

RNA-Seq Analysis of Sulfur-Deprived *Chlamydomonas* Cells Reveals Aspects of Acclimation Critical for Cell Survival^W

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The *Chlamydomonas reinhardtii* transcriptome was characterized from nutrient-replete and sulfur-depleted wild-type and *snrk2.1* mutant cells. This mutant is null for the regulatory Ser-Thr kinase SNRK2.1, which is required for acclimation of the alga to sulfur deprivation. The transcriptome analyses used microarray hybridization and RNA-seq technology. Quantitative RT-PCR evaluation of the results obtained by these techniques showed that RNA-seq reports a larger dynamic range of expression levels than do microarray hybridizations. Transcripts responsive to sulfur deprivation included those encoding proteins involved in sulfur acquisition and assimilation, synthesis of sulfur-containing metabolites, Cys degradation, and sulfur recycling. Furthermore, we noted potential modifications of cellular structures during sulfur deprivation, including the cell wall and complexes associated with the photosynthetic apparatus. Moreover, the data suggest that sulfur-deprived cells accumulate proteins with fewer sulfur-containing amino acids. Most of the sulfur deprivation responses are controlled by the SNRK2.1 protein kinase. The *snrk2.1* mutant exhibits a set of unique responses during both sulfur-replete and sulfur-depleted conditions that are not observed in wild-type cells; the inability of this mutant to acclimate to S deprivation probably leads to elevated levels of singlet oxygen and severe oxidative stress, which ultimately causes cell death. The transcriptome results for wild-type and mutant cells strongly suggest the occurrence of massive changes in cellular physiology and metabolism as cells become depleted for sulfur and reveal aspects of acclimation that are likely critical for cell survival.

INTRODUCTION

The dominant form of sulfur (S) in terrestrial and aquatic habitats is usually the sulfate anion (SO₄²⁻), the most oxidized form of S. Animals do not have the enzymatic machinery needed for reducing SO₄²⁻ to sulfide (S²⁻), which is required to synthesize most S-containing compounds. Plants and microbes have specific transporters that efficiently import SO₄²⁻ into cells, where it is activated and then reduced to S²⁻ for incorporation into S-containing amino acids and other molecules, such as S-adenosylmethionine (SAM), GSH, FeS clusters, thionucleosides, sulfolipids, vitamins, and enzyme cofactors, such as CoA (CoA), molybdopterin, thiamine, or biotin. However, most organisms have a low capacity to store S and thus have developed diverse acclimation strategies that optimize S use and balance the rate of S metabolism with S availability. These processes allow organisms to remain viable even when S is severely limiting to growth.

The alga *Chlamydomonas reinhardtii* (*Chlamydomonas* throughout) has been used as a model organism for studying

responses of photosynthetic eukaryotes to S deprivation. S-responsive processes in this alga include rapid changes in cell size (Zhang et al., 2002), elevated production of hydrolytic extracellular enzymes (de Hostos et al., 1989; Takahashi et al., 2001), alterations in cell wall structure (Takahashi et al., 2001), changes in the activities and composition of the photosynthetic apparatus (Collier and Grossman, 1992; Wykoff et al., 1998; Zhang et al., 2004), elevated SO₄²⁻ transport activity (Yildiz et al., 1994; Pootakham et al., 2010), and the synthesis of enzymes (plus the encoding transcripts) required for efficient S assimilation (Ravina et al., 1999, 2002; González-Ballester and Grossman, 2009). Moreover, in the last 5 years, S-depleted *Chlamydomonas* cells have been used for microarray-based RNA abundance studies (Zhang et al., 2004; Nguyen et al., 2008), determination of metabolite profiles (Bolling and Fiehn, 2005), and sustained production of H₂ (Ghirardi et al., 2007). Many studies of S-deprived plants have also been performed (Lewandowska and Sirko, 2008; Kopriva et al., 2009); some of these studies include determinations of transcript levels and metabolite profiles (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003, 2005a, 2005b; Lunde et al., 2008).

A number of regulatory elements controlling S deprivation responses in *Chlamydomonas* have been identified. The sulfur acclimation gene *SAC1* encodes a polypeptide with homology to Na⁺/SO₄²⁻ transporters (SLC13 family). However, the protein appears to function as a sensor that responds to extracellular SO₄²⁻ levels

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rather than as a transporter (Davies et al., 1996; Moseley and Grossman, 2009). The *sac1* mutant exhibits marked reduction in accumulation of many transcripts encoding proteins associated with S acclimation responses (Takahashi et al., 2001; Ravina et al., 2002; Zhang et al., 2004) and rapidly loses viability following exposure to S deprivation; the loss of viability is a consequence of the inability of the cells to decrease photosynthetic electron transport activity (Wykoff et al., 1998). However, transcription from some S-responsive genes does not show an absolute dependence on SAC1. For example, the *sac1* mutant still develops a considerable level of high affinity SO_4^{2-} transport activity (Davies et al., 1996) and accumulates transcripts encoding SO_4^{2-} transporters in response to S deprivation (González-Ballester et al., 2008).

Two other genes encoding regulatory proteins that control S deprivation responses have been extensively characterized; they are the plant-specific SNF-1 related kinases *SNRK2.1* and *SNRK2.2* (the latter is also known as *SAC3*) (Davies et al., 1999; González-Ballester et al., 2008). *SNRK2.2* is responsible for repression of S-inducible genes when cells are replete for S (Davies et al., 1999; Ravina et al., 2002; González-Ballester et al., 2008). It may also be involved in repression of chloroplast transcription during S deprivation (Irihimovitch and Stern, 2006). The *SNRK2.1* kinase is required for most S-responsive gene expression and for maintaining cell viability during S deprivation. S-responsive gene expression and cell viability are more severely impacted in *snrk2.1* than in the *sac1* mutant during S deprivation (Pollock et al., 2005; González-Ballester et al., 2008). Moreover, while SAC1 and *SNRK2.2* do not show a clear epistatic relationship, *SNRK2.1* is epistatic to *SNRK2.2*. Recently, it has been suggested that SAC1 may act as a negative modulator of *SNRK2.2* activity during S deprivation (Moseley et al., 2009). Figure 1 shows a simplified diagram of the relationship among the different regulatory factors and the putative interactions associated with the acclimation of *Chlamydomonas* cells to S deprivation.

The recently developed RNA-seq technology (Wang et al., 2009) has made genome-wide transcript analyses both sensitive and quantitative. Here, we report on the use of RNA-seq technology to evaluate the responses of wild-type (the parental strain of the mutant) and the *snrk2.1* mutant of *Chlamydomonas* to S deprivation. A comparison of RNA-seq, microarray, and quantitative RT-PCR (qRT-PCR) results demonstrated reduced sensitivity of microarray analyses for estimating changes in mRNA abundance, especially for low abundance transcripts, relative to the RNA-seq platform. Furthermore, these RNA-seq studies have yielded large, quantitative data sets for transcript abundance in wild-type and *snrk2.1* mutant cells, both during S starvation and nutrient-replete growth. The data strongly suggest the occurrence of novel changes in cellular structures and metabolic pathways as wild-type cells acclimate to S deprivation. The data also define detailed responses of the *snrk2.1* mutant to S deprivation.

RESULTS AND DISCUSSION

Design, Statistical, and Comparative Analyses

We examined genome-wide transcript abundance in *Chlamydomonas* wild-type and *snrk2.1* mutant cells during growth in both

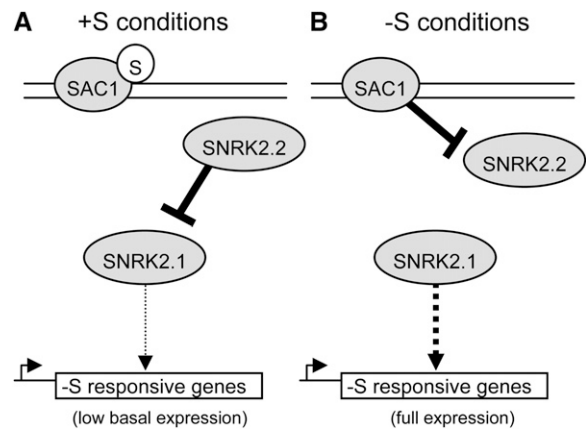


Figure 1. Model for S Deprivation-Responsive Gene Regulation.

(A) and (B) Simplified diagrams of gene regulation in wild-type cells in +S and -S conditions, respectively.

(A) In the presence of S, SAC1 is inactive and *SNRK2.2* inhibits *SNRK2.1*-activated expression of S-responsive genes, leading to basal level expression.

(B) In S-deficient conditions, active SAC1 blocks *SNRK2.2* inhibition of *SNRK2.1*, allowing full expression of S-responsive genes. This model is based on one previously developed by Moseley et al. (2009).

nutrient-replete (designated +S) and S-depleted (-S) conditions. RNA-seq and microarray profiling were used to determine the abundance of transcripts associated with each *Chlamydomonas* gene model (based on Joint Genome Institute [JGI] Chlre v.3.1) for each experimental condition (wt +S, wt -S, *snrk2.1* +S, and *snrk2.1* -S). The abundance of selected transcripts was also determined by qRT-PCR. Total RNA isolated from cells 6 h after they were transferred to -S and +S medium was used for RNA-seq. By 6 h after the cells were placed in -S conditions, the abundances of most of the induced transcripts had peaked. The same RNA preparations were also used for both microarrays and qRT-PCR analyses. Microarray hybridizations were also performed with RNA isolated from cells that were transferred from +S to both -S and +S conditions for 1 and 24 h. Genome-wide RNA-seq coverage graphs are available on a public installation of the UCSC genome browser at <http://genomes.mccdb.ucla.edu/CreSulfur/>. A browser image of a 14-kb region of the *Chlamydomonas* genome is shown in Figure 2.

Fold changes in transcript abundances using each of the techniques are presented in Supplemental Data Set 1 online. Transcripts with significant fold changes, as determined by RNA-seq, are grouped into categories, as shown in Figure 3 and Supplemental Data Set 2 online. An overview of the physiological functions related to these transcript categories is presented in Table 1, with an emphasis on differences between wild-type and *snrk2.1* mutant cells. Finally, some genes have been grouped according to their putative functionalities, independent of their relative expression levels (Supplemental Data Set 3 online).

Table 2 and Figure 4 show the correlations as fold change of transcript abundance for cells transferred from +S to -S conditions measured by RNA-seq, microarray hybridizations, and qRT-PCR. The levels of moderate and high abundance

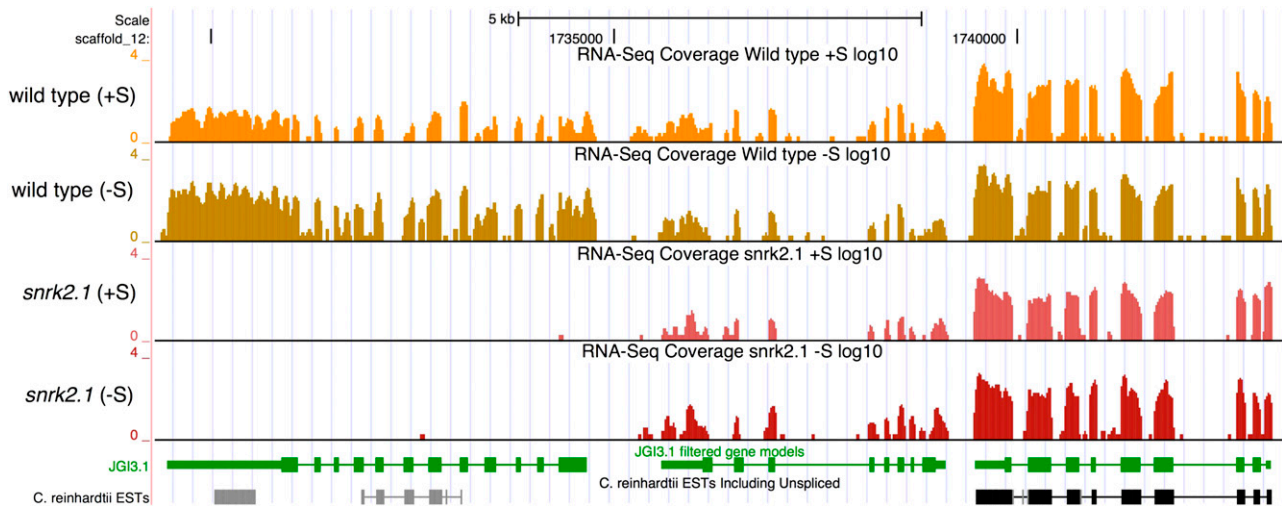


Figure 2. RNA-Seq Coverage for a 14-kb Region of the *Chlamydomonas* Genome as Displayed by the UCSC Genome Browser.

Individual tracks for each *Chlamydomonas* strain and condition discussed in the article are displayed in different colors. The x axis represents a genome fragment with the linear arrangement of gene models along that fragment, while the y axis provides a representation of the degree of transcript coverage in log scale. JGI v.3.1 gene models and ESTs tracks are displayed at the bottom. See Supplemental Methods online for more details. The most 5' gene model (left-hand side) corresponds to the *AOT4* locus, a putative amino acid/polyamine transporter with increased expression in S-deprived wild-type cells that is barely expressed in *snrk2.1* cells (under both +S and –S conditions).

transcripts were similar for the RNA-seq and qRT-PCR results; there was significantly more variability in quantification of low abundance transcripts. For microarray values, the direction of change was the same compared with the other quantification methods, although the data exhibited a smaller dynamic range of fold change than RNA-seq or qRT-PCR (e.g., less of an increase at the upper range and much lower sensitivity at the lower range). Furthermore, low-abundance transcripts that could be quantified by RNA-seq and qRT-PCR were below the detection limits of the microarray. Finally, the most recent *Chlamydomonas* array queries <10,000 unique transcripts, while RNA-seq potentially covers the entire transcriptome. These results demonstrate that RNA-seq represents an excellent genome-scale platform for analyzing transcript levels, yielding fold changes that agree well with qRT-PCR quantifications. Unless otherwise noted, the results discussed in this manuscript will be from the +S and 6 h –S samples analyzed by RNA-seq, with many of the trends corroborated by qRT-PCR.

Sulfur Metabolism

Sulfur Acquisition and Assimilation

S deprivation of *Chlamydomonas* cells elicits an increase in the abundance of many transcripts encoding proteins involved in SO_4^{2-} acquisition and assimilation (Figure 5, Table 3). For example, levels of transcripts for a number of SO_4^{2-} transporters exhibited dramatic changes in response to S deprivation; transcripts encoding two putative $\text{Na}^+/\text{SO}_4^{2-}$ transporters (SLT1 and SLT2) and one putative $\text{H}^+/\text{SO}_4^{2-}$ transporter (SULTR2) markedly increased, while transcripts for three other putative SO_4^{2-} transporters (SLT3, SULTR1, and SULTR3) remained approximately

the same or declined during S deprivation; these results suggest that SLT1, SLT2, and SULTR2 are the main contributors to the inducible high-affinity SO_4^{2-} transport activity (Yildiz et al., 1994), a hypothesis that has recently been experimentally confirmed (Pootakham et al., 2010). In the *snrk2.1* mutant, there was no or little elevation in the levels of transcripts encoding proteins involved in SO_4^{2-} acquisition and assimilation, confirming previous results demonstrating that SNRK2.1 activity is crucial for regulating S acclimation responses (González-Ballester et al., 2008).

Moreover, there are transcripts encoding enzymes involved in S assimilation and the synthesis of S metabolites that show either little change in abundance or that decline under –S conditions. For example, transcripts encoding three isoforms of the O-acetylserine(thiol)-lyase (OASTL1, OASTL2, and OASTL3), APS kinase (APK), APS reductase (APR), sulfite reductase (SIR3), SAT2, and enzymes required for GSH synthesis and degradation did not significantly change under –S conditions (Figure 5, Table 3; see Supplemental Data Set 3 online). Interestingly, transcripts encoding proteins required for the Met/SAM cycle and for the biosynthesis of the S-containing cofactors thiamine and biotin decreased in abundance under –S conditions (Figure 5, Table 3). Similarly, the level of a transcript in *Arabidopsis thaliana* that likely functions in thiamine biosynthesis was diminished under –S conditions (Maruyama-Nakashita et al., 2003), although some transcripts encoding enzymes of the Met/SAM cycle increased in S-deprived *Arabidopsis* plants (Nikiforova et al., 2003). There have been no previous reports demonstrating transcriptional regulation of biotin biosynthesis in plants during S deprivation. Reduced levels of transcripts encoding enzymes required for Met biosynthesis in *Chlamydomonas* are congruent with the low levels of free Met present in S-deprived *Chlamydomonas* cells

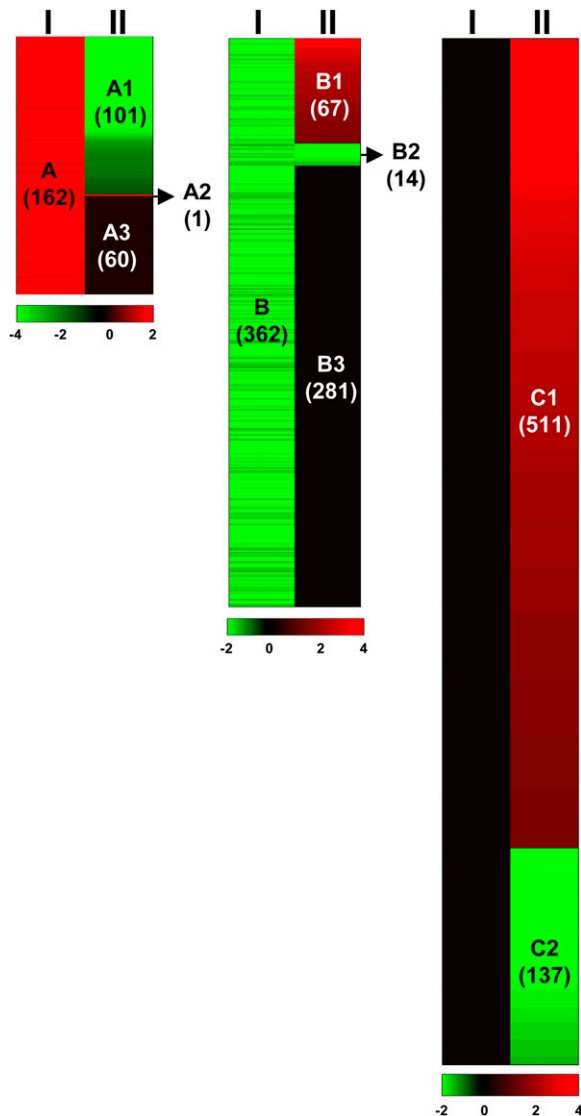


Figure 3. Cluster Diagrams Depicting the Fold Change in Transcript Levels Determined by RNA-Seq.

The \log_2 relative expression values of 1.5 and -1.5 were selected as the thresholds to designate the categories of transcripts that accumulate (or are more abundant) and decline (or are less abundant), respectively, for wild type $-S$ /wild type $+S$ (I) and *snrk2.1* $-S$ /wild type $-S$ (II) conditions. For more details about each of the categories, see Methods. The number of different transcripts in each category is indicated in the appropriate sector. Areas are proportional to the numbers of genes within the specific category. A short description of each category is given below. A, transcripts that accumulate in wild-type cells in $-S$ conditions; A1, transcripts that accumulate in wild-type cells in $-S$ conditions and that are relatively less abundant in the mutant in $-S$ conditions; A2, transcripts that accumulate in wild-type cells in $-S$ conditions and that are relatively more abundant in the mutant in $-S$ conditions; A3, transcripts that accumulate in wild-type cells to approximately the same extent as in mutant cells in $-S$ conditions; B, transcripts that decline in wild-type cells in $-S$ conditions; B1, transcripts that decline in wild-type cells in $-S$ conditions and that are relatively more abundant in mutant cells in $-S$ conditions; B2, transcripts that decline in wild-type cells in $-S$ conditions

(Bolling and Fiehn, 2005), while Met levels in *Arabidopsis* plants subjected to S deprivation do not decline (Nikiforova et al., 2005b).

Like the transcriptional regulator SLIM1 from *Arabidopsis* (Maruyama-Nakashita et al., 2006), the *Chlamydomonas* SNRK2.1 kinase controls expression of many genes encoding proteins critical for SO_4^{2-} uptake, reductive assimilation of SO_4^{2-} to Cys, and S recycling (see section below: Redistribution and Recycling of S). However, transcriptional regulation of thiamine biosynthesis and Met/SAM cycle genes does not appear to be directly controlled by SNRK2.1, and there is no evidence suggesting that SLIM1 controls these genes in *Arabidopsis* (Maruyama-Nakashita et al., 2006); the activity of these genes may be impacted by the levels of S metabolites in both *Chlamydomonas* and *Arabidopsis*.

Despite the finding that Met levels in *Arabidopsis* do not decrease during S deprivation, SAM levels in *Arabidopsis* do decline (Nikiforova et al., 2005b); we would also expect the SAM levels in *Chlamydomonas* to decline, although they were not directly measured. SAM potentially represents a large drain on the Met pool; 80% of the Met synthesized in *Lemna paucicostata* cells may be consumed in the SAM pathway, with only $\sim 20\%$ routed to protein synthesis (Giovanelli et al., 1985). Hence, reduced levels of SAM may impact several metabolic pathways including those responsible for the synthesis of thiamine, chlorophyll, polyamines, and lipids. Therefore, not unexpectedly and as previously observed in plants (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003), the levels of numerous transcripts encoding proteins associated with these biosynthetic pathways in *Chlamydomonas* were significantly affected during S deprivation (see Supplemental Data Set 2 online).

In this study, we also identified two transcripts encoding putative amino acid/polyamine transporters (AOT2 and AOT4) that increased in S-deprived wild-type cells. The absolute abundance of the AOT4 transcript was ~ 8.5 times higher than that of AOT2. Interestingly, the AOT4 transcript was almost undetectable in *snrk2.1* cells maintained in either $+S$ or $-S$ conditions (Figure 2, Table 3), indicating that this gene is under stringent SNRK2.1 control. An increase in the AOT4 transcript level did not occur in phosphorous-depleted ($-P$) or nitrogen-depleted ($-N$) cells (Figure 6A). Although the only amino acid-specific transport activity that has been demonstrated in *Chlamydomonas* is for Arg (Kirk and Kirk, 1978), the existence of additional amino acid transporters has been proposed (Perez-Alegre and Franco, 1998), and recently we demonstrated that wild-type *Chlamydomonas* cells, unlike the *snrk2.1* mutant, were able to grow slowly

and that are relatively less abundant in mutant cells in $-S$ conditions; B3, transcripts that decline in wild-type cells and decline to approximately the same extent in mutant cells in $-S$ conditions; C1, transcripts that are more abundant in mutant cells than in wild-type cells in $-S$ conditions and that do not change significantly in wild-type cells in $-S$ conditions; C2, transcripts that are less abundant in mutant cells than in wild-type cells in $-S$ conditions and that do not change significantly in wild-type cells in $-S$ conditions. Additional information for all genes included in this figure can be found in Table 1 and Supplemental Data Set 2 online.

Table 1. General Overview of Metabolic Pathways and Cellular Processes for Which Related Transcripts Accumulate or Decline in Wild-Type and *snrk2.1* Mutant Cells during –S Conditions

Specific in the Wild Type	Common in the Wild Type and <i>snrk2.1</i> Mutant	Specific in <i>snrk2.1</i> Mutant
Upregulated		
A1	A2+A3	C1
S acquisition and assimilation (to Cys) Routing and recycling of S Remodeling of PSII antenna (LHCBM9) Cell wall and periplasmic proteins (HAPs, ARSs, and ECPs) SQDG synthesis Oxidative pentose phosphate pathway <i>CAH9</i> Others	Stress-related LHC proteins Others	Oxidative stress (singlet oxygen) Proteolysis-related genes Vacuolar and vesicular transport Apoptosis Lipid degradation Pro degradation Putrescine biosynthesis Low CO ₂ -inducible genes ^a Shikimate biosynthesis (<i>SHKG1</i>) CoA biosynthesis Others
Downregulated		
B1	B2+B3	C2
tRNA biosynthesis ^b Transcription and translation processes ^b Polyamine biosynthesis Others	Met, SAM, thiamine, and biotin biosynthesis Photosynthesis and chlorophyll biosynthesis Carbon and folate metabolism Plastid ribosomes ^c Amino acid biosynthesis ^c Lipid metabolism Purine and pyrimidine metabolism Others	S assimilation (<i>APK</i> and <i>APR</i>) Urea/Arg cycle Carbon fixation Others

A1, A2, A3, B1, B2, B3, C1, and C2 designate the same group categories described in Figure 3 and Supplemental Data Set 2 online. Footnotes indicate exceptions.

^aSome transcripts were more abundant in *snrk2.1* mutant relative to wild-type cells in +S conditions.

^bSome transcripts were also diminished in *snrk2.1* mutant cells.

^cSome transcripts were not diminished in *snrk2.1* mutant cells.

with Cys, but not with Met, taurine, or homocysteine, as a sole S source (see Supplemental Figure 1 online). These results raise the possibility that the AOT2 and AOT4 transporters function in the uptake of Cys from the environment. Alternatively, they may participate in the redistribution of internal S-containing amino acids and/or polyamines among the different cellular compartments.

Redistribution and Recycling of S

Several proteins encoded by transcripts that accumulated during –S conditions may be related to Cys degradation and/or to the recycling and redistribution of intracellular S (Figure 7). Cys dioxygenase (CDO) catalyzes the conversion of Cys to 3-sulfinoalanine (Figure 7), a key metabolite implicated in the control of Cys homeostasis and taurine biosynthesis in animal cells. The transcript from a putative *CDO1* gene increased in *Chlamydomonas* wild-type cells by ~28-fold during –S conditions (Table 3). Despite the potential importance of recycling S-containing amino acids, there are no reports to our knowledge that demonstrate CDO activity in plants, and the *CDO* gene has only recently been identified in bacteria (Dominy et al., 2006). Interestingly, the *CDO1* gene of *Chlamydomonas* is clustered in a tail-to-tail orientation with the *PWR1* gene (Pro-Trp-Arg motif) of unknown function whose transcript increased in abundance by ~76-fold during –S conditions. The levels of transcripts for both *CDO1* and *PWR1* are under the control of the SNRK2.1 kinase.

Taurine/α-ketoglutarate dioxygenase (TAUD), which catalyzes the conversion of taurine to SO₃²⁻ and aminoacetaldehyde, may also be integral to the Cys degradation pathway (Figure 7). Two putative TAUD genes (*TAUD1* and *TAUD2*) were found on the *Chlamydomonas* genome; the transcripts from these genes increased by 142- and 37-fold, respectively, in S-deprived wild-type cells (Table 3). Exposure of the cells to –P and –N conditions did not alter *TAUD1* or *TAUD2* transcript levels (Figure 6B). The *TAUD1* and *TAUD2* genes are clustered together with another gene of unknown function (ID: 143415) that is also responsive to S levels; S deprivation elicited a 128-fold increase in the transcript from the latter gene. Furthermore, all three of the transcripts are under SNRK2.1 kinase control. While no TAUD homologs have been identified in plants or animals, the putative TAUD proteins of *Chlamydomonas* have significant similarity to bacterial and fungal TAUD/TfdA family proteins (~19 to 35% identity), as shown in Supplemental Figure 2 online. Moreover, the *Escherichia coli* TauD, the *Saccharomyces cerevisiae* YLL057c, and the *Pseudomonas syringae* AtsK α-ketoglutarate dioxygenases can efficiently metabolize various sulfonates in addition to taurine (Eichhorn et al., 1997; Hogan et al., 1999; Kahnert et al., 2000). Similarly, the putative TAUD proteins of *Chlamydomonas* might use alkanesulfonates other than taurine. For example, it is possible that TAUD functions in degrading chloroplast sulfolipids such as sulfoquinovosyl diacylglycerol (SQDG) (Figure 7); much of the sulfolipid may represent an S

Table 2. Comparative Quantification of Transcript Levels

ID ^a	Gene ^b	Wild Type –S/Wild Type +S			<i>snrk2.1</i> /Wild Type (+S)			<i>snrk2.1</i> /Wild Type (–S)		
		M	S	Q	M	S	Q	M	S	Q
205496	<i>ARS1</i>	3.6	10.5	9.5	nd	–1.2	–0.5	–3.0	–16.1	–12.0
205502	<i>SLT1</i>	2.6	6.9	7.9	nd	–6.3	–7.3	–5.1	–15.0	–20.4
205501	<i>SLT2</i>	4.2	6.3	9.3	nd	–2.5	–1.0	–4.4	–9.8	–10.3
150514	<i>SULTR2</i>	2.5	5.3	7.7	–0.9	–4.5	–3.9	–1.2	–6.4	–7.5
196910	<i>ATS1</i>	1.9	1.6	1.6	nd	–1.2	–1.3	–1.3	–3.0	–2.9
133924	<i>ATS2</i>	1.1	2.8	3.6	nd	0.0	0.0	–1.7	–4.4	–3.6
189320	<i>OASTL4</i>	2.0	2.9	5.6	–0.5	–0.3	–0.3	–1.6	–5.0	–6.6
205985	<i>SAT1</i>	3.9	5.2	4.2	nd	–0.7	0.1	–2.7	–4.2	–4.3
143892	<i>CDO1</i>	2.5	4.7	4.5	nd	–1.4	–4.0	nd	–7.3	–8.4
127464	<i>TAUD1</i>	3.3	7.1	5.6	nd	–3.5	–5.3	–1.3	–11.7	–12.5
184479	<i>LHCBM9</i>	2.9	10.0	8.8	nd	–2.6	–10.1	–3.8	–12.8	–20.5
130684	<i>ECP76</i>	3.0	10.7	10.3	nd	–0.8	–0.4	–2.5	–13.1	–11.8
143696	<i>HAP2</i>	5.2	8.4	7.1	nd	–0.1	–0.6	–5.6	–11.6	–11.5
183511	<i>RDP3</i>	2.8	4.1	4.4	nd	–0.8	–2.7	–0.8	–4.0	–6.6
182845	<i>SBDP</i>	2.1	5.4	5.0	–0.5	0.1	–1.6	–2.6	–8.3	–8.4
206105	<i>AOT4</i>	1.3	3.9	4.0	–2.6	–8.0	–8.8	–2.1	–13.0	–13.1

Fold change (\log_2 values) of selected transcripts under specified conditions as determined by microarray (M), RNA-seq (S), and qRT-PCR (Q). Values are from cells maintained in nutrient-replete medium and exposed to –S conditions for 6 h. nd, not enough high-quality data for statistical tests.

^aJGI protein accession number.

^bThe protein name associated with each of the genes is given in Table 3.

storage compound that can be rapidly mobilized when cells become S limited (Sugimoto et al., 2007). However, neither CDO nor TAUD polypeptides of *Chlamydomonas* appear to have presequences, suggesting that they function in the cytosol.

The *Chlamydomonas* genome also contains a number of other genes encoding putative sulfurtransferases that may be involved in the turnover of S compounds in the cell or in synthesizing new S-containing compounds. The rhodanases are sulfurtransferases thought to be involved in the synthesis of a diverse set of compounds, including molybdopterin, thiamin, CoA, biotin, lipoic acid, and iron-sulfur clusters (Kessler, 2006). *Chlamydomonas* has three genes (*RDP1*, 2, and 3) encoding small polypeptides that are part of a relatively poorly characterized family of rhodanase proteins (Bordo and Bork, 2002). The *RDP3* transcript rapidly and specifically accumulates when cells are deprived of S (~17-fold increase); this response is SNRK2.1 dependent (Table 3, Figure 6C). Moreover, Cys degradation can also occur via a 3-sulfinoalanine-independent pathway that generates a 3-mercaptopyruvate intermediate, which can be reduced by a mercaptopyruvate sulfotransferase (MST; designated TST in *Chlamydomonas*) (Figure 7). MST activity may also be part of the mechanism involved in detoxification of H₂S in *Arabidopsis* (Riemenschneider et al., 2005). The MSTs/TSTs are related to rhodanases, with common sequence motifs and similar physicochemical properties (Colnaghi et al., 2001; Bordo and Bork, 2002), raising the possibility that *RDP3* is involved in the 3-sulfinoalanine-independent metabolism (Figure 7).

Cys desulfurases (CSDs), like the rhodanases, have also been proposed to act as sulfurtransferases. Like *RDP3*, the *Chlamydomonas* *CSD2* transcript increased when cells were deprived of S; this increase was SNRK2.1 dependent (Table 2). Hence, *RDP3* and *CSD2* may both be associated with redistribution of S

among pools of metabolites, which could be crucial under conditions in which cells are limited for S.

Alternatively, rhodanases and CSDs, which are able to bind selenium, may function in the detoxification of selenate or as selenium delivery proteins for the synthesis of selenocysteine, as has been suggested by others (Ogasawara et al., 2005; Mueller, 2006). The link between selenium and S homeostasis is supported by the findings that transcripts encoding several selenoproteins, including SELW1, MSP1, and two selenium binding proteins, accumulated in *Chlamydomonas* cells deprived of S and that this accumulation was dependent on SNRK2.1 (see Supplemental Data Set 2A online).

A major S-containing metabolite generated by pathways that recycle S from Cys is SO₃²⁻, which at high concentrations is cytotoxic. However, SO₃²⁻ can be detoxified by oxidation to SO₄²⁻ by sulfite oxidase (SUOX), as has been suggested for *Arabidopsis* SUOX (Hansch et al., 2007). *Chlamydomonas* has a *SUOX* gene that encodes an animal-type enzyme with a putative mitochondrial targeting sequence. Increased accumulation of the *SUOX* transcript during –S conditions supports the hypothesis that S deprivation in *Chlamydomonas* promotes the degradation of Cys and other S-containing metabolites. The SO₄²⁻ generated by SUOX could be routed back into chloroplasts where it can be either incorporated into Cys by reductive assimilation or directly used for sulfation of molecules through the PAPS pathway. SUOX activity allows for SO₄²⁻ regeneration in S-depleted cells and therefore may be required to maintain PAPS-dependent metabolism during –S conditions (Figure 7).

Although the substrate specificities of CDO1, TAUD1, TAUD2, RDP3, CSD2, and SUOX1 need to be clarified, the coordinated increases in their transcript levels in wild-type cells experiencing S deprivation indicates the critical nature of this group of

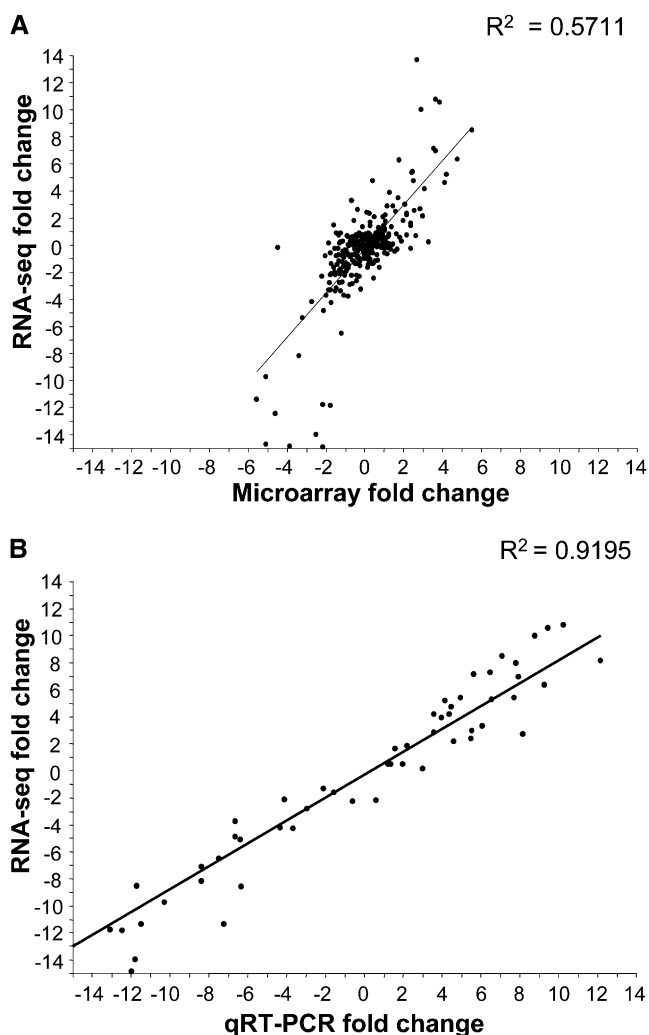


Figure 4. A Comparison of Fold Change (\log_2) Values of Transcript Levels Measured by RNA-Seq, Microarrays, and qRT-PCR.

(A) Fold change (\log_2) values for individual transcripts between two experimental conditions in which the RNA levels were quantified by microarray analyses (x axis) and were then compared with corresponding values calculated from RNA-seq experiments (y axis).

(B) Fold change (\log_2) values for individual transcripts between two experimental conditions in which the RNA levels were quantified by qRT-PCR analyses (x axis) and then compared with corresponding values obtained by RNA-seq experiments (y axis). The pairs of experimental conditions compared include wild type $-S$ /wild type $+S$; *snrk2.1* $-S$ /wild type $-S$; *snrk2.1* $+S$ /wild type $+S$, with all time points for $-S$ corresponding to 6 h after the onset of S deprivation.

enzymes for the catabolism of Cys and/or sulfolipids, and redistributing intracellular S as the cells achieve a new homeostasis. Furthermore, the redistribution and recycling of S-containing molecules is likely under SNRK2.1 control since *CDO1*, *TAUD1*, *TAUD2*, *SUOX1*, *RDP3*, and *CSD2* transcripts are significantly diminished in the *snrk2.1* mutant relative to wild-type cells (Table 3).

Sulfolipid Degradation and Synthesis

Whereas there is a rapid decline in the SQDG level in S-deprived *Chlamydomonas* cells (Sugimoto et al., 2007), the enzymes that catalyze SQDG degradation have not been identified, although they appear to be synthesized de novo after the cells are deprived of S (Sugimoto et al., 2009). The genes involved in this process may be under SNRK2.1 control and among those placed in category A1 in Figure 3 and in Supplemental Data Set 2 online. Transcripts potentially involved in sulfolipid degradation during exposure of *Chlamydomonas* to $-S$ conditions encode TAUD1, TAUD2 (discussed above), and a putative lipase (JGI ID: 144554) (Table 3), although this possibility will require experimental validation. *Arabidopsis* plants also show some increase in the catabolism of sulfolipids during S deprivation. However, this degradation may reflect a general increase in lipid catabolism rather than a specific mechanism for recycling S (Nikiforova et al., 2005b); indeed, the SQDG levels in *Arabidopsis* plants transferred to $-S$ conditions do not significantly decline (Okazaki et al., 2009). Instead, in *Arabidopsis* and other members of the Brassicaceae, glucosinolates may serve as an intracellular S reserve that can be rapidly catabolized under conditions of S limitation (Maruyama-Nakashita et al., 2003; Nikiforova et al., 2005b).

Surprisingly, S deprivation elicited an increase in three transcripts that have been associated with SQDG synthesis: *SQD1*, *SQD2*, and *LPB1* (Figure 7, Table 3). Increases in these transcripts were not observed in the *snrk2.1* mutant (this work); an increase in *SQD1* mRNA was also demonstrated to be impacted by both the *sac1* and *sac3* lesions (Zhang et al., 2004; Sugimoto et al., 2009). *SQD1* and *SQD2* encode UDP-sulfoquinovose synthase and sulfolipid synthase, respectively (Sato et al., 1995). *LPB1* (for Low Phosphate Bleaching protein) (Chang et al., 2005) is similar to UDP-glucose pyrophosphorylase 3 of *Arabidopsis*, which is involved in the synthesis of UDP-glucose, the precursor of sulfolipids (Okazaki et al., 2009). The *LPB1* transcript was elevated in cells exposed to $-S$, but not in cells exposed to $-P$ or $-N$ conditions (Figure 6D). Elevated levels of *SQD1*, *SQD2*, and *LPB1* transcripts during $-S$ conditions are congruent with the increased capacity for SQDG biosynthesis previously observed in S-deprived *Chlamydomonas* cells (Sugimoto et al., 2009).

Photosynthesis

S-deprived *Chlamydomonas* cells exhibit a marked decline in photosynthetic electron transport that has been associated with a SAC1-dependent decrease in photosystem II (PSII) activity (Wykoff et al., 1998). Moreover, *sac1* and *snrk2.1* mutants photobleach and die after ~ 3 to 6 d of being transferred to medium devoid of S, while wild-type cells can survive for significantly longer periods (Davies et al., 1996; González-Ballester et al., 2008). These results suggest that both SAC1 and SNRK2.1 are important for tuning photosynthetic activity when the cells are starved for S and that this tuning is critical for cell viability.

Many transcripts encoding proteins associated with PSII, photosystem I (PSI), light-harvesting complexes (LHC) of PSI (LHCA) and PSII (LHCB), subunits of the cytochrome *b₆f* complex, photosynthetic electron transport, and chlorophyll biosynthesis declined when *Chlamydomonas* was exposed to $-S$

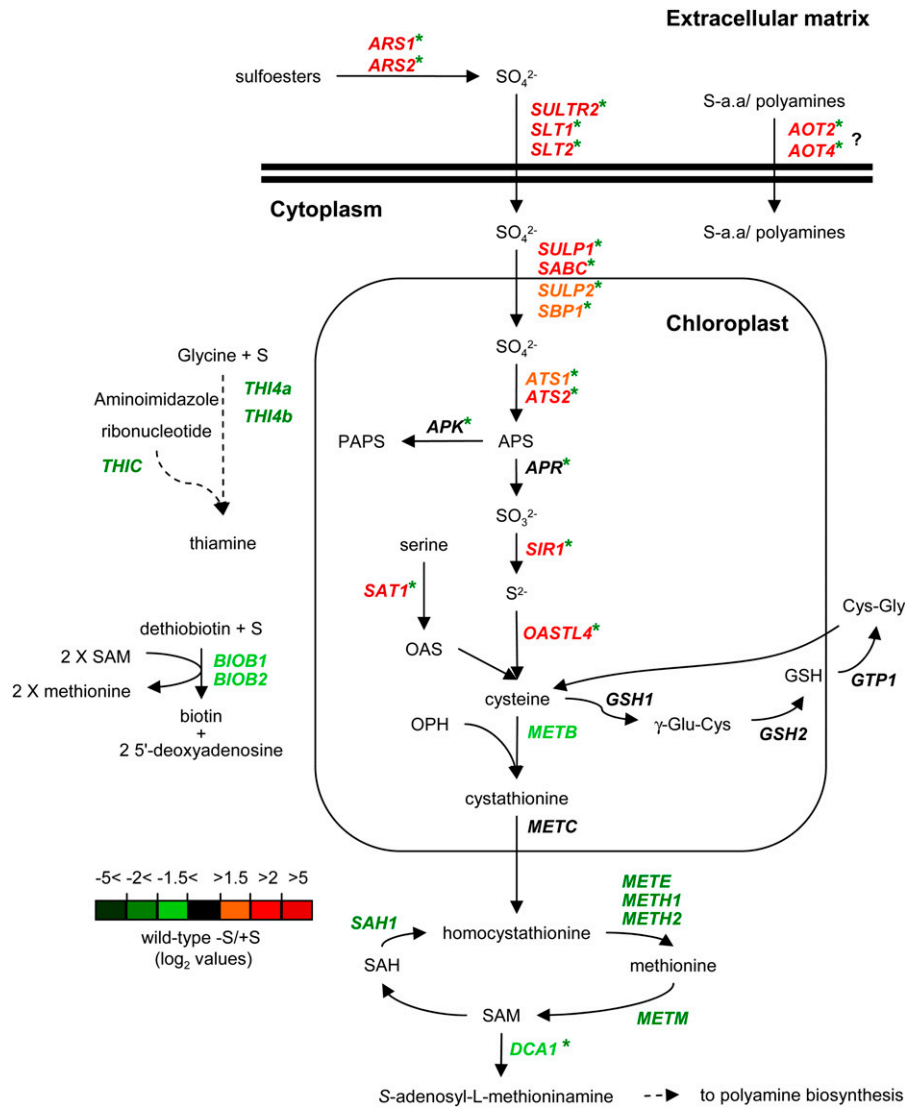


Figure 5. Pathways for S Acquisition and Assimilation.

Genes encoding the proteins of these pathways are labeled with colors, indicating the fold change (\log_2) of their transcript levels under S deprivation relative to nutrient-replete conditions for wild-type cells based on RNA-seq data (color code is given in the figure). The proteins encoded by each of the genes represented in the pathway diagram are given in Table 3. Dashed lines represent multiple metabolic steps. Asterisks represent genes that showed altered expression in *snrk2.1* mutant relative to wild-type cells upon S deprivation. APS, adenosine 5'-phosphosulfate; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; S^{2-} , sulfide; OAS, O-acetyl-serine; OPH, O-phosphohomoserine; SAH, S-adenosyl-homocysteine; S-a.a, sulfur-containing amino acids.

conditions (see Supplemental Data Sets 2B and 3 online). A similar decrease in chlorophyll content and transcripts encoding proteins of the photosynthetic apparatus also occur in plants. Among the transcripts most sensitive to S deprivation were those encoding highly conserved members of the LHCBM protein family (Elrad and Grossman, 2004) that comprise the bulk of the peripheral PSII antennae. The mRNAs encoding LHCBM1-8 polypeptides declined after 6 h of S deprivation, with a more severe decline after 24 h (Figure 8A, Table 3). Levels of these transcripts were even lower in the *snrk2.1* mutant than in wild-type cells (Figure 8B, Table 3). By contrast, the LHCBM9 tran-

script, which was barely detectable when *Chlamydomonas* cells were grown in +S medium, increased by >1000-fold during S deprivation (see Supplemental Data Set 2 online) and was the second most abundant mRNA in wild-type S-depleted cells (see Supplemental Data Set 1 online). Recently, an increase in the *LHCBM9* transcript level during S deprivation was noted by others (Nguyen et al., 2008). This -S-dependent increase was not observed in the *snrk2.1* or *sac1* mutants (Figure 8C), suggesting that *LHCBM9* is a direct target of the S-sensing regulatory pathway; there was no increase in *LHCBM9* mRNA when wild-type cells were exposed to -P or -N conditions (Figure 6E).

Table 3. Expression Levels of Selected S-Regulated Genes

ID	Name/Description	Fold Change Expression (Log ₂)						Transcript Abundance (RPKM) ^a			
		Wild Type -S/ Wild Type +S		<i>snrk2.1</i> /Wild Type (+S)		<i>snrk2.1</i> /Wild Type (-S)		Wild Type	Wild Type +S	<i>snrk2.1</i> -S	<i>snrk2.1</i> +S
		S	Q	S	Q	S	Q	-S	+S	-S	+S
Sulfur acquisition and assimilation											
205496	<i>ARS1</i> , arylsulfatase (ext)	10.5	9.5	-1.2	-0.5	-16.1	-12.0	2717	2	0	1
55757	<i>ARS2</i> , arylsulfatase (ext)	8.2	12.2	1.0	-2.9	-11.1	-15.1	84	0	0	1
205502	<i>SLT1</i> , Na ⁺ /SO ₄ ²⁻ transporter type (p)	6.9	7.9	-6.3	-7.3	-15.0	-20.4	2341	20	0	0
205501	<i>SLT2</i> , Na ⁺ /SO ₄ ²⁻ transporter type (p)	6.3	9.3	-2.5	-1.0	-9.8	-10.3	1339	17	1	3
150514	<i>SULTR2</i> , H ⁺ /SO ₄ ²⁻ transporter type (p)	5.3	7.7	-4.5	-3.9	-6.4	-7.5	152	4	2	0
196910	<i>ATS1</i> , ATP sulfurylase	1.6	1.6	-1.2	-1.3	-3.0	-2.9	2137	723	275	305
133924	<i>ATS2</i> , ATP sulfurylase (chl)	2.8	3.6	0.0	0.0	-4.4	-3.6	254	36	12	37
128906	<i>SULP1</i> , chl. SO ₄ ²⁻ transport system	2.4		-0.6		-3.4		147	28	14	18
116547	<i>SULP2</i> , chl. SO ₄ ²⁻ transport system	1.5		-0.7		-2.2		56	20	12	12
182359	<i>SBP1</i> , chl. SO ₄ ²⁻ transport system	1.5		-1.1		-2.7		79	28	12	13
194724	<i>SABC</i> (CysA), chl. SO ₄ ²⁻ transport system	2.4		-0.3		-2.8		20	4	3	3
184419	<i>APK</i> , APS kinase (chl.)	0.4	1.3	0.5	-0.7	-1.7	-1.6	39	29	12	41
131444	<i>APR</i> (MET16), APS sulforeductase (chl.)	0.5	1.4	-0.9	0.8	-2.3	-0.6	285	197	57	105
206154	<i>SIR1</i> , ferredoxin-sulfite reductase (chl.)	2.0		-1.1		-2.3		124	31	26	14
205485	<i>SIR2</i> , ferredoxin-sulfite reductase	2.2		-0.7		-2.2		597	126	132	80
189320	<i>OASTL4a</i> , O-acetylserine(thiol)- lyase (chl.)	2.9	5.6	-0.3	-0.3	-5.0	-6.6	976	128	31	103
205985	<i>SAT1a</i> , serineacetyl transferase (chl.)	5.1	4.2	-0.7	0.1	-4.3	-4.3	794	23	40	14
24268	<i>CGS1</i> (METB), cystathionine g-synthase (chl.)	-1.6		-0.6		1.4		23	71	63	49
127384	<i>THS1</i> , Thr synthase (chl.)	-1.4		-0.7		-0.3		54	138	42	84
196483	<i>METC</i> , cystathionine b-lyase (chl.)	-0.5		-0.5		1.3		24	34	57	24
154307	<i>METE</i> , Met synthase	-		-0.6		0.2		46	606	54	395
76715	<i>METH1</i> , Met synthase (cobalamin)	-		-0.9		-0.2		33	134	29	72
195332	<i>METH2</i> , Met synthase (cobalamin)	-		-0.8		1.1		30	211	65	123
182408	<i>METM</i> , S-adenosylmethionine synthetase	-		-0.2		-0.3		526	2288	420	1976
129593	<i>SAH1</i> , S-adenosylhomocysteine hydrolase	-		-0.4		-0.9		152	1298	83	952
183928	<i>DCA1</i> , S-adenosylmethionine decarboxylase	-		-0.3		1.5		54	184	149	146
206104	<i>AOT2</i> , a.a/polyamine transporter (p)	4.1	3.6	-5.5	-2.4	-9.8	-6.3	36	2	0	0
206105	<i>AOT4</i> , a.a/polyamine transporter (p)	3.9	4.0	-8.0	-8.8	-13.0	-13.1	311	21	0	0
185190	<i>THI4a</i> , thiazole biosynthetic enzyme	-		-0.1		-2.8		203	1859	29	1682
196899	<i>THI4b</i> , THI4 regulatory protein	-		-0.1		-2.8		188	1715	27	1557
192720	<i>THICa</i> , hydroxymethylpyrimidine phosphate synthase	-		-0.4		-3.3		6	95	1	74
196900	<i>THICb</i> , hydroxymethylpyrimidine phosphate synthase	-		-0.4		-3.4		6	92	1	72
142289	<i>BIOB1</i> , biotin synthase	-		1.1		-0.5		2	10	1	23
97943	<i>BIOB2</i> , biotin synthase	-		-0.1		-1.6		23	86	8	77

(Continued)

Table 3. (continued).

ID	Name/Description	Fold Change Expression (Log ₂)						Transcript Abundance (RPKM) ^a			
		Wild Type -S/ Wild Type +S		<i>snrk2.1</i> /Wild Type (+S)		<i>snrk2.1</i> /Wild Type (-S)		Wild Type -S	Wild Type +S	<i>snrk2.1</i> -S	<i>snrk2.1</i> +S
		S	Q	S	Q	S	Q				
181975	<i>GSH1</i> , γ-glutamylcysteine synthetase (chl.)	0.1		0.0		0.1		35	33	38	34
189020	<i>GSH2</i> , glutathione synthetase (chl.)	-0.3		-0.3		-1.4		20	25	8	20
170508	<i>GTP1</i> , γ-glutamyl transpeptidase	0.2		0.7		0.7		25	23	41	37
Redistribution and recycling of S											
143892	<i>CDO1</i> , Cys dioxygenase	4.7	4.5	-1.4	-4.0	-7.3	-8.4	138	5	1	2
127464	<i>TAUD1</i> , taurin dioxygenase	7.1	5.6	-3.5	-5.3	-11.7	-12.5	1417	10	0	1
77600	<i>TAUD2</i> , taurin dioxygenase	5.2	6.6	-0.4	0.9	-5.1	-6.4	632	17	19	13
59800	<i>SUOX1</i> , sulfite oxidase, mit.	2.0		-0.2		-1.8		149	36	42	31
183511	<i>RDP3</i> , small rothhanase	4.1	4.4	-0.8	-2.7	-4.0	-6.6	369	21	24	12
132010	<i>TST</i> , mercaptopyruvate sulfurtransferase	0.2		0.4		-1.5		22	19	8	26
167884	<i>CSD2 (NIFS2)</i> , Cys desulfurase	1.6		-0.2		-2.0		43	14	10	12
98369	D-Cys desulfhydrase	2.2		0.2		-2.5		37	8	7	9
Lipid metabolism											
144554	Esterase/lipase/thioesterase	3.1		-0.5		-1.6		65	7	21	5
27658	<i>SQD1</i> , UDP-sulfoquinovose synthase	1.7		-0.9		-4.1		336	102	19	57
206163	<i>SQD2</i> , sulfolipid synthase	1.5		-0.3		-5.1		53	19	2	16
196477	<i>LPB1</i> , low phosphate bleaching	4.1	4.2	-1.6	-0.7	-5.5	-5.8	1711	98	39	33
Photosynthesis											
Stress-related genes											
148916	<i>ELIP3</i> , chlorophyll <i>a/b</i> binding protein	2.9		0.0		0.2		45	6	50	6
184724	<i>LHCSR1</i> , light-harvesting stress-related protein (chl.)	1.8	2.2	0.6	1.2	-1.3	-2.1	631	181	255	277
184731	<i>LHCSR2</i> , light-harvesting stress-related protein (chl.)	2.2	4.6	1.4	5.9	0.5	2.0	262	58	366	158
184730	<i>LHCSR3</i> , light-harvesting stress-related protein (chl.)	2.4	5.5	1.2	7.0	0.1	3.0	680	133	743	312
Major light-harvesting proteins of PSII											
185533	<i>LHCBM1</i> (chl.)	2.9		0.0		0.0		42	6	42	6
184067	<i>LHCBM2</i> (chl.)	1.8	2.2	0.6	1.2	-1.6	-2.1	596	174	202	257
195162	<i>LHCBM3</i> (chl.)	2.1	4.6	1.4	5.9	0.4	2.0	244	56	315	148
191690	<i>LHCBM4</i> (chl.)	2.3	5.5	1.1	7.0	-0.1	3.0	611	128	564	275
184775	<i>LHCBM5</i> (chl.)	-0.6		0.2		-1.1		5425	8019	2584	9327
184490	<i>LHCBM6</i> (chl.)	-0.2		0.2		-0.6		2705	3188	1740	3774
184071	<i>LHCBM7</i> (chl.)	-1.7		0.5		-0.2		676	2262	606	3112
205752	<i>LHCBM8</i> (chl.)	-0.3		0.3		-2.2		747	951	163	1160
184479	<i>LHCBM9</i> (chl.)	-1.4		0.6		-2.6		564	1485	95	2292
Carbon metabolism											
196354	<i>SHMT2</i> , Ser hydroxymethyltransferase 2	-3.0		-0.4		0.5		31	254	43	196
101528	6-Phosphogluconolactonase-like protein	2.0		-0.2		-1.3		401	100	160	85
163301	<i>GLD2-C</i> , glucose-6-phosphate dehydrogenase	2.3		-0.3		-2.1		77	16	18	13
192597	<i>GND1a</i> , 6-phosphogluconate dehydrogenase (decarboxylating) (chl.)	1.9		-0.2		-1.6		308	83	101	74
158911	<i>GND1b</i> , 6-phosphogluconate dehydrogenase (decarboxylating) (cyt.)	1.9		-0.2		-1.6		313	86	102	74
163238	<i>RIBA</i> , bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase	1.7		-0.7		-2.4		83	26	15	16
196834	<i>CAH9</i> , carbonic anhydrase (cyt?)	5.0		-1.1		-6.8		1952	62	18	28

(Continued)

Table 3. (continued).

ID	Name/Description	Fold Change Expression (Log ₂)						Transcript Abundance (RPKM) ^a			
		Wild Type –S/ Wild Type +S		<i>snrk2.1</i> /Wild Type (+S)		<i>snrk2.1</i> /Wild Type (–S)		Wild Type	Wild Type +S	<i>snrk2.1</i> –S	<i>snrk2.1</i> +S
		S	Q	S	Q	S	Q	–S	+S	–S	+S
Folate metabolism											
206121	<i>MTDH/MTCH2</i> , methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase	–1.6		–0.2		–1.5		13	40	5	35
127560	<i>FTFHS</i> , 10-formyltetrahydrofolate synthetase	–2.5		0.3		–1.8		10	59	3	71
30522	<i>ADCL1</i> , aminotransferase related to 4-amino-4-deoxychorismate lyase	–1.7		–0.2		0.2		23	73	26	65
111330	<i>MTHFR</i> , 5,10-methylenetetrahydrofolate reductase	–3.1		–0.1		0.1		11	92	12	86
182461	<i>GGH1</i> , γ -glutamyl hydrolase	3.9		–0.6		–6.2		36	2	0	2
Extracellular/cell wall proteins											
168785	<i>HAP1</i> , vanadium haloperoxidase	7.9	7.8	–0.2	–2.0	–8.4	–11.7	739	3	2	3
143696	<i>HAP2</i> , vanadium haloperoxidase	8.4	7.1	–0.1	–0.6	–11.6	–11.5	1044	3	0	3
182794	<i>HAP3</i> , vanadium haloperoxidase	7.1	6.5	–3.2	–1.0	–11.1	–7.2	1456	10	1	1
130684	<i>ECP76</i> , extracellular protein	10.7	10.3	–0.8	–0.4	–13.1	–11.8	899	1	0	0
137329	<i>ECP88</i> , extracellular protein	13.4		–2.3		–15.9		2295	0	0	0
119420	<i>ECP61</i> , extracellular protein	8.5		–0.6		–11.0		268	1	0	0
194201	<i>ECP56</i> , extracellular protein	8.6		–4.2		–13.0		317	1	0	0

Fold change (log₂ values) in transcript levels under specified conditions as determined by RNA-seq (S) and, when available, by qRT-PCR (Q). Values above 1.5 or below –1.5 are marked in bold type. Some genes for which expression is not affected by –S conditions are included for informative purposes. Putative subcellular localizations are denoted in parentheses after the transcript names as cyt, cytosolic; mit, mitochondria; chl, chloroplast; and ext, extracellular. ID, JGI protein accession number; p, putative; nd, not enough high-quality data for statistical tests.

^aTranscript abundance obtained from RNA-seq data is indicated as RPKMs (see Methods).

These results demonstrate that –S conditions elicit a massive rise in *LHCBM9* mRNA that is specifically controlled by previously identified S regulatory elements and that this rise is concurrent with a loss in transcripts encoding *LHCBM1-8*. The possible reduction in *LHCBM1-8* polypeptide synthesis may be balanced by an increase in *LHCBM9* synthesis. These results could also explain the photosynthetic phenotypes of the *snrk2.1* and *sac1* mutants; a severe reduction in *LHCBM1-8* transcripts without the concomitant increase in *LHCBM9* in S-deprived *snrk2.1* may compromise the overall integrity of PSII and elicit elevated production of reactive oxygen species (ROS), and more specifically the photoproduction of singlet oxygen (¹O₂). This hypothesis is supported by the findings that transcripts encoding proteins important for ameliorating the consequences of ¹O₂ production are elevated in the S-starved *snrk2.1* mutant, but not in S-starved wild-type cells (see section: ROS Detoxification and Cellular Redox). Elevated ¹O₂ levels can cause photooxidative damage, reduce cell viability, and elicit programmed cell death; genes encoding proteins potentially involved in apoptosis are elevated in *snrk2.1* but not in wild-type cells under –S conditions (see section: Apoptosis and Autophagy). A model depicting correlative relationships between photosynthetic electron flow, *LHCBM9*, ¹O₂, and programmed cell death during S deprivation in the mutant and wild-type cells is shown in Figure 9; more work is needed to substantiate this model.

Similar to *LHCBM9* mRNA, transcripts encoding four unique LHC polypeptides (*LHCSR1*, *LHCSR2*, *LHCSR3*, and *ELIP3*) that are not associated with light-harvesting function markedly increased in cells experiencing –S conditions. *LHCSR2* and *LHCSR3* transcript levels increased to a similar extent in the S-starved wild type and the *snrk2.1* mutant, although the levels of these transcripts during nutrient-replete growth were also somewhat higher in the mutant cells. By contrast, like *LHCBM9* mRNA, the increase in the *LHCSR1* transcript observed in wild-type cells under –S conditions was not observed in the *snrk2.1* mutant (Table 3). *Chlamydomonas* LHCSR polypeptides have no homologs in vascular plants and have been associated with photoprotection during stress conditions (Richard et al., 2000; Elrad and Grossman, 2004; Peers et al., 2009), and, in a previous study, the level of *LHCSR2* mRNA was shown to increase in cells deprived of S (Zhang et al., 2004).

Carbon Metabolism

Transcripts encoding key proteins of the Calvin-Benson cycle (reductive pentose phosphate pathway), the glyoxylate cycle, and the C₄ dicarboxylic acid cycle declined in both wild-type and *snrk2.1* mutant cells experiencing S deprivation; this decline was generally more severe in mutant than wild-type cells (see

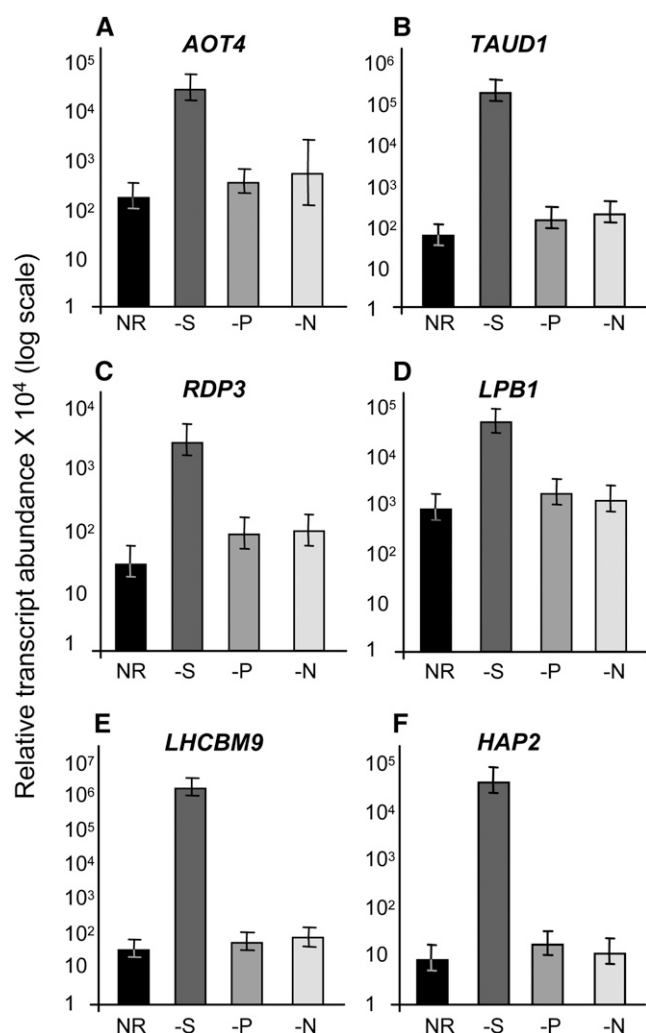


Figure 6. Abundances of Six Selected Transcripts under Different Nutrient Deprivation Conditions.

RNA samples were extracted from wild-type cells for qRT-PCR analysis at 6 h following their transfer to the different media (NR, nutrient-replete; -S, S-depleted; -P, phosphorus-depleted; -N, nitrogen-depleted). Levels of individual transcripts noted in (A) to (F) are given as relative fold abundance with respect to the housekeeping control gene (*CBLP*) and then multiplying relative target gene abundances by a factor of 10⁴. Each of the values was from at least three replicates, with the error bars representing 1 SD. Graphics are in log scale; note the different scales for the different analyses.

Supplemental Data Set 2B online). Moreover, transcripts for most proteins required for folate biosynthesis also declined, while the transcript encoding γ -glutamyl hydrolase (GGH1), an enzyme involved in folate catabolism (Scott et al., 2000), increased following the imposition of -S conditions (Table 3). Various folate derivatives, including tetrahydrofolates (THFs), are one-carbon donors and acceptors required for many cellular processes, including the synthesis of Gly from Ser, methylation of dUMP to form dTMP, the biosynthesis of pantothenate, purine biosynthesis, formylation of fMet-tRNA, and Met biosynthesis. In

plants, the majority of folate (5-methylTHF) is used for the synthesis of Met/SAM (Hanson and Roje, 2001). The decline in transcript levels for enzymes involved in THF synthesis parallels the decline in Met levels (Bolling and Fiehn, 2005) and in the levels of those transcripts encoding Met/SAM cycle enzymes (Table 3) in S-deprived *Chlamydomonas*. Furthermore, since THF is required for the Ser-Gly interconversion reaction of the photorespiration/glyoxylate cycle, it is likely that photorespiratory activity declines in S-deprived cells. A reduction in photorespiration is also suggested by a decrease in the level of the transcript encoding Ser hydroxymethyltransferase (SHMT2) (Table 3), a key enzyme of the pathway that requires a THF cofactor. Similarly, a decline in photorespiration and folate metabolism as consequence of reduced SAM levels in S-depleted *Arabidopsis* plants has been proposed (Nikiforova et al., 2005b), although no empirical data were presented to substantiate this conjecture.

Transcripts encoding proteins of the oxidative pentose phosphate (OPP) pathway increased in S-deprived *Chlamydomonas* cells, which may be reflected by accumulation of OPP metabolites (Bolling and Fiehn, 2005). In plants, an increased flux of metabolites through OPP is stimulated by stress conditions and is linked to ROS detoxification (Couee et al., 2006). Alternatively, elevated OPP activity may provide cells with an additional capacity to generate NADPH under conditions of diminished photosynthetic activity. The increase in transcripts encoding OPP enzymes was less in *snrk2.1* relative to wild-type cells (Table 3). Furthermore, S deprivation elicited a rise in the level of a transcript encoding the bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase (RIBA). This enzyme uses ribulose-5-P and GTP to catalyze the first step of riboflavin biosynthesis. Elevated RIBA levels may route excess ribulose-5-P formed as the final product of OPP into riboflavin, FMN, and FAD.

A number of other interesting observations have been noted that suggest significant modifications in carbon metabolism upon exposure of *Chlamydomonas* to -S conditions. S-starved algal cells accumulate high levels of starch (Zhang and Melis, 2002), suggesting a strong redirection of fixed carbon into storage, most likely because the cells cease dividing and have reduced anabolic metabolism. However, there is no significant increase in transcripts for enzymes involved in starch synthesis. Similar results have been observed for rice (*Oryza sativa*) plants (Lunde et al., 2008). We also noted that transcripts encoding the putative carbonic anhydrases CAH7 and CAH9 (Moroney and Ynalvez, 2007) increased in S-deprived cells; the CAH9 transcript exhibited a 31-fold increase that was SNRK2.1 dependent (Table 3). A CAH gene was also found to be highly upregulated when *Arabidopsis* plants were deprived of S (Maruyama-Nakashita et al., 2003). Although intriguing, the role of these CAHs during acclimation of organisms to -S conditions is unexplored and not easily explained at this time.

Extracellular Proteins

Extracellular arylsulfatases (ARSs) and putative extracellular cell wall polypeptides (ECP76 and ECP88), as well as transcripts encoding these peptides, specifically accumulated when *Chlamydomonas* was deprived of S (de Hostos et al., 1988; Takahashi

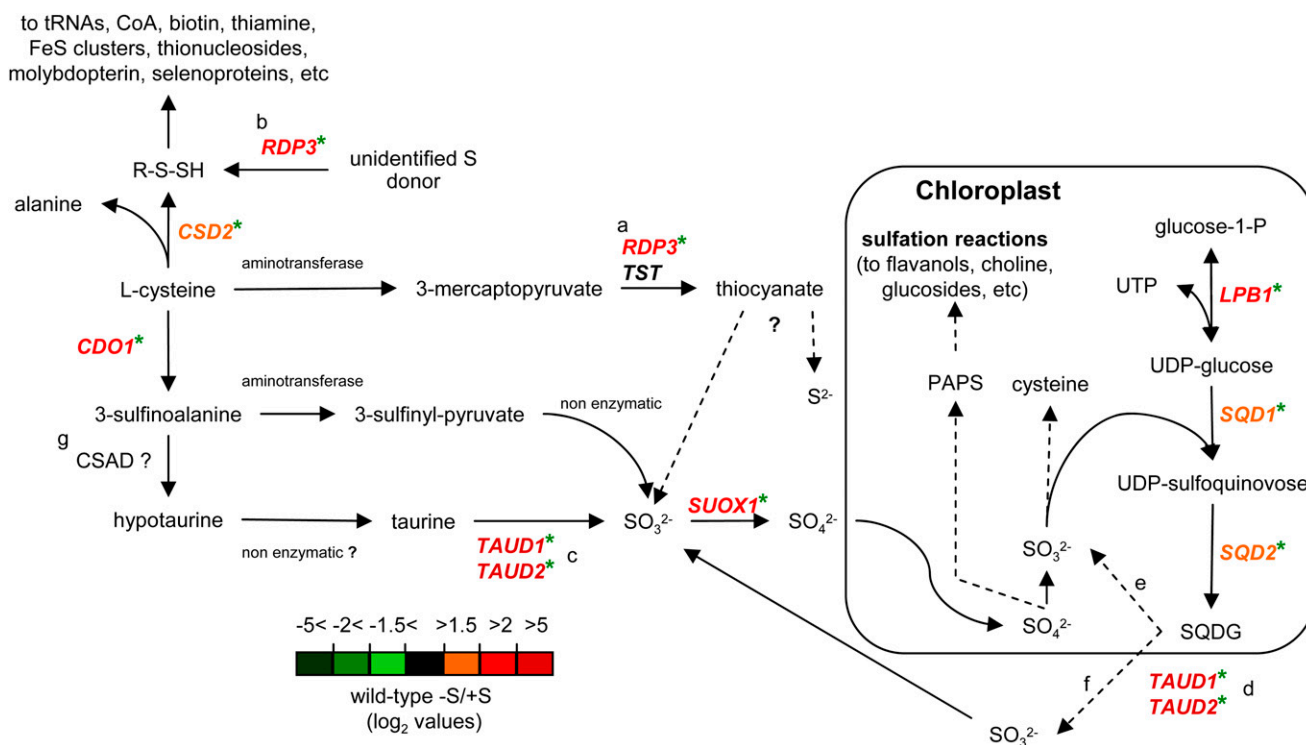


Figure 7. Proposed Pathways for Redistribution and Recycling of S.

The “a” and “b” denote two possible functions for RDP3; “c” and “d” denote two possible functions for TAUD1 and TAUD2; “e” and “f” denote two possible subcellular compartments in which SQDG degradation may occur; “g”, no gene encoding sulfinoalanine decarboxylase (CSAD) was identified on the *Chlamydomonas* genome. Other details are as in Figure 5. PAPS, adenosine 3'-phosphate 5'-phosphosulfate; S²⁻, sulfide.

et al., 2001). In this study, we identified five new transcripts encoding putative extracellular/cell wall proteins that increase during S deprivation; two encode ECP-like proteins, while the remaining three encode putative extracellular vanadium haloperoxidases (HAPs) (Table 3).

The two transcripts encoding novel ECP-like proteins, ECP61 and ECP56 (protein IDs 119420 and 194201, respectively; the deduced molecular masses follow the ECP designation), rose by up to 268- and 317-fold, respectively, in cells experiencing -S conditions (Table 3). The increase in all ECP transcripts was completely abolished in the *snrk2.1* mutant. Like ECP88 and ECP76, these new putative proteins have features similar to those of cell wall, Hyp-rich glycoproteins with very low S-containing amino acid content (Table 4). While the synthesis of the S-responsive ECPs is likely a means to economize on the use of limited available S, it may also have significant physiological consequences (e.g., rigidity of the wall and protection of the cell body during conditions of growth limitation).

HAP1, *HAP2*, and *HAP3* transcripts increased by up to 246-, 348-, and 145-fold, respectively, in wild-type cells experiencing S deprivation; these increases were strictly dependent on SNRK2.1 (Table 3). Interestingly, based on microarray data, the *HAP2* mRNA increased within 1 h of transferring cells to -S conditions (see Supplemental Data Set 2A online), but did not increase in response to -P or -N conditions (Figure 6F). HAPs, often closely related to acid phosphatases and mostly localized

outside of the cell body, can use H₂O₂ to catalyze the oxidative halogenation of organic compounds. These enzymes may also be responsible for extracellular peroxidase activity previously noted in *Chlamydomonas* (Torkelson et al., 1995). However, HAPs generally have broad substrate specificity and in addition to halide oxidation, may catalyze sulfoxidation, epoxidation, and phosphodiesterase reactions (Hemrika et al., 1997; Dembitsky, 2003). Because of their broad potential reaction scope and substrate specificity, it is difficult to assign HAPs a specific physiological function. Putative roles for these enzymes under -S conditions would include (1) participation in the biosynthesis/degradation of cell wall polyphenols (Vreeland et al., 1998; Ortiz-Bermudez et al., 2007) in reactions linked to H₂O₂ production (Slesak et al., 2007); such activities may alter cell wall architecture. (2) They function as an extracellular phosphatase in S-deprived cells. Such an activity, which is both SNRK2.1 and SAC1 dependent, has been previously observed (Moseley et al., 2009), although its physiological significance is not known. (3) They contribute to the conversion of sulfides to sulfoxides, possibly the initial step in a novel mechanism for scavenging external sulfides. Sulfides frequently present in the environment include Met, SAM, cystathionine, dimethylsulfide, and 3-methylpropionate. The latter two compounds are intermediary metabolites in dimethylsulfoniopropionate degradation, a potentially abundant, ecologically important molecule in the environment (Bentley and Chasteen, 2004).

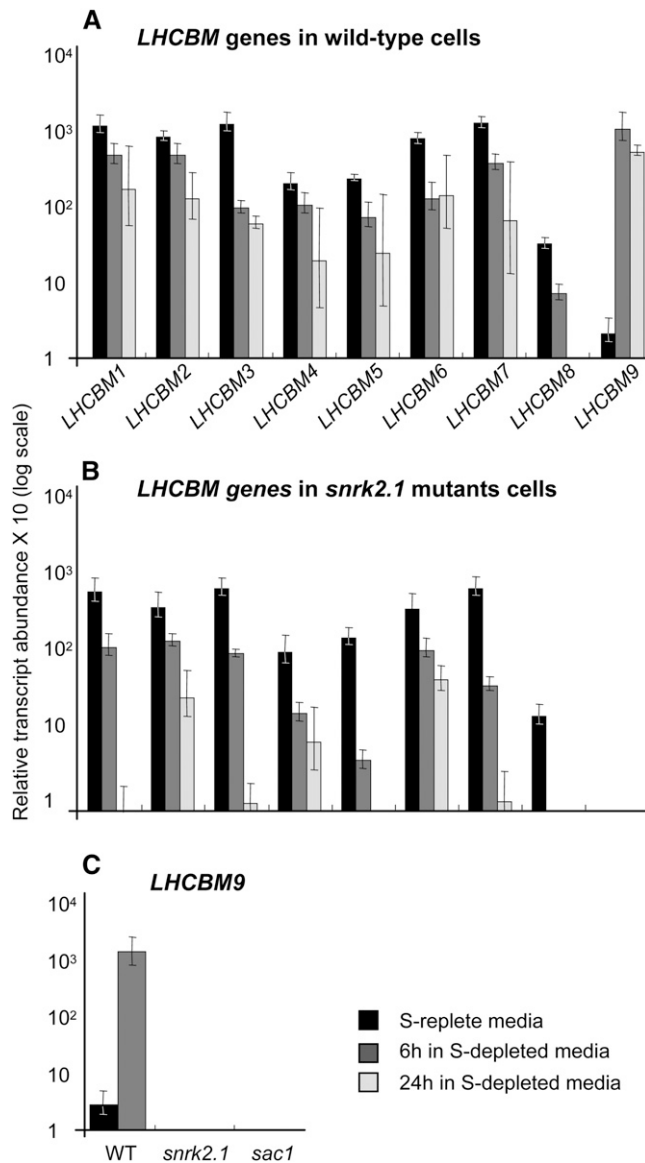


Figure 8. Abundances of *LHCBM* Transcripts Based on qRT-PCR.

(A) and (B) RNA samples were extracted from the wild type (A) and the *snrk2.1* mutant (B) and analyzed for all nine of the *LHCBM* transcripts. (C) Comparisons of *LHCBM9* expression levels in wild-type, *snrk2.1*, and *sac1* cells. Levels of individual transcripts are given as relative fold abundances with respect to the housekeeping gene (*CBLP*) and then multiplying the relative target gene abundances by a factor of 10. Graphics are in log scale. Each of the values was from at least three replicates, with the error bars representing 1 SD. None of the values obtained were much below 1, and those values that were below 1 are represented as 0 on the graph.

S Sparing at the Proteome Level

As proposed previously (Mazel and Marliere, 1989; Takahashi et al., 2001; Pereira et al., 2008), replacing high S content proteins with others that have low S content represents an S-sparing response that would help conserve cellular S re-

sources. In *Chlamydomonas*, the swapping of proteins with high S content for ones with low S content was previously proposed with respect to the putative cell wall polypeptides ECP76 and ECP88 (Takahashi et al., 2001) and, as discussed above, can now be extended to ECP61 and ECP56 (Table 4). Interestingly, the *LHCBM9* polypeptide also has a low content of S-containing amino acids. There are three invariant S-containing amino acids present in all mature *LHCBM* polypeptides except *LHCBM9* (Figure 10). Hence, mature *LHCBM9* has at least three fewer S-containing amino acids than the other *LHCBM*s (Table 4). Since *LHCBM* polypeptides are abundant cellular constituents, substitution of S-rich *LHCBM1-8* with the S-poor *LHCBM9* would allow for significant S conservation. An analogous situation has been described for cyanobacterial LHC proteins (Mazel and Marliere, 1989). Moreover, many other *Chlamydomonas* transcripts that accumulated during -S conditions encode proteins that contain relatively few S-containing amino acids (e.g., ARSSs, HAPs, TAUDs, among others), and the average S amino acid content of proteins encoded by the transcripts that accumulate during S deprivation is lower (3.32%) than that of the average *Chlamydomonas* proteome (4.19%) (Table 5). This decrease in S amino acid content is even more dramatic if the percentages of these proteins are weighted by the abundance of their corresponding transcripts in S-deprived cells (2.33%) (Table 5). The SNRK2.1 kinase is critical for this S-sparing response (Table 5). A similar S-sparing phenomenon has been described for yeast (Fauchon et al., 2002). While these data are not quantitative with respect to the relative amount of S-containing amino acids used for protein biosynthesis (absolute mRNA levels and/or identification and quantification of the proteins in the proteome would be required), they do suggest that proteins that accumulate under -S conditions tend to have a reduced S amino acid content, which likely helps optimize the use of the limiting resource.

Other Significant Processes

S deprivation also elicited an increase in transcripts encoding several putative plasma membrane transporters and 83 RNAs and/or proteins of unknown function (see Supplemental Data Set 2A online), along with a decrease in the levels of transcripts for proteins associated with amino acid biosynthesis, lipid metabolism, some transport processes and translocons, tRNA biosynthesis, chaperones, translation (especially chloroplast ribosomal proteins), and transcription (see Supplemental Data Set 2B online). A decrease in the levels of mRNAs encoding proteins associated with a variety of metabolic processes may reflect an overall slowing of metabolism as the cells stop dividing.

S Deprivation Responses Specific to the *snrk2.1* Lesion

Transcripts of wild-type and *snrk2.1* mutant cells that were responsive to S deprivation were categorized based on their transcriptome profiles (Figure 3; see Supplemental Data Set 2 online). Those genes that appear to be under SNRK2.1 control (directly or indirectly) are in categories A1 and B1 in Figure 3 and Supplemental Data Set 2 online. Transcripts that change in abundance independently of SNRK2.1 are in categories A2-A3

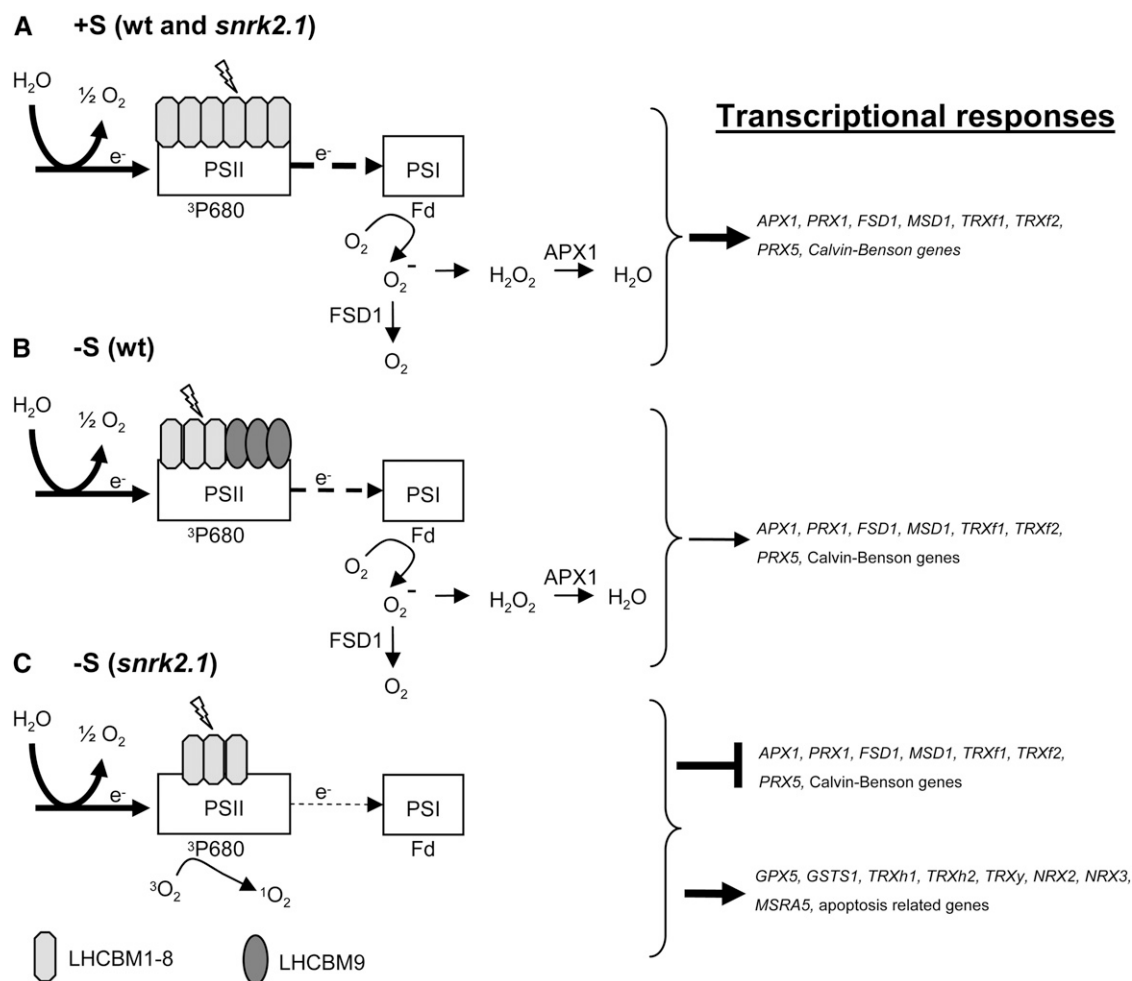


Figure 9. Proposed Model for How the *snrk2.1* Lesion Affects Photosynthesis and ROS Production Processes.

Wild-type (wt) and mutant cells in nutrient-replete medium **(A)** perform photosynthesis at optimal levels with the consequent production of superoxide (O_2^-) and H_2O_2 . Under these conditions, transcripts encoding proteins related to O_2^- and H_2O_2 detoxification are relatively abundant, along with transcripts encoding Calvin-Benson-Bassham cycle-related enzymes. In wild-type cells that experience S deprivation **(B)**, photosynthetic activity declines (most photosynthesis, chlorophyll biosynthesis, and Calvin-Benson-Bassham cycle-related transcripts decline), and the LHCBM1-8 proteins are partially replaced by LHCBM9. The production of O_2^- and H_2O_2 may be diminished because of reduced PSII activity and electron flow to PSI. Transcripts encoding proteins related to the detoxification of these specific ROS also decline. In the *snrk2.1* mutant experiencing S deprivation **(C)**, LHCBM9 is not synthesized and the PSII antenna may become reduced in size. This may impair the proper assembly of PSII and its capacity for photoprotection, triggering the production of singlet oxygen (1O_2) and of genes specifically associated with 1O_2 accumulation. Aberrant PSII architecture, 1O_2 accumulation, and severe S deficiency could dramatically impair PSII function in the *snrk2.1* mutant, resulting in a marked decline in electron flow to PSI. This decline in the photosynthetic activity would result in a decrease in the levels of transcripts encoding Calvin-Benson-Bassham cycle enzymes as well as those related to O_2^- and H_2O_2 accumulation. Accumulation of 1O_2 may also trigger expression of genes potentially involved in the initiation of apoptosis (listed in Table 6). Thickness of the arrow between PSII and PSI depicts a qualitative representation of the electron flow rate. See “ROS Detoxification and Cellular Redox” section for more details.

and B2-B3. However, there is also a large set of transcripts that changed in abundance in S-deprived *snrk2.1* but not in S-deprived wild-type cells. Included in these mutant-specific changes, presented as categories C1 and C2, are transcripts encoding proteins associated with oxidative stress, proteolysis, and apoptosis. Some genes encoding proteins critical for the biosynthesis of the S-containing cofactor CoA also increased in S-deprived mutant relative to S-deprived wild-type cells (Table 6). All these changes potentially reflect the severe S deficiency

and the highly aberrant physiological state exhibited by the mutant because of its inability to acclimate to S-limiting conditions.

ROS Detoxification and Cellular Redox

Transcripts encoding GSH-peroxidase 5 (GPX5), GSH-transferase 1 (GSTS1), and thioredoxin h1 (TRXh1) proteins increased in the *snrk2.1* mutant relative to wild-type cells following the

Table 4. S-Containing Amino Acid Content of Cell Wall and PSII Light-Harvesting Proteins

ID	Protein	Length	C+M ^a	C+M (%) ^b
Cell wall proteins				
130684	ECP76, cell wall protein	583	1 (+1)	0.2
137329	ECP88, cell wall protein	595	0 (+4)	0.0
119420	ECP61, cell wall protein	577	1 (+1)	0.2
194201	ECP56, cell wall protein	532	1 (+1)	0.2
195768	GP2, cell wall protein	1182	48 (+1)	4.1
196402	PHC2, cell wall protein	505	36 (+2)	7.1
196403	PHC3, cell wall protein	443	25 (+1)	5.6
195824	VSP4, cell wall protein	993	43 (+2)	4.3
196399	PHC1, cell wall protein	479	36 (+2)	7.5
130359	VSP6, cell wall protein	951	46 (+1)	4.8
148333	PHC15, cell wall protein	184	10 (+3)	5.4
194264	PHC12, cell wall protein	407	28 (+3)	6.9
187643	PHC14, cell wall protein	566	42 (+1)	7.4
193780	GAS31, cell wall protein	507	37 (+1)	7.3
Peripheral PSII antennae proteins				
184479	LHCBM9	254	2 (+2)	0.8
185533	LHCBM1	256	5 (+1)	2.0
184067	LHCBM2	249	5 (+2)	2.0
195162	LHCBM3	257	5 (+1)	1.9
191690	LHCBM4	254	6 (+1)	2.4
184775	LHCBM5	268	5 (+2)	1.9
184490	LHCBM6	253	5 (+2)	2.0
184071	LHCBM7	249	5 (+2)	2.0
205752	LHCBM8	254	5 (+2)	2.0

The S-containing amino acid levels of the ECP and LHCBM9 proteins are compared with the LHCBM1-8 proteins and the 10 most abundant putative cell wall proteins (according to RNA-seq data).

^aTotal number of Cys and Met residues (C+M) for the mature proteins, with the numbers between parentheses indicating the C+M content in the putative signal peptides.

^bC+M percentages are for the mature proteins.

imposition of S deprivation (Table 6); these proteins are known to mitigate the effects of ROS-elicited lipid peroxidation and DNA damage (Goyer et al., 2002; Sarkar et al., 2005; Dayer et al., 2008) by ameliorating the toxic effects of ¹O₂ (Ledford et al., 2007). Furthermore, *TRXh1* and *GPX5* transcript accumulation in *Chlamydomonas* is either not affected or affected to a minor extent by elevated superoxide (O₂⁻) or H₂O₂ concentrations (Lemaire et al., 1999; Sarkar et al., 2005; Ledford et al., 2007). Ledford et al. (2007) identified 14 transcripts that changed in abundance when *Chlamydomonas* cells experienced high ¹O₂ levels. These transcripts displayed a similar change in abundance in S-deprived *snrk2.1* but not in S-deprived wild-type cells (see Supplemental Table 1 online), suggesting that S deprivation of the mutant elicits sustained production of ¹O₂. In photosynthetic organisms, ¹O₂ is mainly produced as a consequence of PSII electron flow, especially when the organisms are absorbing excess excitation energy, and is thought to be the main cause of photooxidative damage and cell death in plants (Apel and Hirt, 2004; Asada, 2006; Triantaphylides et al., 2008; Triantaphylides and Havaux, 2009). As proposed in the section "Photosynthesis" and in Figure 9, an aberrant light-harvesting architecture in *snrk2.1* during -S conditions (likely associated with a deficit in LHCBM9) may severely impact the activity of PSII and its

capacity for photoprotection, which in turn could cause elevated ¹O₂ production.

Interestingly, transcripts encoding proteins more specific to combating the lethal effects of superoxides and H₂O₂, including L-ascorbate peroxidases 1 and 3 (APX1 and APX3), peroxiredoxin 1 (PRX1), mitochondrial superoxide dismutase 1 (MSD1), and chloroplast superoxide dismutase 1 (FSD1), were slightly reduced in wild-type cells, but their reduction was dramatic in *snrk2.1* cells deprived of S (Table 6). These data suggest that the production of O₂⁻ and H₂O₂ declined during S deprivation, especially in the *snrk2.1* strain. The production of O₂⁻ and H₂O₂ are mostly generated as a consequence of peroxisome metabolism, respiration, and hyperreduction of PSI (Apel and Hirt, 2004; Asada, 2006). In wild-type cells during -S conditions, photosynthetic electron transport is downregulated (Wykoff et al., 1998), which would likely reduce the production of O₂⁻ and H₂O₂ because of reduced electron flow to PSI. In S-deprived *snrk2.1*, the flow of electrons to PSI would potentially be more severely diminished, as depicted in Figure 9.

Other proteins encoded by transcripts elevated exclusively in the *snrk2.1* mutant during S deprivation include TRXh2, TRXy, Met sulfoxide reductase 5 (MSRA5), glutaredoxin 2 (GRX2), and nucleoredoxins 2 and 3 (NRX2 and NRX3). By contrast, S deprivation of the mutant cells caused a decline in the levels of *PRX5*, *TRXf1*, and *TRXf2* transcripts; the level of *TRXf2* mRNA also declined in -S wild-type cells, although absolute levels were significantly lower in the mutant (Table 6). Elevated *MSRA5* transcripts levels may reflect protein damage by oxidative conditions. GRX activity is required for protein deglutathionylation; the role of this enzyme in glutathionylation is not understood (Rouhier et al., 2008). Many chloroplast proteins are nonenzymatically glutathionylated during oxidative stress (including TRXf, PRX1, and Calvin-Benson cycle enzymes), resulting in inhibition of their activities and/or protection from irreversible oxidation (Michelet et al., 2005, 2008). Thus, the high levels of the GRX2 may alter the glutathionylation state of many chloroplast proteins during -S conditions in the *snrk2.1* mutant. TRXf polypeptides, located in chloroplasts, are reductively activated by ferredoxin (Fd) and participate in light-dependent activation/deactivation of key carbon metabolism enzymes, especially those of the Calvin-Benson cycle. Decreased TRXf transcript levels during S deprivation may reflect a decrease in photosynthetic electron flow through PSI (Figure 9); this decrease was more pronounced in *snrk2.1* relative to the wild type (Table 6). Additionally, the redox state of TRXf is expected to decrease, which would alter its activity and the activities of its target proteins.

Apoptosis and Autophagy

The S-deprived *snrk2.1* mutant exhibited an increase in the levels of six transcripts encoding proteins previously associated with apoptosis, as well as a decline in a transcript encoding the antiapoptosis protein (DAD1) (Table 6). There were also 33 transcripts for proteins related to autophagy and vesicular and vacuolar transport (including the exocyst complex, GTPases, R-SNARE and Q-SNARE family proteins, among others) that increased specifically in *snrk2.1* cells (and not in wild-type cells) in response to S deprivation (see Supplemental Data Set 2C1

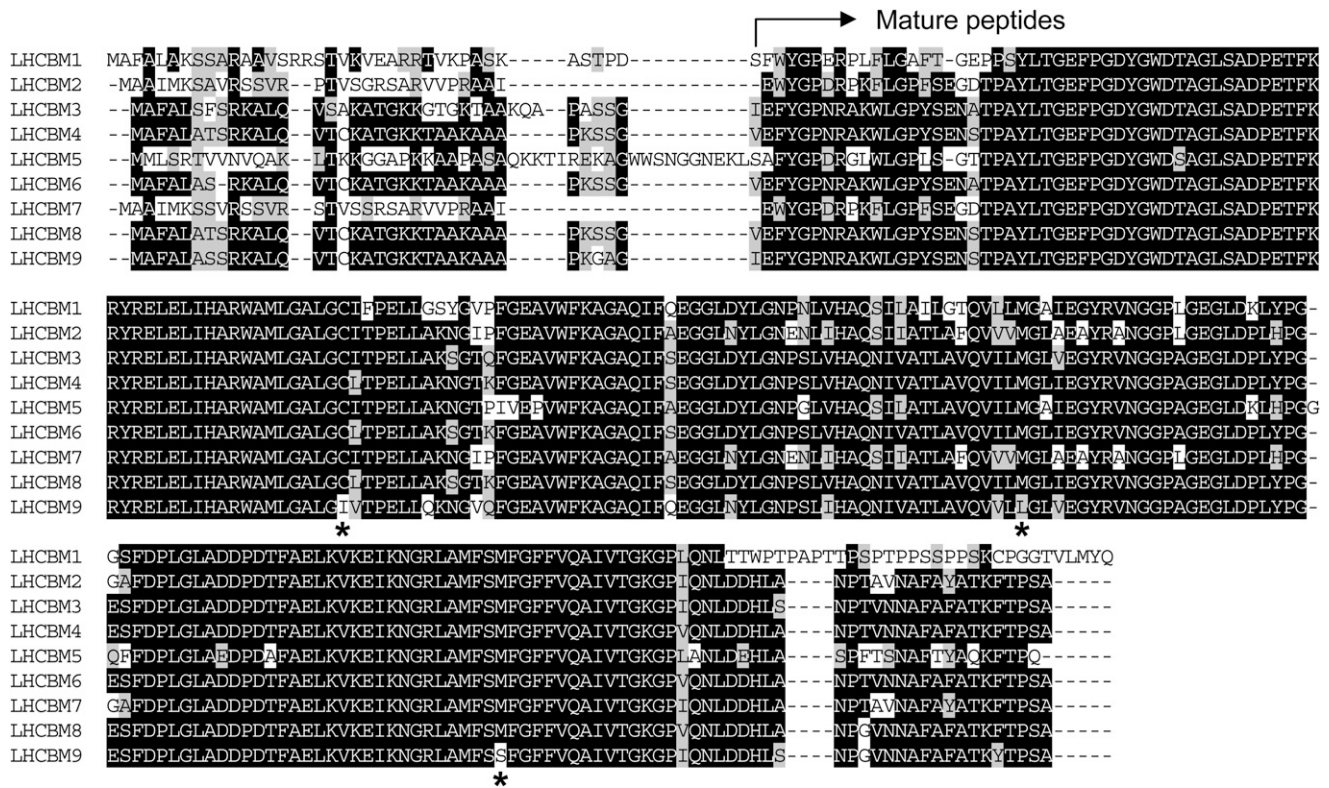


Figure 10. Alignments of the Amino Acid Sequences of *Chlamydomonas* LHCBM Proteins.

The LHCBM9 polypeptide has substitutions at positions in which there are conserved S-containing amino acids in the other LHCBM proteins; these are Met157Leu, Met213Ser, and Cys101Ile; they are marked with an asterisk. The black and gray boxes indicate identical and similar amino acids, respectively. Alignments were performed using BioEdit 7.0.5.3 software.

online); plant cells experiencing apoptosis display intense autophagy, with the vacuole acting like an animal autophagosome (Williams and Dickman, 2008). Finally, numerous transcripts encoding proteins associated with proteolysis, nuclease activity, chromatin condensation, RNA binding, DNA binding, and lipid degradation also increased specifically in the *snrk2.1* mutant under -S conditions (see Supplemental Data Set 2C1 online). The proteins encoded by all of these transcripts are potentially involved in apoptosis/autophagy, which could explain the severe bleaching and rapid death of *snrk2.1* cells during -S conditions (González-Ballester et al., 2008). Apoptosis-like processes have been previously noted for *Chlamydomonas* cells exposed to UV-C irradiation (Moharikar et al., 2006, 2007) and for plants experiencing photooxidative damage and elevated levels of ¹O₂ (Triantaphylides et al., 2008; Williams and Dickman, 2008). Thus, these data support the hypothesis that aberrant light-harvesting architecture, elevated ¹O₂ production, and a loss of cell viability are all linked to the inability of the *snrk2.1* mutant to acclimate to -S conditions (Figure 9).

Putrescine Biosynthesis

The transcript for Orn decarboxylase (ODC2) increased by up to 10-fold specifically in the *snrk2.1* mutant during S deprivation (Table 6). ODC2 catalyzes the conversion of Orn to putrescine,

the precursor of the polyamines spermine and spermidine. Although Orn biosynthesis occurs mostly through the acetyl cycle of Arg, the increase in *ODC2* transcript levels in *snrk2.1* during -S conditions was accompanied by a decrease in most transcripts encoding enzymes involved in Arg biosynthesis; the only exception was that of the *ARG9* transcript, which encodes an *N*-acetylornithine aminotransferase (NAOAT) (Table 6, Figure 11). An alternative route to Orn/polyamine synthesis involves Pro dehydrogenase (PRD), coupled with Orn aminotransferase activity (OAT) (Slocum, 2005) (Figure 11). In the *snrk2.1* mutant, *PRD* transcript levels increased under -S conditions, although the level of the *OAT1* transcript showed little change. Interestingly, several reports indicate that some NAOATs can also use the nonacetylated compounds Orn and Glu-5-semialdehyde as substrates (Billheimer et al., 1976; Heimberg et al., 1990; Ledwidge and Blanchard, 1999). Therefore, it is possible, although still speculative, that *Chlamydomonas* NAOAT (*ARG9*) also works as an OAT. Additionally, the level of the transcript encoding Glu-5-semialdehyde dehydrogenase (*GSD1*) increased in S-starved *snrk2.1* cells (Table 6, Figure 11). The coordinated activities of PRD, *GSD1*, *ARG9* (acting as an OAT), and *ODC2* may define an alternative pathway for the synthesis of putrescine from Pro and Glu in -S *snrk2.1* cells (Figure 11). This pathway could be predominant over the acetyl cycle of Arg under conditions in which levels of the S-containing CoA, a cofactor

Table 5. S-Amino Acid Content of *Chlamydomonas* Proteins

List of Transcripts ^a	C+M % (nwt) ^b	C+M% Weighed by Transcript Abundance ^b					
		+S (Wild Type)	–S (Wild Type)	+S (<i>snrk2.1</i>)	–S (<i>snrk2.1</i>)	+P (Wild Type)	–P (Wild Type)
All	4.19	4.55	4.32	4.36	4.54	4.20	4.25
–S up (wild type)	3.32	3.83	2.33	3.28	3.09	4.08	3.13
–S down (wild type)	4.05	3.93	3.89	3.79	4.24	3.64	3.49
–S up (<i>snrk2.1</i>)	4.18	4.25	4.02	4.28	4.28	4.45	4.28
–S down (<i>snrk2.1</i>)	3.98	3.66	3.68	3.57	3.64	3.64	3.62
–P up (wild type)	4.18	4.42	3.12 ^c	4.73	4.29	4.43	4.06
–P down (wild type)	4.18	4.61	4.48	4.37	4.07	4.32	4.30

Phosphorous deprivation (–P) was used as internal control. nwt, data not weighted by transcripts abundance.

^aThe list of transcripts that accumulate (up) or decline (down) under the different conditions were selected as described in Figure 3. Subsequently, the genes encoding proteins with no significant homology to a known protein (unknown genes) were filtered out because many of them represent inaccurate v.3.1 gene models and would introduce a strong bias into the data.

^bS amino acid content percentages are calculated for proteins in the unprocessed state (containing putative signal peptides).

^cThe low values observed under this condition reflects the fact that some transcripts that increase during S deprivation also show a slight increase during –P conditions (D. González-Ballester and A.R. Grossman, unpublished data).

required for the acetyl cycle of Arg, are diminished. Accumulation of transcripts required for the biosynthesis of CoA in the *snrk2.1* mutant during –S conditions (Table 6) may be indicative of a CoA deficiency. While elevated levels of *ARG9* and *GSD1* gene products could also contribute to the production of Pro, a plant stress-related metabolite, this seems unlikely since there is essentially no increase in the transcript encoding pyrroline-5-carboxylate reductase (PCR1) (Table 6, Figure 11).

Putrescine and other polyamines can also be synthesized via agmatine (Figure 11), although only one transcript of this pathway was elevated in S-deprived *snrk2.1* cells (the agmatine iminohydrolase, *AIH1*), and its absolute level was much lower than that of *ODC2* (Table 6). Hence, as proposed previously (Voigt et al., 2000), the most prominent pathway for putrescine biosynthesis in *Chlamydomonas* is likely via Orn.

The above observations suggest a greater capacity for putrescine synthesis in S-deprived *snrk2.1* than in wild-type cells. Accumulation of putrescine was previously observed in *Arabidopsis* plants deprived of S (Hirai et al., 2003; Nikiforova et al., 2005b). A SAM deficiency was proposed to be the reason for this accumulation since the synthesis of the putrescine-derived polyamines spermidine and spermine requires SAM. In *Chlamydomonas* wild-type cells, transcripts encoding enzymes for spermine and spermidine synthesis (*S*-adenosylmethionine decarboxylase, *DCA1*; spermidine synthase, *SPD1*; and spermine synthase, *SPS1*) declined following S deprivation. This decline was not observed in the *snrk2.1* mutant (Table 6, Figure 11), although spermidine and spermine biosynthesis in the S-starved mutant cells may still be very low as these cells are likely to be deficient for SAM. While the potential accumulation of putrescine in S-deprived *snrk2.1* mutant cells may be a consequence of reduced SAM levels, this accumulation may also be important for acclimation of the organism to its extreme environment. Some have suggested roles for polyamines in the stabilization of membranes, scavenging of ROS, retardation of senescence, and hypersensitive cell death (Yoda et al., 2003; Groppa and Benavides, 2008). Polyamines have also been found to associate with LHCb proteins, influencing nonphotochemical quenching,

photophosphorylation, and organization of PSII. Moreover, treatment of plants with UV-B irradiation and high light can cause an increase in the levels of polyamines associated with LHCb (Del Duca et al., 1995; Della Mea et al., 2004; Ioannidis et al., 2006; Ioannidis and Kotzabasis, 2007; Navakoudis et al., 2007; Sfichi-Duke et al., 2008). Overall, both our work (presented here) and the work of others suggest that oxidative stress, cell death, PSII architecture, and putrescine accumulation may be processes that are closely related.

Effects of the *snrk2.1* Lesion on Transcript Accumulation during Nutrient-Replete Conditions

Some transcripts exhibited aberrant levels in the *snrk2.1* mutant relative to wild-type cells even when the cells were maintained in nutrient-replete conditions (see Supplemental Data Sets 2D and 2E online). The high number of transcripts in this group (89 in total) suggests that regulation via SNRK2.1 is not simply an on-off switch, but may be more similar to a rheostat that controls cellular responses over a dynamic range of S concentrations. Transcripts elevated in *snrk2.1* relative to wild-type cells maintained in either +S or –S conditions include those encoding a pyruvate ferredoxin oxidoreductase (PFR1), a specific apoferritin (FDX5), an iron hydrogenase (HYD1), the hydrogenase assembly factors HYDEF and HYDG, and several prolyl 4-hydroxylases (Table 6; see Supplemental Data Sets 2C1 and 2D online). The protein products of these genes have previously been associated with cells experiencing anoxia and H₂ production (Mus et al., 2007). The link between SNRK2.1 kinase and repression of genes regulated by anoxic conditions is intriguing but could reflect an indirect impact of S deprivation on the physiological state of the cells. For example, a decrease in photosynthetic activity and an increase in *HYD* transcript accumulation and H₂ production was observed in *Chlamydomonas* transformants harboring antisense constructs of the chloroplast SO₄²⁻ permease (SULP1) grown in nutrient-replete medium (Chen et al., 2005). The authors suggested that the reduced permease activity in the transformants limited the supply of S to

Table 6. Expression Levels of Selected Genes That Show Aberrant Behavior in the *snrk2.1* Mutant

ID	Name/Description	Fold Change Expression (Log ₂)						Transcript Abundance (RPKM)			
		Wild Type –S/ Wild Type +S		<i>snrk2.1</i> / Wild Type (+S)		<i>snrk2.1</i> / Wild Type (–S)		Wild	Wild	<i>snrk2.1</i>	<i>snrk2.1</i>
		S	Q	S	Q	S	Q	Type–S	Type+S	–S	+S
Redox processes and singlet oxygen stress-related genes											
142363	<i>PRX1</i> , 2-cys peroxiredoxin, thioredoxin dependent peroxidase (chl.)	–2.2		–0.2		–3.1		177	843	20	759
157173	<i>PRX5</i> , peroxiredoxin type II, thioredoxin dependent peroxidase (chl.)	–0.2		–0.6		–1.5		105	124	38	83
186597	<i>APX1</i> , L-ascorbate peroxidase (chl.)	0.3		–0.2		–2.4		103	85	20	74
165193	<i>APX3</i> , L-ascorbate peroxidase (chl.)	–1.4		–0.6		–1.7		41	108	13	70
182933	<i>FSD1</i> , superoxide dismutase [Fe] (chl.)	–0.5		0.3		–1.7		171	241	53	293
53941	<i>MSD1</i> , superoxide dismutase (mito.)	0.0		0.0		–1.7		381	393	120	391
158519	Singlet oxygen stress-related gene	0.8		1.3		–2.4		259	149	48	355
143122	<i>GPXH</i> (GPX5), glutathione peroxidase (cyt.)	0.7		–0.2		2.0		125	78	502	68
193661	<i>GSTS1</i> , glutathione S-transferase	0.1		–0.3		3.7		37	34	494	28
195887	<i>TRXh1</i> , thioredoxin (cyt.)	–0.2		0.0		1.8		190	216	643	210
196117	<i>TRXh2a</i> , thioredoxin (cyt.)	0.3		0.8		2.7		60	49	385	86
196116	<i>TRXh2b</i> , thioredoxin (cyt.)	0.3		0.8		2.7		71	57	455	102
196118	<i>TRXh2c</i> , thioredoxin (cyt.)	0.3		0.8		2.7		75	61	483	108
134747	<i>TRXy</i> , thioredoxin (chl.)	–0.3		0.2		1.9		16	21	60	23
182094	<i>NRX2</i> , nucleoredoxin	–1.7		1.5		7.2		5	15	701	42
141568	<i>NRX3</i> , nucleothioredoxin	–0.8		2.3		6.6		2	3	150	12
195611	<i>GRX2</i> , glutaredoxin, CPYC type (cyt.)	0.5		–0.1		1.8		111	79	384	73
187796	<i>MSRA5</i> , peptide Met sulfoxide reductase	0.4		0.4		1.5		19	14	54	19
139781	<i>TRXf1</i> , thioredoxin f1 (chl.)	0.1		–0.1		–1.8		185	176	52	170
195886	<i>TRXf2</i> , thioredoxin f2 (chl.)	–1.0		–0.4		–2.2		55	111	12	83
Apoptosis-related genes											
141763	<i>DAD1</i> , antiapoptosis gene	–0.6		0.4		–1.7		80	118	24	159
182542	<i>DDI1</i> , DNA damage-inducible v-SNARE binding protein	–0.3		0.2		1.8		9	11	30	12
174932	<i>LAG1</i> , longevity assurance protein	0.0		0.5		3.7		21	20	276	29
205713	Metacaspase, type I	0.4		0.6		2.0		9	6	33	10
205701	<i>PHD1</i> , V1AF phosphatase-like protein	–0.8		0.4		4.5		20	35	437	47
190610	<i>PDCD6/ALG-2</i> , programmed cell death-related gene	0.1		0.6		1.6		23	22	68	33
179155	Senescence-associated gene	–0.1		0.8		4.5		13	14	281	24
Arg/Om/polyamine biosynthesis											
206062	<i>ODC2</i> , Orn decarboxylase 2	0.7		0.2		2.7		45	27	289	30
139007	<i>ARG9</i> , acetylornithine aminotransferase	–0.4		–0.6		2.4		33	45	174	29
183928	<i>DCA1</i> , S-adenosylmethionine decarboxylase	–1.8		–0.3		1.5		54	184	149	146
206050	<i>SPD1</i> , spermidine synthase	–0.9		–0.5		0.7		12	24	20	17
206069	<i>SPS1</i> , spermine synthase	–2.6		–1.0		2.0		6	34	22	17
193221	<i>PRD</i> , Pro dehydrogenase	0.5		1.0		2.8		14	10	100	19
192364	<i>PCR1</i> , pyrroline-5-carboxylate reductase	–1.3		0.2		1.0		15	37	29	42
130812	<i>GSD1</i> , Glu-5-semialdehyde dehydrogenase	–0.6		–0.5		2.0		42	62	164	45
195386	<i>OAT1</i> , Orn aminotransferase	1.6		0.9		–1.2		48	16	20	30
13028	<i>AIH1</i> , agmatine iminohydrolase	–0.2		–1.0		3.5		3	3	28	2
Hydrogen production-related genes											
156833	<i>FDX5</i> , Apoferreredoxin	–2.6		6.2		7.7		1	5	174	367
122198	<i>PFR1</i> , pyruvate-ferreredoxin oxidoreductase	1.8		6.7		6.5		1	0	103	33
183963	<i>HYD1</i> , iron hydrogenase	0.4		1.9		2.0		49	36	190	130
128256	<i>HYDEF</i> , iron hydrogenase assembly protein	0.8		2.0		1.6		12	7	35	26
196226	<i>HYDG</i> , hydrogenase assembly factor	1.0		1.7		1.4		61	30	158	97
Carbon metabolism											
115491	Acetate transporter (p)	–1.7		–1.5		–1.6		699	2285	228	806
196311	<i>ACS3</i> , Acetyl CoA synthetase, mit	–1.6		–1.4		–1.1		233	710	112	271

(Continued)

Table 6. (continued).

ID	Name/Description	Fold Change Expression (Log ₂)						Transcript Abundance (RPKM)			
		Wild Type –S/ Wild Type +S		<i>snrk2.1</i> / Wild Type (+S)		<i>snrk2.1</i> / Wild Type (–S)		Wild Type–S	Wild Type+S	<i>snrk2.1</i> –S	<i>snrk2.1</i> +S
		S	Q	S	Q	S	Q				
24120	<i>CAH1</i> , carbonic anhydrase (ext.)	–2.1	–4.1	1.9	6.4	2.4	8.2	328	1375	1727	5057
24552	<i>CAH4</i> , carbonic anhydrase	–2.2	0.6	2.1	8.3	3.0	6.1	83	368	655	1614
55019	<i>LC11</i> , low CO ₂ -inducible gene	–2.9		2.5		3.3		20	150	199	843
189430	<i>CCP1</i> , chl. envelope protein (chl.)	–0.7		1.9		1.9		139	230	529	830
128766	<i>CCP2</i> , chl. envelope protein (chl.)		0.3	2.2		2.3		90	76	453	346
135648	<i>NAR1.2</i> , bicarbonate transporter (p)	–0.7		2.0		1.6		132	214	401	849
205996	Limiting CO ₂ -inducible gene	–0.4		1.9		2.5		25	33	139	124
CoA biosynthesis											
116788	Phosphopantothenate Cys ligase	0.0		–0.7		4.1		6	6	103	4
196344	PANB, ketopantoate hydroxymethyltransferase	–0.7		0.4		4.3		3	5	56	6

Details are as given in Table 3.

chloroplasts, which caused a decline in PSII activity and a decrease in O₂ evolution. This situation could result in a rate of respiratory O₂ uptake that exceeds the rate of O₂ evolution, and the cells would experience anaerobic conditions even in the light growing on complete medium. The *snrk2.1* mutant cultures may also be experiencing a low level of S limitation during nutrient-replete growth since the levels of many transcripts encoding proteins required for efficient uptake and assimilation of SO₄^{2–} were significantly reduced in the mutant relative to wild-type cells (Table 3). Thus, *snrk2.1* may have a greater tendency to become anoxic and produce H₂ than wild-type cells during nutrient-replete growth.

Interestingly, even when *snrk2.1* cells were maintained in acetate-containing, nutrient-replete medium, which normally would partially suppress low CO₂ responses, many CCM-related transcripts became highly abundant. These transcripts include those encoding the periplasmic carbonic anhydrase *CAH1*, mitochondrial *CAH4*, the HCO₃[–]/nitrite transporter *NAR1.2* (Mariscal et al., 2006), and several previously identified low CO₂-inducible genes (see Supplemental Data Sets 2C1 and 2D online). The *CAH1* transcript level was strongly repressed in the *snrk2.1* mutant in acetate-free medium bubbled with 4% CO₂ in air (similar to wild-type cells), confirming that the *snrk2.1* lesion has an indirect effect on the regulation of this gene and potentially other CCM genes in acetate-containing medium (see Supplemental Figure 3 online). Interestingly, the *snrk2.1* mutant grown in nutrient-replete medium exhibited low levels of transcripts (relative to wild-type cells) encoding proteins required for acetate assimilation, including a putative acetate transporter (ID 115491) and the acetyl CoA synthetase (*ACS3*) (Table 6). These data suggest that the *snrk2.1* strain may favor the use of atmospheric CO₂/HCO₃[–] even when growing in acetate-containing medium. Normally, *Chlamydomonas* cells grown in acetate-containing medium exhibit decreased rates of CO₂ fixation and O₂ evolution (Heifetz et al., 2000). This bias of *snrk2.1* cells toward the fixation of CO₂ rather than toward the assimilation of acetate may in some way link to the greater tendency of the mutant culture to become anoxic. Elevated CO₂ fixation would

potentially increase O₂ evolution and reduce the level of anoxia in mutant cultures grown in the light on nutrient-replete, acetate-containing medium. These relationships and their physiological implications need to be more thoroughly established.

Conclusion

In this study, the use of RNA-seq technology yielded deep coverage for most individual transcripts in *Chlamydomonas* and provided a genome-scale, quantitative view of how a changing S environment impacts transcript patterns. Analysis of the transcriptome profiles of both the wild type and the *snrk2.1* mutant, which is unable to acclimate to S deprivation, clearly identified the set of –S-responsive genes, and those –S-responsive genes under the control of the regulatory SNRK2.1 kinase. S-deprived *Chlamydomonas* cells accumulated transcripts involved in the scavenging of environmental S and in the reductive assimilation of SO₄^{2–} to Cys, while transcripts encoding enzymes required for the synthesis of Met, SAM, thiamine, and biotin declined. This result suggests the importance of maintaining an efficient capacity for Cys biosynthesis relative to other S-containing metabolites. Moreover, the results of this study also indicate a massive redistribution of the S resources of the cells as exemplified by the coordinated S deprivation-elicited accumulation of the *CDO1*, *TAUD1*, *TAUD2*, *RDP3*, *CSD2*, and *SOUX1* transcripts. While the enzymes encoded by these transcripts have been associated with the redistribution of S through the catabolism of Cys and other S-containing molecules (e.g., sulfolipids), their exact substrate specificities have not been defined. Indeed, the precise ways that these enzymes participate in creating a new S homeostasis during S deprivation have not yet been established for any photosynthetic organism.

The critical nature of the redistribution of S resources is also suggested by changes in the organization of cellular processes during S deprivation. A massive increase in the transcript encoding LHCBM9, a light-harvesting protein with a reduced S amino acid content relative to other LHCBM polypeptides,

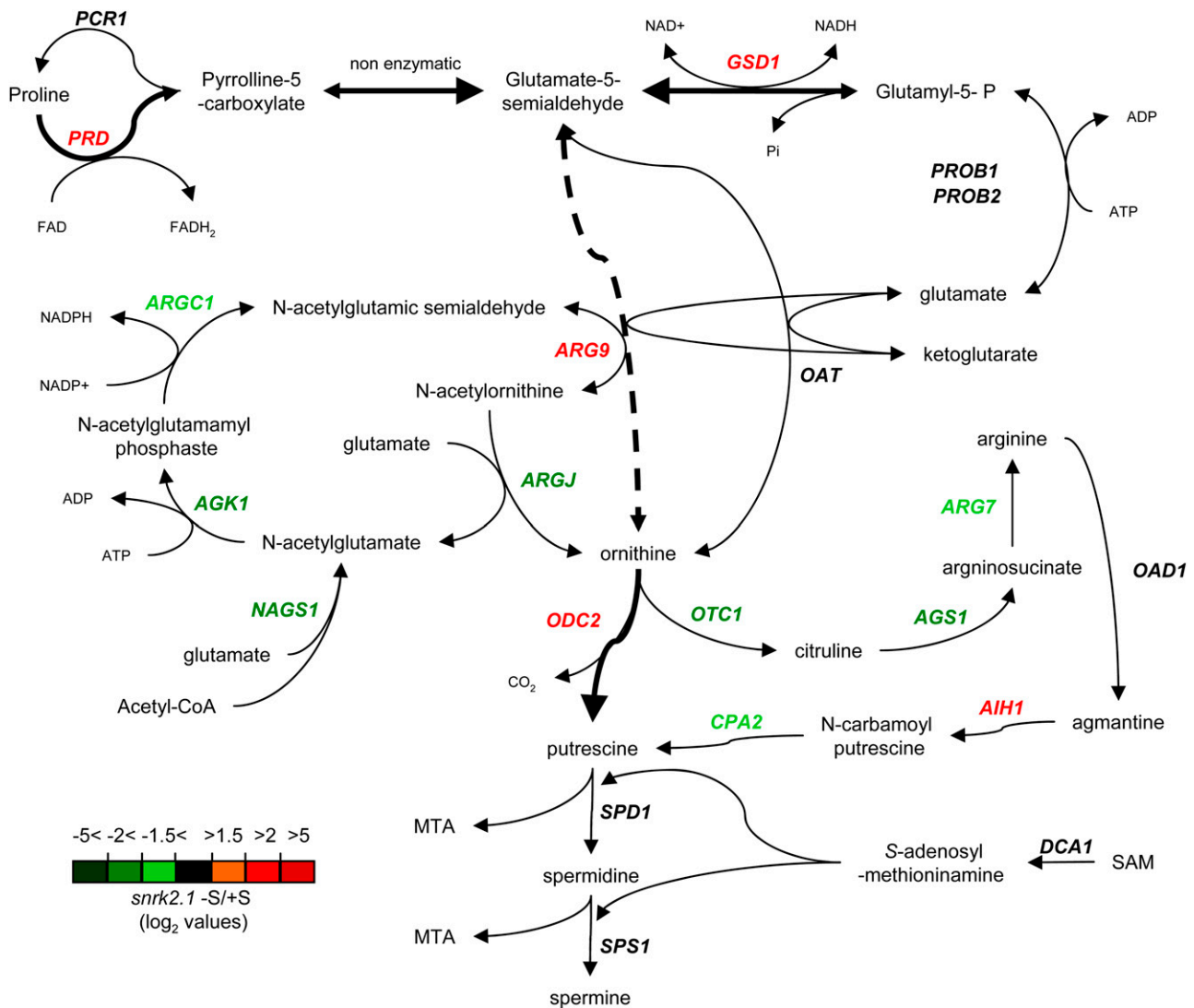


Figure 11. Potential Pathways for Polyamine Biosynthesis.

Genes encoding the proteins of the pathways are labeled with colors, indicating the fold change (\log_2) of their transcript levels under S deprivation conditions relative to nutrient-replete conditions for *snrk2.1* cells based on RNA-seq data (color code is given in the figure). The dashed line indicates the alternative activities proposed for the ARG9 protein. The protein encoded by each of the genes represented in the pathway diagram is given in Table 6. MTA, S-methyl-5'-thioadenosine.

paralleled a decline in the level of transcripts encoding all of the other LHCBM s. This shift in antenna protein production could lead to significant S conservation and a change in the ways in which the alga uses light energy. Indeed, at a global level, proteins that accumulate during $-S$ conditions tend to have a reduced S amino acid content; such proteins include the ECPs, HAPs, ARSs, and TAUDs. Based on these findings, it is likely that a recycling of S-containing amino acids and their reallocation among proteins with low S amino acid content represents an extreme economy of S use that can extend cell viability when S resources become scarce.

Another important aspect of this work addresses the question of what a cell does when it is unable to implement the $-S$

acclimation program, as is the case of the *snrk2.1* mutant. The inability of mutant cells to acclimate to $-S$ conditions likely prevents efficient use of external SO_4^{2-} (free and organic), the recycling of internal S (SQDG and Cys), and the redistribution of the S resources among different S-containing metabolites (e.g., CoA and SAM); this in turn results in aberrant control of cellular metabolism. Moreover, the absence of transcripts encoding LHCBM9 and LHCSR1 in the *snrk2.1* mutant during $-S$ conditions may alter the structure of the photosynthetic apparatus. These metabolic and structural aberrations can result in the accumulation of metabolites and reactive molecules (e.g., putrescine and 1O_2) that have extreme consequences, ultimately leading to diminished cell viability. Indeed, when the *snrk2.1*

mutant is starved for S, it appears to show molecular responses, such as the accumulation of transcripts associated with oxidative damage, ROS production, and apoptosis that are typical of organisms experiencing extreme environmental conditions.

METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii strains used for this study were D66 (wild-type strain; *nit2 cw15 mt+*) (Pollock et al., 2003), *ars11 (snrk2.1cw15mt+)* (González-Ballester et al., 2008), 21gr (wild-type strain; *nit5 mt-*) (Harris, 1989), and *sac1 (sac1mt+)* (Davies et al., 1994). The *ars11* strain was designated as the *snrk2.1* mutant throughout since the lesion is in the *SNRK2.1* gene. Cells were cultured under continuous light of $\sim 60 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ at 23°C in liquid and on solid Tris-acetate-phosphate (TAP) medium (Harris, 1989). To impose S deprivation, cells in mid-logarithmic growth phase were washed twice with liquid TAP medium without S (TAP-S) (Harris, 1989), and equal numbers of cells were resuspended in TAP or TAP-S. Cell aliquots were collected for RNA isolation just prior to and 1, 6, and 24 h after being transferred to TAP and TAP-S medium.

RNA Isolation, Synthesis of cDNA, and qRT-PCR

Isolation of total RNA using a phenol-chloroform procedure, cDNA synthesis, and qRT-PCR was performed by standard methods as previously described (Schloss et al., 1984; González-Ballester et al., 2008). Ct values were determined for three independent technical experiments, with at least two measurements of the same sample in each experiment. Relative fold differences were calculated based on the ΔCt method ($2^{-(\text{Ct}_{\text{sample}} - \text{Ct}_{\text{control gene}})}$) (Livak and Schmittgen, 2001) using the *CBLP* amplification product as an internal standard. Primer pairs used for qRT-PCR analyses are in Supplemental Table 2 online. Sizes of amplification products were 100 to 300 bp.

Microarray Analysis

Oligonucleotides (~ 70 nucleotides length) representing $\sim 10,000$ unique ESTs were used to print the arrays. The design and generation of the microarrays chips used in this work was described previously (Eberhard et al., 2006). The following RNA samples were compared in the microarrays: wild type 1 h -S compared with wild type 1 h +S, wild type 6 h -S compared with wild type 6 h +S, wild type 24 h -S compared with wild type 24 h +S, wild type 0 h -S compared with *snrk2.1* 0 h -S, wild type 1 h -S compared with *snrk2.1* 1 h -S, wild type 6 h -S compared with *snrk2.1* 6 h -S, and wild type 24 h -S compared with *snrk2.1* 24 h -S. Preparation of the fluorescent microarray probes and hybridization to the arrays was performed as described previously (Eberhard et al., 2006; Mus et al., 2007). Between two and four biological replicates were used for each sample, and at least four microarray slides for each comparison (additionally, each slide has two copies of each array element). Arrays were scanned using a GenePix 4000B scanner (Molecular Devices). Photomultiplier tube voltages were adjusted manually to balance Cy5 and Cy3 signals (1:1 ratio) and to avoid spot saturation. Resulting images were imported into SpotReader (version 1.3.0.5; Niles Scientific), and spot positions were defined and fluorescence signal intensities determined. Spot quality signals were automatically flagged as reliable (good), non-reliable (absent), or empty based on the following parameters: saturated foreground, 70%; uneven color, 0.5; bright specks, 4; low foreground, 1.4; high background, 10; low background, 0.1; variable background, 5. Additionally, spot signals that were distorted by dust, locally high backgrounds, or printing flaws were manually flagged as nonreliable (absent). Spot backgrounds were automatically subtracted; a radius of 2 times the

average radius for each spot (from the spot center) was defined, and the region of the field within this radius and between the new diameter and the original spot diameter was considered background. Subsequent data analyses were performed using GeneSpring 7.3.1 (Agilent Technologies). Normalizations of spot intensities were achieved using the software standard "per spot and per chip intensity-dependent (Lowess)" normalization (<http://stat-www.berkeley.edu/users/terry/zarray/Html/normspie.html>). Only spots flagged as reliable (good) were analyzed. Error models were computed based on replicates. Signal ratios were considered only if they passed Student's *t* test for significance with a P value of ≤ 0.05 .

Microarray probe sequences were mapped to the *Chlamydomonas* v.3.1 genome to find their correspondence with gene models. Gapped alignment using BLAT (Kent, 2002) was performed for a set of 9998 microarray probes, of which 6745 overlapped with JGI v.3.1 gene models. At least one matching probe was found for 5948 gene models (out of 14,598). When genes have multiple matching probes, the probe with a more significant fold change was selected when comparing two samples. Correspondences between microarray probes and JGI v.3.1 gene models are included in Supplemental Data Set 1 online.

RNA-Seq Technology

Sample Preparation

Total RNA from wild-type (D66) and *snrk2.1* mutant cells 6 h after being transferred to -S and +S medium were submitted to Illumina for sequencing using their proprietary Genome Analyzer. cDNA libraries were assembled according to the manufacturer's RNA-seq protocol, loaded, and sequenced as 35-mers in a total of 11 lanes of the sequencer (see Supplemental Table 3 online). Raw image files were collected by the sequencer and analyzed using the standard Illumina pipeline.

Mapping Reads to the Genome

Processed sequence files from the Illumina pipeline output were aligned against the v.3.1 assembly of the *Chlamydomonas* genome. The SOAP alignment program (Li et al., 2008) was used to map the short reads in two steps (see Supplemental Methods online for details). In a first round, the raw 35-mers sequences were aligned to the genomic sequence with a tolerance of up to two mismatches and no indels. Approximately 77% of the reads mapped to at least one genomic location on either of the two strands, and of these, 89% mapped uniquely. This percentage compares favorably with previously reported RNA-seq studies (Marioni et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008). For those sequences that did not align to the genome, a second round alignment was performed in which we recursively trimmed one base at a time (up to 21-mers) at either the 5' or 3' end of the reads until we found a (perfect) match to the genome. This procedure allowed us to recover some misalignments caused by the presence of exon-exon junctions, sequencing errors, adaptor sequences, and poly(A) tails in the sequence reads. About 50% of the reads that were not aligned in the initial analysis were recovered, bringing the total fraction of mappable reads to $\sim 88\%$ (see Supplemental Table 3 online).

All alignments were tagged as either unique or not unique and then combined. The most 5' position of each alignment was recorded along with the length *k* of the aligned read (*k-hit* throughout). For differential expression analysis, per-base unique *k-hits* from both strands were pooled (as the cDNA library lacks strand specificity) and summed across the genome to obtain gene counts.

Transcript Abundance

Relative transcript abundances were determined as follows (see Supplemental Methods online for notation): for each particular gene model *j* in

the JGI annotation and each read length k (in the range 21 to 35 in this work), we determined the set of unambiguously mappable bases (M_{jk}^m) at a given multiplicity threshold m , and its cardinality $M_{jk}^m = |M_{jk}^m|$ denotes the number of mappable bases per gene or m -mappability. The mappable set M_{jk}^1 includes all of the exonic bases that are uniquely mappable with k -mers, except for those that overlap with neighbor gene models or are located in a window of size $k - 1$ at the 3' end of the exon. For each lane i , we quantified the number of unique k hits associated with each gene (H_{ijk}^1), normalized the value using the corresponding 1-mappability, and summed over all k values using the total number of aligned sequences H_i (a measure of sequencing depth) as a global scaling factor. The transcript relative abundance T_{ij}^1 (TRA) is expressed in units of reads per kilobase of mappable exon length per million mapped reads (RPKM), where the constant factor c is equal to 1×10^6 (Mortazavi et al., 2008). More details are shown online in Supplemental Methods, Supplemental Figure 6, and Supplemental Figure 7.

$$T_{ij}^1 = \frac{c}{H_i} \sum_k \frac{H_{ijk}^1}{M_{jk}^1}$$

Reproducibility of the Data

The total number of hits H_{ij}^1 for a particular gene was modeled as a random variable from a Poisson distribution. The significance of hits-per-gene variation between different lanes, runs, or experiments was calculated as described previously (Audic and Claverie, 1997) (see Supplemental Methods online for details): the conditional probability, $p(H_{a(ij)}^1 | H_{b(ij)}^1)$ for the number of hits in two different groups of lanes ($a(i), b(i)$) for the same gene model j is computed by means of a uniform prior for all values of the parameter of the Poisson distribution. Thus, the cumulative distribution function of $p(H_{a(ij)}^1 | H_{b(ij)}^1)$ is used to estimate the probability (designated P value throughout) that the observed hits fall outside the confidence interval defined by $H_{a(ij)}^1$ and $H_{b(ij)}^1$.

Based on the previous statistical framework, pairwise comparisons were performed between and within runs and/or samples to get a sense of the quality and reproducibility of the sequencing process. The null hypothesis (that the number of hits from both lanes follows the same random distribution) can be tested from deviations of the P value distribution from uniformity. Supplemental Figure 4 online shows qq plots (quantile probability plots) of hits-per-gene statistics for several pairs of lanes. Supplemental Figure 5 online shows normalized expression fold changes relative to overall expression. The data shows that lane-to-lane or run-to-run comparisons show slight overrepresentations of small P values. However, sample-to-sample variations are much more pronounced. Additional details regarding the false discovery rate for differential expression can be found in Supplemental Methods online.

Estimation of Differential Expression

Due to small but detectable lane and flow cell effects, we chose to take the mean ($\bar{T}_{a(ij)}^1$) of single-lane TRA values for each condition as if they were true experimental replicates to average out the impact of sequencing bias in expression estimates. By contrast, P values were computed after pooling all lanes for the same sample to improve detection of low abundance transcripts and minimize the effect of different levels of sequencing depth across samples. Moreover, this choice also allows us to make use of true count data using the aforementioned Poisson approach.

Fold changes in expression between conditions (wild type –S/wild type +S, *snrk2.1* –S/*snrk2.1* +S, *snrk2.1* –S/wild type –S, and *snrk2.1* +S/wild type +S) were calculated using the TRAs \log_2 ratios. For those gene models with null TRA values, a value equal to the minimum on each experiment was imputed. In that case, fold changes must be interpreted as lower bounds of the actual value. The sets of genes that were

considered for further analysis in each comparison were selected after applying the following filters: (1) TRA \log_2 ratios were considered significant if ≥ 1.5 or ≤ -1.5 ; (2) positive TRA \log_2 ratios that had numerators below 30 were not considered; (3) negative TRA \log_2 ratios that had denominators below 30 were not considered; (4) the P value for differential expression was set to be ≤ 0.05 . After applying the three first filters (1 to 3), >99.9% of the genes passed the fourth filter. Transcripts that passed all the filters were used to derive the information in Figure 3 and Supplemental Data Set 2 online.

Sequence Analysis

Gene annotations were retrieved from the *Chlamydomonas* genome browser, which was developed by the JGI (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>) in collaboration with the *Chlamydomonas* community (Merchant et al., 2007). For gene models with no associated annotation, BLAST searches were performed to identify sequence similarities. Sequences were analyzed using DNASTar software v.4.05 (Laser-gene Navigator), Bioedit Sequence Alignment Editor version 5.0.9 (Department of Microbiology, North Carolina State University), the National Center for Biotechnology Information BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>), and the iPSORT and PSORT servers (<http://hc.ims.u-tokyo.ac.jp/iPSORT/> and <http://psort.ims.u-tokyo.ac.jp/form.html>).

Accession Numbers

JGI v.3.1 protein accession numbers are provided in Supplemental Data Set 1 online with the corresponding Web link to the JGI browser. RNA-seq raw data (see Supplemental Data Sets 1 to 3 online) are available at the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE17970 and at <http://genomes.mcdb.ucla.edu/CreSulfur/>.

Supplemental Data

Supplemental Figure 1. *Chlamydomonas* Growth with Different S-Containing Amino Acids as Sole S Source.

Supplemental Figure 2. Alignment of Putative Taurine Dioxygenases (TAUD).

Supplemental Figure 3. *CAH1* Transcript Accumulation in Wild-Type and *snrk2.1* Cells under Fixed and Inorganic Carbon Conditions (Based on qRT-PCR).

Supplemental Figure 4. qq-Plots Comparing P Values from Selected Pairwise Comparisons of the Total Number of Hits per Gene from RNA-Seq Individual Lanes (See Supplemental Table 3 Online for Details).

Supplemental Figure 5. Logarithmic Scatterplots of Normalized Expression Fold Changes (y Axes) versus Overall Expression Measures (x Axes).

Supplemental Figure 6. Definition of Mappable Sets and Assignment of k -Hits per Gene.

Supplemental Figure 7. Coverage Data for a 3-kb Region of the *GAP3* Locus.

Supplemental Table 1. *Chlamydomonas* Singlet Oxygen Stress-Related Genes and Their Expression in the Wild Type and *snrk2.1* Mutant in –S Conditions.

Supplemental Table 2. Primers Used for qPCR Analyses.

Supplemental Table 3. Summary Statistics for RNA-Seq Samples and Sequence Alignment.

Supplemental Data Set 1. Expression Levels of All Analyzed Transcripts.

Supplemental Data Set 2. Relative Levels of Transcripts Represented in Figure 3.

Supplemental Data Set 3. Expression Levels of Genes Grouped by Putative Functions.

Supplemental Methods.

Supplemental References.

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