Defects in a New Class of Sulfate/Anion Transporter Link Sulfur Acclimation Responses to Intracellular Glutathione Levels and Cell Cycle Control1[W][OPEN]

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We previously identified a mutation, suppressor of mating type locus3 15-1 (smt15-1), that partially suppresses the cell cycle defects caused by loss of the retinoblastoma tumor suppressor-related protein encoded by the MAT3 gene in Chlamydomonas reinhardtii. smt15-1 single mutants were also found to have a cell cycle defect leading to a small-cell phenotype. SMT15 belongs to a previously uncharacterized subfamily of putative membrane-localized sulfate/anion transporters that contain a sulfate transporter domain and are found in a widely distributed subset of eukaryotes and bacteria. Although we observed that smt15-1 has a defect in acclimation to sulfur-limited growth conditions, sulfur acclimation (sac) mutants, which are more severely defective for acclimation to sulfur limitation, do not have cell cycle defects and cannot suppress mat3. Moreover, we found that smt15-1, but not sac mutants, overaccumulates glutathione. In wild-type cells, glutathione fluctuated during the cell cycle, with highest levels in mid G1 phase and lower levels during S and M phases, while in smt15-1, glutathione levels remained elevated during S and M. In addition to increased total glutathione levels, smt15-1 cells had an increased reduced-to-oxidized glutathione redox ratio throughout the cell cycle. These data suggest a role for SMT15 in maintaining glutathione homeostasis that impacts the cell cycle and sulfur acclimation responses.

Cell cycle progression is coordinated with the cellular redox environment, which also undergoes periodic cycling. In budding yeast (Saccharomyces cerevisiae), DNA synthesis and mitosis occur during the reductive phase and cell division initiates in the oxidative phase (Tu et al., 2005). Restriction of DNA replication to the reductive phase of the metabolic cycle is important to ensure genome integrity (Chen et al., 2007). In mammals, low levels of reductive oxygen species stimulate cell cycle entry (Lee et al., 1998; Martindale and Holbrook, 2002; Boonstra and Post, 2004) by activating cell cycle regulators (Shackelford et al., 2000; Boonstra and Post, 2004; Macleod, 2008; Burhans and Heintz, 2009). Moreover, alterations in redox homeostasis can cause defects in cell cycle progression (Esposito et al., 1997; Reichheld et al., 1999; Alic et al., 2001; Menon et al., 2003; Markovic et al., 2009; Tsukagoshi et al., 2010). Glutathione is a thiol-containing tripeptide whose function is not only important to maintain redox homeostasis when coping with biotic and abiotic stresses (Cobbled et al., 1998; Ball et al., 2004; Rouhier et al., 2008; Foyer and Noctor, 2009; Mhamdi et al., 2010; Dubreuil-Maurizi and Poinssot, 2012; Shanmugam et al., 2012) but also acts as a redox signal or sensor for cell cycle control (Chiu et al., 2011; Chiu and Dawes, 2012). Therefore, defects in glutathione-mediated redox balance can lead to aberrant cell cycle progression and, subsequently, defects in growth and development (Vernoux et al., 2000; Cairns et al., 2006; Jiao et al., 2013). However, the molecular mechanism that connects glutathione-mediated cellular redox state and cell cycle regulation is not fully understood.

Retinoblastoma-related proteins (RBRs) are evolutionarily conserved cell cycle regulators with a central role in controlling the initiation of DNA replication and cell cycle entry. The canonical retinoblastoma (RB) pathway involves the cell cycle-regulated interaction of RBRs with a heterodimeric E2 promoter binding factor (E2F)/Dimerization partner (DP) transcription factor. The RB-associated E2F/DP protein complex represses the transcription of cell cycle genes, and this repression is released by the removal or modification of RBRs via phosphorylation. Subsequently, E2F/DP-dependent transcription...
transcription of cell cycle genes promotes S phase entry and cell cycle progression. Because of its central role in controlling the transcription of cell cycle genes, RB serves as a convergence point for regulating the cell cycle in response to internal and external mitogenic signals (Nakagami et al., 2002; Stevaux et al., 2002; Cobrinik, 2005; Dimova and Dyson, 2005; Wikenheiser-Brockamp, 2006; Jullien et al., 2008; van den Heuvel and Dyson, 2008; Borghi et al., 2010; Henriques et al., 2010; Johnston et al., 2010; Chen et al., 2011; Gutzat et al., 2011, 2012; Weimer et al., 2012). Recent studies also provide evidence that RB depletion causes metabolic reprogramming and suggest that the RB pathway in animals exerts part of its effect on cell proliferation through the control of Gln metabolism (Nicolay et al., 2013; Reynolds et al., 2014).

Chlamydomonas reinhardtii is a unicellular green alga that proliferates using a multiple fission cell cycle. Its mitotic cell cycle starts with a long G1 phase, during which cells can grow manyfold in size. At the end of G1, mother cells undergo n rapid rounds of alternating S phase (DNA synthesis) and M phase (mitosis) to produce 2^n daughter cells. Two size checkpoints are integrated into this mitotic cell cycle. In early/mid G1, cells pass commitment, the first size checkpoint, which requires cells to acquire sufficient mass to be able to complete the cell cycle. The second size checkpoint occurs during S/M, where mother cells, whose sizes can be highly variable, undergo an appropriate number of division cycles to produce uniformly sized daughters (Craigie and Cavaliersmith, 1982; Donnan and John, 1983). Because C. reinhardtii mother cell division can occur in the absence of concurrent growth, daughter cell size can be conveniently used to assess the cell size checkpoint function during S/M phase (Umen, 2005).

Our previous studies showed that the RB pathway in C. reinhardtii is important for size checkpoint control and size-mediated cell division (Umen and Goodenough, 2001; Fang et al., 2006). The C. reinhardtii RBR homolog is encoded by a single gene, MAT3. mat3 mutants pass commitment at a smaller size than normal but remain in G1 for a normal period of time before entering S/M, where they undergo supernumerary cell divisions to produce tiny daughter cells. Mutations in the C. reinhardtii E2F1 and DP1 genes could suppress the mat3 size defect, indicating that the overall architecture of the RBR pathway is conserved in C. reinhardtii, with MAT3/RBR serving as a negative regulator of E2F- and DP-related proteins (Fang et al., 2006; Olson et al., 2010).

Besides mutations in E2F1 and DP1, several additional extragenic suppressors of mat3 (smt) mutants were isolated that are weaker suppressors than e2f1 and dp1 (Fang and Umen, 2008). smt15-1 mat3-4 double mutants are larger than mat3-4 mutants but smaller than wild-type cells. This finding suggested that SMT15 might be a positive regulator of cell division. However, smt15-1 single mutants had reduced daughter cell size compared with wild-type cells, indicating a potential negative regulatory role for SMT15 in controlling size-dependent cell division. These results suggest a nonlinear relationship between the MAT3/RB pathway and the pathway(s) that are impacted by SMT15.

In this study, we identified the SMT15 gene and showed that it encodes a member of a conserved but uncharacterized family of proteins with homology to sulfate/anion transporters. Although smt15 strains showed defects in acclimation to sulfur limitation, canonical sulfur acclimation mutants did not show cell cycle defects and were unable to suppress mat3, indicating that general defects in sulfur metabolism do not impact the cell cycle. Instead, we found that glutathione, an end product of sulfur assimilation, overaccumulated in the smt15-1 mutant, which also showed attenuated induction of sulfur acclimation genes. These results identify a link between glutathione-mediated redox regulation and the cell cycle in C. reinhardtii and suggest a potentially new mechanism for glutathione homeostasis mediated through a conserved family of membrane transporters.

RESULTS

Characterization of the SMT15 Locus

smt15-1 was isolated as a recessive suppressor of mat3-4 in a genetic screen using a paromomycin resistance marker as an insertional mutagen. Linkage between the paromomycin marker and suppression of the mat3-4 size phenotype was established previously (Fang and Umen, 2008). In this study, the flanking sequence adjacent to the inserted paromomycin resistance marker in smt15-1 was identified, and the insertion was located in a gene encoding a putative transporter (Fig. 1A). Because the genome assembly of sequences surrounding the SMT15 locus was incomplete, we used reverse transcription (RT)-PCR and RACE-PCR to isolate and deduce the structure of the SMT15 mRNA and the predicted SMT15 protein (see “Materials and Methods”). RT-PCR using primers flanking the insertion site amplified a product of the predicted size for SMT15 complementary DNA (cDNA) prepared from wild-type RNA but not from smt15-1 (Fig. 1B).

The predicted SMT15 protein is homologous to a family of sulfate/anion transporters comprising a sulfate transporter domain (Pfam 00916), a sulfate transporter and anti-sigma factor antagonist (STAS) domain (Pfam 01740), and a cyclic nucleotide-binding domain (Pfam 00027; Fig. 1C). Like other transporters in this superfamily, SMT15 is predicted to encode an integral membrane protein with 10 transmembrane helices (Supplemental Fig. S1; Sonnhammer et al., 1998). A phylogenetic tree constructed from previously identified sulfate transporters and representatives identified in BLAST searches revealed that SMT15 belongs to members of tribe 1 of eukaryotic sulfate/anion transporters, which includes plant Sulfate transporter (SULTR), metazoan Solute Carrier 26 transporter (SLC26), and fungal Sulfate Permease (SUL) families (Takahashi et al., 2012). The sequence alignment of the sulfate transporter domain (Pfam 00916) of tribe 1 of eukaryotic sulfate/anion transporters is shown in Supplemental
Figure S2. However, SMT15 and its orthologs are distantly related to the major families of tribe 1 transporters and, therefore, are classified as a new group, family C (Fig. 1D). Members of family C were found in diverse taxa, including chlorophycean green algae such as Volvox, Chlorella, and Coccomyxa spp., but undetectable in the more basal prasinophyte algae or in land plants (Supplemental Fig. S3). However, family C homologs were found outside the green lineage in opisthokonts, including choanoflagellates and fungi (but not metazoans), and stramenopiles, including diatoms, brown algae, and oomycetes, as well as dinoflagellates (Fig. 1D;
Supplemental Fig. S3). Among prokaryotes, SMT15 homologs were found in cyanobacteria and a subset of proteobacteria but not in archaea (Fig. 1D; Supplemental Fig. S3). A sequence alignment of family C SMT15 homologs is shown in Supplemental Figure S4. A notable discordance in the family C lineage is the split affilation of the opisthokont members, where the choanoflagellate (M. brevicollis) homolog groups with heterokonts and the fungal homologs group with bacteria (Fig. 1D). This discordance is not observed in the family A1 lineage, where the fungal, choanoflagellate, and metazoan transporters form a monophyletic clade, as expected for normal vertical inheritance of A1 family members. Most of the SMT15 homologs identified in this study contain the three conserved domains found in SMT15, but there are exceptions, such as Emiliania huxleyi (EOD04916) and P. infestans (EYE60920 and EYE64284), two of which are missing a detectable cyclic nucleotide-binding domain (Supplemental Table S1).

Complementation of smt15

Complementation was used to confirm that disruption of the SMT15 gene by insertion of the paromomycin resistance marker was responsible for the previously reported smt15-1 phenotypes (Fang and Umen, 2008). The smt15-1 mutant strain was transformed with plasmid pSMT15.1 containing the wild-type SMT15 gene including its predicted promoter region and sequences downstream of its 3′ untranslated region (see “Materials and Methods”). Four hundred fifty-eight transgenic lines were generated, and three transformants (lines 57, 62, and 64) showed noticeable restoration of growth to various degrees (Table 1). RT-PCR showed that SMT15 mRNA abundance was restored to 2.4%, 19.4%, and 78.9% of wild-type levels in lines 57, 62, and 64, respectively (Fig. 2). Line 64 (smt15-1 pSMT15.1), with 78.9% restored SMT15 RNA, showed rescue to near wild-type growth rate (Table 1) and was used for further experiments. The nonlinear correlation between mRNA levels and growth rate in lines 57 and 62 suggests that very low (line 57) to low (line 62) expression levels of SMT15 may be enough to alleviate some of the growth defect. Line 64 was crossed to a mat3-4 strain to generate a population of smt15-1 mat3-4 pSMT15.1 and smt15-1 mat3-4 progeny. All the smt15-1 mat3-4 progeny that received the pSMT15.1 had mat3-like small cell size distributions, while progeny that did not receive the complementing plasmid showed suppression of the mat3-4 small-cell phenotype (Table II), as reported previously for smt15-1 mat3-4 double mutants (Fang and Umen, 2008). Taken together, these data confirmed that disruption of SMT15 is responsible for suppression of the mat3-4 size phenotype and for the slow-growth phenotype of smt15-1 single mutants.

SMT15 mRNA Levels Are Light Regulated

To investigate how SMT15 mRNA was regulated and whether its accumulation was controlled by the cell cycle-dependent or by diurnal/circadian rhythms, we used RT-PCR to monitor its expression in samples collected from synchronous cultures. Wild-type cells were synchronized under a 14-h-light/10-h-dark (14L:10D) regime, and the culture synchrony was assessed by measurements of cell size, mitotic index, and periodic expression of the S/M phase marker gene Cyclin-Dependent Kinase B1 (CDKB1; Fang et al., 2006). SMT15 mRNA abundance was measured under the 14L:10D synchrony regime or in portions of the culture that were removed and darkened at 10 h or left in continuous light. In the 14L:10D culture, SMT15 mRNA accumulated steadily during the light phase and remained elevated until the dark phase, when it declined (Fig. 3A). In the culture that was darkened at 10 h, SMT15 mRNA levels declined 4 h earlier than in the 14L:10D culture, indicating that maintenance of the highest SMT15 expression is light dependent. On the other hand, continuous light could not maintain high expression of SMT15 after 14 h, although it stayed higher than in both dark cultures (Fig. 3A). To more directly examine the influences of light on SMT15 mRNA accumulation, its levels were monitored in asynchronous cultures before and after dark incubation. As shown in Figure 3B, SMT15 mRNA levels in cells growing under continuous illumination (T0) were comparable to samples collected at 12 h from synchronized cultures. Importantly, the levels of SMT15 transcript declined within 2 h after

Table 1. Growth rates of complemented smt15-1 strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Doubling Time (h) ± SE (n = 4 or 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>smt15-1</td>
<td>9.1 ± 0.5</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 57</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 62</td>
<td>7.5 ± 0.6</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 64</td>
<td>6.9 ± 0.7</td>
</tr>
</tbody>
</table>

Figure 2. Complementation of the smt15-1 mutant. Expression levels are shown for SMT15 mRNA in the wild type (wt), smt15-1, and three independently generated smt15-1 transformants that were complemented with pSMT15.1 (lines 57, 62, and 64).
switching to dark (T2 and T4) and increased again after reillumination (T6, T8, and T10), indicating that SMT15 expression is light regulated. No correlation was found between transcript levels of SMT15 and the S/M phase marker CDK1i in unsynchronized cultures.

The Sulfur Acclimation Response Is Affected in smt15-1 Cells

Because SMT15 is predicted to encode a potential sulfate/anion transporter and might play a role in sulfur uptake or response to sulfur limitation (Footakham et al., 2010), we asked whether the smt15-1 mutant showed any aberrant responses to sulfur limitation and whether SMT15 mRNA abundance was regulated by the availability of sulfur. Viability tests of wild-type and smt15-1 cultures during sulfur starvation (−S) conditions showed an enhanced loss of viability in smt15-1 after 3 d, when less than 50% of the mutant cells were alive compared with more than 80% of the wild-type cells (Fig. 4A). To determine whether the loss of viability was specific to −S, mutant and wild-type cells were starved for nitrogen (−N) and phosphate (−P) as well. smt15-1 had decreased viability relative to wild-type cells in −N, although the defect was not as severe as in −S, while the viability of the mutant was not significantly affected by −P (Supplemental Fig. S5). We next examined whether SMT15 mRNA levels respond to nutrient deprivation. As shown in Figure 4B, SMT15 mRNA was transiently elevated severalfold after switching to −S conditions but showed much less change in response to −P conditions. SMT15 mRNA was slightly elevated 2 h after −N treatment. Because −N responses can be very rapid (Boyle et al., 2012; Blaby et al., 2013), we also monitored SMT15 mRNA with short time intervals. Our results showed that less than 3-fold change was observed within the first 6 h of −N treatment (Supplemental Fig. S6).

The reduced ability of smt15-1 to cope with −S and the early and transient induction of SMT15 mRNA by −S suggested that SMT15 might participate in the sulfur starvation acclimation responses. To further test this idea, RNA sequencing (RNA-seq) was used to determine genome-wide transcript abundance in the wild type and smt15-1 under sulfur-replete (+S) conditions or after 6 h in −S. Normally, SULFUR ACCLIMATION (SAC) genes are up-regulated to allow cells to cope with sulfur starvation stress (Zhang et al., 2004; González-Ballester et al., 2010). We found that the induction of SAC genes was either attenuated or decreased in smt15-1 (Table III). The defects of smt15-1 in inducing SAC-responsive genes were verified using quantitative RT-PCR and compared with those of the sulfur acclimation mutant sac1, which is severely impaired in its SAC response (Davies et al., 1994; Table III; Fig. 4C). While the transcriptional induction of SAC markers was almost completely blocked in the sac1 mutant, it was only attenuated in smt15-1 compared with its full induction in wild-type cells (Fig. 4C).

Among the SAC-responsive genes that were mis-regulated, most notable were the genes encoding sulfur uptake and assimilation proteins, such as arylsulfatases (ARS1 and ARS2), sulfate transporters (SULTR2, SLT1, and SLT2), and ATP sulfurylases (ATS1 and ATS2; for details, see Table III). Instead of being up-regulated after −S, transcripts of sulfite reductases (SIR1 and SIR2) and serine acetyl transferase (SAT1) of smt15-1 were down-regulated at least 40-fold. In the case of SAT1, its transcript levels were too low to be detected by quantitative RT-PCR after −S (data not shown). Transcripts encoding enzymes involved in sulfur assimilation but not classified as SAC-responsive genes, such as ADENYLYLPHOSPHOSULFATE REDUCTASE1 and ADENOSINE 5′-PHOSPHOSULFATE KINASE1 (González-Ballester et al., 2010), showed little or no change in abundance between wild-type and smt15-1 strains in either +S or −S conditions (Supplemental Table S2).

SMT15 mRNA levels did not show significant change in the sac1 mutant in either −S or +S medium, indicating that its transcriptional regulation is independent of SAC1 signaling (data not shown). Because smt15-1 has a cell size defect, we examined the expression of core cell cycle genes under +S and −S conditions compared with the wild type but found no significant alterations (Supplemental Table S2). Although not related to defects in smt15-1, there was one cyclin-dependent kinase-encoding gene, CDK2, whose transcript showed a strong up-regulation in the wild type under −S conditions. Whether CDK2 up-regulation is part of the SAC response or possibly a general stress response remains to be determined.

General Defects in Sulfur Acclimation Do Not Affect Cell Cycle Regulation

To assess whether sac mutants in general have cell cycle defects or can suppress mat3 mutants, we examined their cell size and also generated double mutants with mat3-4. SAC1 encodes a protein that shares similarity with ion transporters (Pollock et al., 2005) and has been shown to be the major positive regulator of SAC in C. reinhardtii (Davies et al., 1996). SAC3 encodes a Ser/Thr protein kinase that is related to the Snf1p kinase of budding yeast and has been shown to play a negative role in controlling sulfur acclimation responses (Davies et al., 1999). Unlike smt15-1, neither sac1 nor sac3 showed

Table II. Modal sizes and σ of of complemented smt15-1 mat3-5 and related strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Daughter Cell Size (μm)</th>
</tr>
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<tbody>
<tr>
<td>mat3-5</td>
<td>23.5 ± 2.0</td>
</tr>
<tr>
<td>smt15-1 mat3-5</td>
<td>37.6 ± 1.6</td>
</tr>
<tr>
<td>smt15-1 mat3-5 pSMT15.1</td>
<td>26.5 ± 2.5</td>
</tr>
</tbody>
</table>

σ values were derived from five independent smt15-1 mat3-5 pSMT15.1 strains, three independent smt15-1 mat3-5 strains, and three independent mat3-5 strains.
any cell size defects as single mutants (Supplemental Table S3). Moreover, \textit{sac1 mat3-4} and \textit{sac3 mat3-4} strains had size distributions indistinguishable from \textit{mat3-4} single mutants, meaning that these two SAC regulators could not suppress \textit{mat3-4} when mutated (Table IV; Supplemental Fig. S7). These results show that defects in the \textit{Sac} acclimation pathway in general do not affect cell size control or the MAT3/RB pathway in \textit{C. reinhardtii}. Although \textit{smt15-1} is defective in sulfur stress responses, its effect on cell size regulation and its ability to suppress \textit{mat3-4} must be unrelated to general sulfur stress responses.

**Elevated Glutathione Levels in \textit{smt15-1} Affect the Sulfur Acclimation Response**

Because a sulfur acclimation response was activated in \textit{−S} conditions in \textit{smt15-1} but did not reach full strength, we suspected that the mutant's effect on SAC might be indirect. A literature search for sulfur-related metabolites that influence the cell cycle led us to hypothesize that glutathione levels might be involved in the \textit{smt15-1} cell cycle and sulfur stress phenotypes (Diaz Vivancos et al., 2010a; Markovic et al., 2011; Noctor et al., 2012; García-Giménez et al., 2013). It has
been reported that glutathione, one of the end products of the sulfur assimilation pathway, is capable of repressing sulfur assimilation and uptake in plants (Herschbach and Rennenberg, 1994; Lappartient et al., 1999; Vauclare et al., 2002; Buchner et al., 2004). Additionally, glutathione has been shown to play a role in cell cycle progression in plants and animals (Thelander and Reichard, 1979; Menon et al., 2003; Menon and Goswami, 2007; Diaz Vivancos et al., 2010b). Therefore, we speculated that SMT15 might connect sulfur acclimation to cell cycle control through the misregulation of glutathione homeostasis. To test this idea, we compared glutathione levels in wild-type, smt15-1, and sac1 strains in +S and −S conditions. Under sulfur-replete conditions, smt15-1 accumulated approximately 50% higher levels of glutathione than the wild type (33 versus 20 pmol mg⁻¹). After 16 h, −S wild-type cells and sac1 cells both had glutathione levels of approximately 1.5 pmol mg⁻¹; while smt15-1 had glutathione levels that were 2-fold higher (approximately 3 pmol mg⁻¹; Fig. 5A; Table V). To verify linkage between the SMT15 lesion and the glutathione defect, we monitored glutathione levels in the progeny of crosses between smt15-1 and wild-type strains. The smt15-1 mutation is caused by the insertion

Figure 4. A, Viability of wild-type (wt) and smt15-1 strains following S deprivation in liquid medium. B, Expression patterns of SMT15 mRNA in −S, −P, or −N medium. C, Relative expression levels of sulfur acclimation genes in −S conditions in wild-type, smt15-1, and sac1 strains.
Table III. Comparative transcript abundances of selected sulfur acclimation marker genes (González-Ballester et al., 2010) from wild-type (wt) and smt15-1 strains grown in the presence (+S) and absence (−S) of sulfur in RNA-seq and quantitative RT-PCR analyses

wt−S/wt+S, Fold change of the selected transcripts for the wild type in +S and −S medium; smt15-1−S/wt+S, fold change of the selected transcripts between the smt15-1 strain in −S medium and the wild-type in +S medium; smt15-1−S/smt15-1+S, fold change of the selected transcripts between the smt15-1 strain in −S medium and smt15-1 in +S medium; smt15-1+S/wt+S, fold change of the selected transcripts between the smt15-1 strain in +S medium and the wild-type in +S medium. Fold change of the selected transcripts under specified conditions was determined by RNA-seq (R) or quantitative RT-PCR (Q). Values are from cells exposed to the −S condition for 0 h (R0 or Q0), 2 h (Q2), or 6 h (R6 or Q6). Samples for RNA-seq and quantitative RT-PCR analyses were derived from independent experiments. Quantitative RT-PCR values showed similar trends to those of RNA-seq, except for SAT1 (Cre10.g466750.t1.3). Asterisks indicate that the RNA-seq data showed significant differences in fold change from the quantitative RT-PCR analyses. NT, Not tested.

<table>
<thead>
<tr>
<th>Identifier in Version 5 (Version 4)</th>
<th>Name and Description</th>
<th>wt−S/wt+S</th>
<th>smt15-1−S/wt+S</th>
<th>smt15-1−S/smt15-1+S</th>
<th>smt15-1+S/wt+S</th>
<th>RPKM Values</th>
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<tr>
<td></td>
<td></td>
<td>R6 (Q2, Q6)</td>
<td>R6 (Q2, Q6)</td>
<td>R6 (Q2, Q6)</td>
<td>R6 (Q2, Q6)</td>
<td>wt+S</td>
</tr>
<tr>
<td>Sulfur uptake</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cre16.g671400.t1.1</td>
<td>ARS1, periplasmic arylsulfatase</td>
<td>3307 (3.452, 5.637)</td>
<td>1407 (887, 3093)</td>
<td>703 (830, 2891)</td>
<td>2.0 (1.1)</td>
<td>0.3</td>
</tr>
<tr>
<td>Cre16.g671350.t1.2</td>
<td>ARS2, periplasmic arylsulfatase</td>
<td>5010 (1.348, 4.235)</td>
<td>560 (22, 60)</td>
<td>17 (7.3, 17)</td>
<td>2.0 (0.6)</td>
<td>0.2</td>
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<tr>
<td>Cre17.g723350.t1.2</td>
<td>SULTR2, H+/SO42− transporter type</td>
<td>69 (72, 114.2)</td>
<td>2.8 (5.5, 13)</td>
<td>17 (7.3, 17)</td>
<td>0.2 (0.8)</td>
<td>12</td>
</tr>
<tr>
<td>Cre12.g502600.t1.2</td>
<td>SIR1, Na′/SO42− transporter type</td>
<td>1430 (534, 958)</td>
<td>229 (230, 547)</td>
<td>23 (60, 142)</td>
<td>10 (3.9)</td>
<td>0.7</td>
</tr>
<tr>
<td>Cre07.g348600.t1.3</td>
<td>SLT1, Na+/SO42− transporter</td>
<td>9.3 (11.3, 13.6)</td>
<td>2.0 (6.0, 1.6)</td>
<td>2.4 (10.2, 2.8)</td>
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<td>6</td>
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<tr>
<td>Cre14.g6616900.t1.1</td>
<td>SLT2, Na+/SO42− transporter</td>
<td>5.8 (5.6, 4.8)</td>
<td>2.0 (4.3, 2.0)</td>
<td>2.2 (8.2, 3.8)</td>
<td>0.9 (0.5)</td>
<td>23</td>
</tr>
<tr>
<td>Cre06.g257000.t1.2</td>
<td>SLP3, sulfate-binding protein</td>
<td>5.1 (3.6, 3.0)</td>
<td>1.3 (1.7, 0.5)</td>
<td>1.4 (3.5, 0.9)</td>
<td>0.9 (0.5)</td>
<td>15</td>
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<tr>
<td>Cre06.g273750.t1.3</td>
<td>SUA1, chloroplastic sulfate peroxidase</td>
<td>7.7 (5.2, 7.7)</td>
<td>2.5 (4.4, 6.4)</td>
<td>2.2 (6.0, 8.9)</td>
<td>1.1 (0.7)</td>
<td>20</td>
</tr>
<tr>
<td>Cre02.g145750.t1.1</td>
<td>ATS2, ATP sulfurylase</td>
<td>7.4 (3.2, 3.8)</td>
<td>1.5 (2.2, 1.6)</td>
<td>2.8 (5.4, 4.0)</td>
<td>0.5 (0.4)</td>
<td>292</td>
</tr>
<tr>
<td>Cre02.g107450.t1.2</td>
<td>ATS2, ATP sulfurylase</td>
<td>10 (7.4, 12)</td>
<td>2.6 (5.8, 3.3)</td>
<td>2.0 (6.3, 3.5)</td>
<td>1.3 (0.9)</td>
<td>27</td>
</tr>
<tr>
<td>Cre16.g15615.t1</td>
<td>SIR1, ferredoxin-sulfite reductase</td>
<td>4.9 (4.4, 6.0)</td>
<td>0.05 (&lt;0.05, &lt;0.05)</td>
<td>2.0 (&lt;0.05, &lt;0.05)</td>
<td>&lt;0.05 (0.9)</td>
<td>41</td>
</tr>
<tr>
<td>Cre16.g56868.t1</td>
<td>SIR2, ferredoxin-sulfite reductase</td>
<td>3.3 (3.8, 4.2)</td>
<td>0.05 (&lt;0.05, &lt;0.05)</td>
<td>1.0 (&lt;0.05, 0.1)</td>
<td>&lt;0.05 (0.4)</td>
<td>53</td>
</tr>
<tr>
<td>Cre16.g685550.t1.2</td>
<td>ASL4, O-acetyl-Ser-thiol-lyase/Cys synthesis</td>
<td>9.2 (7.4, 17)</td>
<td>3.1 (5.1, 6.2)</td>
<td>4.0 (6.0, 7.2)</td>
<td>0.8 (0.9)</td>
<td>140</td>
</tr>
<tr>
<td>Cre10.g466750.t1.3</td>
<td>SAT1, Ser acetyl transferase</td>
<td>3.2 (26, 42)</td>
<td>3.1* (&lt;0.05, &lt;0.05)</td>
<td>6.0* (&lt;0.05, &lt;0.05)</td>
<td>0.5 (1.0)</td>
<td>17</td>
</tr>
</tbody>
</table>

(Table continues on following page.)
of a paromomycin resistance marker in the SMT15 locus (Fig. 1A; Fang and Umen, 2008), and the paromomycin resistance phenotype was used to identify six smt15-1 progeny along with six wild-type progeny that were paromomycin sensitive. Elevated glutathione levels segregated with the paromomycin resistance phenotype in all 12 progeny, confirming that the smt15-1 mutation increases glutathione levels (Fig. 5B). Moreover, glutathione levels decreased and became closer to those of the wild type in the complemented smt15-1 strains under +S conditions (Fig. 5A; Table V). Restoration of glutathione levels was more modest in the complemented lines versus the parental smt15-1 strain, possibly due to a more stringent requirement for SMT15 function under –S conditions.

Glutathione Levels Cycle in Synchronous Cultures

Glutathione levels have been reported to be cell cycle regulated in plants and animals (Menon and Goswami, 2007; Diaz Vivancos et al., 2010a; García-Giménez et al., 2013). To investigate whether glutathione levels are cell cycle regulated in *C. reinhardtii*, we monitored glutathione content in synchronized cultures. In a synchronous culture, cells normally divide at the end of the light period or the beginning of the dark period, making it difficult to uncouple the effects of light/dark transitions from cell cycle transitions. In order to circumvent this issue, we first synchronized cultures in a 12-h/12-h light/dark cycle and then released the synchronized cells into continuous light. We sampled the cultures starting at time zero, which corresponds to the end of the last dark period, and continued through the completion of the cell cycle in continuous light. Culture synchrony was evaluated by scoring passage through commitment and mitotic index (Fig. 6A) and by measuring the periodic expression of the cell cycle marker genes CDKB1 and Proliferating Cell Nuclear Antigen (PCNA; Fig. 6B). In synchronized wild-type cultures, glutathione concentration doubled (from approximately 13 to approximately 28 pmol mg$^{-1}$) during the first few hours in the light, reached its peak around the time of commitment, and then dropped gradually for the remainder of the cell cycle, reaching basal levels just before S/M (Fig. 6C). In synchronized smt15-1, glutathione accumulation followed a similar pattern to the wild type during the first 8 h in the light, corresponding to early

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**Table IV.** Modal daughter cell sizes and $s_0$ of mat3-4, smt15-1 mat3-4, sac1 mat3-4, and sac3 mat3-4 strains

$s_0$ values were derived from four independent experiments for smt15-1 mat3-4 and sac3 mat3-4 strains and five independent experiments for mat3-4 and sac1 mat3-5 strains.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Daughter Cell Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>mat3-4</td>
<td>23.1 ± 1.1</td>
</tr>
<tr>
<td>smt15-1 mat3-4</td>
<td>38.4 ± 3.4</td>
</tr>
<tr>
<td>sac1 mat3-4</td>
<td>23.7 ± 1.5</td>
</tr>
<tr>
<td>sac3 mat3-4</td>
<td>19.6 ± 1.3</td>
</tr>
</tbody>
</table>
and mid G1 phases, but its levels did not decrease toward the onset of cell division and instead remained elevated during the time that glutathione levels dropped in wild-type cultures during S/M (Fig. 6C). These data indicate that rhythmic glutathione accumulation in smt15-1 is defective and shows its greatest departure from the wild type during cell division (S/M).

Glutathione can exist in an oxidized dimeric state (GSSG) or a reduced monomeric state (GSH). To investigate whether GSH-GSSG redox ratios are diurnally or cell cycle regulated, we measured them in synchronized wild-type and smt15-1 cultures. In wild-type cultures, the GSH-GSSG ratio was approximately 16 in early G1 and declined during the light period to between 5 and 6 at the time of cell division (S/M; Fig. 6D). The temporal changes in GSH-GSSG ratios in synchronous smt15-1 cultures were similar to those in the wild type, except that the values were about double that of the wild type at every time point tested (Fig. 6D). Taken together, our data show that smt15-1 has both elevated total glutathione levels and elevated GSH-GSSG ratios compared with the wild type.

DISCUSSION

SMT15 Belongs to a Distinct Subfamily of Putative Sulfate/Anion Transporters

smt15-1 was isolated as a suppressor of the small cell size defect of mat3 (Fang and Umen, 2008). Here, we characterized the smt15-1 mutant and verified that disruption of SMT15, which encodes a novel sulfate/anion transporter family member, caused growth and size defects in C. reinhardtii. Phylogenetic analysis indicates that SMT15 belongs to a distinct subfamily of sulfate/anion transporters whose origins are difficult to discern because of its patchy distribution among eukaryotes and prokaryotes (Supplemental Fig. S3; Takahashi et al., 2012). However, the general phylogenetic coherence we observed among prokaryotic and eukaryotic members suggests limited amounts of horizontal transfer of SMT15-like/family C genes between kingdoms, with the only exception being the split affiliation of the opisthokont members, where fungal homologs are closest to the eubacterial group while the choanoflagellate homolog is closest to other eukaryotic members (Fig. 1D). Overall, the phylogeny of family C is consistent with the early acquisition of SMT15/family C genes at the base of the eukaryotic lineage (Supplemental Fig. S2), perhaps through an endosymbiotic event, followed by multiple independent losses.

Few members of tribe 1 superfamily transporters have been characterized. Some of them in the SLC26 family function as Cl⁻/HCO₃⁻ transporters and others are SO₄²⁻ transporters (Satoh et al., 1998; Melvin et al., 1999; Soleimani et al., 2001; Wang et al., 2002). Intriguingly, down-regulation of a diastrophic dysplasia sulfate transporter (SLC26A2) is tightly associated with high rates of proliferation in colon cancer cells (Yusa et al., 2010), suggesting a connection between this family of transporters and the control of cell proliferation. Plant

Table V. Glutathione concentrations of the complemented smt15-1 strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total Glutathione Content (+S) pmol mg⁻¹</th>
<th>Total Glutathione Content (−S) pmol mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>21gr (wild type)</td>
<td>20.0 ± 0.7</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>smt15-1</td>
<td>33.5 ± 2.8</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>sac1</td>
<td>17.5 ± 1.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 57</td>
<td>24.3 ± 3.1</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 62</td>
<td>23.7 ± 2.3</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>smt15-1 pSMT15.1 line 64</td>
<td>20.6 ± 2.6</td>
<td>2.2 ± 0.7</td>
</tr>
</tbody>
</table>
**Figure 6.** A, Graph showing passage through commitment (dashed lines) and mitotic index (solid lines) of synchronous cultures. Wild-type (wt) culture entered S/M phase at approximately 12 h; smt15-1 culture entered S/M phase at approximately 10 h. The cell cycle phases are indicated by bars above the graph. The wild type is labeled in blue and smt15-1 is labeled in red. Because daughter cells of wild-type (6145-Y1) and smt15-1 strains failed to hatch from the mother cells in the first few hours after cultures were switched to light, the cell size and commitment assays were omitted at the early time points. Synchronized cultures were maintained in 12-h-light/12-h-dark cycles. Cultures were kept under light during sampling. B, Expression of S/M phase markers PCNA and CDKB1 determined by quantitative RT-PCR, with SE shown by error bars. C, Total glutathione levels of the synchronized wild-type and smt15-1 strains with SE indicated. D, GSH-GSSG ratios of synchronized wild-type and smt15-1 strains with SE indicated.
transporters in family P are mainly \( \text{SO}_4^{2-} \) transporters. The rest of tribe 1 transporters remain uncharacterized. Our study represents, to our knowledge, the first phenotypic characterization of a family C member outside of budding yeast, whose homolog is YGR125W, a sulfate transporter domain (Pfam 00916) containing membrane protein. Like smt15-1, YGR125W mutants have growth defects as measured by competition assays (Breslow et al., 2008). Moreover, YGR125W interacts genetically with mutations in SUL1 and SUL2 that encode sulfate transporters, but its specific roles in budding yeast in sulfate metabolism or other aspects of cell physiology have not been investigated.

SMT15 and SAC Responses

The reduction of viability of smt15-1 cells in –S and its inability to activate a full SAC response indicates a role for SMT15 in sulfur starvation, but this role is likely to be indirect. We found that smt15-1 overaccumulates glutathione (Fig. 5A), whose elevated levels are known to suppress the SAC response (Herschbach and Rennenberg, 1994; Lappartient et al., 1999; Vauclare et al., 2002; Buchner et al., 2004). It is likely, therefore, that elevated levels of glutathione in smt15-1 strains attenuate the SAC response in –S conditions. Our RNA-seq data set showed that mRNAs of \( \gamma \)-GLUTAMYL-CYSTEINE SYNTHETASE and GLUTATHIONE SYNTHETASE, two major enzymes required for glutathione biosynthesis, were not altered significantly in smt15-1 (Supplemental Table S2), suggesting that SMT15 affects glutathione homeostasis by a posttranscriptional mechanism.

C. reinhardtii encodes several –S-inducible sulfate transporters whose functions have been partially characterized (Poothakham et al., 2010). SMT15 mRNA levels were induced under –S conditions, but not to the extent of the messages for known sulfur transporters, such as SULTR2 and SLTI2 (Table III). The transporters responsible for sulfate uptake under +S conditions are not known, but if the growth defects of smt15-1 in +S were due to inadequate sulfate uptake, it would be expected to show a constitutive SAC response under +S conditions, which is not the case (Fig. 4C; Table III). A plausible transport function for SMT15 might be in maintaining metal ion homeostasis through its function as a cotransporter (Lee et al., 2014; Srinivasan et al., 2014). Glutathione binds to and helps detoxify heavy metals and typically accumulates in response to elevated metals (Cobbett and Goldsborough, 2002; Jozefczak et al., 2012; Zagorchev et al., 2013), so its elevated levels in smt15-1 could reflect a response to altered metal ion levels. An alternative possibility is that SMT15 is not a transporter but a sensor/signaling protein linked to sulfur metabolism, glutathione metabolism, and/or metal stress. Precedent for such a function comes from the Sac1 protein of C. reinhardtii, which has homology to transporters but likely serves as a sensor to activate SAC responses (Davies et al., 1996; Pollock et al., 2005). Future work aimed at determining the substrate(s) of SMT15 should help clarify its role in sulfur metabolism and ion transport.

SMT15, Glutathione, and Cell Cycle Control

Our findings lead to the question of what substrates, if any, are transported by SMT15 and why defects in its function cause glutathione overaccumulation and cell cycle defects? Although smt15-1 has a clear sulfur acclimation defect, this defect is unlikely to be linked to its effect on cell cycle control, because mutants with more severe defects in SAC responses had no detectable impact on the cell cycle (Table IV; Supplemental Fig. S7). Our finding that glutathione overaccumulates in smt15-1 but not in sac mutants is a possible clue for understanding the cell cycle defects in this mutant. Glutathione levels and subcellular localization have been linked to cell cycle control in both plants and animals (Markovic et al., 2007; Pallardó et al., 2009; Pellny et al., 2009; Díaz Vivancos et al., 2010a, 2010b), although its specific impact on cell cycle-related processes is not completely understood. Changing the redox state of cell cycle regulators through glutathionylation has also been shown to influence the cell cycle (Chiu and Dawes, 2012). A particularly striking finding in our study was the cell cycle-regulated fluctuations in glutathione levels and their disruption in smt15-1. In synchronized wild-type cells, glutathione levels peaked in mid G1 and then declined during cell division, while in smt15-1, they remained elevated (Fig. 6C). The glutathione redox ratio (GSH-GSSG) also fluctuated during the wild-type cell cycle, reaching its lowest levels around the time of cell division (Fig. 6D). While this cyclical pattern was mirrored in smt15-1, the mutant GSH-GSSG ratio was consistently around 2-fold higher than in the wild type, suggesting that a more reduced state of cell cycle regulators through glutathionylation occurred in smt15-1 cells for reactions that are in equilibrium with glutathione.

Based on these findings, we speculate that aberrant glutathione levels and redox homeostasis cause the increased division and small-cell phenotypes observed in the smt15-1 mutant. Supporting this hypothesis are numerous reports on the close association between cell proliferation and elevated glutathione levels and high GSH-GSSG ratios (Mauro et al., 1969; Kosower and Kosower, 1978; Suthanthiran et al., 1990; Sánchez-Fernández et al., 1997; May et al., 1998; Nkabyo et al., 2002). We have not been able to examine temporal redox control at a finer scale in wild-type and smt15-1 cells, as has been done in budding yeast, where redox and respiratory activity cycle with a period of about 40 min (Tu et al., 2005). It may be revealing to examine C. reinhardtii cells at these time scales to see if there are shorter scale periodicities in its redox metabolism.

In Arabidopsis (Arabidopsis thaliana), glutathione levels were found to cycle in synchronized cell cultures but appeared to peak during cell division, which is different from what we found for C. reinhardtii, where glutathione levels were near their lowest levels during cell division.
(Fig. 6C). However, the increased glutathione during S and G2 phases occurred in a partially synchronous cell population generated from starvation and refeeding (Diaz Vivancos et al., 2010b). This raises the question of whether the peak of glutathione seen was due to cell cycle phasing or is a general response to proliferation induced by refeeding starved cultures. A similar caveat has been raised for measurements of glutathione in synchronous mammalian cell culture experiments (Markovic et al., 2010). In our hands, starvation and refeeding of C. reinhardtii also caused a transient increase in glutathione levels that are unrelated to cell cycle phasing (Supplemental Fig. S8). Therefore, it is premature to conclude that our results conflict with those from Arabidopsis or other organisms in which glutathione cycling has been examined with respect to the cell cycle.

Although the established connections between glutathione-mediated redox state and cell cycle control make redox defects a plausible explanation for the cell size phenotype, we cannot exclude the possibility that other defects account for its cell size phenotype. It is clear that the mutant does not cause the misregulated expression of core cell cycle genes (Supplemental Table S2) and is most likely causing a posttranscriptional change in cell cycle control. The molecular mechanism connecting glutathione flux and cell cycle control remains to be determined.

Even though the accumulation of SMT15 mRNA was in sync with cell cycle phasing (Fig. 3A), it is also light regulated. It is well known that light reactions of photosynthesis generate abundant reactive oxygen species (Balsera et al., 2014; Schmitt et al., 2014) and may activate the transcription of SMT15 in order to maintain glutathione homeostasis and thereby mitigate cellular damage and stress caused by reactive oxygen species. Equally possible is that glutathione levels are directly coupled to the glutathionylation/deglutathionylation of proteins required for photosynthetic activities as cells grow (Zaffagnini et al., 2012; Michelet et al., 2013).

Recent studies on cancer cell metabolism indicate that cancer cells are metabolically different from normal cells. The increased demand of glutathione is important for RB-defective cells to cope with oxidative stress (Nicolaï et al., 2013; Reynolds et al., 2014). At least in the mouse system, increases in γ-glutamyl-Cys ligase partly contribute to increased glutathione levels in RB−/− cancer cells (Reynolds et al., 2014). The connection between increased cell division and defective glutathione control in smt15-1 and tumor cells suggests that cell cycle-dependent cellular redox regulation may be similar in both systems, and smt15-1 may provide a unique tool for understanding redox-regulated cell cycle control.

MATERIALS AND METHODS

Strains and Growth Conditions

Chlamydomonas reinhardtii strains 21gr (CC1690, mating type plus [MT+]), 6145-Y1 (CC1691-Y1, mating type minus [MT−]), smt15-1 (Fang and Umen, 2008), sac1 (CC3801, MT); Davies et al., 1996), and sac1 (CC3799, MT); Gonzalez-Ballester et al., 2008) were used. sac1 and sac3 were ordered from the C. reinhardtii stock center (http://chlamycollection.org/). 6145 (CC1691) was backcrossed to 21gr twice to remove a y/l mutation (Bednarek and Hoober, 1985; White and Hoober, 1994) and was then designated as 6145-Y1 (CC1691-Y1). 6145-Y1 was used in the experiment presented in Figure 6. 21gr or mutants derived from the 21gr strain background were used in the majority of experiments. sac1 and sac3 were backcrossed to 6145-Y1, and MT− sac progeny were backcrossed to 21gr to generate sac1−g and sac3−g progeny of both mating types. sac1−g and sac3−g were used for the experiments in this study.

For segregation analysis, bulk meiotic progeny were germinated on high-salt medium (HSM) plates, resuspended and serially diluted in Tris-acetate-phosphate (TAP), and plated for single colonies to obtain meiotic segregants.

Cells were grown in HSM (Sueoka, 1960) or TAP (Gorman and Levine, 1965) under illumination at 250 to 300 μmol photons m−2 s−1 aerated with 0.5%/v/v CO2. Culture synchronization was achieved by growing the 12-h-light/12-h-dark cycles for several weeks. The cultures were then transferred to 14L:10D cycles to optimize synchrony. Synchronized cultures were maintained in 14L:10D cycles unless mentioned otherwise.

For −5 treatment, the sulfate salts in HSM was replaced with chloride salts (HSM-S). Sulfur-free trace elements were prepared as described by the C. reinhardtii Resource Center (http://chlamycollection.org/hutners-trace-elements-recipe). For sulfur deprivation experiments, the cells were grown to logarithmic phase in +S medium, washed twice with 250 mL of HSM-S, and then resuspended in HSM-S.

Identification of Genomic DNA Sequences Flanking the smt15-1 Insertion

The isolation of smt mutants was described previously (Fang and Umen, 2008). The following protocol for identifying insertion sites was provided by Steve Pollock (personal communication) with details to be published elsewhere. Briefly, genomic DNA was digested with Sool to generate blunt-ended fragments. A blunt-ended asymmetric adaptor consisting of a 48-bp DNA oligonucleotide and a 10-bp oligonucleotide was then ligated to the digested genomic DNA. An insert-specific primer, IMP-3 (5′-CGATTTCGCGCCCATTTGGTGA-3′), and an adaptor primer, AP1 (5′-GAATACGATCATACTAGCAT-3′), were used to amplify the genomic flanking region adjacent to the smt15-1 insertion. The insert-specific primer IMP-2 (5′-ATCCATTCGCCATCTACGG-3′) and the adaptor primer AP2 (5′-ACTATAGATACGGCTGTG-3′) were used for nested PCR. PCR fragments were amplified by Taq DNA polymerase in a final volume 20 μL in the presence of 1× ExTaq buffer (Takara Bio), 1 μm primers, 80 μm deoxynucleotide triphosphate (dNTP), and 2% (v/v) dimethyl sulfoxide (DMSO). PCR conditions were as follows: 96°C for 3 min, followed by 36 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. PCR products were gel purified and sequenced.

Strain Genotyping

One microliter of genomic DNA prepared as described (http://www.chlamy.org/methods/quick_pcr.html) was used for PCR amplification. PCR fragments were amplified using Taq DNA polymerase in a final volume of 20 μL in the presence of 1× ExTaq buffer (Takara Bio), 1 μm primers, 80 μm dNTP, 0.5 μm betaine, and 3% (v/v) DMSO. Primer pairs used for PCR-based genotyping are listed in Supplemental Table S4. PCR conditions were as follows: 96°C for 2 min, followed by 42 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45s.

RACE-PCR and Isolation of the Full-Length SMT15 cDNA

Total RNA was isolated as described previously (Fang et al., 2006). Five micrograms of total RNA was used for cDNA synthesis. cDNA was synthesized with a mixture of oligo(dT) and random primers (9:1 ratio) at 55°C for 70 min using the Thermoscript RT-PCR kit (Invitrogen) following the manufacturer’s instructions. PCR was used to amplify different parts of the SMT15 cDNA under the following conditions: 20 μL RT-PCR with 0.5 μL of cDNA, 4 μL of 5× Phusion HF buffer, 200 μL dNTPs, 0.5 μm primers, 3% (v/v) DMSO, 0.5 μm betaine, and 0.4 unit Phusion High-Fidelity DNA polymerase (New England Biolabs). PCR amplification conditions were as follows: 98°C for 1 min, and then 40 cycles of 98°C for 10 s, 65°C for 20 s, and 72°C for 30 s. An approximately 1.78-kb SMT15 cDNA fragment was amplified using primers 5′-ATGCCGCTCAGCTAAGGCAC-3′ and 5′-CGGAATTCGCGCCATTTGGTGA-3′. An approximately 1.32-kb SMT15 cDNA fragment was amplified using primers 5′-AAGCTTGGACGACCTTCA-3′ and 5′-TTGGCGAAGATGACGTTGAC-3′. The amplified cDNA fragments were then cloned into pGEM-T easy vector (Promega) separately and sequenced.
SMT15 Governs Sulfur Stress, Glutathione, and the Cell Cycle

Dark-Shift Experiments and Cell-Size Measurements

Dark-shift experiments and daughter cell size measurements were performed as described previously (Fang and Umen, 2008). Briefly, liquid cultures were grown under continuous light in HSM, and cell density was maintained between 10^6 and 10^7 cells mL^{-1} before dark incubation. After incubating cultures in the dark for 16 to 18 h, cells were fixed with a final concentration of 0.2% (v/v) glutaraldehyde and 0.005% (v/v) Twem 20. Cell size distributions were measured using a Coulter Counter (MULTISIZER 3; Beckman-Coulter) set to count at least 300 events in the modal channel. Modal daughter cell size was determined from at least three independent cultures as described previously (Fang and Umen, 2008).

Cell Viability Analysis

A total of 1 x 10^6 cells were collected by brief centrifugation at 2,000 g in a microcentrifuge tube. Cells were resuspended in 1 mL of HSM with 5 μM of the vital fluorescent dye carboxymethylfluorescein diacetate (CMFDA; Invitrogen; Johnson et al., 2013). Cells were kept in the dark at room temperature for 30 to 40 min and then washed two times with phosphate-buffered saline (PBS). Fluorescence and differential interference contrast images were taken on a Zeiss Axio Scope A1 fluorescence microscope equipped with an AxioCam MRc camera (Zeiss). Viability was measured as (number of fluorescent cells)/(number of total cells) x 100. Glutathyldehydrated-treated or heat-treated cells were used as negative controls and showed no signal after CMFDA staining. The viability data obtained with CMFDA staining was consistent with what was described previously using a different method (Chang et al., 2005).

RNA-seq

Approximately 30 μg of DNA-free RNA samples isolated from wild-type (21g) and smt15-1 mutant cells 6 h after being transferred to 5- or 5.5 medium was submitted to Genomics BioSci & Tech, Taiwan (http://www.genedragon.com.tw/site_ngs.php#menu03). Total RNA was converted into a library template for sequencing using an mRNA sequencing sample preparation kit (catalog no. RS-930-1001; Illumina). Briefly, mRNA was isolated by Sera-Mag Magnetic Oligo(dT) Beads, washed, and then fragmented using divalent cations under elevated temperature. First and second strand cDNAs were synthesized and end repaired to convert overhangs into blunt ends. The 3' ends were then adenylated for ligation of the Illumina adapters. cDNA templates were purified by gel isolation for a size isolation of approximately 200 ± 25 bp and amplified via PCR. Products were isolated by the QiAgquick PCR Purification Kit (Qiagen) and added to the flow cell for sequencing. Illumina HiSeq2000 technology was used. The raw sequencing data were filtered to remove low-quality sequences, including ambiguous nucleotides, adaptor sequences, and repeat sequences. A total of 50 million reads were then subjected to the Illumina pipeline output were aligned against the version 4.1 assembly of the C. reinhardtii genome using Bowtie software (Langmead et al., 2009). One mismatch was allowed during the alignment. More than 76% of the reads were mapped to annotated transcripts of the version 4.1 C. reinhardtii genome. The statistics on read analyses are summarized in Supplemental Table S5. Expression estimates for each sample were provided in units of reads per kilobase of exon per million aligned reads (RPKM, Mortazavi et al., 2008). Two-fold or 4-fold differences were used as cutoffs for identifying differentially expressed genes. Genes with differential expression patterns were subjected to Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses using the online Algal Functional Annotation Tool (Lopez et al., 2011; http://pathways.mcbio.ucd.edu/algal/index.html).

Quantitative PCR

One or 3 μg of total RNA was used for cDNA synthesis. DNA-free RNA was reverse transcribed in the presence of a mixture of dT and random primers (9:1 ratio) using Thermoscript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Ten-microliter RT-PCR contained 2.5 μL of 1:20 diluted cDNA, 0.2 or 0.25 μM primers, and 5 μL of 2× KAPA SYBR FAST master mix (KAPA Biosystems). Real-time PCR was performed using a CFX96 Real-Time PCR detection system (Bio-Rad). Quantitative analysis was calculated based on the comparative cycle threshold method 2^-ΔΔct using G protein Beta Subunit-Like Protein as an internal standard by CFX Manager Software following the manufacturer’s instructions (Bio-Rad). Primers used for quantitative PCR are listed in Supplemental Table S4.

Phylogenetic Tree Construction

To construct the phylogenetic tree in Figure 1D, the SMT15 protein sequence (KF709427) was used for a BLASTp query of the National Center for Biotechnology Information (NCBI). The high-scoring positives (E-value of 1e-25 was set as an arbitrary cutoff) were selected to generate Figure 1D. An independent BLAST search on the Phytozome database (http://www.phytozome.net/) was conducted to check for the presence of SMT15 homologs in other green algae. The locus numbers or gene identification numbers in Figure 1D are indicated according to Joint Genome Institute (http://www.jgi.doe.gov/). Phytozome (http://www.phytozome.net/), Saccharomyces Genome Database (http://yeastgenome.org/), The Arabidopsis Information Resource (http://www.arabidopsis.org/), or NCBI (http://www.ncbi.nlm.nih.gov/ protein/). Locus numbers or gene identification numbers used to generate phylogenetic trees are listed in Supplemental Table S1. Members of families A1, A2, and P from tribe 1 sulfani/anison transporters (Takahashi et al., 2012), including Aspergillus niger, Arabidopsis (Arabidopsis thaliana), C. reinhardtii, Coccomyxa subellipsoidea, Monosiga brevicollis, Saccharomyces cerevisiae, and Volvox carteri, were included as an outgroup for tree construction. For the family C group, proteins containing both the sulfite permease domain (Pfam 00916) and the STAS domain (Pfam 01740 or cd07042) were selected for tree construction. Full-length protein sequences were aligned by ClustalW. The resulting alignments were used to construct phylogenetic trees in MEGA 5.22 (Tamura et al., 2011). The neighbor-joining method was used to generate phylogenetic trees, and 1,000 replicates were used for bootstrapping. Bootstrap values of 50% or higher were shown for each clade. The evolutionary distances were computed using the JTT matrix (Jones et al., 1992).

To search for distant SMT15 homologs in different phyla (Supplemental Fig. S2), SMT15 or its homologs were BLAST searched against nonredundant protein sequences, ESTs, and transcriptome assemblies in NCBI. E values of 1e-15 or higher were shown for each clade. The evolutionary distances resulting alignments were used to construct phylogenetic trees in MEGA 5.22 (Tamura et al., 2011). The neighbor-joining method was used to generate phylogenetic trees, and 1,000 replicates were used for bootstrapping. Bootstrap values of 50% or higher were shown for each clade. The evolutionary distances were computed using the JTT matrix (Jones et al., 1992).

Complementation of smt15-1

Bacterial artificial chromosome clone pTSQ801 (generated by Dr. Peter Leleboevre at the University of Minnesota and available from the Clemson University Genomics Institute) was digested with BstBI and NheI, and a 13.675-kb DNA fragment containing the predicted SMT15 gene was gel purified and ligated to BstBI- and NheI-digested pSP124 plasmid that had been modified to contain BstBI and NheI restriction enzyme sites (Lumbrares et al., 1998) to generate pSMT151. BstBI-linearized pSMT151 was transformed into smt15-1 by electroporation (http://nutneg.eastern.edu/~adams/ChlamyTeach/experiments. html), and transformants were selected on TAP containing 5 μg mL^{-1} zeocin (Invitrogen).

Growth Rate Measurements

Liquid cultures were grown in continuous light, and cell density was maintained between 10^8 to 10^9 cells mL^{-1} by dilution into fresh HSM. Cultures at around 1 to 2 x 10^7 cells mL^{-1} were used for the initial sampling point, and additional samples were collected every 3 h for 12 h. Chlorophyll content was used as a measure of culture density as described previously (Harris, 1989), and each growth experiment was repeated at least three times.
Glutathione Measurements

For total glutathione measurements, 2 × 10^7 cells were collected from C. reinhardtii cultures and washed with 1× PBS twice. The cell pellet was then resuspended in 500 μL of cold 5% (w/v) metaphosphoric acid and homogenized by vortexing in the presence of 500 μL of zirconium beads (BioSpec Products) at 4°C for 30 min. The suspension was transferred to a 1.5-mL tube and centrifuged at 12,000g for 14,000g for 5 min at 4°C. The supernatant was transferred into a clean 1.5-mL tube and stored at −80°C before analysis. Total glutathione was measured enzymatically using a glutathione detection kit (Enzo Life Sciences). Briefly, glutathione reductase was added to reduce GSSG to GSH. Total GSH reacts with 5,5′-dithiobis-2-nitrobenzoic acid and produces a yellow 5-thio-2-nitrobenzoic acid that absorbs at 405 nm. GSH content was extrapolated based on a standard curve generated by GSSG with a known concentration (provided in the kit). Total glutathione was normalized to total protein content. For protein content measurement, 1 × 10^8 cells were collected and heated in 2% (w/v) SDS solution at 100°C for 10 min. A 2-fold dilution of the crude protein extract was used for protein content measurement. Protein content was measured with a detergent-compatible DC protein assay kit (Bio-Rad) following the manufacturer’s instructions. To determine the ratio of GSH to GSSG, total glutathione and GSSG were measured. A total of 7.5 × 10^9 cells were collected and washed with 1× PBS twice. Cell pellets were resuspended in 1,000 μL of cold 5% (w/v) metaphosphoric acid (Sigma) and homogenized by vortexing in the presence of 500 μL of zirconium beads at 4°C for 45 min. The suspension was transferred to a 1.5-mL tube and centrifuged at 12,000g for 14,000g for 5 min at 4°C. The supernatant was transferred to a clean 1.5-mL tube and stored at −80°C before analysis. For measurement of GSSG, 1 μL of 2 μM 4-vinylpyridine (Sigma) was added per 50 μL of sample before measurement. 4-Vinylpyridine blocks free thiols present in the reaction, thus eliminating any contribution of GSH. Samples without 4-vinylpyridine treatment were used for measurement of total glutathione. Total glutathione and GSSG were measured using a glutathione detection kit (Enzo Life Sciences) as described previously. The ratio of GSH to GSSG was deduced from (total glutathione − GSSG)/GSSG.

Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Prediction of transmembrane topology of SMT15 protein by TransMembrane prediction using Hidden Markov Models (TMHMM 2.0).

**Supplemental Figure S2.** ClustalW alignment of the sulfate transporter domain (Pam 00916) of listed proteins in Figure 1.

**Supplemental Figure S3.** Diverse distribution of C family SMT15 homologs in eukaryotic and prokaryotic phyla.

**Supplemental Figure S4.** ClustalW alignment of parts of Family C sulfate/union transporters.

**Supplemental Figure S5.** Viability of wild-type and smt15-1 strains following N deprivation or P deprivation in liquid medium.

**Supplemental Figure S6.** Expression patterns of SMT15 mRNA under nitrogen-deplete conditions.

**Supplemental Figure S7.** Size distributions of dark-shifted cells from sac1 mat3-4, sac3 mat3-4, and mat3-4.

**Supplemental Figure S8.** Glutathione concentration and mitotic index of wild-type cells after being diluted into fresh medium.

**Supplemental Table S1.** Accession number, locus number, or gene identities, and structure organization of representative proteins used for constructing the phylogenetic tree.

**Supplemental Table S2.** Comparative quantification of transcript abundance (RPKM) of selected cell cycle genes and genes involved in S assimilation and glutathione biosynthesis by RNA-seq.

**Supplemental Table S3.** Daughter cell size distribution of wild-type, sac1, and sac3 strains.

**Supplemental Table S4.** List of primer pairs used for quantitative RT-PCR.

**Supplemental Table S5.** Statistics of RNA-seq reads.

**LITERATURE CITED**


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