Reactive oxygen species modulate the differential expression of methionine sulfoxide reductase genes in *Chlamydomonas reinhardtii* under high light illumination

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Illumination of *Chlamydomonas reinhardtii* cells at 1000 (high light, HL) or 3000 (very high light, VHL) μmol photons m⁻² s⁻¹ intensity increased superoxide anion radical (O₂⁻⁻) and hydrogen peroxide (H₂O₂) production, and VHL illumination also increased the singlet oxygen (¹O₂) level. HL and VHL illumination decreased methionine sulfoxide reductase A4 (CrMSRA4) transcript levels but increased CrMSRA3, CrMSRA5 and CrMSRB2.1 transcripts levels. CrMSRB2.2 transcript levels increased only under VHL conditions. The role of reactive oxygen species (ROS) on CrMSR expression was studied using ROS scavengers and generators. Treatment with dimethylthiourea (DMTU), a H₂O₂ scavenger, suppressed HL- and VHL-induced CrMSRA3, CrMSRA5 and CrMSRB2.1 expression, whereas H₂O₂ treatment stimulated the expression of these genes under 50 μmol photons m⁻² s⁻¹ conditions (low light, LL). Treatment with diphenylamine (DPA), a ¹O₂ quencher, reduced VHL-induced CrMSRA3, CrMSRA5 and CrMSRB2.2 expression and deuterium oxide, which delays ¹O₂ decay, enhanced these gene expression, whereas treatment with ¹O₂ (rose bengal, methylene blue and neutral red) or O₂⁻⁻ (menadione and methyl viologen) generators under LL conditions induced their expression. DPA treatment inhibited the VHL-induced decrease in CrMSRA4 expression, but other ROS scavengers and ROS generators did not affect its expression under LL or HL conditions. These results demonstrate that the differential expression of CrMSR genes under HL illumination can be attributed to different types of ROS. H₂O₂, O₂⁻⁻ and ¹O₂ modulate CrMSRA3 and CrMSRA5 expression, whereas H₂O₂ and O₂⁻⁻ regulate CrMSRB2.1 and CrMSRB2.2 expression, respectively. ¹O₂ mediates the decrease of CrMSRA4 expression by VHL illumination, but ROS do not modulate its decrease under HL conditions.

**Abbreviations** – DAB, 3,3′-diaminobenzidine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMTU, dimethylthiourea; DPA, diphenylamine; EST, expressed sequence tag; H₂DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; HL, high light; LL, low light; MB, methylene blue; MEN, menadione; MSR, methionine sulfoxide reductase; MV, methyl viologen; NBT, nitroblue tetrazolium; NR, neutral red; O₂⁻⁻, singlet oxygen; O₂⁻⁻, superoxide anion radical; PCR, polymerase chain reaction; PET, photosynthetic electron transport; PS, photosystem; RB, rose bengal; RES, reactive electrophile species; R-MetO, methionine R-sulfoxide; ROS, reactive oxygen species; S-MetO, methionine S-sulfoxide; SOD, superoxide dismutase; SOSG, singlet oxygen sensor green; TAP, Tris-acetate-phosphate; TBARS, thiobarbituric acid reactive substance; VHL, very high light.
Introduction

Upon exposure of photosynthetic organisms to excessive light, the photosynthetic electron transport (PET) components become reduced if the energy supply exceeds the capacity of final electron acceptors to accept electrons, and this causes excess production of reactive oxygen species (ROS) (Asada 1994). Oxidative damage occurs when the amount of pro-oxidants exceeds scavenging capacity, resulting in oxidation of cellular components and loss of cellular function and the following metabolism reactions (Wise and Naylor 1987, Asada 1994, Foyer and Noctor 2005). Once formed, ROS can promote protein oxidation. For example, mild oxidation of peptide methionine (Met) leads to the formation of two diastereomers of methionine sulfoxide, methionine S-sulfoxide (S-MetO) and R-MetO, which results in a change in protein structure and function (Davies 2003). To prevent dysfunction of proteins caused by Met oxidation, plants have evolved mechanisms (Davies 2003) to deal with the repairing of oxidized Met.S-MetO by Met oxidation, plants have evolved mechanisms (Davies 2003).

MSR genes have been discovered in most organisms from bacteria to human (Kryukov et al. 2002). Algae, including microalgae, Chlamydomonas reinhardtii (Tarrago et al. 2009), Euglena gracilis (Brot et al. 1981), Guillardia theta (Goldschmidt 1998) and Ostreococcus spp. (Tarrago et al. 2009), and macroalgae such as Gracilaria gracilis (NCBI Genbank no. AF121271), Porphyra yeoensis (gi—16329216, the EST library, Department of Plant Genome Research, Kazusa DNA Research Institute, Japan) and Ulva fasciata (Hsu and Lee 2010) contain MSRA and MSRB genes. Based on the genome database information, the unicellular green alga C. reinhardtii possesses five MSRA (CrMSRA) and four MSRB (CrMSRB) isoforms (Tarrago et al. 2009). The sequence prediction for the deduced proteins of CrMSR suggests that CrMSR isoforms display different subcellular localizations. CrMSRA1 and CrMSRB2.2 proteins might be localized in the cytosol; CrMSRA2, CrMSRA3, CrMSRA4, CrMSRB1.1, CrMSRB1.2 and CrMSRB2.1 proteins might be localized in the mitochondrion; and CrMSRA5 is possibly targeted to the chloroplast (Tarrago et al. 2009). However, these predicted localizations may not be exact. Several studies have shown that CrMSR can be activated by stress. The mRNA levels of CrMSRA2 and CrMSRA5 can be increased by manganese (Allen et al. 2007) and sulfur (González-Ballester et al. 2010) deficiency.

This study examined whether HL illumination (1000μmol photons m⁻² s⁻¹) modulates CrMSR expression and the factors potentially involved in the regulation of CrMSR expression by HL illumination. Because ROS are considered signaling molecules for the regulation of gene expression under HL conditions (Karpinski et al. 1997, 1999), they are possible candidates. In addition, because singlet oxygen (¹⁰O₂) produced in the chloroplast could activate the expression of nuclear genes in A. thaliana through carotenoid oxidation products, for example, β-cyclocitril (Ramel et al. 2012), and also in C. reinhardtii cells under 3000μmol photons m⁻² s⁻¹ conditions (op den Camp et al. 2003, Fischer et al. 2006, 2007), the role of ¹⁰O₂ in the regulation of CrMSR expression was examined at very high light (VHL) intensity (3000μmol photons m⁻² s⁻¹). First, the levels of superoxide anion radical (O₂⁻•⁻), H₂O₂ and ¹⁰O₂ were determined under HL or VHL conditions. Next, ROS scavengers and generators were treated to algal cells and CrMSR transcript levels were determined. A change in the redox state of the PET chain can modulate the expression of genes to adjust HL responses (Escoubas et al. 1995, Pfannschmidt et al. 2001, Sheraamenti et al. 2002, Kimura et al. 2003). The role of PET in CrMSR expression was also examined using specific inhibitors of photosystem II (PSII) and cyt b6f complexes, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), respectively.

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Materials and methods

Algal culture and treatments

*Chlamydomonas reinhardtii*, strain CC-125 (mt⁻), cells were cultured in Tris-acetate-phosphate (TAP) medium (Harris 1989) with trace element solution in 125-ml flasks (PYREX® Laboratory Glassware, Lowell, MA) and agitated on an orbital shaking incubator (model OS701, TKS Company, Taipei, Taiwan) at a speed of 150 rpm. Before treatment, 50 ml of culture was grown to a cell density of 3–5 × 10⁶ cells ml⁻¹ and centrifuged (Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). The cells were washed and then incubated in the dark with 5 μM H₂DCFDA for 5 min. After cleavage of the acetate group by intracellular esterases, H₂DCFDA remains in the cells and reacts with ROS, mainly H₂O₂ (manual from Invitrogen Inc.).

Fresh TAP medium was added to resuspend the pellet, and it was centrifuged again. The pellet was resuspended in fresh TAP medium to obtain the cell density of 3 × 10⁶ cells ml⁻¹. Ten milliliters of culture was transferred to a 100-ml beaker (internal diameter: 3.5 cm) for preincubation at 25°C under low light (LL) conditions for 1.5 h in an orbital shaker (model OS701, TKS Company) at a speed of 150 rpm. Then, the algal cells were exposed to LL, HL (1000 μmol photons m⁻² s⁻¹) or VHL (3000 μmol photons m⁻² s⁻¹) at 25°C with or without chemical treatment. Each treatment had three replicates (n = 3). Samples before (0 min) and after treatment (30, 60 or 180 min) were collected and samples were collected also in cultures non-treated with chemicals, then centrifuged at 3000 g for 5 min and the pellet was fixed in liquid nitrogen and stored in a −70°C freezer until analysis.

To verify the role of H₂O₂ on CrMSR expression, dimethylthiourea (DMTU), a H₂O₂ scavenger (Levine et al. 1994), was added to TAP medium at a final concentration of 75 mM before high-intensity illumination. In addition, H₂O₂ was also added at a final concentration of 0.2, 0.5 or 1.0 mM to the medium under LL conditions to test the role of H₂O₂ in the modulation of CrMSR expression. O₂•⁻ generators, MV (for the generation of O₂•⁻ in the chloroplast) (Farrington et al. 1973) at 0.5 or 5 μM and menadione (MEN) at 10 or 50 μM, were also added separately in the medium under LL conditions. The role of ¹O₂ in the regulation of CrMSR expression was explored by the addition of 15 μM diphenylamine (DPA), a ¹O₂ scavenger (Kruk and Trebst 2008), or 50% deuterium oxide (D₂O), which can increase ¹O₂ lifetime, to the medium. Additional treatments with photosensitizers were performed, including neutral red [NR, a typical type I photosensitizer that generates ¹O₂ and other ROS (Marks et al. 1984)], rose bengal (RB) (Lee and Rodgers 1987) and methylene blue (MB) (Gabrielli et al. 2004) (RB and MB, typical type II photosensitizers, which are known to mainly generate ¹O₂ when excited by light in the visible range), under LL conditions and in the dark, respectively.

ROS determination

ROS (H₂O₂) were initially assayed using cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen Inc., Carlsbad, CA). The cells were washed and then incubated in the dark with 5 μM H₂DCFDA for 5 min. After cleavage of the acetate group by intracellular esterases, H₂DCFDA remains in the cells and reacts with ROS, mainly H₂O₂ (manual from Invitrogen Inc.). Then, the fluorescence of dichlorofluorescein was detected with a fluorescence microscope (Eclipse Ni, Nikon, Tokyo, Japan) at an excitation wavelength of 488 nm with an FITC filter (Nikon). To avoid photo-oxidation of the indicator dye, fluorescence images were collected by a single rapid scan with identical parameters, such as contrast and brightness, for all samples. In addition, the fluorescence level was also measured as the peak height at 525 nm (excitation 488 nm) by fluorescence spectrophotometry (F-2500, Hitachi, Tokyo, Japan).

H₂O₂ and O₂•⁻ were detected using 3,3′-diaminobenzidine (DAB)-HCl (Sigma, St. Louis, MO) and nitroblue tetrazolium (NBT; Sigma) (Hema et al. 2007), respectively. The cells were pretreated with NBT and DAB before high-intensity illumination. The cells were centrifuged at 3000 g (Centrifuge 5810R, Eppendorf AG) using a swing-bucket rotor (F-34-6-38, Eppendorf AG) for 3 min at room temperature. The pellet was resuspended in new TAP medium containing 0.5 mM NBT or 5 mM DAB for a 10-min incubation in the dark. Subsequently, the cells were illuminated under LL, HL or VHL conditions for 10, 30 and 60 min. At each time point, the cells were filtered onto glass microfiber filters (diameter 45 mm, GF/C, Whatman, GE Healthcare, Piscataway, NJ). The pigments were completely removed following the wash of the filter discs twice with methanol. After drying, the filters were scanned as digital images and staining intensities were estimated using IMAGE software (free software from http://rsbweb.nih.gov/ij/index.html) and compared between treatments. To confirm the detection of O₂•⁻ and H₂O₂ by NBT and DAB, 100 U ml⁻¹ bovine erythrocytes superoxide dismutase (SOD) (Sigma) was added together with NBT and 100 U ml⁻¹ bovine liver catalase (Sigma-Aldrich) was also added together with DAB. SOD and catalase could effectively reduce the blue color (see Fig. S1, Supporting Information) and the brown color (see Fig. S2) in the algal cells illuminated under VHL conditions for 30 min, respectively. This indicates that the color development after DAB and NBT staining was mainly due to H₂O₂ and O₂•⁻, respectively.
\[^1\text{O}_2\] was detected using singlet oxygen sensor green (SOSG) (Molecular Probes, Invitrogen Inc.). After exposure to VHL for 10, 30 or 60 min, an aliquot of 1 ml was taken and supplemented with 5 \(\mu\text{M}\) SOSG and then stained in LL, HL or VHL conditions for another 5 min. After being washed with new TAP medium following centrifugation, the pellet was resuspended in 1 ml of new TAP medium, and the fluorescence level was measured at 525 nm (excitation 488 nm) in a fluorescence spectrophotometer (F-2500, Hitachi). SOSG fluorescence was also observed under fluorescence microscopy (Eclipse Ni, Nikon) with excitation at 488 nm with an FITC filter (Nikon). The fluorescence images were acquired using a CCD camera (Nikon’s Digital Sight DS-U3), and final composite images were constructed using Adobe Photoshop (Adobe Systems, San Jose, CA).

**RNA isolation, cDNA synthesis and mRNA quantification by real-time PCR**

Algal cells in 10-ml cultures were harvested by centrifugation (Centrifuge 5810R, Eppendorf AG) using a swing-bucket rotor (F-34-6-38, Eppendorf AG), and the total RNA of the algal cells was extracted using TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. RNA integrity was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, using 1% agarose gel electrophoresis and ethidium bromide staining. RNA sample concentration was adjusted to 2.95 \(\mu\text{g} \text{ml}^{-1}\) total RNA. After treating with DNase (TURBO DNA-free™ Kit, Ambion Inc. The RNA Company) to remove residual DNA, 1.5 \(\mu\text{g}\) of total RNA was used for the preparation of cDNA. cDNA was amplified from the poly-(A)\(^+\) end using an Oligo(dT)\(_{12-18}\) by Verso™ cDNA Kit (Thermo Fisher Scientific Inc., Waltham, MA), and the volume was adjusted starting with an initial denaturation at 95\(^\circ\)C for 15 s. The dissociation curves were generated followed by 50 amplification cycles including annealing at 60\(^\circ\)C for 10 s, elongation at 72\(^\circ\)C for 10 s, and fluorescence measurement and then denaturation at 95\(^\circ\)C for 15 s. The dissociation curves were generated after the PCR, and the fluorescence was analyzed using a LightCycler® 480 system. The product was analyzed by 1% agarose gel electrophoresis to assess the presence of a unique final product. After purification from gel and cloning into a T-vector for sequencing, the presence of a unique final product. After purification from gel and cloning into a T-vector for sequencing, the sequences of the PCR products were determined to be in agreement with the predicted gene fragment. Software with auto CT (cycle threshold) was used to determine the threshold of each gene, and the 2\(^{-\Delta\Delta\text{CT}}\) method was used to calculate the \(\Delta\text{CT}\) values, in which the relative change in the transcript level was normalized to reference genes, elongation factor 1 alpha (CrEF-1\(\alpha\), NCBI: XM_001696516.1) and ubiquitin-conjugating enzyme E2 isoform (CrUBCX, NCBI: AY062935) (see Table S1), and the fold of increase was calculated relative to the RNA sample from the control without chemical treatment at 0 min. All results represent the
averages of three replicates. Because the results based on CrUBCX were similar to those based on CrEF-1α, the mRNA expression levels were presented as a fold change in mRNA level, normalized to CrEF-1α as an endogenous reference gene, relative to the RNA sample of the initial control at 0 min or the LL control.

Statistics
All experiments were repeated at least thrice and because they showed a similar trend, only one of the individual experiments is discussed in this article. The statistics were analyzed using spss (spss 15.0 for Windows Evaluation Version, Inc., Chicago, IL). Significant differences between sample means were analyzed using a Student’s t-test or Duncan’s new multiple range test following analysis of variance of the controls and treatments at a $P < 0.05$ significance level.

Results

Induction of CrMSR expression and ROS production by HL and VHL illumination

The relative change in the transcript level of the CrMSRA isoforms in relation to the CrMSRA1 transcript level at 0 min under LL conditions indicated that CrMSRA4 had the highest abundance among isoforms, followed by CrMSRB2.1, CrMSRA5, CrMSRB2.2 and CrMSRA3, whereas CrMSRA1, CrMSRA2, CrMSRB1.1 and CrMSRB1.2 were less abundant ($P < 0.001$). The transcripts of all CrMSRs remained unchanged after 60 min of incubation under LL conditions ($P = 0.725$) (Fig. 1B), whereas those of CrMSRA3, CrMSRA5 and CrMSRB2.1 increased gradually after exposure to HL or VHL illumination and in contrast, the CrMSRA4 transcript level decreased continuously ($P < 0.001$) (Fig. 1B). The CrMSRB2.2 transcript level was not changed by HL illumination but increased after VHL illumination ($P < 0.001$) (Fig. 1A). Because the cells illuminated under VHL conditions for >120 min started to bleach, the CrMSR transcript levels were not determined after 120 min. The change of CrMSR transcript levels increased as light intensity increased. In addition, the inhibition of the HL- and VHL-induced increase of CrMSR transcript levels by a transcriptional inhibitor, actinomycin-D ($P < 0.001$) (see Fig. S3), suggests that the increase of the CrMSR transcript levels by HL or VHL treatment could be at least partially attributable to increased transcriptional activity. However, the increase of the CrMSR transcript levels through mRNA stabilization cannot be excluded.

The detection of ROS in C. reinhardtii cells using O$_2$$^•$– (NBT), H$_2$O$_2$ (H$_2$DCFDA and DAB) and 1O$_2$ (SOSG) dyes indicates that illumination at HL intensity could increase the H$_2$O$_2$ (Fig. 2A, C) and O$_2$$^•$– (Fig. 2B and Fig. S6) levels but did not affect the 1O$_2$ level (Fig. 2D). Compared to HL illumination, VHL illumination not only induced a higher increase in the O$_2$$^•$– (Fig. 2B) and H$_2$O$_2$ (Fig. 2C) levels but also triggered an increase of 1O$_2$ level (Fig. 2D). We observed that different types of ROS appear to have different time-course dynamics. In Fig. 2, the increase of the O$_2$$^•$– and H$_2$O$_2$ levels occurs at the same time after 10 min and not at different time points as stated, whereas the increase of the 1O$_2$ level occurs 60 min after VHL illumination.

Differential expression of CrMSRA and CrMSRB under high-intensity illumination is modulated by different types of ROS

DMTU, an H$_2$O$_2$ scavenger, was used to determine the role of H$_2$O$_2$ in the regulation of CrMSR expression by
high-intensity illumination. First, the ability of DMTU to scavenge H$_2$O$_2$ was tested at different concentrations at 15, 50 and 75 mM. Treatment with 75 mM DMTU effectively inhibited the increase of H$_2$O$_2$ level under HL or VHL conditions (Fig. 3A), whereas a DMTU concentration of 15 or 50 mM demonstrated reduced effects (data not shown). Next, 75 mM DMTU was used to test the role of H$_2$O$_2$ on CrMSR expression. We observed that treatment with 75 mM DMTU could inhibit the increase of CrMSRA3 (Fig. 3B), CrMSRA5 (Fig. 3D) and CrMSRB2.1 (Fig. 3E) transcript levels under HL or VHL illumination but did not affect CrMSRB2.2 (Fig. 3F) and CrMSRA4 (Fig. 3C) transcript levels and also did not affect CrMSRA1, CrMSRA2, CrMSRB1.1 and CrMSRB1.2 transcript levels (data not shown). In addition, treatment with H$_2$O$_2$ under LL conditions could increase CrMSRA3 (Fig. 4C), CrMSRA5 (Fig. 4E), CrMSRB1.1 (Fig. 4F), CrMSRB1.2 (Fig. 4G) and CrMSRB2.2 (Fig. 4H) transcript levels. Thus, the H$_2$O$_2$ overproduced in C. reinhardtii cells is associated with the induction of CrMSRA3, CrMSRA5 and CrMSRB2.1 expression under high-intensity illumination.

The role of 1O$_2$ in the regulation of CrMSR expression under VHL illumination was examined by treatment with DPA, a 1O$_2$ scavenger, and by incubation in a medium containing D$_2$O, which could prolong the lifetime of 1O$_2$ in aqueous solutions (Fischer et al. 2004). The results obtained from microscopic observation (Fig. 5A) and spectrophotometric determination (Fig. 5B) of SOSG fluorescence dye indicate that the increase in the 1O$_2$ level under VHL conditions was inhibited by 15 μM DPA treatment and enhanced by 50% D$_2$O incubation. A change in 1O$_2$ level by DPA or D$_2$O treatment under VHL conditions causes a change in CrMSR expression.
Fig. 4. The effects of H$_2$O$_2$ (0.2, 2 and 6 mM) on the transcript levels of CrMSRA1 (A), CrMSRA2 (B), CrMSRA3 (C), CrMSRA4 (D), CrMSRA5 (E), CrMSRB1.1 (F), CrMSRB1.2 (G), CrMSRB2.1 (H) and CrMSRB2.2 (I) of Chlamydomonas reinhardtii cells grown under 50 μmol photons m$^{-2}$ s$^{-1}$ conditions. The data are presented as the mean ± SD (n = 3), and different letters indicate significant differences (P < 0.05).

DPA treatment inhibited the increase of CrMSRA3 (Fig. 6A), CrMSRA5 (Fig. 6C) and CrMSRB2.2 (Fig. 6E) transcript levels and the decrease of the CrMSRA4 transcript level (Fig. 6B) under VHL illumination, whereas incubation in the medium containing 50% D$_2$O resulted in contrasting result (Fig. 6F–K). The increase of the CrMSRB2.1 transcript level under VHL illumination was not affected by DPA (Fig. 6D) or D$_2$O (Fig. 6I) treatment. The CrMSRA1, CrMSRA2, CrMSRB1.1 and CrMSRB2.1 transcript levels under VHL conditions were also not affected by DPA or D$_2$O treatment (data not shown).

1O$_2$ generators, NR (10 μM), RB (1 μM) and MB (10 μM), were used for treatments under LL conditions to confirm the role of 1O$_2$ in CrMSR expression, and NR, RB or MB treatment increased the CrMSRA3 (Fig. 6L), CrMSRA5 (Fig. 6N) and CrMSRB2.2 (Fig. 6P) transcript levels by treatment the CrMSR4 transcript (Fig. 6M) level. The CrMSRB2.1 transcript level was not affected by these 1O$_2$ generators (Fig. 6O). Because 1O$_2$ generators are photosensitizers that release 1O$_2$ under light conditions (Marks et al. 1984, Lee and Rodgers 1987, Gabrielli et al. 2004), NR, RB and MB were added in the dark to verify whether 1O$_2$ is the factor in initiating CrMSR expression. Treatment with NR, RB or MB in the dark did not affect the CrMSRA (see Table S2) and CrMSRB (see Table S3) transcript levels, reflecting that 1O$_2$ at least partially contributes to the modulation of CrMSR expression with these 1O$_2$ generators. Overall, the data obtained from the changes of CrMSR expression through the modifications of the cellular 1O$_2$ level via treatment with DPA, D$_2$O and 1O$_2$ generators demonstrate that 1O$_2$ is a factor involved in the induction of CrMSRA3, CrMSRA5 and CrMSRB2.2 under VHL illumination and the suppression of CrMSRA4 expression by VHL illumination.

The role of O$_2$•$^-$ on CrMSR expression was tested using O$_2$•$^-$ generators, MV and MEN, under LL conditions. MV (0.5 or 10 μM) or MEN (10 or 50 μM) treatment
increased the CrMSA3 (Fig. 7C), CrMSRA5 (Fig. 7E) and CrMSRB2.2 (Fig. 7I) transcript levels but decreased the CrMSRA1 (Fig. 7A) and CrMSRA2 (Fig. 7B) transcript levels. The CrMSRA4 (Fig. 7D), CrMSRB1.1 (Fig. 7F), CrMSRB1.2 (Fig. 7G) and CrMSRB2.1 (Fig. 7H) transcript levels were not affected by MV or MEN treatment.

**PET is not involved in CrMSR expression under high-intensity illumination**

DCMU, an inhibitor at the Qb site of PSII (Allen and Bennett 1981), and DBMIB, a PQ antagonist that blocks electron flow from PQH2 to the cyt b6f complex (Allen et al. 1981), that is, the quinol oxidase (Qo) site of the cyt b6f complex, and prevents the reduction of cyt b6f complex (Schoepp et al. 1999), were used for the functional dissection of the PET system in relation to CrMSR expression. Treatment with DCMU or DBMIB under LL, HL or VHL conditions inhibited PSII activity (Fm/Fv and Fm’/Fv’) (see Fig. S4). DCMU treatment did not affect the CrMSRA3, CrMSRA4, CrMSRA5, CrMSRB2.1 and CrMSRB2.2 transcript levels. In contrast, DBMIB treatment could increase the CrMSRA3, CrMSRA5 and CrMSRB2.2 transcript levels under LL, HL or VHL conditions (see Fig. S5). Recently, it has been observed that DBMIB can modulate the expression of genes in C. reinhardtii cells in the dark through other chemical properties and mechanisms, such as a lipophilic reactive electrophile species (RES) that induces an electrophilic stress response (Fischer et al. 2012). Thus, DBMIB was administered in the dark to confirm its specific role in CrMSR expression through PET blockage or owing to its RES property. The results in Fig. S5 indicate that the CrMSRA3, CrMSRA5 and CrMSRB2.2 transcript levels are significantly increased by DBMIB treatment in the dark. The extent of induction of CrMSR expression by DBMIB treatment was similar between light and dark conditions.

**Discussion**

MSR plays a role under oxidative stress conditions (Romero et al. 2004, Zhang and Weissbach 2008). As MSR has been identified as an important cellular defense mechanism against oxidative stress in plants...
Fig. 7. The effects of MV (0.5 and 10 μM) and MEN (10 and 50 μM) on the transcript levels of CrMSRA1 (A), CrMSRA2 (B), CrMSRA3 (C), CrMSRA4 (D), CrMSRA5 (E), CrMSRB1.1 (F), CrMSRB1.2 (G), CrMSRB2.1 (H) and CrMSRB2.2 (I) of Chlamydomonas reinhardtii cells grown under 50 μmol photons m$^{-2}$ s$^{-1}$ conditions. The data are presented as the mean ± SD (n = 3), and different letters indicate significant differences (P < 0.05).

(Rodrigo et al. 2002, Bae et al. 2003, Bechtold et al. 2004, Romero et al. 2004, Oh et al. 2005, Kwon et al. 2007), it can be speculated that the induction of CrMSR expression in Chlamydomonas by ROS generated under excess light conditions provides a defense mechanism against oxidative stress. The CrMSRA and CrMSRB genes are selectively expressed in C. reinhardtii upon exposure to high-intensity illumination. Illumination under both HL and VHL conditions inhibited the expression of CrMSRA4, the most abundant CrMSR isoform in terms of mRNA level but stimulated CrMSRA3, CrMSRA5 and CrMSRB2.1 expression in a time-dependent manner. CrMSRB2.2 expression increased only after 60 min of VHL treatment. The induction of CrMSR gene expression by VHL illumination is similar to the findings in our recent study (Chang et al. 2013). In addition, we also observed that illumination at 300 μmol photons m$^{-2}$ s$^{-1}$ also induced an increase in the CrMSRA3, CrMSRA5 and CrMSRB2.1 transcript levels and a decrease in the CrMSRA4 transcript level, but this occurred to a lesser extent compared with 1000 and 3000 μmol photons m$^{-2}$ s$^{-1}$ illumination (data not shown). The induction of MSR expression by HL irradiance has been observed in the higher plant Arabidopsis. In particular, AtMSRA4 (Romero et al. 2004) and AtMSRB1 (Vieira Dos Santos et al. 2005) protein levels are increased upon exposure to increased illumination. Recently, we observed that exposure to HL levels (300–1200 μmol photons m$^{-2}$ s$^{-1}$) resulted in increased transcript levels of both UiMSRA and UiMSRB in the intertidal macroalga U. fasciata (Hsu and Lee 2010, 2012). To the best of our knowledge, this study is the first to demonstrate that CrMSR isoforms are differentially expressed in C. reinhardtii cells following high-intensity illumination.

Both HL and VHL illumination increased the H$_2$O$_2$ level, whereas the 1O$_2$ level increased only under VHL conditions. The O$_2$•$^-$ level does not increase under HL conditions, probably because O$_2$•$^-$ could be fast detoxified to H$_2$O$_2$ or scavenged by antioxidants. The significant increase of 1O$_2$ level in C. reinhardtii cells under VHL conditions but not increase under HL conditions is in agreement with the studies of op den Camp et al. (2003) and Fischer et al. (2006, 2007). Using isolated spinach thylakoid membranes, it has been recently proposed that light could induce the formation of carbon-centered radicals (R•) formed by oxidation of lipids and proteins and 1O$_2$ may be generated in the donor-side photoinhibition of PSII via the Russell mechanism for the recombination of two peroxyl radicals (ROO•) formed by the interaction of R• with O$_2$ (Yadav and Pospíšil 2012). Because the level of lipid peroxide (reflected by high thiobarbituric acid reactive substance (TBARS) contents) in C. reinhardtii cells becomes high after 60 min of VHL illumination (data not shown), it is possible that 1O$_2$ could also be produced from lipid peroxide after 60 min through the Russell mechanism. However, the TBARS contents were not increased under HL conditions (data not shown). Thus, the production of 1O$_2$ from lipid peroxide through the Russell mechanism under HL conditions may be scarce. Alternatively, 1O$_2$ produced in the chloroplast could activate the expression of nuclear genes in A. thaliana through carotenoid oxidation products, for example, β-cyclocitral (Ramel et al. 2012).

The experiments using ROS scavengers and generators suggest that the differential expression of CrMSR genes under HL conditions can be attributed to ROS that exhibit different accumulation patterns under HL conditions. The data indicate that the decrease of the CrMSRA4 transcript level following VHL treatment was suppressed and enhanced by DPA and D$_2$O treatments, respectively, and that treatment with 1O$_2$ generators (RB and MB) under LL conditions suppressed CrMSRA4 expression,
suggesting that the inhibition of CrMSRA4 expression under VHL illumination is mediated by $^{1}\text{O}_2$. Because DMTU treatment under HL or VHL conditions and H$_2$O$_2$ treatment under LL conditions do not affect CrMSRA4 expression, H$_2$O$_2$ is not responsible for the inhibition of CrMSRA4 expression by HL or VHL illumination. Furthermore, treatment with O$_2^{-}$•• generators (MV and MEN) under LL conditions did not affect CrMSRA4 expression. Evidently, both H$_2$O$_2$ and O$_2^{-}$•• are not involved in the regulation of CrMSRA4 expression under HL conditions.

The inhibition of both the HL- and VHL-induced increases of CrMSRA3 and CrMSRA5 transcript levels by DMTU treatment and the activation of their expression by H$_2$O$_2$ treatment under LL conditions clearly demonstrate that H$_2$O$_2$ is involved in the induction of CrMSRA3 and CrMSRA5 expression by both HL and VHL illumination. Because of the relatively low reactivity of H$_2$O$_2$ compared with other ROS (Halliwell and Gutteridge 1985), H$_2$O$_2$ has been implicated as a signaling molecule during the acclimation of plants to HL stress (Mullineaux et al. 2006). In this study, the inhibition of the VHL-induced increase of CrMSRA3 and CrMSRA5 transcript levels in the presence of DPA and the enhancement of their expression by D$_2$O incubation suggest that $^{1}\text{O}_2$ contributes to the induction of CrMSRA3 and CrMSRA5 expression by VHL illumination. Furthermore, treatment with $^{1}\text{O}_2$ generators (NR, MB and RB) under LL conditions can increase CrMSRA3 and CrMSRA5 transcript levels. The current results are in agreement with previous studies that have suggested that $^{1}\text{O}_2$ is a signaling molecule involved in the induction of the expression of nuclear genes in C. reinhardtii cells under illumination with 3000 $\mu$mol photons m$^{-2}$ s$^{-1}$ (op den Camp et al. 2003, Fischer et al. 2006, 2007). In addition, the stimulation of CrMSRA3 and CrMSRA5 expression by treatment of O$_2^{-}$•• generators under LL conditions indicates that O$_2^{-}$•• overaccumulation under HL or VHL conditions may also be involved in the regulation of CrMSRA3 and CrMSRA5 expression. Although it decays swiftly, O$_2^{-}$•• acts as a signaling molecule in plants, such as in the activation of the expression of defense genes and in phytoalexin synthesis (Jabs et al. 1997). We have recently demonstrated, in a similar manner, that both H$_2$O$_2$ and O$_2^{-}$•• are involved in the induction of UfMSRA expression in the green macroalga U. fasciata when exposed to excessive CuSO$_4$ (Wu et al. 2009). Our current results demonstrate that the regulation of both CrMSRA3 and CrMSRA5 expression in C. reinhardtii cells under high levels of light can be attributed to all types of ROS examined in this study, H$_2$O$_2$ and O$_2^{-}$•• for HL treatment and $^{1}\text{O}_2$, H$_2$O$_2$ and O$_2^{-}$•• for VHL treatment.

The evidence indicates that different types of ROS are responsible for differential expression of CrMSRB2.1 and CrMSRB2.2 under HL and VHL conditions. The CrMSRB2.1 transcript level is increased by both HL and VHL illumination, whereas the CrMSRB2.2 transcript level is increased only under VHL conditions. Treatment with DPA or D$_2$O under VHL conditions does not affect CrMSRB2.1 expression. In addition, treatment with MV or MEN under LL conditions produces a similar effect. However, CrMSRB2.1 expression under VHL conditions was inhibited by DMTU treatment, and its expression under LL conditions was induced by H$_2$O$_2$ treatment. Hence, both HL- and VHL-induced CrMSRB2.1 expression is specifically modulated by H$_2$O$_2$ but not by $^{1}\text{O}_2$ and O$_2^{-}$••. The role of H$_2$O$_2$ in the regulation of the expression of antioxidant defense-related genes has been reported (Levine et al. 1994, Karpinski et al. 1999, Morita et al. 1999), and a cDNA microarray study has also identified 175 non-redundant expressed sequence tags (ESTs) that are regulated by H$_2$O$_2$ in Arabidopsis, in which 113 ESTs are induced and 62 ESTs are repressed by H$_2$O$_2$ (Desikan et al. 2001, Gadjev et al. 2006). Thus, we suggest that the H$_2$O$_2$-dependent signaling pathways are activated during HL and VHL illumination, resulting in the induction of CrMSRB2.1 expression in C. reinhardtii cells. In contrast to CrMSRB2.1, $^{1}\text{O}_2$ and O$_2^{-}$••, rather than H$_2$O$_2$, are involved in the VHL-induced increase of CrMSRB2.2 expression. CrMSRB2.2 expression under VHL conditions was inhibited and enhanced by DPA and D$_2$O treatment, respectively, and treatment with MV or MEN under LL conditions induces increased CrMSRB2.2 expression. O$_2^{-}$•• is a signaling molecule involving in defense responses that executes its function independently of H$_2$O$_2$ (Pastori and Foyer 2002, Vranova et al. 2002). In rice embryo cultures, MV treatment induces the expression of a cytosolic APX gene through H$_2$O$_2$ instead of O$_2^{-}$•• (Morita et al. 1999). O$_2^{-}$••, but not H$_2$O$_2$, initiates the runaway cell death of Arabidopsis lsd1 mutant (Jabs et al. 1996). Our results, together with previous studies, indicate that the expression of some stress-related genes in C. reinhardtii is modulated by O$_2^{-}$•• independently of H$_2$O$_2$, for example, the expression of CrMSRB2.2 under VHL conditions.

The expression of CrMSRB1.1 and CrMSRB1.2 was increased by exogenously applied H$_2$O$_2$ under LL conditions, whereas DMTU treatment did not affect CrMSRB1.1 and CrMSRB1.2 expression under HL or VHL conditions. We speculate that the signaling pathway involved in the modulation of CrMSRB1.1 and CrMSRB1.2 expression by external H$_2$O$_2$ differs from the signaling network governed by internal H$_2$O$_2$.
generated under VHL conditions. Similarly, CrMSRA1 and CrMSRA2 expression can be decreased by O$_2$•− generators under LL conditions but was not affected under HL or VHL conditions. Consequently, the O$_2$•−-dependent signaling pathways involved in the negative regulation of CrMSRA1 and CrMSRA2 expression were activated by extracellular O$_2$•− but did not respond to intracellular O$_2$•−.

The subcellular protein localization of some CrMSRs predicted according to their deduced peptide sequences (Tarrago et al. 2009) is linked to localized ROS formation under HL conditions (Table 1). 1O$_2$ can be produced in the chloroplast in the PSII reaction centers (Krieger-Liszkay 2005) and/or accumulated from the photodynamic compound, protochlorophyllide (op den Camp et al. 2003). In addition, this molecule is also observed in the cytosol of C. reinhardtii cells upon illumination at 3500 μmol m$^{-2}$ s$^{-1}$ (Fischer et al. 2007). Because 1O$_2$ is the main factor causing oxidative damage in C. reinhardtii cells under HL conditions (Fischer et al. 2006, Ledford et al. 2007), it is believed that 1O$_2$ signaling allows induction of MSR genes under oxidative stress conditions, such as the induction of chloroplastic CrMSRA5 and cytosolic CrMSRB2.2 expression. The expression of CrMSRA3 localized in the mitochondrion could also be activated by 1O$_2$ existed in the chloroplast and in the cytosol. It is clear that mitochondrial CrMSRA3 is not linked to the site of 1O$_2$ production. O$_2$•− and H$_2$O$_2$ generated in chloroplasts in the presence of triplet Chl and electron transfer chains in PSI and PSII (Asada 2006) and in the mitochondrial electron transfer chain (Vranova et al. 2002) are also responsible for the activation of the expression of chloroplastic CrMSRA5 and mitochondrial CrMSRA3 and CrMSRB2.1 against O$_2$•− and/or H$_2$O$_2$-mediated oxidative damage to peptide methionine in these two organelles. It has been reported in Arabidopsis that the chloroplastic AtMSRB1 and AtMSRB2 are known to play a role in the preservation of photosynthetic antennae (Laugier et al. 2010). Possibly, CrMSRA5 targeting in the chloroplast prevents oxidative damage related to PSII. However, this remains to be proven.

PET is not involved in the modulation of CrMSR expression under high-intensity illumination. Using DCMU and DBMIB, we have previously shown that the expression of UfMSRA and UfMSRB genes of the marine green macroalga U. fasciata was upregulated by light intensity (300 μmol photons m$^{-2}$ s$^{-1}$) via a modification in the redox status of the PET components (Hsu and Lee 2010). Although the PET components display marked changes in their redox state depending on photosynthetic electron flow, the signals originating from electron transport play decisive roles in the regulation of nuclear gene expression in the adjustment of photosynthetic performance (Dietz 2003, Pfannschmidt 2003) and antioxidant enzymes, such as cytosolic ascorbate peroxidase genes (APX1 and APX2) (Karpinski et al. 1997, Mullineaux et al. 2000). However, we did not verify an effect of DCMU on CrMSR expression. Although treatment with DBMIB under HL or VHL conditions increased the CrMSRA3, CrMSRA5 and CrMSRB2.2 transcript levels, we observed that treatment with DBMIB in the dark triggered a similar increase in the CrMSRA3, CrMSRA5 and CrMSRB2.2 transcript levels. These results reflect that the effect of DBMIB on the induction of CrMSR expression is not due to the blockage of PET. We speculate it may be an electrophilic stress response. In a recent study examining the components involved in signal transduction and activation of the 1O$_2$-mediated response in a 1O$_2$ resistant (sor1) mutant, 2 or 5 μM DBMIB induced the expression of genes in C. reinhardtii cells in the dark mainly owing to other chemical properties and mechanisms, such as lipophilic RES that can induce an electrophilic stress response (Fischer et al. 2012). Because 1O$_2$ can form reactive electrophiles by lipid peroxidation, the slow induction of CrMSRB2.2 expression under VHL conditions may be caused indirectly by 1O$_2$-mediated reactive electrophile products. Because MEN act as oxidant and electrophile (Monks and Jones 2002, Rodriguez et al. 2004), the profound effect of MEN on the inhibition of CrMSRA1 and CrMSRA2 expression compared to MV can be attributable to the electrophile property of MEN. The role of electrophiles on the regulation of CrMSR expression is now undertaken. Although Shao et al. (2008) demonstrated that PET is involved in the H$_2$O$_2$-dependent signaling pathway in C. reinhardtii cells, PET is not linked to the CrMSR gene expression mediated by H$_2$O$_2$ signaling. Our finding shows that the redox status of photosynthetic complexes and an increase in ROS production resulting from electron transfer interruption and overoxidation of photosynthetic components are involved in the regulation of the expression of CrMSR genes.

In conclusion, this present study demonstrated that CrMSR isoforms are selectively and differentially modulated under HL conditions through different type of ROS rather than PET. CrMSRA3, CrMSRA5 and CrMSRB2.1 expression are increased under both HL and VHL illumination, whereas CrMSRB2.2 expression is increased only under VHL illumination. 1O$_2$, H$_2$O$_2$ and O$_2$•− induce CrMSRA3 and CrMSRA5 expression, whereas H$_2$O$_2$ and 1O$_2$ induce CrMSRB2.1 and CrMSRB2.2 expression, respectively. The VHL-induced inhibition of CrMSRA4 expression is mediated by O$_2$•−, but the suppression of CrMSRA4 expression is not mediated by O$_2$•− nor PET. The reactive electrophiles...
may also be involved in the induction of CrMSR expression under VHL conditions, for example, the slow induction of CrMSRB2.2 expression. The cross-talk between the different types of ROS and the involvement of reactive electrophiles in the regulation of CrMSR gene expression and the specific function of individual CrMSR proteins in C. reinhardtii cells to acclimate to HL stress are now being studied.

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References


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**Table 1.** The localization of ROS formation and protein targeting of CrMSRA and CrMSRB isoforms of Chlamydomonas reinhardtii to different types of ROS and under elevated light intensity (HL and VHL).

<table>
<thead>
<tr>
<th>Name</th>
<th>Subcellular localization</th>
<th>O2•− (mitochondrion, plastid and cytosol)</th>
<th>H2O2 (mitochondrion, plastid and cytosol)</th>
<th>1O2 (plastid and cytosol)</th>
<th>HL</th>
<th>VHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrMSRA3</td>
<td>Mitochondrion</td>
<td>△</td>
<td>△</td>
<td>△</td>
<td>△</td>
<td>△</td>
</tr>
<tr>
<td>CrMSRA4</td>
<td>Mitochondrion</td>
<td>△</td>
<td>△</td>
<td>△</td>
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<td>△</td>
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<tr>
<td>CrMSRA5</td>
<td>Plastid</td>
<td>△</td>
<td>△</td>
<td>△</td>
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</tr>
<tr>
<td>CrMSRB2.1</td>
<td>Mitochondrion</td>
<td>△</td>
<td>△</td>
<td>△</td>
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</tr>
<tr>
<td>CrMSRB2.2</td>
<td>Cytosol</td>
<td>△</td>
<td>△</td>
<td>△</td>
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</tr>
</tbody>
</table>

*Treatment with O2•− generators, MV and MEN, under LL conditions.*

*Obtained from the experiments of exogenously treated H2O2 under LL conditions.*

△ indicates the increase of transcript level and ▼ indicates the decrease of transcript level when compared with the control.
Fischer BB, Krieger-Liszkay A, Eggen RIL (2004) Photosensitizers neutral red (Type I) and rose bengal (Type II) cause light-dependent toxicity in Chlamydomonas reinhardtii and induce the Gpxh gene via increased singlet oxygen formation. Environ Sci Technol 38: 6307–6313


Yadav DK, Pospíšil P (2012) Evidence on the formation of singlet oxygen in the donor side photoinhibition of
Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Effects of SOD on the NBT color development in *Chlamydomonas reinhardtii* cells under 50 and 3000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) conditions.

**Fig. S2.** Effects of catalase on the DAB color development in *Chlamydomonas reinhardtii* cells under 50 and 3000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) conditions.

**Fig. S3.** Effects of actinomycin-D on transcripts of *CrMSRA3, CrMSRA4, CrMSRA5, CrMSRB2.1* and *CrMSRB2.2* of *Chlamydomonas reinhardtii* cells under 1000 and 3000 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) conditions.

**Fig. S4.** Effect of DCMU and DBMIB on PSII activity of *Chlamydomonas reinhardtii* cells under 1000 and 3000 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) conditions.

**Fig. S5.** Effect of DCMU and DBMIB on *CrMSRA3, CrMSRA4, CrMSRA5, CrMSRB2.1* and *CrMSRB2.2* transcripts of *Chlamydomonas reinhardtii* cells under 1000 and 3000 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) conditions.

**Fig. S6.** The detection of \( ^1 \text{O}_2 \) by SOSG green fluorescence in *Chlamydomonas reinhardtii* cells illuminated at 50 and 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \).

**Table S1.** Primers of *CrMSR* and internal control genes used for real-time PCR.

**Table S2.** Relative transcript levels of methionine sulfoxide reductase A (MSRA) genes of *Chlamydomonas reinhardtii* treated with photosensitizers in the dark.

**Table S3.** Relative transcript levels of methionine sulfoxide reductase B (MSRB) genes of *Chlamydomonas reinhardtii* treated with photosensitizers in the dark.