Tiered Regulation of Sulfur Deprivation Responses in *Chlamydomonas reinhardtii* and Identification of an Associated Regulatory Factor[1][C][W]

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During sulfur (S) deprivation, the unicellular alga *Chlamydomonas reinhardtii* exhibits increased expression of numerous genes. These genes encode proteins associated with sulfate (SO_4^{2-}) acquisition and assimilation, alterations in cellular metabolism, and internal S recycling. Administration of the cytoplasmic translational inhibitor cycloheximide prevents S deprivation-triggered accumulation of transcripts encoding arylsulfatases (ARS), an extracellular polypeptide that may be important for cell wall biosynthesis (ECP76), a light-harvesting protein (LHCBM9), the selenium-binding protein, and the haloperoxidase (HAP2). In contrast, the rapid accumulation of transcripts encoding high-affinity SO_4^{2-} transporters is not affected. These results suggest that there are two tiers of transcriptional regulation associated with S deprivation responses: the first is protein synthesis independent, while the second requires de novo protein synthesis. A mutant designated *ars73a* exhibited low ARS activity and failed to show increases in *ECP76*, *LHCBM9*, and *HAP2* transcripts (among others) in response to S deprivation; increases in transcripts encoding the SO_4^{2-} transporters were not affected. These results suggest that the ARS73a protein, which has no known activity but might be a transcriptional regulator, is required for the expression of genes associated with the second tier of transcriptional regulation. Analysis of the *ars73a* strain has helped us generate a model that incorporates a number of complexities associated with S deprivation responses in *C. reinhardtii*.

Sulfur (S) is an essential macronutrient that is integral to proteins, lipids, electron carriers, and redox controllers (Leustek et al., 2000). Most organisms, including *Chlamydomonas reinhardtii*, have a limited capacity to store S and thus depend on a continual supply of an external source of this nutrient. The majority of S that is taken up from the soil solution by microbes and plants is in the form of the sulfate anion (SO_4^{2-}). Assimilation of SO_4^{2-} involves reduction to sulfide and subsequent incorporation into the amino acids Cys and Met (Grossman and Takahashi, 2001).

The ability of microorganisms to acclimate to limited amounts of nutrients, including S, is critical to their survival in the natural environment. *C. reinhardtii* exhibits both general and specific responses when experiencing S deprivation. The general responses are common to a number of stress conditions and include the cessation of cell division, the accumulation of storage starch, and a decrease in metabolic processes including photosynthesis. In contrast, the specific responses are those associated with the deprivation of a single nutrient and include an elevated rate of SO_4^{2-} uptake, the synthesis of extracellular arylsulfatases (ARS), and an increased capacity to assimilate SO_4^{2-} by increasing the levels of enzymes needed for Cys biosynthesis (de Hostos et al., 1988; Yildiz et al., 1994; Ravina et al., 2002). Changes in genome-wide transcript accumulation as *C. reinhardtii* experiences S deprivation were recently reported (González-Ballester et al., 2010). The results of that study suggest that there are marked alterations in the activities of pathways associated with the biosynthesis of S compounds and that specific mechanisms have evolved to limit the synthesis of proteins with high-S amino acid content; this process has been termed S sparing (Fauchon et al., 2002; González-Ballester et al., 2010). Changes in the levels of a number of specific proteins encoded by S-responsive transcripts have also been observed (Takahashi et al., 2001; Pootakham et al., 2010).

ARS, an activity first detected approximately 3 h after the transfer of cells to medium lacking S (de

[1] This work was supported by the National Science Foundation (grant no. MCB–824469 to A.R.G.).

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Arthur R. Grossman (arthurg@stanford.edu).

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www.plantphysiol.org/cgi/doi/10.1104/pp.113.214593

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Hostos et al., 1988), is secreted into the periplasmic space of *C. reinhardtii* cells, where it hydrolyzes soluble SO_4^{2-} esters in the medium, releasing free SO_4^{2-} for uptake and assimilation. The identification and characterization of ARS polypeptides led to the cloning of two ARS-encoding genes, *ARS1* and *ARS2*. Transcripts from both of these genes accumulate in response to S deprivation (de Hostos et al., 1989; Ravina et al., 2002; Zhang et al., 2004). S deprivation of *C. reinhardtii* also elicits an increase in SO_4^{2-} uptake, which is a consequence of the de novo synthesis of specific SO_4^{2-} transport systems (Yildiz et al., 1994). The SO_4^{2-} transporters encoded by *SULTR2* (for *Sulfate Transporter2*), *SLT1* (for *SAC1-like transporter1*), and *SLT2* are strongly up-regulated at the transcript and protein levels almost immediately following the imposition of S deprivation (Pootakham et al., 2010). The initial rate of SO_4^{2-} uptake increases as early as 1 h following the removal of S from the medium and becomes maximal after approximately 6 h. An increase in the affinity of the transport system for SO_4^{2-} could also be detected within 1 h of S deprivation (Yildiz et al., 1994). Interestingly, S-starved cells show increased SO_4^{2-} uptake prior to the detection of ARS activity, suggesting that the control of these two processes is differentially sensitive to the level of S in the environment.

C. reinhardtii also has mechanisms to conserve and recycle intracellular S during S-limiting conditions. The degradation of proteins and lipids that are not essential under S-deficient conditions can supply cells with a limited amount of S (Ferreira and Teixeira, 1992). S-starved *C. reinhardtii* cells degrade most of the chloroplast sulfolipid to redistribute S for protein synthesis and other processes (Sugimoto et al., 2007). Four prominent extracellular polypeptides, ECP56, ECP61, ECP76, and ECP88, are synthesized in response to S deprivation (Takahashi et al., 2001; González-Ballester et al., 2010). While the functions of these polypeptides have not been established, they contain almost no S-containing amino acids and exhibit features similar to those of cell wall, Hyp-rich glycoproteins. These findings suggest that the amino acids of S-rich cell wall proteins present during S-replete growth can be replaced by the ECPs; the S-containing amino acids of the S-rich cell wall proteins would become available for recycling (Takahashi et al., 2001). S deprivation also triggers a potential change in the subunit composition of light-harvesting complexes, favoring the synthesis of complexes containing polypeptides with few S amino acids (Nguyen et al., 2008; González-Ballester et al., 2010).

A number of S starvation-elicited responses appear to be controlled at the level of transcript abundance and gene activity. Transcripts encoding SO_4^{2-} transporters, ARS, ECPs, LHCBM9 (for light-harvesting protein), and enzymes involved in SO_4^{2-} assimilation [e.g. ATP sulfurylase, Ser *O*-acetyl transferase, *O*-acetyl Ser (thiol) lyase] are markedly elevated in cells deprived of S (de Hostos et al., 1989; Yildiz et al., 1996; Takahashi et al., 2001; Ravina et al., 2002; Zhang et al., 2004). Phenotypic screening for mutants unable to properly acclimate to S limitation led to the identification of three key regulators of S deprivation responses, SAC1 (for sulfur acclimation), SNRK2.1 (for Snf1-related protein kinase), and SNRK2.2 (Davies et al., 1994; Pollock et al., 2005; González-Ballester et al., 2008). *SAC1* encodes an integral membrane protein similar to an Na^+/SO_4^{2-} cotransporter. However, SAC1 is a positive regulator critical for the activation of many genes involved in scavenging and assimilating S from the environment, including those encoding ARS, ECPs, a number of proteins important for SO_4^{2-} assimilation (Davies et al., 1996; Ravina et al., 2002; Zhang et al., 2004), and proteins associated with the potential restructuring of the photosynthetic apparatus (Davies et al., 1994; Wykoff et al., 1998). The *SNRK2.2* gene, previously designated *SAC3* (Davies et al., 1999), encodes a Ser/Thr kinase that acts as a negative regulator of S deprivation-responsive genes. A *snrk2.2* mutant exhibits low constitutive ARS activity and expresses elevated basal levels of S deprivation-responsive genes during S-replete growth (Davies et al., 1994, 1999; Ravina et al., 2002; González-Ballester et al., 2008). SNRK2.2 is also required for the proper down-regulation of chloroplast transcriptional activity in S-deprived cells (Irihimovitch and Stern, 2006). Finally, another Ser/Thr kinase, SNRK2.1, has recently been shown to play a central role in controlling S deprivation responses. The *snrk2.1* mutant fails to activate the expression of most S deprivation-responsive genes and cannot maintain normal basal levels of expression of some genes under nutrient-replete conditions (González-Ballester et al., 2008, 2010).

In this study, we discovered that cycloheximide, an inhibitor of translation on 80S cytosolic ribosomes, did not block the induction of SO_4^{2-} transporter genes (accumulation of the mRNA was not affected) but did block the induction of a subset of S deprivation-responsive genes, most of which are associated with S scavenging and recycling. This finding suggests the sequential, temporal regulation of subsets of S deprivation-responsive genes and that this tiered regulatory response may be dependent upon the accumulation of a specific protein(s) synthesized during the early stages of S deprivation. In a previous screen for mutants defective in the synthesis of active ARS (Pollock et al., 2005), a mutant designated *ars73a* was identified. The phenotype of this mutant is congruent with its involvement in the second tier responses of *C. reinhardtii* to S deprivation.

RESULTS

Identification of the *ars73a* Mutant

The acclimation of *C. reinhardtii* cells to S deprivation can be monitored by assaying the cells for the production of extracellular ARS activity. Wild-type *C. reinhardtii* cells grown on solid medium containing a limited amount of SO_4^{2-} export the ARS enzyme into

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the periplasmic space or into the agar medium (in strains lacking a cell wall). The activity of this enzyme can be detected using the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulfate; hydrolysis of this substrate by ARS results in the formation of a blue precipitate. This assay was employed in a screen to identify insertional mutant strains (using the *AphVIII* gene as an insertion marker that confers transformants with paromomycin resistance) that cannot synthesize ARS following S deprivation (Pollock et al., 2005). One of the mutants identified in this screen, designated *ars73a*, exhibited almost no ARS activity following growth on Tris-acetate-phosphate medium without sulfur (TAP-S; Fig. 1A, both *ars73a* and BC; the latter is the backcrossed *ars73a* strain), while high levels of activity are detected in the parental strain. The mutant displayed no obvious phenotype when grown on S-replete medium (e.g. cell growth was similar to that of the parental strain) except for a loss of motility, which was unlinked to the insertion of the *AphVIII* gene (see below).

To determine whether the *ars*$^-$ mutant phenotype (dramatic reduction in ARS activity) cosegregated with the introduced *AphVIII* gene, the original *ars73a* strain was crossed to a wild-type *C. reinhardtii* strain. Approximately 500 random progeny were scored for growth on paromomycin-containing medium and ARS activity on medium devoid of S. The loss of ARS activity always cosegregated with the paromomycin resistance phenotype, demonstrating a strong linkage between the *AphVIII* insertion and the loss of ARS activity. Linkage was also analyzed using the backcrossed *ars73a* mutant. The original *ars73a mt*$^+$ was backcrossed three times to 21gr (another wild-type strain), the *ars73a mt*$^-$ progeny from the third backcross were crossed to 21gr *mt*$^+$, and 15 full tetrads were analyzed to determine if the paromomycin resistance phenotype was linked to the *ars*$^-$ phenotype. All 15 tetrads showed a 1:1 segregation of the paromomycin-resistant to paromomycin-sensitive phenotype, indicating a single insertion of the *AphVIII* gene. Furthermore, the resistant phenotype always cosegregated with the loss of ARS activity. The phenotype of the progeny of four of the analyzed tetrads is shown in Figure 1B. These results demonstrate linkage between the *ars*$^-$ phenotype and insertion of the marker gene. Furthermore, the paralyzed flagella phenotype, which was observed in the original mutant strain, segregated from the paromomycin-resistant and *ars*$^-$ phenotypes, demonstrating that a lesion causing flagella dysfunction in the original mutant background was not linked to the *AphVIII* insertion. Finally, western-blot analysis demonstrated that *ars73a* did not synthesize any ARS polypeptide following S deprivation of the cells (Fig. 1C).

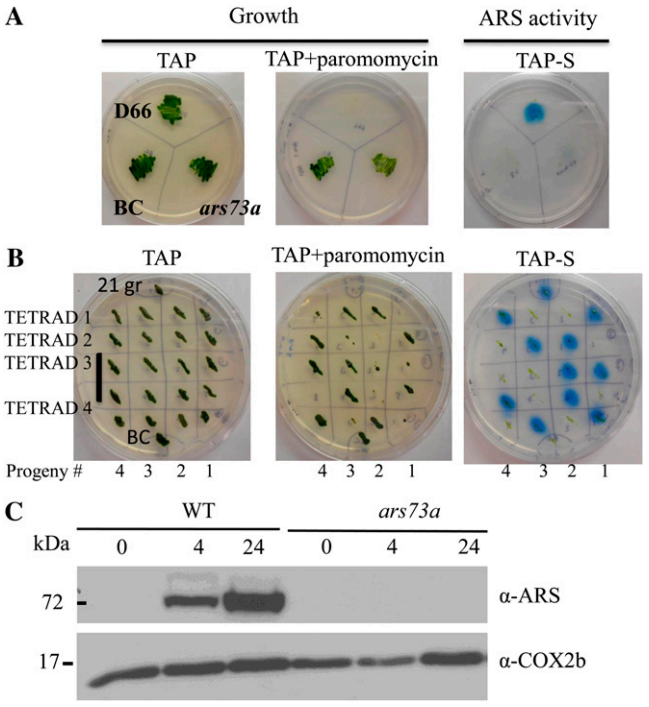


Figure 1. ARS activity and genetic linkage analysis. A, Phenotypic analysis of the *ars73a* mutant determined by assaying strains for ARS activity on solid medium. The parental, wild-type strain D66, the *ars73a* mutant, and the backcrossed mutant (BC; backcrossed to 21gr) were spread on TAP-S solid medium, and after the cells grew for 7 d, they were sprayed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl sulfate. Note that the *ars73a* mutant has almost no ARS activity, while essentially no activity is detected in the BC strain. Only the original mutant and the mutant derived from the backcross grew on paromomycin, as expected. B, Linkage analysis of the BC *ars73a* strain. The BC mutant was crossed to 21gr *mt*$^+$, and 15 full tetrads were analyzed for both paromomycin resistance and ARS activity. All of the paromomycin-resistant progeny exhibited an *ars*$^-$ phenotype. Results from four of the tetrads are shown. Tetrad 3 has eight progeny, while all of the others have four progeny. C, Western-blot analysis of the ARS polypeptide in the wild type (WT; 21gr) and the *ars73a* mutant. *C. reinhardtii* cells were grown in TAP medium and then transferred to TAP-S medium. Samples were collected just before and 4 and 24 h (indicated above the blot) after the removal of S from the medium. The COX2b subunit of cytochrome *c* oxidase served as a loading control. Molecular masses (in kD) are shown on the left.

Mapping Insertion Sites in the *ARS73a* Gene

Initially, adaptor ligation-mediated PCR and inverse PCR methods (Pollock et al., 2003) were used to locate the *AphVIII* marker gene (Fig. 2A) in the genome of the *ars73a* mutant. The sequence of the PCR product (Pollock et al., 2005) indicated that the marker gene insertion was in the sixth intron of gene model C_523864 (Chlre v4; http://genome.jgi-psf.org/chlamy/chlamy.info.html). To further characterize the insertion site and to determine whether the mutant harbors a single copy of the *AphVIII* gene (e.g. a single restriction fragment with an inserted paromomycin cassette), we performed DNA-blot hybridizations to genomic DNA digested with *Pst*I (this enzyme does not cut in the *AphVIII* gene) using the entire *AphVIII* cassette as a hybridization probe as well as a probe that hybridizes to most of intron 6 and 283 nucleotides of exon 7 of *ARS73a* (designated "probe" in Fig. 2B). The *AphVIII* cassette (Fig.

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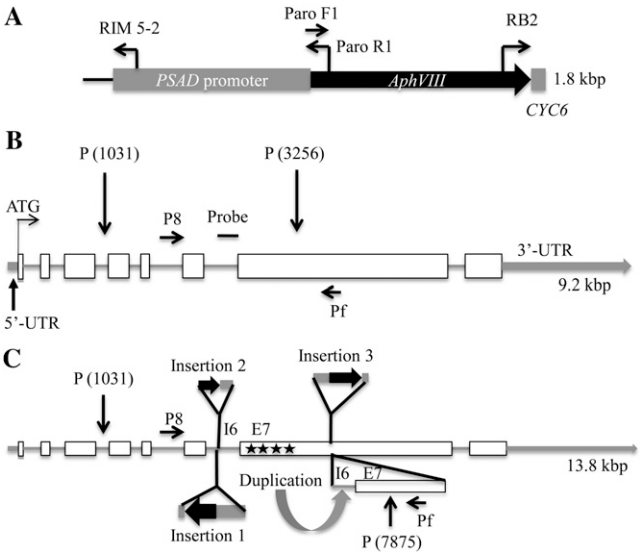


Figure 2. Maps showing the insertion cassette and the *ARS73a* genomic regions in wild-type cells and the *ars73a* mutant. A, Schematic representation of the *AphVIII* cassette with the positions of the primers used for genome walking, the location of the *PSAD* promoter at the 5′ end, and the location of the *CYC6* sequence at the 3′ end. B, Schematic representation of the *ARS73a* gene model in wild-type cells. Exons are represented with white boxes, introns with gray lines, and UTRs with thick gray lines. The position of the probe used for DNA-blot hybridizations is shown as a horizontal line. P8 and Pf are the primers used for genotyping. C, Schematic representation of insertions in the genomic DNA of the *ars73a* mutant. Genome walking was performed using PCR from both sides of *AphVIII* (present in insertions 1 and 3) and into the *ARS73a* sequence (see "Materials and Methods"); the resulting PCR products were sequenced and aligned to the sequence on the genome browser. Alignment of the sequenced reads suggested the presence of three insertions in the gene. Insertion 1 (in intron 6) was a complete cassette inserted in the opposite orientation relative to *ARS73a*. Insertions 2 (also in intron 6) and 3 (in exon 7) were in the same orientation as the *ARS73a* gene, but they represented partial cassette sequences. Duplication of part of intron 6 (I6) and exon 7 (E7) is shown with a curved arrow. *Pst*I sites that are not susceptible to digestion are marked with black stars. The 6.8-kb *Pst*I (P) fragment from P(1031) to P(7875) is the hybridizing band shown in the DNA-blot hybridization in Supplemental Figure S1. Drawings are approximately to scale. For the precise positions of the insertions, see text and Supplemental Figure S3.

2A) also contains a 57-bp intron sequence of the *CYC6* gene (at the 3′ end of *AphVIII*) and the *PSAD* gene promoter. DNA-blot hybridization with these probes revealed a unique hybridizing band of approximately 6.8 kb that is only present in the mutant (hybridization results using the *AphVIII* cassette probe are shown in Supplemental Fig. S1), suggesting that the mutant has a single *AphVIII* insertion; the hybridization signal at approximately 5 kb, which is observed in both the wild-type and mutant strains, is a genomic fragment containing the endogenous *PSAD* gene. The paromomycin cassette is approximately 1.8 kb, and the sequence has no *Pst*I restriction site; therefore, a single insertion into the 2.2-kb *Pst*I fragment of wild-type cells [predicted size of the fragment based on the genomic sequence and represented in Fig. 2B between P(1031) and P(3256)] would yield a hybridizing band of approximately 4 kb. The larger molecular mass of the hybridizing fragment (approximately 6.8 kb) suggested that the fragment contained more than one cassette and/or had experienced a rearrangement near the insertion site.

Extensive PCR and full sequence data (of the 6.8-kb fragment from the mutant) demonstrated the presence of multiple insertions as well as a duplication of part of the genomic DNA. An insertion of the full-length cassette (in the opposite orientation relative to *ARS73a*) in intron 6 of *ARS73a* (insertion 1 in Fig. 2C) was immediately adjacent to a small insertion (insertion 2 in Fig. 2C) derived from the 3′ end of the cassette, followed by a third insertion (insertion 3 in Fig. 2C) of a 5′ truncated cassette in exon 7. This last insertion contains the complete *AphVIII* coding sequence but is missing part of the *PSAD* promoter (0.7 kb at the 5′ region of the cassette). The region of the *ARS73a* gene that was duplicated (indicated with a curved arrow labeled "Duplication") was from nucleotides 2,564 to 4,013 of *ARS73a*; the full *ARS73a* gene sequence is given in Supplemental Figure S2. Finally, the four *Pst*I sites present within the 6.8-kb *Pst*I fragment from mutant genomic DNA, all of which are in a 600-bp region of exon 7 (Fig. 2C, E7 and four adjacent stars), do not appear to be susceptible to endonuclease digestion. This loss of susceptibility could be a consequence of RNA interference-mediated methylation around the site of insertion. Multiple *AphVIII* insertions may elicit the synthesis of double-stranded RNA or RNA hairpins that could promote methylation (Huettel et al., 2007). An unusual CAG methylation found in plants can prevent *Pst*I digestion (Gruenbaum et al., 1981), although this has not been experimentally examined for *C. reinhardtii*. A map that shows the complex structure of the 6.8-kb *Pst*I fragment with the various insertions is presented in Figure 2C. The full nucleotide sequence of the 6.8-kb fragment (from the *ars73a* mutant) is given in Supplemental Figure S3. We cannot rule out the possibility that other fragments of the cassette integrated into other regions of the genome, although if this were the case the integrated fragments would likely be very small, since no other restriction fragments were observed to hybridize to the *AphVIII* cassette.

The results presented in this section clearly demonstrate a disruption of the *ARS73a* gene, with insertions in both intron 6 and exon 7. These insertions in the mutant strain are likely to completely eliminate the synthesis of a functional ARS73a protein. Furthermore, these results emphasize that some lesions generated during the insertional inactivation of genes could result in complex changes in the genomic DNA in the neighborhood of the insertion.

Cycloheximide Impacts the Expression of a Subset of S Deprivation-Responsive Genes

When *C. reinhardtii* is deprived of S, several transcripts, including those encoding the high-affinity SO_4^{2-}

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transporters, ARS, and enzymes integral to SO_4^{2-} assimilation, increase in abundance. We explored the induction kinetics of subsets of S deprivation-responsive genes in both wild-type cells and the *ars73a* mutant. Transcripts encoding the SO_4^{2-} transporters (SULTR2, SLT1, and SLT2), a putative amino acid transporter (AOT4), and the selenobinding protein (SBDP) increased and reached peak levels very quickly (within approximately 30 min) following the imposition of S deprivation; the levels of these transcripts remained high over the 24-h deprivation period imposed on the cells (Fig. 3). In contrast, transcripts encoding arylsulfatases (ARS1 and ARS2), *O*-acetyl-Ser (thiol)lyase (OASTL4), an extracellular polypeptide (ECP76), a light-harvesting polypeptide (LHCBM9), and a putative haloperoxidase (HAP2) gradually increased during the first 4 h of S deprivation, while the mRNA encoding SULTR1 gradually declined (Fig. 3).

As expected, the accumulation of all transcripts was markedly inhibited by administration of the transcriptional inhibitor actinomycin D to the culture at the time of S removal (compare Fig. 4, A and B; note the log scale on the *y* axis). Incomplete inhibition of transcript accumulation may be a consequence of the completion of transcription that had already been initiated and/or a decrease in the degradation rate in the presence of the antibiotic. The results are strikingly different when cycloheximide, which inhibits translation on 80S cytoplasmic ribosomes, was added to the culture medium as the cells were depleted of S. The inhibition of cytosolic protein synthesis by cycloheximide strongly inhibited the S deprivation-elicited increase in the levels of transcripts encoding *ARS1*, *ARS2*, *ECP76*, *LHCBM9*, *SBDP*, and *HAP2* (compare Fig. 4, A and C); the levels of these transcripts were similar in actinomycin D- and cycloheximide-treated cells. Indeed, the cells were behaving as if the S deprivation-dependent increase in mRNAs normally observed for this set of transcripts was blocked. In contrast, increases in the level of transcripts encoding the high-affinity SO_4^{2-} transporters (SULTR2, SLT1, and SLT2) and a putative amino acid transporter (AOT4) were not altered in the presence of cycloheximide (but were strongly altered when actinomycin D was added to the medium).

Although cycloheximide did not significantly impact the expression of *SULTR2*, *SLT1*, and *SLT2*, it did cause an elevated accumulation of the *SULTR1* transcript (Fig. 4C). This result may reflect the translational inhibitor-mediated stabilization of the transcripts, possibly by direct inhibition of turnover or by protection of the transcripts in ribosomal complexes (Lopez et al., 1998). The mRNA level of the housekeeping gene *CBLP* was shown to remain constant during S deprivation (Chang et al., 2005); however, an increase in the *CBLP* transcript level was also observed when cycloheximide was administered to cells following the removal of S from the medium (data not shown), supporting the argument that cycloheximide can generally impact mRNA stability. Finally, chloramphenicol, a translational inhibitor of 70S ribosome (inhibits translation in mitochondria and plastids), had essentially no impact on the expression of S deprivation-responsive genes (Fig. 4D).

The results concerning the impact of inhibitors of translation on transcript abundance suggest that there are at least two tiers of transcriptional regulation in response to S deprivation. The first tier of regulation controls the transcription of the SO_4^{2-} transporters and does not require protein synthesis following the imposition of deprivation conditions. In contrast, a second, protein synthesis-dependent tier of regulation controls many genes encoding enzymes involved in SO_4^{2-} assimilation and restructuring of the cell during S deprivation.

Expression of S Deprivation-Responsive Genes in *ars73a*

To study the responses of *ars73a* to S deprivation, transcript abundances from several genes previously shown to be controlled in wild-type cells by the S status of the medium (*SULTR1*, *SULTR2*, *SLT1*, *SLT2*, *AOT4*, *ARS1*, *ARS2* for ARS, *ECP76*, *SBDP*, *LHCBM9*, *HAP2*, and *OASTL4*), as presented in Figure 3, were analyzed in the *ars73a* mutant (Fig. 5). As expected from measurements of ARS activity in the *ars73a* mutant, *ARS1* and *ARS2* transcripts did not markedly increase (2–3 orders of magnitude less than in wild-type cells) after transferring the mutant to medium devoid of S. The levels of the *ECP76*, *LHCBM9*, *HAP2*, and *SBDP* transcripts were also markedly diminished in the mutant relative to the wild-type strain (compare Fig. 5, A and C). In contrast, the abundance of transcripts for the three inducible SO_4^{2-} transporters (*SULTR2*, *SLT1*, and *SLT2*) and the putative

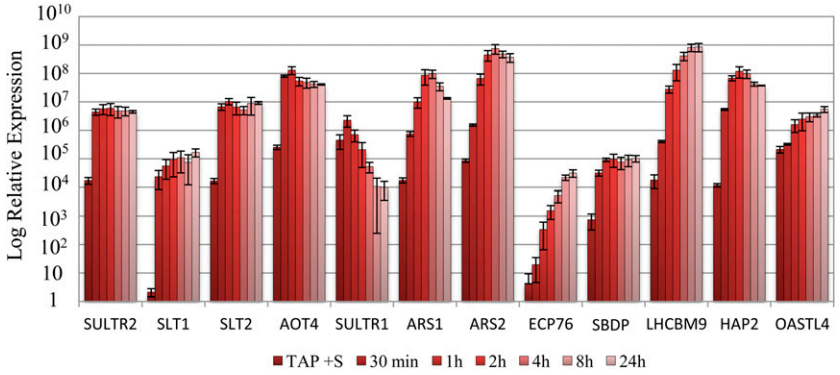


Figure 3. Changes in transcript abundances for various S deprivation-responsive genes following the removal of S from the medium. Levels of transcripts encoding SULTR2, SLT1, SLT2, AOT4, SULTR1, ARS1, ARS2, ECP76, SBDP, LHCBM9, HAP2, and OASTL4 were measured by RT-qPCR. RNA samples were isolated from wild-type cells (21gr) grown in S-replete medium and after being transferred to S-depleted medium for 30 min and 1, 2, 4, 8, and 24 h. [See online article for color version of this figure.]

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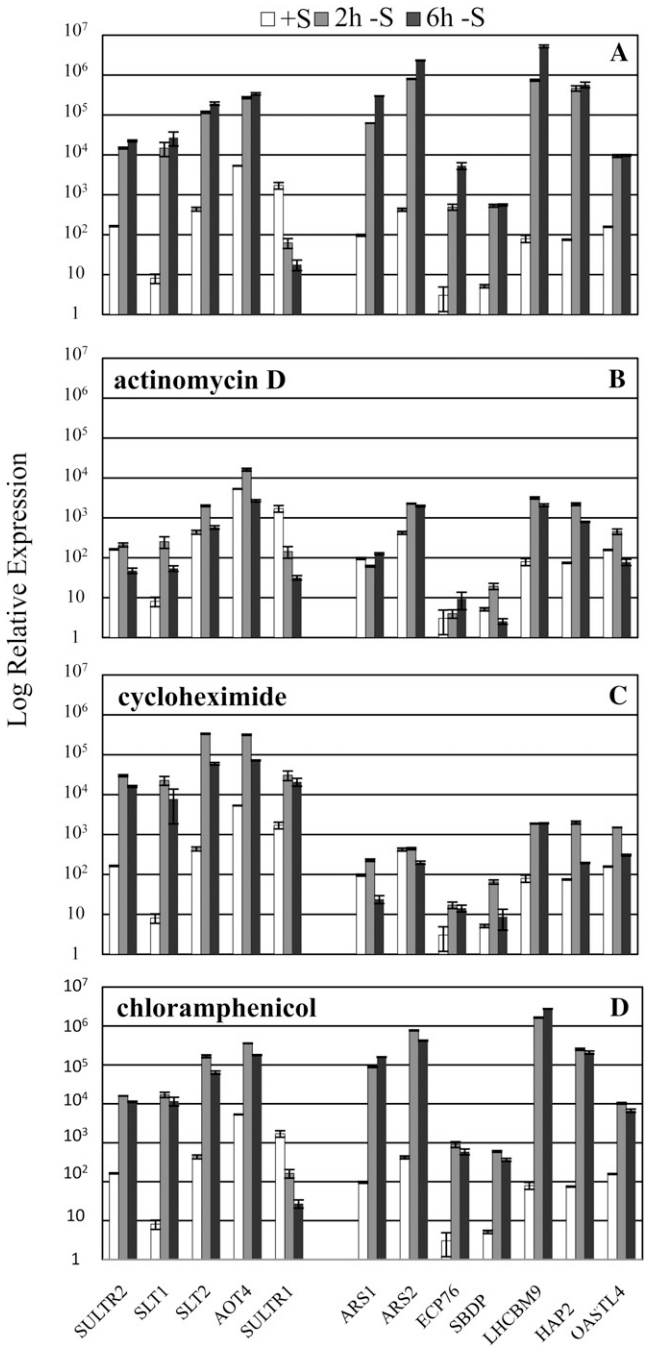


Figure 4. Effects of transcriptional and translational inhibitors on the accumulation of S deprivation-responsive transcripts. Levels of transcripts encoding SULTR2, SLT1, SLT2, AOT4, SULTR1, ARS1, ARS2, ECP76, SBDP, LHCBM9, HAP2, and OASTL4 were measured by RT-qPCR. Wild-type cells (21gr) were grown in TAP medium and then transferred to TAP-S medium either in the absence of an inhibitor (A) or in the presence of the transcriptional inhibitor actinomycin D (B), the cytosolic translational inhibitor cycloheximide (C), or the organellar translational inhibitor chloramphenicol (D). Inhibitors were added to the cultures at the same time as S was removed. Samples were taken prior to starvation and at 2 and 6 h after removal of S from the medium, which is shown with white, gray, and black bars, respectively.

amino acid transporter (*AOT4*) were not significantly affected in the mutant (compare Fig. 5, A and C). Even more remarkable, the pattern of gene expression observed in the mutant was very similar to that of wild-type cells treated with cycloheximide (compare Fig. 5, B and C). For some S deficiency-induced genes (e.g. *ARS1*, *ARS2*, *SBDP*, and *LHCBM9*), a further reduction (although generally small) in transcript levels occurred following the addition of cycloheximide to *ars73a* cells deprived of S (Fig. 5D), suggesting the possible participation of another regulatory factor(s) in controlling the activity of these genes.

In accord with the transcript abundance measurements, SULTR2, SLT1, and SLT2 polypeptides accumulated to approximately the same extent in wild-type cells and the *ars73a* mutant during S deprivation (for epistasis analysis, see below). Furthermore, there was essentially no accumulation of ARS proteins in *ars73a* upon S deprivation (Fig. 1C), as expected from measurements of both ARS activity and transcript levels.

In sum, our results suggest that (1) there are two tiers of S deprivation responses, one independent of (first tier) and the other requiring (second tier) protein synthesis, and (2) the ARS73a protein is required for manifestation of the majority of second tier regulation.

Rescue of the Mutant Phenotype

Over a period of several months, we noted that the *ars73a* mutant exhibited some suppression of the *ars*$^-$ phenotype: low levels of ARS activity were observed in colonies of S-deprived mutant cells. Therefore, we backcrossed the mutant with 21gr to remove putative suppressor mutations, regenerating progeny with essentially no ARS activity. We then cotransformed the backcrossed strain with BAC39M22 and the plasmid pSP124S; the latter contains the bacterial *ble* gene, which provides a selection (resistance to zeocin) for potential cotransformants. BAC39M22 is small (approximately 42 kb of genomic DNA from chromosome 6 plus the approximately 12-kb pBACmn vector) and contains the *ARS73a* genomic locus as well as part of an upstream gene (model C_523863, encoding a putative amino acid transporter) and a downstream gene (model C_523865, encoding a protein with significant homology to ARS73a; an alignment of the two sequences is presented in Supplemental Fig. S4). To specifically test if disruption of the *ARS73a* gene was responsible for the mutant phenotype, BAC39M22 DNA was digested with *Sma*I, which does not cut within *ARS73a* but does cleave the downstream and upstream genes, and the digested DNA plus pSP124S were introduced into *ars73a* by electroporation; transformants were selected on Tris-acetate-phosphate (TAP) medium containing 5 to 10 μg mL^{-1} zeocin. Zeocin-resistant colonies were picked, transferred onto solid TAP medium, and allowed to grow for approximately 1 week. Cells from each colony were then transferred onto solid TAP-S medium, allowed to grow for 7 d, and then assayed for ARS

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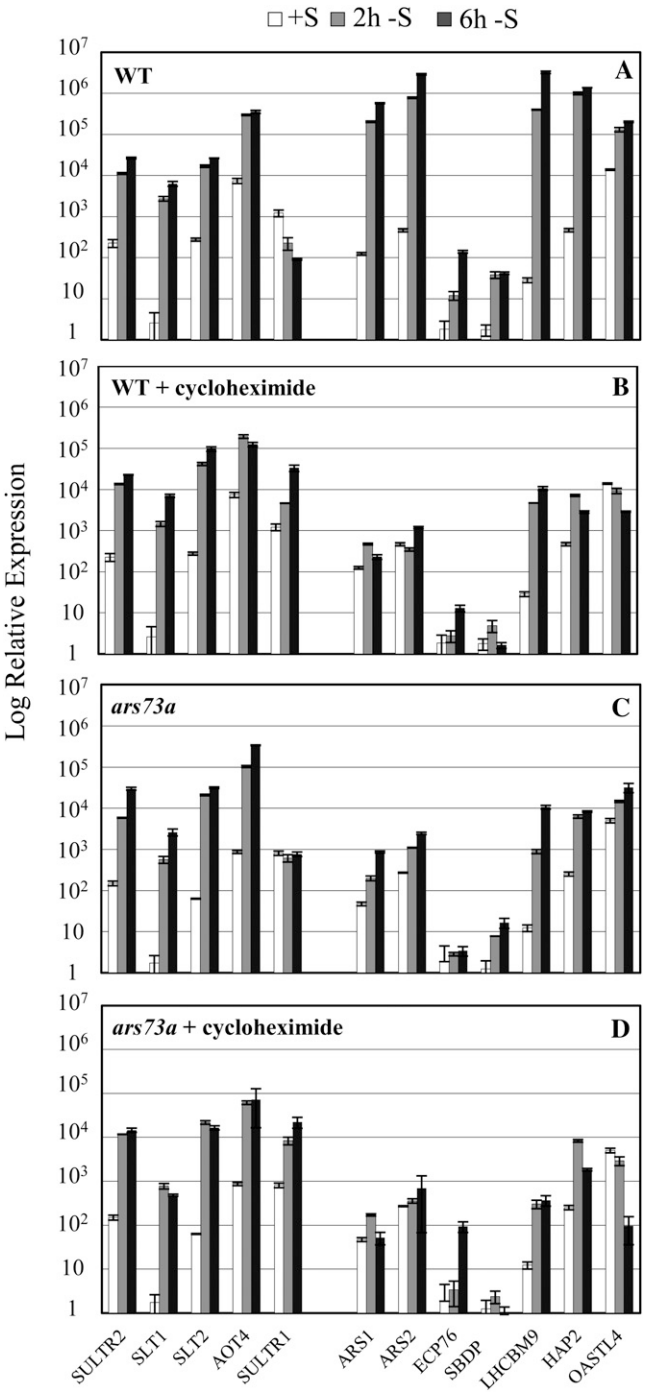


Figure 5. Transcript abundance analyzed by RT-qPCR in wild-type cells (WT; 21gr) and the *ars73a* mutant. Accumulation of *SULTR2, SLT1, SLT2, AOT4, SULTR1, ARS1, ARS2, ECP76, SBDP, LHCBM9, HAP2,* and *OASTL4* transcripts in wild-type cells (A and B) and the *ars73a* mutant (C and D) was monitored. RNA samples were collected prior to and 2 and 6 h after the cells were transferred from TAP to TAP-S medium in the presence (B and D) or absence (A and C) of the cytosolic translational inhibitor cycloheximide.

activity. Eleven strains out of approximately 500 zeocin-resistant transformants tested exhibited ARS activity. These strains were genotyped by PCR using primers between exon 4 and exon 7 of *ARS73a* (the primer pairs flank all the insertions) to identify strains with wild-type and mutant copies of *ARS73a*. Based on this analysis, of the 11 ARS^+ strains 10 contained a copy of the wild-type *ARS73a* gene. The transformant that did not have a wild-type copy of the gene exhibited much lower ARS activity relative to the 10 other ARS^+ transformants, suggesting the occurrence of low-frequency second-site suppression that shows partial restoration of ARS activity, which is consistent with what we observed for the original *ars73a* mutant population. We also transformed the mutant with the pSP124S vector alone and observed some transformants (less than 1%) with very low-level ARS activity (none of the suppressor strains ever had high ARS activity). The ARS activity in these strains was always less than 10% of that observed in wild-type cells or in *ars73a* mutant cells harboring a wild-type copy of *ARS73a*. These findings provide further evidence for suppression of the ars^- phenotype at a significant frequency. Future analyses of these suppressor strains might help elucidate processes that contribute to the expression of S-responsive genes.

To further confirm that the *ARS73a* gene was responsible for rescue of the mutant phenotype, we crossed one of the rescued *ars73a* mutants to the original *ars73a* mutant and analyzed 17 zygotes for segregation of paromomycin resistance, ARS activity, and the presence of the introduced copy of *ARS73a* (the wild-type copy of the gene introduced into the rescued mutant). One-half of the progeny showed high levels of ARS activity, and the other one-half had no ARS activity. Furthermore, based on PCR genotyping (generation of a 2,250-bp product using primers that amplify the gene sequence from exon 4 to exon 7, containing the entire region with the insertions), only those progeny that exhibited ARS activity retained the wild-type copy of *ARS73a*; progeny devoid of ARS activity had lost the wild-type copy of the gene, which was manifest as a lack of a PCR product (the region between the primers in the mutant strain is too long to be amplified with the extension time used; they would generate a 6.9-kb product). An analysis of two tetrads of the cross described above is shown in Figure 6. All of the progeny of the tetrads were paromomycin resistant, with only two of the four expressing ARS activity (Fig. 6A). The same colonies that exhibited ARS activity also harbored the introduced, wild-type copy of the *ARS73a* gene (Fig. 6B). The rescued strain (for ARS activity), 2-3A, and its rescued progeny, P3, also showed normal expression of *LHCBM9* and *ECP76* (Fig. 6C), while the original *ars73a* mutant and P1, a mutant progeny, had lower transcript levels. Increased mRNA levels following S deprivation were observed for all rescued strains; an increase in ECP76 transcript levels was especially notable. These results demonstrate that the *ARS73a* gene product is a major element that controls second tier regulation of the S deprivation responses in *C. reinhardtii*.

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ARS73a Transcripts

We performed 5′ and 3′ RACE to identify the ends of the *ARS73a* mRNA. Based on these results, the 3′ untranslated region (UTR) is 24 nucleotides shorter than what has been reported in the *C. reinhardtii* database (http://genome.jgi-psf.org/chlamy/chlamy.info.html), while the 5′ RACE product terminated 299 bp upstream of the predicted translation start site.

To assay for the presence of *ARS73a* mRNA in *ars73a* and the 21gr wild-type strain, reverse transcription (RT)-PCR was performed with a pair of primers, one of which anneals to exon 4 and the other to exon 7; the expected size of the wild-type PCR product is 596 bp, while the amplified product in the *ars73a* mutant would potentially include part of insertion 1 (which is inserted into intron 6).

Under nutrient-replete conditions, 21gr and the rescued strain (2-3A) had low but detectable levels of *ARS73a* mRNA, represented by a faint band at 596 bp. The level of this band increased dramatically when the cells were shifted to S deprivation conditions (Fig. 7). The identity of the 596-bp band amplified from RNA extracted from cells grown under nutrient-replete and S deprivation conditions was confirmed by sequencing the isolated band. A lower molecular mass RNA observed in nutrient-replete 21gr and the 2-3A rescued strain, also present in low abundance, was not sequenced; the identity of this product is not known. In contrast, little mRNA was detected in either the original or backcrossed *ars73a* mutant when the cells were grown under nutrient-replete conditions (Fig. 7). However, when the mutant cells were shifted to S deprivation conditions, they accumulated low levels of two transcripts: one transcript is approximately 596 bp (the expected size) and the other is 733 bp (Fig. 7). The two different transcripts in the mutant cells were sequenced. The 596-bp product had the expected sequence. The higher molecular mass product contained a partial *AphVIII* sequence in the opposite orientation relative to *ARS73a* (in accord with our finding that the insertion in intron 6 is in the opposite orientation relative to *ARS73a*; Fig. 2C); this sequence was located between exons 6 and 7. We also observed a partial deletion of exon 6 in the aberrant 733-bp mRNA (data not shown). The sequences from the amplified RNA segments identified in mutant cells are presented in Supplemental Figure S5.

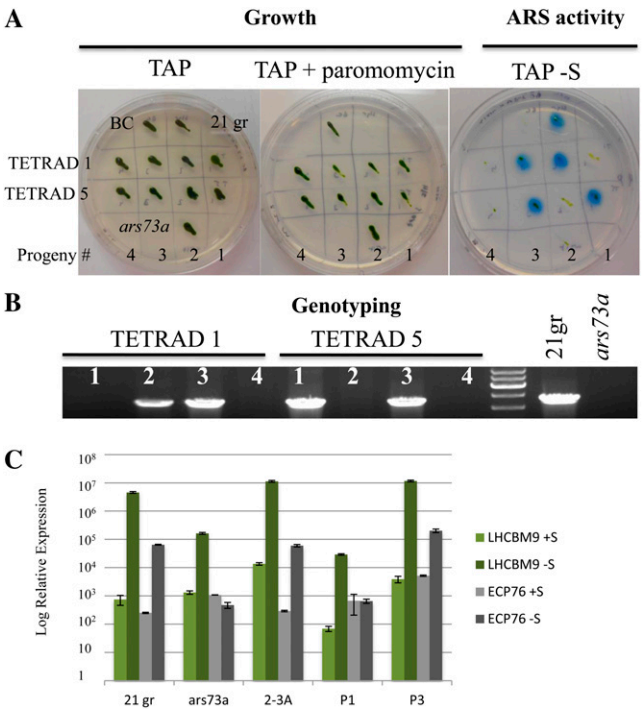


Figure 6. Analysis of progeny from the complemented strain (2-3A). A, A rescued mt^- strain was crossed to *ars73a* mt^+, and the progeny were analyzed for both growth on paromomycin and ARS activity, which is shown for two tetrads (TETRAD 1 and TETRAD 5). B, All of the progeny that displayed ARS activity harbored a wild-type copy of *ARS73a*, as indicated by the presence of a 2,250-bp PCR product. Progeny devoid of ARS activity did not have a wild-type copy of *ARS73a* (no PCR product detected; see text). PCR results from genomic DNA isolated from 21gr (wild-type cells) and the *ars73a* mutant are shown as controls. C, Analysis of the rescued strain (2-3A) and its rescued progeny (P3 of TETRAD 1) for levels of *LHCBM9* and *ECP76* transcripts after 6 h of S deprivation. Similar transcript analyses for progeny that are not rescued (P1 of TETRAD 1), for 21gr, and for *ars73a* are also shown.

We also examined the accumulation of transcripts from the gene (C_523865) downstream of *ARS73a* and found that it was the same in D66 and the *ars73a* mutant under both S-replete and S-depleted conditions, with no aberrant mRNAs detected based on a number of different primer pairs used for RT-PCR analyses (data not shown). These results suggest that the insertions in *ars73a* did not impact expression of the downstream gene. The control for all of these experiments was RT-PCR of the transcript encoding CBLP, a *C. reinhardtii* G-protein β-subunit-like protein. There was no difference in the level of this transcript under nutrient-replete and S deprivation conditions or between the mutant and the D66 parental strain.

Regulation of *ARS73a* Expression

We also examined whether the *ARS73a* gene exhibited a first or a second tier response to S deprivation.

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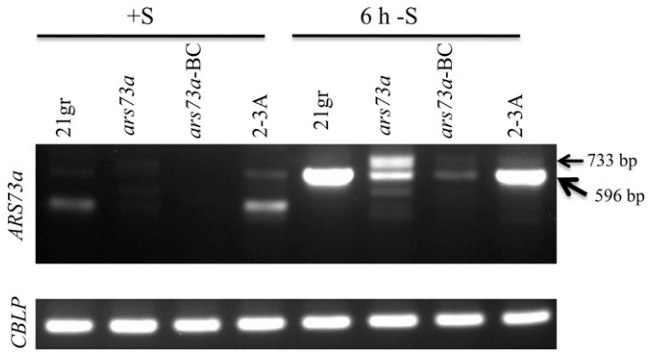


Figure 7. *ARS73a* mRNA in 21gr (wild type), *ars73a*, *ars73a*-BC (backcrossed *ars73a*), and the rescued strain (2-3A). The cells were grown under S-replete conditions or exposed to 6 h of S-depleted conditions prior to RNA isolation. First-strand cDNA was amplified with primers flanking the insertion in intron 6 (amplifying exons 4–7). The expected product derived from the wild-type transcript is 596 bp. A longer 733-bp product is observed in the *ars73a* mutant and the backcrossed mutant under S deprivation conditions. The *CBLP* transcript was used as a loading control. The sequences of these two mRNAs are given in Supplemental Figure S5.

RT-quantitative PCR (qPCR) was performed to determine *ARS73a* transcript abundances in S-replete, S-depleted for 6 h, or S-depleted and cycloheximide-treated cultures (Fig. 8). The *ARS73a* transcript level was elevated in S-depleted conditions in 21gr and the complemented strain (2-3A) but not in *ars73a*. While this experiment showed an increase in the transcript level after 6 h of S deprivation, in other experiments we observed increased transcript accumulation after 30 min of deprivation (data not shown). Cycloheximide treatment did not block this increased transcript accumulation, suggesting that up-regulation of *ARS73a* does not require de novo protein synthesis. Therefore, we concluded that the *ARS73a* gene itself is up-regulated as a first tier gene during S deprivation conditions.

Epistasis Analysis of the Double Mutants *sac1 ars73a*, *snrk2.1 ars73a*, and *snrk2.2 ars73a*

To place the ARS73a protein into the context of the S deprivation network of regulators that have already been examined, we analyzed the phenotype of *ars73a* in the *sac1*, *snrk2.1*, and *snrk2.2* genetic backgrounds. *SAC1* encodes a putative transmembrane SO_4^{2-} sensor (Davies et al., 1996). The SNRK2.1 and SNRK2.2 proteins both encode protein kinases, and the phenotypes of *snrk2.1* and *snrk2.2* mutants were described previously (Davies et al., 1999; González-Ballester et al., 2008).

The *sac1* mutant exhibits little increase in transcript levels from any of the S-responsive genes upon S deprivation (Fig. 9A; Zhang et al., 2004). Since the *ars73a* mutant also shows a strong depression of the level of second tier transcripts under S deprivation conditions (Fig. 5C), it is not possible to determine an epistasis relationship between *SAC1* and *ARS73a* based on the levels of second tier transcripts. However, up-regulation of first tier genes is generally low in the *sac1 ars73a* double mutant (Fig. 9C) relative to *ars73a* (Fig. 5C); the double mutant does not express first tier genes to the same extent as either *ars73a* or wild-type cells upon S deprivation. These results suggest that *SAC1* is epistatic to *ARS73A* with respect to the expression of first tier genes. Furthermore, the *snrk2.1* phenotype is clearly epistatic to *ars73a*; the double mutant exhibits little expression of any of the S-responsive transcripts that were analyzed (Fig. 9D), which is similar to what was observed for the *snrk2.1* single mutant (Fig. 9B). Interestingly, a whole-transcriptome analysis performed using RNA-seq showed that *snrk2.1* had almost no *ARS73A* transcript under S-replete conditions and had truncations of the *ARS73A* transcript in the 5′ and 3′ UTRs under S-depleted conditions (see RNA-seq data on the University of California, Santa Cruz genome browser at http://genomes.mcdb.ucla.edu/; Supplemental Fig. S6). These results suggest that *ARS73A* expression may be controlled by the SNRK2.1 kinase.

Finally, we generated a *snrk2.2 ars73a* double mutant. *SNRK2.2* encodes a Ser/Thr kinase that acts as a negative regulator of S deprivation-responsive genes under nutrient-replete conditions (Davies et al., 1999). The *snrk2.2* mutant has low constitutive ARS activity under nutrient-replete conditions. The double mutant has an *ars*$^-$ phenotype similar to *ars73a*; there is little constitutive accumulation of ARS activity under S-replete conditions, and ARS activity does not markedly increase under S-depleted conditions (Fig. 10A). Furthermore, transcripts from genes associated with the second tier of regulation do not accumulate to high levels when the double mutant is deprived of S (compare Fig. 10, B and C). These results suggest that *ARS73a* is epistatic to *SNRK2.2* with respect to the regulation of second tier genes. The increases in SULTR2, SLT1, and SLT2 transcripts were similar in the *snrk2.2* single mutant and the

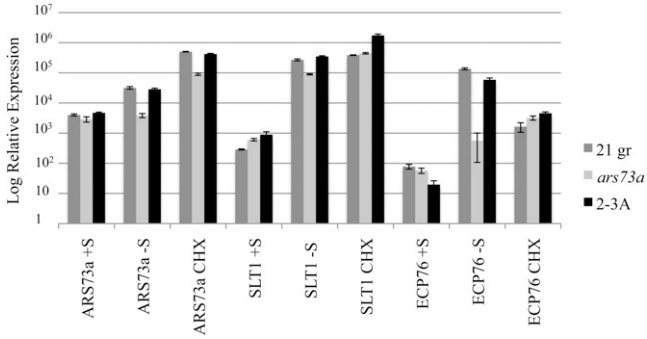


Figure 8. *ARS73a* transcript regulation. RNA was isolated from cultures of 21gr (wild type), *ars73a*, and 2-3A (complemented strain) grown in S-replete medium (+S) or 6 h after they were transferred to S-depleted medium without cycloheximide (−S) or with cycloheximide (CHX). Levels of transcripts for *ARS73a*, *SLT1* (as a control for first tier genes), and *LHCBM9* (as a control for second tier genes) were measured by RT-qPCR.

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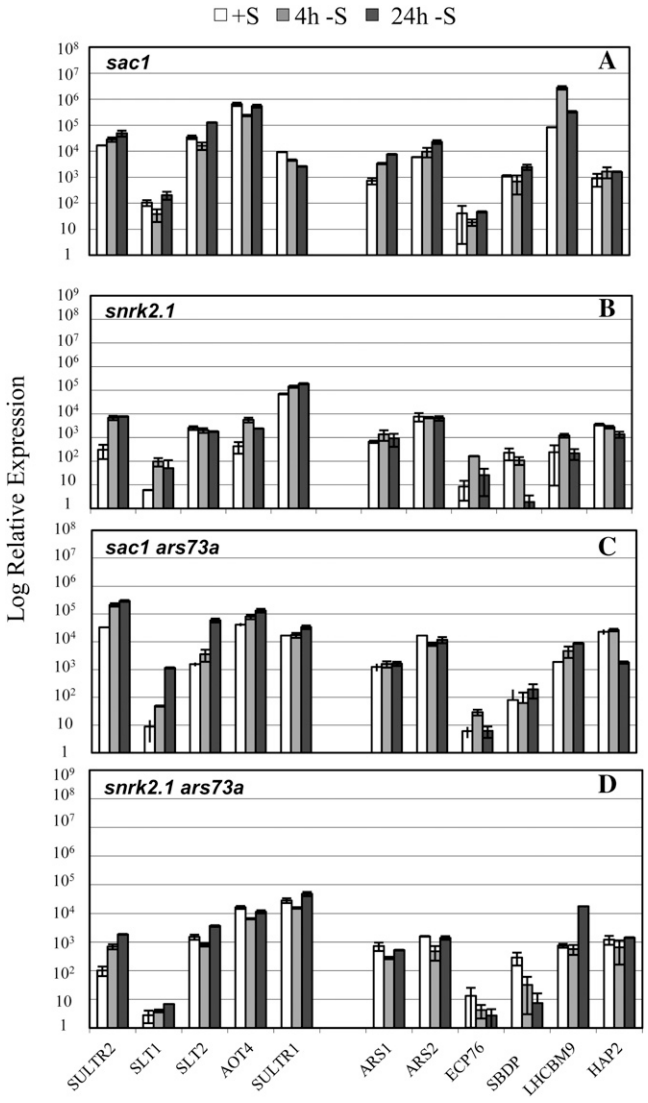


Figure 9. RT-qPCR analysis of transcript accumulation in *sac1*, *snrk2.1*, and the double mutants *sac1 ars73a* and *snrk2.1 ars73a*. Transcripts encoding *SULTR2*, *SLT1*, *SLT2*, *AOT4*, *SULTR1*, *ARS1*, *ARS2*, *ECP76*, *SBDP*, *LHCBM9*, and *HAP2* were quantified by RT-qPCR. RNA samples were isolated from cells grown in S-replete medium (white bars) and at 4 h (gray bars) and 24 h (black bars) following the transfer of cells to medium devoid of S.

snrk2.2 ars73a double mutant (compare Fig. 10, B and C). This is not surprising, since *ARS73a* does not appear to control the *SLT* or *SULTR* genes (Figs. 5, A and C, and 6) while *SNRK2.2* does (González-Ballester et al., 2008). It is interesting that while the *snrk2.2* mutant shows low-level constitutive expression of the transporter mRNA in S-replete medium and normal induction during S deprivation (González-Ballester et al., 2008), only low levels of the transporter proteins accumulate in the membranes in both the *snrk2.2* single mutant and the *snrk2.2 ars73a* double mutant (Fig. 11A), and both strains have low rates of SO_4^{2-} uptake (Fig. 11B) under S-replete and S-depleted conditions. These results suggest a further role (in addition to the suppression of transporter and *ARS* genes) of the SNRK2.2 kinase in the biogenesis of the transporters (for model, see Fig. 12).

DISCUSSION

Plants and algae have evolved a number of different acclimation strategies to accommodate changing levels of S in the environment. The utilization and assimilation of SO_4^{2-} by plants and other photosynthetic organisms and alterations in cellular processes linked to reduced S availability in the environment have recently been reviewed (Takahashi et al., 2011). Responses of *C. reinhardtii* to S deprivation have been studied for many years (de Hostos et al., 1988, 1989;

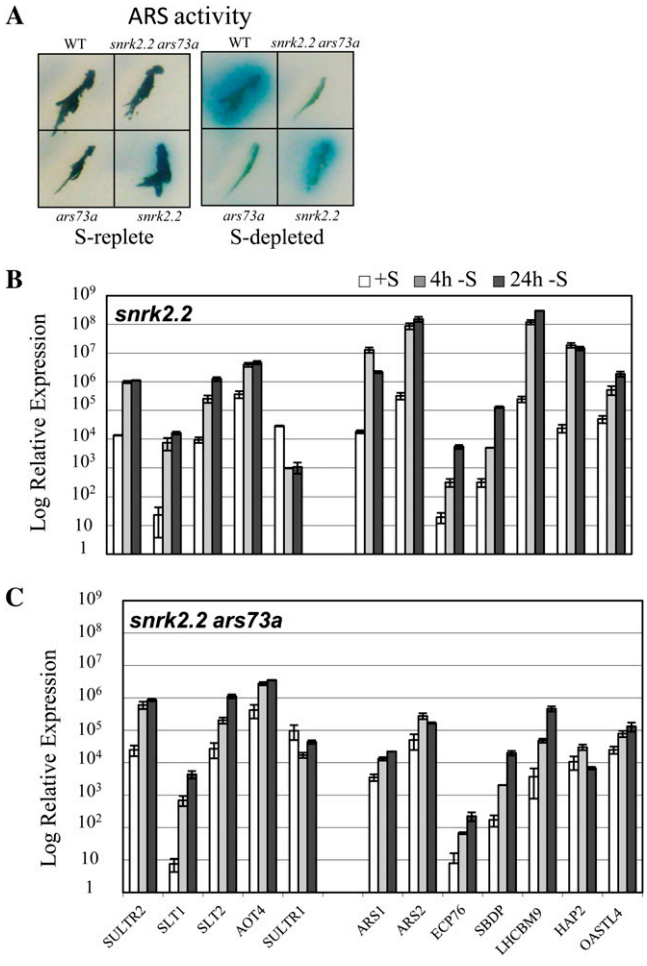


Figure 10. Epistasis analysis of *ARS73a* and *SNRK2.2*. A, ARS activity in the wild type (WT; 21gr), the individual mutants *snrk2.2* and *ars73a*, and the *snrk2.2 ars73a* double mutant on S-replete and S-depleted medium. B and C, RT-qPCR showing the levels of *SULTR2*, *SLT1*, *SLT2*, *AOT4*, *SULTR1*, *ARS1*, *ARS2*, *ECP76*, *SBDP*, *LHCBM9*, *HAP2*, and *OASTL* transcripts in the *snrk2.2* single mutant (B) and *snrk2.2 ars73a* double mutant (C).

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Davies et al., 1994, 1996, 1999; Yildiz et al., 1994, 1996; Takahashi et al., 2001; Zhang et al., 2004; Chang et al., 2005; González-Ballester et al., 2008, 2010; Moseley et al., 2009; Pootakham et al., 2010), and one of the first responses of this alga to S limitation was shown to be the activation of genes encoding high-affinity SO_4^{2-} transporters (Yildiz et al., 1994; González-Ballester et al., 2010; Pootakham et al., 2010). Increased expression from high-affinity SO_4^{2-} transporters has been noted to occur within 30 min of the removal of S from the culture medium (Fig. 3; Pootakham et al., 2010). Other S deprivation responses, including the synthesis and secretion of ARS, require longer periods of acclimation to be detected; the ARS transcript is detected 30 min following the imposition of S deprivation, but it continues to increase for an additional approximately 3 h (Fig. 3).

To better understand the temporal expression of S-responsive genes and their regulatory features, we measured the expression levels of various genes that have been associated with the S deprivation responses, with or without inhibitors of transcription and translation. Interestingly, cycloheximide treatment had little impact on the accumulation of transcripts encoding the previously identified, inducible SO_4^{2-} transporters (SULTR2, SLT1, and SLT2) and AOT4, a putative amino acid transporter. However, administration of cycloheximide immediately prior to the imposition of S deprivation appeared to block the accumulation of transcripts encoding ARS, ECP76, SBDP, LHCBM9, HAP2, and OASTL4. These results suggest that (1) there are at least two tiers of regulation associated with S deprivation responses; (2) no protein synthesis is required for the first tier, which involves the regulation of genes encoding the high-affinity SO_4^{2-} transporters; and (3) protein synthesis is required for the second tier, which involves regulation of the *ARS* genes as well as genes associated with SO_4^{2-} assimilation and cellular reorganization. Our results demonstrate that the regulatory elements for first tier control are already present in cells under nutrient-replete conditions. First tier regulation appears to be dependent on a putative external sensor of SO_4^{2-}, designated SAC1 (Davies et al., 1996; Zhang et al., 2004), as well as the potential internal regulatory element SNRK2.1 (González-Ballester et al., 2008, 2010). Soon after *C. reinhardtii* cells are transferred to S deprivation conditions, the regulatory elements (e.g. SAC1 and SNRK2.1) become active, potentially through some protein modification and/or changes in ligand binding, and first tier genes are activated. In contrast, de novo protein synthesis is required for the second tier of gene regulation, although we are only just beginning to identify the control elements involved in this process.

The advantage of maintaining a tiered acclimation response is that it would allow the cell to make efficient use of its energy budget by only mobilizing second tier control processes under persistent nutrient limitation conditions. The initial activation of genes encoding SO_4^{2-} transporters would allow for the efficient utilization of even low levels of environmental SO_4^{2-}. If the availability of SO_4^{2-} increases during this initial phase of acclimation, the cells would not have to implement additional strategies associated with scavenging, conservation, and metabolic control. The energetically/metabolically costly synthesis of proteins involved in second tier synthesis may not be necessary in many instances (e.g. short-term fluctuation in S availability). However, if the cells experience long-term S deprivation, then additional modes of scavenging, such as accessing SO_4^{2-} bound to organic molecules in the environment through the synthesis of ARS enzymes and restructuring cellular processes through the synthesis of LHCBM9 and the various ECPs, may help sustain cell viability.

In the course of examining *C. reinhardtii* mutants that were unable to acclimate to S deprivation, we noted that a strain designated *ars73a* exhibited no ARS activity when it was starved for S, but it appeared to express *C. reinhardtii* genes encoding the SO_4^{2-} transporters (Fig. 5), which were previously shown to be required for the efficient uptake of SO_4^{2-} under S deprivation conditions (Pootakham et al., 2010). Further characterization of the mutant strain indicated that while first tier genes were

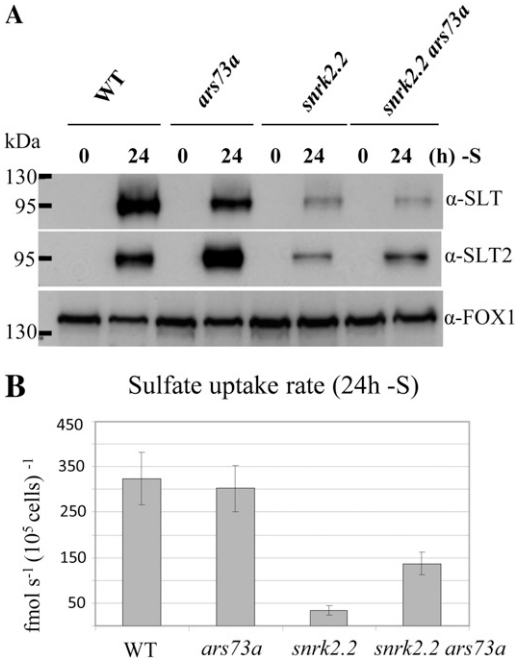


Figure 11. Epistasis analysis of *ARS73a* and *SNRK2.2*. A, Accumulation of proteins encoding the high-affinity SLT SO_4^{2-} transporters in the wild type (WT; 21gr), *ars73a*, *snrk2.2*, and *snrk2.2 ars73a* after 24 h of S starvation. A ferroxidase, FOX1, served as a loading control. B, SO_4^{2-} uptake in wild-type *C. reinhardtii* cells (21gr) and the *ars73a*, *snrk2.2*, and *snrk2.2 ars73a* mutant strains. Uptake rates were determined at 250 μM SO_4^{2-} (specific activity of 330 Ci mol^{-1}). The values are averages of a single experiment with three technical replicates, although similar results were obtained in independent experiments, and the error bars represent 1 SD.

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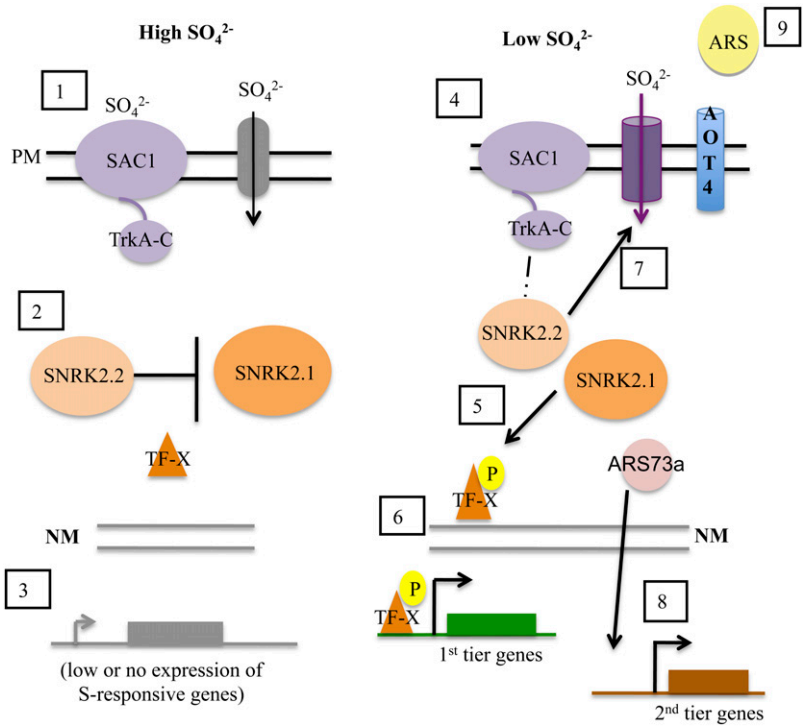


Figure 12. Model depicting regulatory events associated with the acclimation of *C. reinhardtii* cells to S deprivation. (1) Under S-replete conditions (high SO_4^{2-}), the plasma membrane (PM)-located SAC1 senses SO_4^{2-} levels in the environment by binding to SO_4^{2-}. This disengages SAC1 from downstream regulatory events. (2) Cytosolic SNRK2.2 represses the transcription of the S-responsive genes, possibly by inhibiting the activity of the positive regulatory factor SNRK2.1 (e.g. by phosphorylation of SNRK2.1). This interaction ensures that (3) there is low or no expression from S-responsive genes. (4) Under S deprivation conditions (low SO_4^{2-}), SAC1 is free of bound SO_4^{2-}, which allows it to interact with cytosolic SNRK2.2 (hypothesized interaction). This interaction prevents the inhibition of SNRK2.1, which can then (5) become active under S deprivation conditions and phosphorylate a downstream transcription factor (denoted TF-X; it has not been identified). This phosphorylation leads to the activation of first tier genes (6). (7) The SAC1-associated SNRK2.2 functions to help route the SO_4^{2-} transporters to the plasma membrane, integrate them into the membrane, or stabilize them once they are in the membrane. (8) The *ARS73a* transcript and protein are synthesized at high levels, and the protein (which may or may not be modified) directly (by involvement in transcription) or indirectly (by facilitating the translation of a transcription factor) is involved in the transcription of second tier genes. (9) One product of second tier gene activation is ARS, which is secreted to the extracellular space.

up-regulated to a similar extent as in wild-type cells, there was aberrant expression of second tier genes; the increase in expression during S deprivation was markedly reduced relative to wild-type cells (Fig. 5). These results raised the possibility that the ARS73a protein is synthesized along with the SO_4^{2-} transporters during first tier gene activation, followed by its participation in second tier regulation. Therefore, we analyzed ARS73a transcript abundance by RT-PCR with a primer pair that amplifies a region of the transcript between exon 4 and exon 7. Wild-type *C. reinhardtii* cells make the *ARS73a* transcript under both S-replete and S-depleted conditions, although the level significantly increases as the cells become S deprived (Fig. 7). RT-qPCR performed with primers that amplify the exon 2-exon 3 junction also showed that *ARS73a* transcription increases under S-depleted conditions (Fig. 8). These results are in accord with results reported in a genome-wide transcriptome analysis of *C. reinhardtii* cells that were deprived of S; an approximately 3-fold increase in ARS73a mRNA was noted for S-deprived wild-type cells (González-Ballester et al., 2010; Supplemental Fig. S6). Cycloheximide treatment did not block the increase in *ARS73a* transcript accumulation, which suggests that *ARS73a* is a first tier responsive gene (Fig. 8). Hence, the ARS73a protein may already be present in cells grown under nutrient-replete conditions, although it is possible that the protein level is also posttranscriptionally modulated; immunodetection of the protein would enable us to quantify ARS73a protein levels under the different conditions. If the protein is present in nutrient-replete cells, then it may increase in abundance under S deprivation conditions to reach a threshold level that causes gene activation, becomes activated in a protein synthesis-dependent manner when the cells are deprived of S (e.g. a specific modifying protein may be synthesized during first tier protein synthesis that activates ARS73a), or interacts with a partner protein that is synthesized during first tier gene activation. Alternatively, the *ARS73a* transcript, which is present in the cell

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at low levels under nutrient-replete conditions, may not be translated. Activation of specific proteins during S-deprivation may allow *ARS73a* mRNA to be properly processed and translated. The existence of aberrant *ARS73a* mRNAs that are truncated at both 5′ and 3′ UTRs in the *snrk2.1* mutant when the cells are deprived of S lends some support to this possibility; SNRK2.1 or a protein regulated by SNRK2.1 is needed to properly process/translate *ARS73a* mRNA.

With the same primers used for evaluating *ARS73a* transcript levels in wild-type cells, we could barely detect *ARS73a* mRNA in the *ars73a* mutant maintained in nutrient-replete medium (the product generated by these primers spans the first *AphVIII* insertion site in the mutant). However, when the cells were shifted to S-depleted conditions, we observed low levels of two transcripts (Fig. 7). One of these transcripts was identical to the 596-bp RT-PCR product in wild-type cells, and the other was shown to be a fusion between the *ARS73a* mRNA and the sequence of the cassette used for insertional mutagenesis. These results suggest that a transcript is still being synthesized from the mutated gene, that the cassette inserted into intron 6 can be excised from the RNA (although inefficiently), and that aberrant processing of the RNA also occurs, resulting in transcripts that are fused with sequences from the cassette. However, given that an additional insertion of a "partial" cassette has integrated into the *ARS73a* gene downstream of the segment of the transcript amplified by the RT-PCR used to evaluate *ARS73a* mRNA accumulation, it is likely that this strain does not make normal ARS73a protein.

Based on the predicted amino acid sequence, ARS73a has no nuclear localization domain and no known motifs that would allow it to bind DNA and function as a transcription factor. The closest homologs to ARS73a are two proteins encoded by the Volvox gene models Vocar20015096m.g and Vocar20015036m.g. These proteins have strong identity to ARS73a over the first 20% of the protein; the identity is small over the remaining 80% of the protein (data not shown). Both Volvox proteins have ankyrin repeats (not present in ARS73a) but no known physiological functions. Interestingly, ARS73a does have regions with poly-Gln tracks. Proteins with poly-Gln tracks have been shown to be involved in transcriptional regulation in a number of organisms (Ding et al., 2006; Kim et al., 2008; Landeras-Bueno et al., 2011; Atanesyan et al., 2012). Interestingly, another gene that is immediately downstream of *ARS73a*, gene model C_523865, encodes a protein with significant sequence similarity to ARS73a (Supplemental Fig. S4). It is also important to note that the predicted gene model upstream of *ARS73a* encodes a putative amino acid transporter (AOT4) that is likely involved in S acclimation, since the expression of this gene is severely depressed in the *snrk2.1* mutant under both S-replete and S-depleted conditions (González-Ballester et al., 2008, 2010). A mechanistic understanding of the way in which ARS73a impacts second tier regulation, and the possible role of the protein encoded by the related, downstream gene in this regulation, will require the determination of subcellular locations of these proteins and characterizations of their interactions with other proteins in the cell (and potentially with each other).

We have identified a number of regulatory elements important for controlling the responses of *C. reinhardtii* to S deprivation. These proteins, which include SAC1, SNRK2.1, and SNRK2.2, appear to be involved in the first tier of gene regulation. SAC1 has sequence similarity to a Na^+/SO_4^{2-} cotransporter but functions as a sensor protein, while both SNRK2.1 and SNRK2.2 are Ser/Thr kinases. The *sac1* mutant cannot elicit much increased expression of high-affinity transporters and has lower SO_4^{2-} transport activity (Pootakham et al., 2010), but it is also unable to properly up-regulate second tier gene expression. Furthermore, the *sac1* mutant is defective in acclimation to S deficiency and dies after 2 d in S-depleted medium (Davies et al., 1994). The *sac1 ars73a* double mutant has a similar phenotype to that of the *sac1* single mutant; this is not surprising, since the phenotype with respect to the expression of *ARS* is the same for both strains, while SAC1 and not ARS73a is required for expression of the transporter genes.

SNRK2.1 is involved in the regulation of many S-responsive genes, and the *snrk2.1* mutant, like *sac1*, dies within 2 d of S deprivation (González-Ballester et al., 2008, 2010). The levels of both *SAC1* and *SNRK2.2* transcripts are not impacted in the *snrk2.1* mutant, suggesting that these genes are not regulated by *SNRK2.1* (González-Ballester et al., 2010). Interestingly, the *snrk2.1* mutant has almost no *ARS73a* transcript under nutrient-replete conditions, and the transcript present in S-depleted cells has abnormalities: it appears to be truncated at both the 5′ and 3′ UTRs (Supplemental Fig. S6; http://genomes.mcdb.ucla.edu/). These data suggest that *SNRK2.1* has a role in regulating *ARS73a* transcription and/or the stability of the transcript. The *snrk2.1 ars73a* double mutant has a phenotype similar to that of the *snrk2.1* single mutant (Fig. 9A), demonstrating that the *snrk2.1* lesion is epistatic to *ars73a*; the accumulation of all transcripts, those associated with both first and second tier regulation, is blocked in the double mutant, and the levels of the transcripts from all first and second tier genes are extremely low. These results suggest that *SNRK2.1* is involved in the activation of *ARS73a* when the cells are depleted of S.

The *snrk2.2 ars73a* double mutant exhibits an *ars*$^-$ phenotype identical to that of the *ars73a* single mutant (Fig. 9, B and C); the double mutant shows essentially no ARS activity, unlike the *snrk2.2* single mutant, which has constitutive low-level activity (Davies et al., 1999). These findings suggest that *ARS73a* is epistatic to *SNRK2.2* with respect to expression of the *ARS* genes. Interestingly, the *snrk2.2* single mutant was shown to be unable to accumulate SO_4^{2-} transporter proteins in the plasma membrane, and the *snrk2.2 ars73a* double mutant had a similar phenotype (Fig. 11A). This phenotype of the double mutant is in accord with the finding that ARS73a is not involved in controlling the SO_4^{2-} transporter genes (Figs. 5, A and C, and 11). However, these

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findings provide interesting insights into the role of SNRK2.2 in the biogenesis of the *C. reinhardtii* SO_4^{2-} transporters (see the model in Fig. 12 and discussion below). The SNRK2.2 kinase may have a dual role (or a single function that leads to two phenotypes in mutant strains) in the acclimation of the cells to S-deficient conditions; it is involved in the repression of S-responsive genes under nutrient-replete conditions (the *snrk2.2* mutant has low constitutive ARS activity under nutrient-replete conditions) and is required for the trafficking to and/or integration/stability of high levels of transporter protein into the plasma membrane (Fig. 11A); the mutant exhibits little increase in SO_4^{2-} uptake rates and low-level accumulation of the transporter proteins in the plasma membrane under S deprivation conditions (Fig. 11B; Davies et al., 1994; Pootakham et al., 2010). Recently, phosphorylation of the yeast (*Hansenula polymorpha*) nitrate transporter Ynt1 was shown to be essential for its delivery to the plasma membrane when the cells were deprived of nitrogen (Navarro et al., 2008). Similarly, phosphorylation of the yeast plasma membrane ATPase was shown to be critical for its proper maturation and routing to the cell surface (DeWitt et al., 1998). In addition, Phosphate Transporter Traffic Facilitator1, a plant-specific SEC12-related protein, has been demonstrated to play a critical role in allowing the exit of a high-affinity Arabidopsis (*Arabidopsis thaliana*) phosphate transporter from the endoplasmic reticulum (González et al., 2005). Therefore, it is possible that the *snrk2.2* mutant is defective for the maturation and subsequent routing of SO_4^{2-} transporters to the plasma membrane, either because the transporters themselves or a protein involved in transporter biogenesis must be phosphorylated (by SNRK2.2) to function properly.

With the new information generated by these studies of the *ars73a* mutant, we have been able to develop a more detailed model of the way in which S levels regulate acclimation responses in *C. reinhardtii*. This model, shown in Figure 12, integrates the tiered responses that were observed in these studies and the role of ARS73a in those responses. The genes regulated during the first tier of the control, which does not require protein synthesis, include those encoding the high-affinity SO_4^{2-} transporters. The sensor protein SAC1 and the Ser/Thr kinase SNRK2.1 are absolutely necessary for full activation of first tier genes. Expression of the genes involved in SO_4^{2-} scavenging (e.g. *ARS*) and restructuring of the cell (e.g. *ECP76*, *LHCBM9*) requires protein synthesis in a second tier regulatory response. ARS73a is critical for second tier gene expression. Furthermore, SNRK2.2 is required for both complete suppression of first tier gene expression during S-replete conditions and stable accumulation of the transporter proteins in the cytoplasmic membrane; these two phenotypic phenomena may or may not be independent. A detailed diagram of our regulatory model is shown in Figure 12, with a thorough description of the different regulatory events that occur under nutrient-replete and S deprivation conditions presented in the legend.

MATERIALS AND METHODS

Strains and Growth Conditions

The following *Chlamydomonas reinhardtii* strains were used in this work: the wild-type strains 21gr (*CC-1690*, available from the Chlamydomonas Center) and D66 (*CC-4425*, available from the Chlamydomonas Center; Pollock et al., 2005), *snrk2.1* (González-Ballester et al., 2008), and *sac1* and *snrk2.2* (Davies et al., 1994, 1999). Cells were cultured in either S-replete or S-depleted TAP medium under continuous illumination (80 μmol photons m^{-2} s^{-1}) on a rotating platform (200 rpm) at 25°C. TAP-S medium was prepared as described previously (Davies et al., 1994). For S starvation experiments, cells were grown to midlogarithmic phase (2–4 $\times$ 10^6 cells mL^{-1}) in standard TAP medium, washed once with TAP-S medium (2,500*g* for 5 min), and resuspended in TAP-S to the original cell density.

Cell Treatment

Cycloheximide, which inhibits protein synthesis on cytoplasmic 80S ribosomes, was used at a final concentration of 10 μg mL^{-1}, as described previously (Kawazoe et al., 2000). Chloramphenicol was used at a concentration of 300 μg mL^{-1} and actinomycin D at a concentration of 100 μg mL^{-1}.

Assay for ARS Activity

The ARS activity of colonies growing on solid medium was assayed by spraying the colonies with 300 to 500 μL of 10 mM 5-bromo-4-chloro-3-indolyl sulfate (Sigma-Aldrich) in 0.1 M Tris-HCl, pH 7.5, as described by Davies et al. (1996). A blue precipitate formed around each colony producing active ARS protein within 1 to 2 h of exposure of the colonies to the chromogenic substrate.

SO_4^{2-} Uptake Assay

SO_4^{2-} transport assays were performed as described previously (Yildiz et al., 1994).

Southern-Blot Analyses

Genomic DNA was isolated from 100-mL liquid cultures of 21gr and the *ars73a* mutant using a standard phenol-chloroform extraction protocol (Sambrook et al., 1989). Approximately 10 μg of genomic DNA was digested overnight with 10 units of the restriction endonuclease *Pst*I (New England Biolabs). The fragments resulting from the digestion were separated by agarose (0.8%–1%) gel electrophoresis, blotted overnight in 20× SSC onto nitrocellulose membranes (GeneScreen; DuPont-New England Nuclear), and the transferred DNA was cross-linked to the membrane by UV illumination. An alkaline phosphatase-labeled probe was synthesized by chemical cross linking of a thermostable alkaline phosphatase to the nucleic acid template. Probe synthesis and hybridization were performed using the Amersham AlkPhos Direct Labeling and Detection Systems following the manufacturer's protocol (Amersham Biosciences).

RNA Isolation and Quantification

Total RNA was extracted from frozen cell pellets using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase I (Qiagen) to remove residual genomic DNA. First-strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA using oligo$(dT)_{12-18}$ for priming the SuperScript III reverse transcriptase reaction, as described in the manual (Invitrogen). RT-qPCR was performed with a Roche LightCycler 480. PCR, in a final volume of 20 μL, used 10 μL of LightCycler 480 SYBR Green Master Mix (Roche) or SensiMix SYBR-NoROX (Bioline), 5 μL of a 1:50 cDNA dilution, 400 nM of each primer, and distilled water to make up the remainder of the 20-μL volume. The Roche mix conditions used for amplification in the thermocycler were as follows: preincubation at 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s, elongation at 72°C for 20 s, and measurement of fluorescence after 80°C for 5 s (the last step was incorporated into the protocol to avoid background signals resulting from the formation of primer dimers). The Bioline mix preincubation time was increased to 10 min, and denaturation steps were for 15 s, as suggested by the manufacturer.

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Similar results were obtained with both mixes. A melt-curve analysis program (60°C–99°C, heating rate of 2.2°C s^{-1}, and continuous fluorescence measurements) was used to evaluate the specificity of each amplification reaction. All reactions were performed in triplicate with at least two biological replicates. The *CBLP* gene was used as the control housekeeping gene (Chang et al., 2005). The primer pairs used for RT-qPCR were as follows: 5′-CTTCTCG-CCCATGACCAC-3′ and 5′-CCCACCAGGTTGTTCTTCAG-3′ for *CBLP*; 5′-GCAATGTGGCAGGGGCATGGTT-3′ and 5′-GAGCACCGACCTGGATTAC-CAGCT-3′ for *ARS1*; 5′-CTTAATTGCATGCGCGCCGTCA-3′ and 5′-TCA-GAACACCAACGCAAGTTTCCAG-3′ for *ARS2*; 5′-TGGCCATGCTTATCG-TCATCTATG-3′ and 5′-TCGATGCGCATGACCAGGAT-3′ for *SULTR1*; 5′-ACGTGGCATGCAGCTCAT-3′ and 5′-CTTGCCACTTTGCCAGGT-3′ for *SULTR2*; 5′-ACGGGTTCTTCGAGCGAATTGC-3′ and 5′-CGACTGCTTACG-CAACAATCTTGG-3′ for *SLT1*; 5′-GTACGGAGTTCCTTACGCGC-3′ and 5′-TTCTTCGCCACCGATGAGC-3′ for *SLT2*; 5′-CCCAGTCTTTTGGCGG-CAAG-3′ and 5′-GGCCTACTCGCTACCGTACC-3′ for *SLT3*; 5′-CCTCGCT-CTCCTCGCTGCTG-3′ and 5′-CGGCCGACTTGGGTAATTGC-3′ for *ECP76*; 5′-GGACGGCAGCATCATGGTGAGC-3′ and 5′-TCCACACGCCCTTGACC-TTGAG-3′ for *SBDP*; 5′-CCGCCTGGCCATGTTCTCGTC-3′ and 5′-CCGTG-GGCCCTATCCGTGGTA-3′ for *LHCBM9*; 5′-AAGGATTGTGTCAGGCTCG-TCTCG-3′ and 5′-TTCCCACCGCAGCCTAACCACA-3′ for *HAP2*; 5′-GGC-CCTCCTGGAAGCTGAATCA-3′ and 5′-CCCCACCCCACCCATTAGTA-GTC-3′ for *OASTL4*; 5′-TTGGCGGTGAGGTAGGAACAGACG-3′ and 5′-TGCCGCCAGACAGGGAACCAC-3′ for *AOT4*; and 5′-GTTGGAGTTCCTG-GACTGGTTTT-3′ and 5′-GATGGGCAGGCTAGGGAAAGT-3′ for *ARS73a*.

RT-PCR for *ARS73a* and Downstream Gene Model

The primer pair used for RT-PCR to amplify the region of *ARS73a* from exon 4 to exon 7 was R3 (5′-AGGAGTACGCGGTGCGCTGG-3′) and Pb (5′-CAGCCCACTGGACTGGTGGAGCAGCAT-3′). The primers used to detect transcripts for the gene model C_523865 were F1 (5′-TGCAGCCGGCCAC-CATGCACA-3′) and R1 (5′-CGGGCGCTGGCGCCAGCCCA-3′).

Backcrossing and Generation of the Double Mutants *sac1 ars73a*, *snrk2.1 ars73a*, and *snrk2.2 ars73a*

ars73a was backcrossed three times to the wild-type strain 21gr according to the protocol of Harris (1989) to remove most background mutations generated by the transformation procedure and to obtain a mutant strain in the 21gr genetic background. To generate the *snrk2.2 ars73a* double mutant, *snrk2.2* mt^+ (in the 21gr genetic background) was crossed to the third backcrossed progeny of *ars73a* mt^-. The genotype of the progeny was determined by colony PCR using the following primers: 5′-CGTACAAGGCCCATGCGTGAGTC-3′ and 5′-TCGCCGAAAATGACCCAGAGC-3′ for identifying the *snrk2.2* allele and 5′-TACCGGCTGTTGGACGAGTTCTTCTG-3′ (RB2) and 5′-CAGTTCGTGA-CATTCATTCTGACGG-3′ for identifying the *ars73a* allele. A similar method was used to generate the *snrk2.1 ars73a* and *sac1 ars73a* double mutants. The primers used to identify the *snrk2.1* allele were 5′-CAGGATGCGGGA-CAGCAGGTC-3′ and RB2, while those used to identify the *sac1* allele were 5′-GTCGTTGGGCATCTTCATCGC-3′ and 5′-GCGACTCGTTGAACTTCTCC-3′.

Generation of Antibody

Antibodies to ARS were prepared by Covance Research Products. Antibodies that recognize both ARS1 and ARS2 were generated in rabbits against a peptide region common to ARS1 and ARS2 (SDKPQNSKVGLHVD) but not well conserved in other ARS-like proteins. The peptide was conjugated to keyhole limpet hemocyanin via a terminal Cys and injected into rabbits. The preparation of antibodies against the SO_4^{2-} transporters was described previously (Pootakham et al., 2010).

Protein Isolation, SDS-PAGE, and Immunoblot Analysis

C. reinhardtii cells (2–4 × 10^6 cells mL^{-1} in 100 mL) were collected by centrifugation (3,000*g*, 5 min) and resuspended in 0.1 M Na_3PO_4 buffer (pH 7.0). Chlorophyll was extracted from cells into 80% acetone-20% methanol, and its concentration was determined spectrophotometrically (Arnon, 1949) after removal of cell debris by centrifugation. Quantities of cells with equal chlorophyll content (200–300 μg) were pelleted, resuspended in an ice-cold homogenization buffer (0.25 M Suc, 0.1 M HEPES, pH 7.5, 15 mM EGTA, 5% glycerol, and 0.5% polyvinylpyrrolidone) containing a protease inhibitor cocktail (Sigma-Aldrich), and then disrupted by agitation with glass beads (425–600 μm). The lysate was centrifuged briefly (2,000*g*, 5 min) to remove cell debris. For total protein preparations (used for immunoblots), 1 volume of loading buffer (6.25 mM Tris-HCl, pH 6.8, 5% SDS, 6 M urea, 500 mM dithiothreitol, 10% glycerol, and 0.002% bromophenol blue) was added to samples prior to incubation at 100°C for 5 min. To prepare a microsomal fraction (used for immunoblots with anti-SLT and anti-SLT2 antibodies), the supernatant was centrifuged at 100,000*g* for 50 min. The microsomal pellet was resuspended in homogenization buffer containing 1% Triton X-100. An equal volume of loading buffer was added to samples prior to incubation at 42°C for 15 min. Solubilized polypeptides were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes using a wet-transfer method. Blots were blocked in 5% milk in Tris-buffered saline solution with 0.1% Tween 20 prior to a 2-h incubation in the presence of primary antibodies. Dilutions of the primary antibodies used were as follows: 1:3,000 anti-SLT, 1:1,000 anti-SLT2, 1:1,000 anti-FOX1, and 1:2,000 anti-ARS. A 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Promega) was used as a secondary antibody, and the peroxidase activity was detected by an ECL assay (Amersham Biosciences).

Location of the Paromomycin Cassette in Genomic DNA of the *ars73a* Mutant

The *AphVIII* gene (Pollock et al., 2005) was transformed into *C. reinhardtii* cells by electroporation (Shimogawara et al., 1998), and three closely spaced insertions in *ARS73a* were identified (see below). The neighborhood of the insertion was thoroughly analyzed by PCR-based genome walking from the *AphVIII* gene insertions (see below) and sequencing of the genomic fragments generated during the analysis. We used genomic walking from the site of marker insertion 1 (in intron 6, but in the opposite orientation of *ARS73a*; Fig. 2) to the *ARS73a* 5′ UTR using the primers ParoF1 (5′-GTTGTTTGTCAAGGTGG-CAGCT-3′; *AphVIII* coding sequence) and P5 (5′-AACGCTTTCCATCGCAC-GTGGAAGA-3′; 5′ UTR of *ARS73a*). A specific product was only observed when genomic DNA from the mutant was used as a template (Supplemental Fig. S7). This 3.3-kb amplification product was sequenced using the primers ParoF1 and RB2 (5′-TACCGGCTGTTGGACGAGTTCTTCTG-3′), which anneal to the *AphVIII* cassette, plus P5, R3 (5′-AGGAGTACGCGGTGCGCTGG-3′), F2 (5′-TATCTGCACGCACCCTGCCA-3′), F3 (5′-CCCCATTGCCTGTTCAAGC-CGT-3′), and R1 (5′-CAGTTCGTGACATTCATTCTG-3′), which anneal to the 5′ region of *ARS73a*. Full sequence analyses of the region revealed another insertion of the *AphVIII* gene (designated insertion 3) downstream of the first insertion in exon 7 but in the same orientation as *ARS73a* (Fig. 2). We amplified a fragment extending from insertion 3 using ParoF1 and Pf (5′-GCATGCTAAT-CGTGTGCGCTG-3′). Again, a product (2.5 kb) was only obtained from mutant genomic DNA (Supplemental Fig. S7), and this product was sequenced using various primers including ParoF1 and RB2 in *AphVIII* and Pf, Pb (5′-CAGCC-CACTGGACTGGTGGAGCAGCAT-3′), PD (5′-TGGTACTGCGTCAGCTCGT-CCA-3′), and PE (5′-TACAGTAATTCACTGCAGATGGCCG-3) in *ARS73a*. We also performed PCR using RIM5-2 (primes in the 5′ region of insertion 1) and PF1 (5′-TAACTCATAATGACACAGCGC-3′) and PF4 (5′-GTGGACAGGTTCAT-CCAACATA-3′), both of which prime in intron 6 of *ARS73a*; the sequence revealed an additional insertion of a small sequence from the cassette that contains only 0.3 kb of the 3′ end of the *AphVIII* cassette (designated insertion 2). To amplify the region between the two *AphVIII* insertions, we performed PCR using the primers ParoR1 (5′-AGCTGCCACCTTGACAAACAAC-3′; reverse complement of ParoF1) and P3 (5′-ATAATGCAAGCGCTCTGCAACA-3′), which anneals to a sequence in intron 6 of *ARS73a*. We also amplified the region between insertions using RB2 (primes in the 3′ end of insertion 2) and ParoR1. A specific PCR product was generated using genomic DNA from the mutant as template, and the products were sequenced (data not shown). A map showing the entire region of the *ARS73a* gene in the mutant strain, with the three insertions and a partial *ARS73a* gene duplication, is presented in Figure 2, while the entire sequence of the region is given in Supplemental Figure S3.

Rescue of the *ars73a* Mutant Phenotype

The *ars73a* mutant was backcrossed three times, and the mutant phenotype (*ars*$^-$) of the backcrossed strain was rescued by the introduction of BAC39M22 (from the Clemson University Genomics Institute; http://www.genome.clemson.edu/cgi-bin/orders?page=productGroup&service=bacrc& productGroup=162), which has an approximately 42-kb insert containing the *ARS73a* gene; introduction of the bacterial artificial chromosome DNA was by cotransformation

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with pSP124 (*ble* gene encoding zeocin resistance; Lumbreras et al., 1998). Briefly, liquid cultures were chilled on ice for 5 min, and then 10% Tween 20 (1:2,000, v/v) was added to the cultures, which were then gently mixed. Cells were pelleted at 800*g* for 5 min at 4°C and resuspended to a final concentration of 2×10^8 cells mL^{-1} in TAP medium containing 40 mM Suc. Two hundred fifty microliters of the cell suspension was electroporated with 3 to 5 μg of *Sma*I-digested BAC39M22 (which cuts all of the genes on the plasmid except *ARS73a*) and 0.5 μg of *Bam*HI-digested pSP124S. Electroporation was performed with the Bio-Rad GenePulser II at 25 μF and 0.9 kV. Following electroporation, the cells were transferred to 5 mL of TAP Suc medium and grown under low light (approximately 30 μmol photons m^{-2} s^{-1}) overnight to allow for expression of the *ble* gene. Following this incubation, the cells were pelleted by centrifugation at 2,700*g*, resuspended in approximately 500 μL of TAP medium containing 20% starch, and equal volumes were spread onto two petri dishes containing solid TAP medium supplemented with 5 to 10 μg mL^{-1} zeocin. The cells were grown for 10 d prior to testing each zeocin-resistant colony for ARS activity.

Sequence data for ARS73a can be found in Phytozome v9.1 (www.phytozome.net) under accession number Cre06.g298800.

Supplemental Data

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

Supplemental Figure S1. Insertion of the *AphVIII* cassette into the *ARS73a* locus.

Supplemental Figure S2. Complete nucleotide sequence of the *ARS73a* gene.

Supplemental Figure S3. Complete nucleotide sequence of the 6.8-kb *Pst*I fragment containing the disrupted *ARS73a* gene in the *ars73a* mutant.

Supplemental Figure S4. Alignment of ARS73a (C_523864) with the protein encoded by the gene immediately downstream (C_523865).

Supplemental Figure S5. Partial mRNA sequence of *ARS73a* from exon 4 to exon 7 in D66 and *ars73a*.

Supplemental Figure S6. *ARS73a* transcript levels determined by RNA-seq analysis.

Supplemental Figure S7. DNA products generated by genome walking.

ACKNOWLEDGMENTS

We thank the members of the Grossman and Bhaya laboratories for insightful discussions.
Received January 16, 2013; accepted March 8, 2013; published March 12, 2013.

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