recent data based on RNA sequence analysis have shown that a great number of genes were upregulated in *Chlamydomonas* under sulfur deficiency (Gonzalez-Ballester et al. 2011). Because the protein was accumulated to a considerable amount until 12 h of sulfur depletion, and cell growth was also recovered to some extent (this work), we propose that MSRB1.1 is one of the proteins involved in cell adaptation to sulfur deprivation.

The overlapping effect of sulfur-depletion and oxidative stresses on MSRB1.1 revealed in our experiment indicates that regulation of *MSRB1.1* gene expression is complex in *Chlamydomonas*. In root tissue of *Arabidopsis*, it has been observed that the level of ROS is higher under sulfur starvation than under sulfur-sufficient conditions (Lappartient and Touraine 1997), but direct evidence demonstrating that ROS serves as a signaling component in sulfur-starved plants is still lacking (Schachtman and Shin 2007). In *Chlamydomonas*, no experimental data has been reported thus far showing that the level of ROS was increased under sulfur-depletion stress conditions. The overlapping effect of sulfur-depletion and oxidative stresses on MSRB1.1 brings an open question whether this protein exerts its function with the involvement of ROS under this stress condition. Based on proteomic analysis, we have previously suggested that H2O2 could be the major ROS produced at the later stage of H2 photoproduction under anaerobic sulfur deprivation (Chen et al. 2010). Considering that H2 photoproduction is one of the efficient modules for long-term survival of *Chlamydomonas* under anaerobic sulfur-deprivation conditions (Melis et al. 2000), and MSRB1.1 is involved in sulfur-depletion stress (this work), we further compare the capacity of H2 production in wild-type and the MSRB1.1-overexpressing cells under anaerobic sulfur-deprivation conditions. Our finding that MRSR1.1-overexpressing transgenic cells exhibited better capability of H2 production than wild-type at the later stages of the H2-producing process (Figure 7C) may support the hypothesis that MSRB1.1 exerting its role with the involvement of ROS under sulfur depletion. Thus, further elucidation of the relationship between ROS and MSRB1.1 under sulfur deprivation will gain novel insights in the signaling pathways.

In summary, our present work revealed the distinct expression patterns as well as functional significance of *Chlamydomonas* MSRBs under stress conditions. MSRB2.2 protein was found accumulated under high light whereas MSRB1.1 was clearly increased under sulfur-depletion and oxidative stresses. The correlation of MSRB2.2 and MSRB1.1 with cell response to the above stresses was further confirmed by the distinct phenotype observed in wild-type and the transgenic cells. The physiological implications revealed from the present work provide important information for in-depth studies towards comprehensive understanding of MSRBs in the single cell photosynthetic organism.

### Materials and Methods

### Strains, culture conditions, and stress treatments

*Chlamydomonas reinhardtii* wild-type strain, CC400 (mt−) was obtained from the *Chlamydomonas* Center (www.chlamy.org). The algal cells were cultured in TAP medium (Gorman and Levine 1965) at 25°C under 100 μE/m²/sec continuous light. For all experiments, mid-exponentially growing cells (~4–6 × 10⁶ cells/mL) were harvested as described (Chen et al. 2010). The cell pellet was suspended in the same medium and the cell density was adjusted to 2–4 × 10⁶ cells/mL, which...
was used for different stress treatments. For high-light stress treatment, the cultures were transferred into Petri dishes then placed in a growth chamber (DHZ-D, Taicang, China) set at the desired high-light intensity (1,300 μE/m²/s) under constant temperature conditions (25 °C). For other stress treatments (H2O2, sulfur-, iron-depletion), the cells were either directly cultured in TAP medium with H2O2 (1 mmol/L) or collected and washed twice with TAP-S (Melis et al. 2000) or TAP-iron medium (Allen et al. 2007). The cells were then resuspended in respective medium and incubated further as described above. Growth was measured as OD750. For protein and mRNA analysis, cells were harvested and washed once with 0.01 mol/L sodium phosphate buffer (pH 7.4) by centrifugation before being stored at −70 °C.

**Gene cloning and heterologous expression of Chlamydomonas MSRBs**

Gene cloning and heterologous expression was performed as described (Zhou et al. 2008). cDNA of MSRB genes was amplified from total RNA by PCR. Total RNA isolation and purification of Chlamydomonas was performed as described (Chen et al. 2010). Reverse transcription reactions using a poly(A)−-specific primer were performed with M-MLV Reverse Transcriptase (TransGen Biotech, Beijing, China). A total of 0.5 μg RNA was used for reverse transcription reactions. Gene-specific primers and restriction sites are listed in Table S2. cDNAs were amplified with PrimeSTAR HS DNA Polymerase (Takara, Ohtsu, Japan). Thermocycling conditions were 15 s at 98 °C, followed by 32 cycles of 10 s at 98 °C, 15 s at 62 °C, 1 min at 72 °C, and an additional reaction extension for 5 min at 72 °C. The PCR product was cloned directly into pEasy-blunt vector (TransGen Biotech, Beijing, China), which was then transformed into competent E. coli DH5α cells. Positive clones containing the recombinant plasmids were selected and sequenced to ensure the authenticity of the open reading frames (Sunbiotech, Beijing, China). For heterologous expression, the plasmids containing MSRBs were digested with Ndel and EcoRI. The fragments were purified and subcloned into similarly treated vector pET-28a (+) (Merck, Darmstadt, Germany) to generate pET-MSRBs, respectively. The constructed expression plasmids were transformed into expression host E. coli Transetta (DE3) (TransGen Biotech). The cells were grown in LB medium containing 50 mg kanamycin/mL at 37 °C. When the culture reached an A600 of 0.6–0.8, 400 μM isopropyl-β-D-thiogalactopyranoside was added (final concentration, 400 μM) and incubated for 6 h at 30 °C. Following the induction, cells were harvested by centrifugation at 4,400 g for 5 min at 4 °C and washed once with 10 mmol/L sodium phosphate buffer (pH 7.4). Protein concentration was determined by Bradford method (Bradford 1976) using bovine serum albumin (BSA) as standard. The homogeneity of recombinant proteins was confirmed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

**Antibodies production and specificity determination**

Antibodies specific to MSRB1.1, MSRB1.2, MSRB2.1, and MSRB2.2 were supplied by MBL. The synthetic peptides NH2-ATERRWTSPYSFKEKREGC-COOH (MSRB1.1), NH2-KQTKSSRGWAPRDESTPC-COOH (MSRB1.2), NH2-SSDGVKLDKSTPDWSVK-COOH (MSRB2.1), and NH2-MAANAELGKDTPESTWC-COOH (MSRB2.2) were synthesized and used as immunogen. Specificity of each antibody was verified by immunoblot as described (Terauchi et al. 2009). Equal amounts of total proteins extracted from E. coli Transetta (DE3) cells expressing corresponding recombinant MSRB protein were analyzed by immunoblotting.

**Protein extraction, SDS-PAGE, and immunoblot analysis**

Proteins were extracted from Chlamydomonas as described (Dewez et al. 2009). Proteins were separated by SDS-PAGE (12.5% polyacrylamide, w/v) and transferred onto nitrocellulose membrane (GE Healthcare, Cleveland, OH, USA) using electroblotting for immunoblot analysis, as previously described (Zhao et al. 2011). The dilution for the specific antibodies is as follows: anti-MSRB1.1 (1:1,000); anti-MSRB1.2, anti-MSRB2.1, and anti-MSRB2.2 (1:2,000). The immuno-signal was detected using the Pro-light HRP ECL detection system (Tiangen Biotech, Beijing, China). The blots were scanned using UMAX PowerLook 2100XL scanner (Willich, Germany) at a resolution of 600 dpi. Protein quantification was carried out using ImageJ software (http://rsbweb.nih.gov/ij). Protein content was determined by Bradford method (Bradford 1976) using BSA as standard.

**qRT-PCR analysis**

Total mRNAs used for qRT-PCR experiments were isolated from Chlamydomonas as described above. qRT-PCR reactions were performed in triplicate using a model Rotor-Gene 3000 sequence detection system (Qiagen, Hilden, Germany) with gene-specific primers and CBLP as the internal control (Gonzalez-Ballester et al. 2011). Gene-specific PCR primer pairs used for the CBLP and four MSRBs are listed in Table S3. PCR primers were designed using Primer 3 software (http://frodo.wi.mit.edu) and the amplifying program was as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. A melting curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the Rotor-Gene 3000 sequence detection system. A negative control reaction minus cDNA template (non-template control) was also routinely
performed in triplicate for each primer pair. The change in fluorescence of SYBR Green I dye in every cycle was monitored by the Rotor-Gene 3000 system software, and the cycle threshold \( C_0 \) above background for each reaction was calculated. The \( C_0 \) value of \( CBLP \) was subtracted from that of the gene of interest to obtain a \( \Delta C_0 \) value. The \( C_0 \) value of an arbitrary calibrator (e.g. the sample from which the largest cycle threshold \( C_{MSRB2.2} \) were digested with the restriction enzyme \( NdeI \) and \( EcoRI \). The fragments were purified and subcloned into similarly treated expression vector pJR38 containing the paromomycin resistance gene \( APHVIII \) to generate pJR38-MSRB1.1 and pJR38-MSRB2.2. The constructed plasmids were transformed into \( Chlamydomonas \) cells using the glass bead method as described (Kindle 1990). Transformants were selected on TAP plates containing 10 \( \mu \)g/mL paromomycin (Sigma-Aldrich, St Louis, MO, USA).

**Generation of amiRNA and overexpression cell lines**

Generation of amiRNA and overexpression cell lines was performed according to (Molnar et al. 2009; Neupert et al. 2009). For generation of amiRNA cell lines, pChlamyRNA3 vector obtained from the Chlamydomonas Center was used. Target gene-specific oligonucleotide sequences were designed using the WMD3 software (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) and verified using the expressed sequence tag database http://est.kazusa.or.jp/en/plant/chlamy/EST;blast.html. The resulting oligonucleotides that target 3’-untranslated region of \( MSRB1.1 \) and \( MSRB2.2 \) genes are listed in Table S4. Plasmids were constructed by annealing the oligonucleotides followed by ligation into pChlamyRNA3 vector at the unique \( SpeI \) site located in the amiRNA precursor containing the 90 bp oligonucleotides, respectively. Primers AmiRNAprec-f (GGTTGGGTCGGTGTTTTTG) and Spacer-rev (TAGCGCTGATCACCACCACCC) were used to screen the correct orientation of the constructed plasmids (pChlamyRNA3-MSRB1.1 and pChlamyRNA3-MSRB2.2). Thermocycling condition was: one cycle at 94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 30 s. The clones with correct orientation were confirmed by sequencing (Sunbiotech) using the AmiRNAprec-for primer above. The plasmids were then isolated and subjected to the culture and analyzed in a gas chromatograph instrument GC-2014 (Shimadzu, Tokyo, Japan) equipped with a thermal conductivity detector using \( N_2 \) as the carrier gas.

**Chlorophyll a fluorescence measurements**

Chlorophyll a fluorescence parameters were measured using a Maxi-Imaging PAM chlorophyll fluorometer (Walz, Effeltrich, Germany) by following the manufacturer’s instruction. Samples were adjusted to a density of \( 2 \times 10^6 \) cells/mL in TAP medium and were dark-adapted for 15 min before each measurement. \( F_0 \) is the minimal fluorescence in the dark-adapted state (weak illumination with blue light (460 nm); 0.5 \( \mu \)E/m²·s per s). \( F_m \) is the maximum fluorescence in the dark-adapted state (blue light, 460 nm; 2,800 \( \mu \)E/m²·s per s) from a light-emitting diode (LED) lamp. \( F_v \) was calculated as \( F_v = F_m - F_0 \). Maximum quantum yield was calculated as \( \Phi_v/F_m \). The effective photochemical quantum yield of PSII was measured as \( \Phi_{II} \) (\( F_m - F/F_0 \)). Relative photosynthetic ETRs were calculated as ETR = \( 0.5 \times \text{yield} \times 0.84 \times \text{PAR} \) \( \mu \)E/m²·s per s, where PAR is the photosynthetically active radiation.

**H₂ photoproduction via sulfur depletion**

\( H_2 \) photoproduction was achieved as described (Chen et al. 2010) with modifications. Briefly, mid-exponentially growing cells (5–10 \( 10^6 \) cells/mL) were harvested and washed once with TAP-S medium. The culture was transferred to a 100 mL bottle and the cell density was adjusted to 30 \( \mu \)g/mL initial chlorophyll concentration. To measure the amount of \( H_2 \) accumulated, 150 \( \mu \)L of gas was taken from the headspace of the culture and analyzed in a gas chromatograph instrument GC-2014 (Shimadzu, Tokyo, Japan) equipped with a thermal conductivity detector using \( N_2 \) as the carrier gas.

**Acknowledgements**

We thank Dr Ralph Bock (Max Planck Institute, Germany) for nuclear transformation vector pJR38. This work was supported by the Ministry of Science and Technology of China (2009CB220000), Chinese Ministry of Agriculture (2009ZX08009-121B), and the Chinese Academy of Sciences.

Received 27 Apr. 2013  Accepted 28 Aug. 2013

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(Co-Editor: Katie Dehesh)
Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1. Sequence alignment of *Chlamydomonas* B-type methionine sulfoxide reductases (MSRBs) with selected proteins.
Amino acid sequences are: CrMSRB1.1 from *Chlamydomonas reinhardtii* (accession no. XP001693285); CrMSRB1.2 (accession no. XP0016899051), CrMSRB2.1 (accession no. XP001699002); AtMSRB1 from *Arabidopsis thaliana* (accession no. NP564640), AtMSRB2 (accession no. NP567639); Alr3901 from *Anabaena sp. PCC 7120* (accession no. NP487941); OsMSRB1.1 from *Oryza sativa* (accession no. NP001057620) and Sll1680 from *Synechocystis sp. PCC 6803* (accession no. NP440114). Identical residues are highlighted in black. Residues conserved above 80% are shown in gray. Conserved cystein residues are highlighted in red. Gaps introduced to optimize sequence alignment are indicated by hyphen. The sequences are aligned using Clustal W in MEGAV3.1 software.

Figure S2. Heterologous expression of recombinant B-type methionine sulfoxide reductase (MSRB) proteins and specificity of MSRB antibodies.
(A) Sodium dodecylsulfate polyacrylamide gel electrophoresis analysis of proteins extracted from *Escherichia coli* Transetta (DE3) cells.
(B) Specificity detection of MSRB antibodies by immunoblotting.

Figure S3. Growth of *Chlamydomonas* under different stress conditions.
(A) In the absence or presence of H$_2$O$_2$ (1 mmol/L).
(B, C) With or without sulfur/iron supplement.

Figure S4. Growth of wild-type (WT) and MSRB2.2-KD (A)/OE (B) cell lines under sulfur depletion.

Figure S5. Growth of wild-type (WT) and *MSRB1.1* (A)/OE (B) cell lines in the presence of methyl viologen (0.5 μM).

Figure S6. Growth of wild-type (WT) and *MSRB1.1* (A)/OE (B) cell lines in the presence of rose bengal (2.5 μM).

Table S1. Prediction of subcellular location of *Chlamydomonas* B-type methionine sulfoxide reductases (MSRBs).

Table S2. Primers used for cloning and constructing expression plasmids of *MSRB* genes in *Chlamydomonas*.

Table S3. Primers used for real-time quantitative polymerase chain reaction analysis of *MSRB* genes in *Chlamydomonas*.

Table S4. Oligonucleotides used for constructing artificial miRNA (amiRNA) plasmids of *MSRB1.1* and *MSRB2.2* genes in *Chlamydomonas*.

Table S5. Primers used for overexpressing-plasmids of *MSRB1.1* and *MSRB2.2* in *Chlamydomonas*.

Table S6. Maximum quantum yield (Fv/Fm) of wild-type and *MSRB1.1*-transgenic cells lines under control and high-light conditions.