

# The Central Role of a SNRK2 Kinase in Sulfur Deprivation Responses<sup>1[W][OA]</sup>

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In the absence of sulfur (S), *Chlamydomonas reinhardtii* increases the abundance of several transcripts encoding proteins associated with S acquisition and assimilation, conserves S amino acids, and acclimates to suboptimal growth conditions. A positive regulator, SAC1 (for sulfur acclimation protein 1), and a negative regulator, SAC3, were shown to participate in the control of these processes. In this study, we investigated two allelic mutants (*ars11* and *ars44*) affected in a gene encoding a SNRK2 (for SNF1-related protein kinase 2) kinase designated SNRK2.1. Like the *sac1* mutant, both *snrk2.1* mutants were deficient in the expression of S-responsive genes. Furthermore, the mutant cells bleached more rapidly than wild-type cells during S deprivation, although the phenotypes of *ars11* and *ars44* were not identical: *ars11* exhibited a more severe phenotype than either *ars44* or *sac1*. The phenotypic differences between the *ars11* and *ars44* mutants reflected distinct alterations of SNRK2.1 mRNA splicing caused by insertion of the marker gene. The *ars11* phenotype could be rescued by complementation with SNRK2.1 cDNA. In contrast to the nonepistatic relationship between SAC3 and SAC1, characterization of the *sac3 ars11* double mutant showed that SNRK2.1 is epistatic to SAC3. These data reveal the crucial regulatory role of SNRK2.1 in the signaling cascade critical for eliciting S deprivation responses in *Chlamydomonas*. The phylogenetic relationships and structures of the eight members of the SNRK2 family in *Chlamydomonas* are discussed.

Sulfur (S) is an essential element present in proteins, lipids, carbohydrates, electron carriers, various metabolites (some involved in the detoxification of heavy metals and xenobiotics), and signaling molecules (Meister and Anderson, 1983; Gupta et al., 1990; Schultz et al., 1992; Marrs, 1996; Grossman and Takahashi, 2001). The preferred S source for most organisms is sulfate ( $\text{SO}_4^{2-}$ ), which can be limiting in the environment; limitations for  $\text{SO}_4^{2-}$  may result in reduced quality and yield of seeds and cause stunted plant growth (Mahler and Maples, 1986, 1987; Warman and Sampson, 1994). Most organisms are unable to efficiently store S; therefore, they are dependent on a continuous supply of this nutrient.

When organisms become S limited, they exhibit a suite of responses that have been described as either specific or general. The specific responses to nutrient limitation are those associated with deprivation for a

single nutrient and are often involved in scavenging or conserving that specific nutrient. For example, during S deprivation, *Chlamydomonas reinhardtii* synthesizes periplasmic arylsulfatases (ARS) that catalyze the hydrolysis of organic  $\text{SO}_4^{2-}$  esters (de Hostos et al., 1989), develops more efficient  $\text{SO}_4^{2-}$  transport (Yildiz et al., 1994) and assimilation (Yildiz et al., 1996; Ravina et al., 2002), and maximizes S utilization efficiency, which could involve significant changes in cell architecture (Grossman and Takahashi, 2001; Zhang et al., 2004). General responses are those associated with deprivation for any essential nutrient and include the cessation of cell growth and division, the accumulation of storage carbohydrates, and the modulation of metabolic processes, including a decrease in photosynthetic activity. The coordination of metabolic processes in the cell during nutrient limitation is critical because an imbalance between the generation of fixed carbon and reducing equivalents with the potential for the cell to grow and divide can stimulate the production of damaging reactive oxygen species. Managing photosynthetic activities and the accumulation of fixed carbon is particularly important, and a number of investigators have characterized the ways in which photosynthesis is controlled by conditions that limit cell growth (Peltier and Schmidt, 1991; Wykoff et al., 1998).

Very little is known about regulatory mechanisms that control S nutrition in vascular plants and other photosynthetic eukaryotes. The generation of insertional mutants of *Chlamydomonas* has resulted in the identification of two specific regulators of S deprivation responses, SAC1 (sulfur acclimation protein 1)

<sup>1</sup> This work was supported by National Science Foundation Grant MCB 0235878 awarded to A.R.G. and by the Carnegie Institution, the Ministerio de Educación y Ciencia (Spain), and the Marie Curie OIF-6 (European Union).

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[www.plantphysiol.org/cgi/doi/10.1104/pp.108.116137](http://www.plantphysiol.org/cgi/doi/10.1104/pp.108.116137)

and SAC3. SAC1 plays a central role in controlling S deprivation responses (Davies et al., 1996; Zhang et al., 2004), and *sac1* mutants are unable to synthesize ARS and to induce many genes that are normally up-regulated when the cells are starved for S (Yildiz et al., 1996; Takahashi et al., 2001; Ravina et al., 2002; Zhang et al., 2004). In addition, the general responses in *sac1* mutants are impaired, and the cells rapidly bleach and die during S deprivation (Davies et al., 1996). This death is light dependent and has been linked to a failure of the cells to down-regulate photosynthetic electron flow out of PSII, suggesting that the modification of photosynthetic electron transport during S deprivation is critical for cell survival.

SAC1 has significant sequence similarity to the animal S transporters ( $\text{Na}^+/\text{SO}_4^{2-}$  transporters). Three additional genes that encode proteins with strong sequence similarity to mammalian  $\text{Na}^+/\text{SO}_4^{2-}$  transporters (*SLT1-SLT3*) have been identified on the *Chlamydomonas* genome. The physiological effects of the *sac1* mutation strongly imply that SAC1 is a hierarchical regulator critical for the acclimation of cells to S deprivation and raise the possibility that transporter-like proteins might have evolved into sensor proteins that are critical for acclimation processes (Davies et al., 1996).

The SAC3 S deprivation regulator of *Chlamydomonas* is a putative Ser/Thr kinase in the plant-specific SNF1-related protein kinase 2 (SNRK2) family. The *sac3* mutants exhibit low-level constitutive ARS activity in S-replete medium, but like wild-type cells, they accumulate high levels of ARS following exposure of the cells to S deprivation. Other S-responsive genes (in addition to ARS) are also negatively regulated by SAC3 (Ravina et al., 2002). Furthermore, the *sac3* mutant does not show the dramatic decrease in chloroplast transcriptional activity that is observed in wild-type cells during S starvation, and the SAC3 kinase may be required to inactivate chloroplast RNA polymerase sigma factor SIG1 under S deprivation conditions (Irihimovitch and Stern, 2006). The relationship between SAC1 and SAC3 is nonepistatic, since the *sac1 sac3* double mutant maintains the phenotype of both of the parental strains (Davies et al., 1994).

Recently, a collection of mutants that exhibit low ARS activity and that are potentially affected in S deprivation responses was generated by insertional mutagenesis (Pollock et al., 2005). Among this mutant population are two strains, *ars11* and *ars44*, that harbor an interruption in the same gene. This gene, designated *SNRK2.1*, encodes a SNRK2 kinase with sequence similarity to SAC3. In this work, we demonstrate that *SNRK2.1* plays a crucial role in the control of S deprivation responses. The *ars11* lesion dramatically affects S deprivation-responsive gene expression, and the mutant cells rapidly bleach following transfer to medium lacking S. Furthermore, unlike *sac1*, *ars11* is epistatic to *sac3*, reflecting a key position of the *SNRK2.1* kinase in the control of S deprivation responses.

## RESULTS

### Isolation of the *ars* Mutants

Insertional mutants (disrupted with the *AphVIII* marker gene) of *Chlamydomonas* were screened for abnormal levels of extracellular ARS activity after transferring cells to TAP-S medium (for Tris-acetate phosphate medium without S). Two kinds of mutants were identified: those with no or low ARS activity (*ars*<sup>-</sup> mutants) and those that exhibited more ARS activity than the parental strain (*ars*<sup>+</sup> mutants). Approximately 30,000 transformants were screened, and from those 50 strains exhibited an *ars*<sup>-</sup> phenotype and two exhibited an *ars*<sup>+</sup> phenotype (Pollock et al., 2005).

An adaptor-mediated PCR technique (Padegimas and Reichert, 1998) was successfully used to determine the genomic regions flanking the *AphVIII* marker for a number of the *ars* mutants. Data showing ARS activities and the flanking regions of some of these mutants were reported previously (Pollock et al., 2005). Among the *ars*<sup>-</sup> mutants for which flanking sequences were obtained, both *ars11* and *ars44* had an *AphVIII* insertion in the same gene, but at different sites within that gene. The interrupted gene encodes a putative Ser/Thr protein kinase belonging to the SNRK2 plant kinase family (Halford and Hardie, 1998). This gene has been designated *SNRK2.1*.

### Cosegregation of Paromomycin Resistance with the *ars* Phenotype

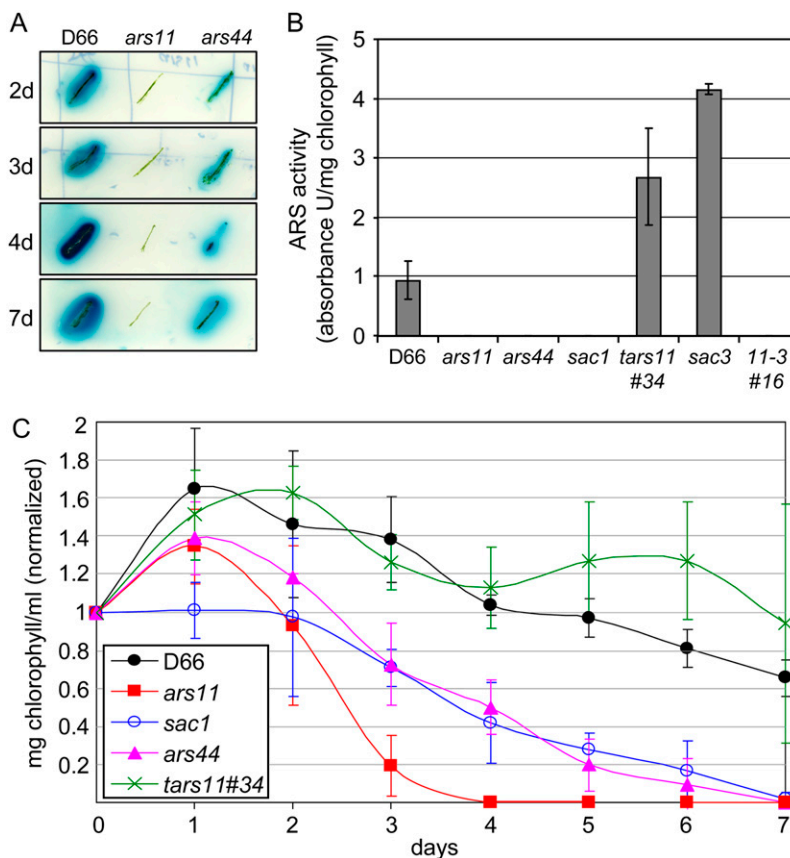
To determine whether the *ars*<sup>-</sup> phenotype was linked to the *AphVIII* insertion, the *ars11* and *ars44* mutants were crossed with the wild-type 21gr strain, which is sensitive to paromomycin. For both mutants, the *paro*<sup>+</sup> progeny (approximately 30 of a total of 60 analyzed) cosegregated with the *ars*<sup>-</sup> phenotype, strongly supporting the conclusion suggested by the occurrence of two alleles with related phenotypes that the insertion was the cause of the mutant phenotype.

### Physiological Characterization of *ars11* and *ars44* Mutants

The *ars11* and *ars44* mutants exhibited no or low ARS activity when the cells were deprived of S, although the phenotypes of the strains were not identical. Like *sac1*, *ars11* had no detectable ARS activity on agar plates, while *ars44* exhibited reduced ARS activity; the *ars44* strain accumulated substantial levels of ARS activity if the cells were maintained on TAP-S plates for a long enough period of time (Fig. 1A). In liquid cultures, *ars44* showed essentially no ARS activity after 24 h of S starvation (Fig. 1B), and even after several days of S starvation, very low-level activity was observed (data not shown).

Davies et al. (1996) showed that the *sac1* strain is defective for the specific and general S deprivation responses. One feature of *sac1* is its rapid loss of chlorophyll and death, relative to wild-type cells,

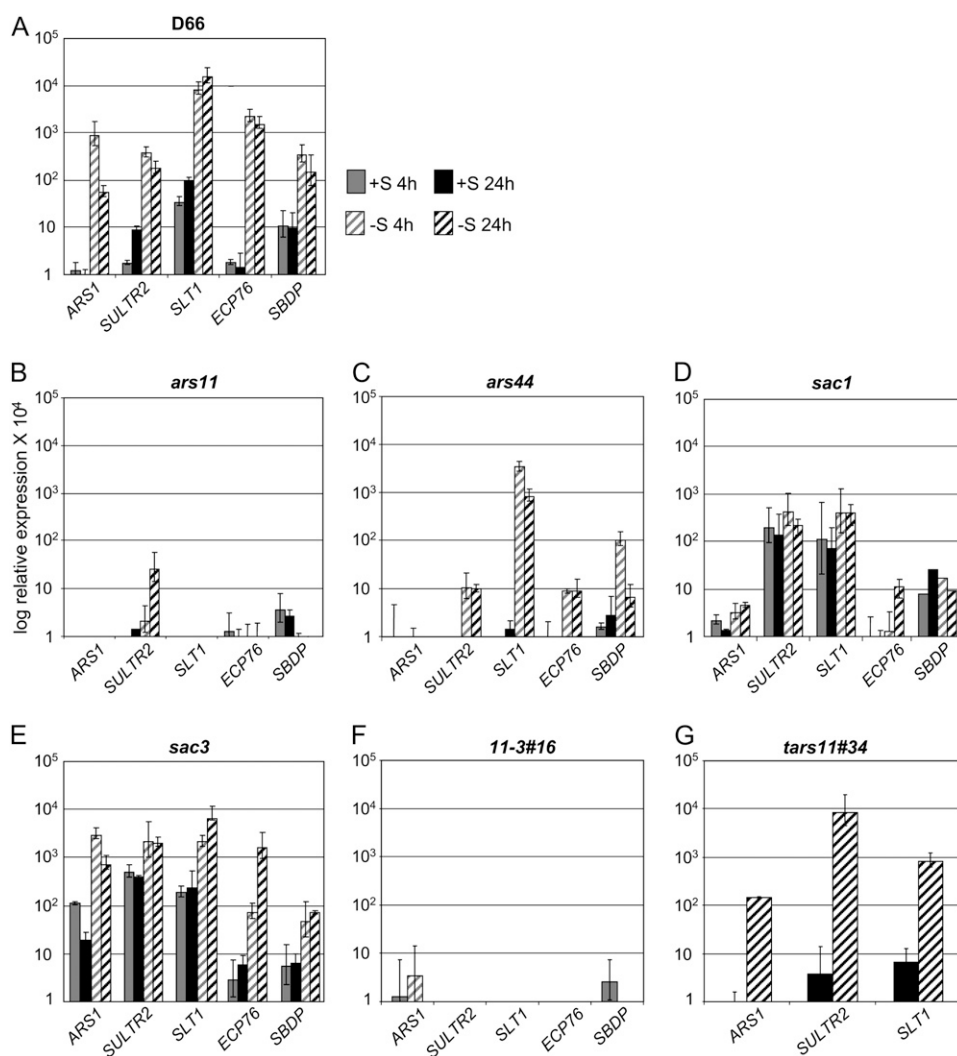
**Figure 1.** ARS activity and chlorophyll in the *ars* mutants. A, ARS activity on solid medium. Cells were streaked onto the surface of solid TAP-S medium and grown for several days before ARS activity was assayed. ARS activity is detected as a blue halo surrounding the colonies. B, ARS activity in liquid medium. ARS activity was measured after 24 h of S starvation. The data are averages of three independent experiments. C, Loss of chlorophyll in S-starved cells. Chlorophyll *a* and *b* content was normalized to the value on day 0 for each strain. Values represent means of three independent experiments. Strains used are D66 (parental strain), *ars11* and *ars44* (*snrk2.1*<sup>-</sup>), *tars11#34* (*ars11* complemented with *SNRK2.1*), *sac1* (*sac1* mutant), *sac3* (*sac3* mutant), and *11-3#16* (*ars11 sac3* double mutant).



following the imposition of S deprivation; this death response may be a consequence of the inability of the mutant to decrease the rate of photosynthetic electron flow through PSII (Davies et al., 1996). The *ars11* and *ars44* mutants also bleached (Fig. 1C) and died much more rapidly than wild-type cells when transferred to TAP-S medium, suggesting that, like *sac1*, these strains are aberrant for both the specific and general S deprivation responses. Interestingly, the *ars11* strain bleached and died significantly more rapidly than either *ars44* or *sac1*.

To further analyze the responses of the *ars11* and *ars44* strains to S deprivation, the levels of transcripts from several genes that were already known to be controlled by the S status of the medium were analyzed; these genes included *ARS1* for the arylsulfatase (de Hostos et al., 1989), *SLT1* for the Na<sup>+</sup>/SO<sub>4</sub><sup>2-</sup>-like transporter (Zhang et al., 2004), *SULTR2* for the H<sup>+</sup>/SO<sub>4</sub><sup>2-</sup>-like transporter (W. Pootakham and A.R. Grossman, unpublished data), *ECP76* for an extracellular protein that is probably associated with the cell wall (Takahashi et al., 2001), and *SBDP* for a putative selenium-binding protein (Zhang et al., 2004; Fig. 2, A–C). Both the *ars11* and *ars44* mutants had lower basal levels for most of these transcripts, with little or no increase (relative to wild-type cells) following the imposition of S deprivation (with some exceptions for *ars44*). However, both the ARS activity assay and the kinetics of the

bleaching response (Fig. 1) demonstrated that the phenotype of *ars44* is significantly less severe than that of *ars11*. This difference in severity is apparent based on the analysis of transcript levels from most of the genes examined and exemplified by the changes in *SLT1* transcript abundance following S deprivation; while no increase in the transcript level was observed in *ars11*, a strong increase (somewhat lower than that of the parental D66 strain) was observed for *ars44*. The pattern of expression for these same genes was also analyzed in *sac1* (Fig. 2D). As in *ars11*, the *sac1* mutant showed no or only a small increase in the levels of known S deprivation-responsive transcripts when the cells were starved for S, although the basal transcript levels in TAP medium in the *sac1* strain were generally higher than in either *ars11* or *ars44*. In contrast, the *sac3* mutant showed relatively high levels of transcripts for a number of the genes tested, but especially for *ARS1*, under S-replete conditions, with increased accumulation when the cells were starved for S (Fig. 2E). These results demonstrate that the SNRK2.1 protein is critical for the accumulation of transcripts from the S deprivation-responsive genes when cells are starved for S. The inability of the mutant cells to respond to S deprivation at the level of transcript accumulation (and likely gene activation) may also lead to the rapid bleaching and loss of viability following the transfer of the mutant strains to TAP-S medium.



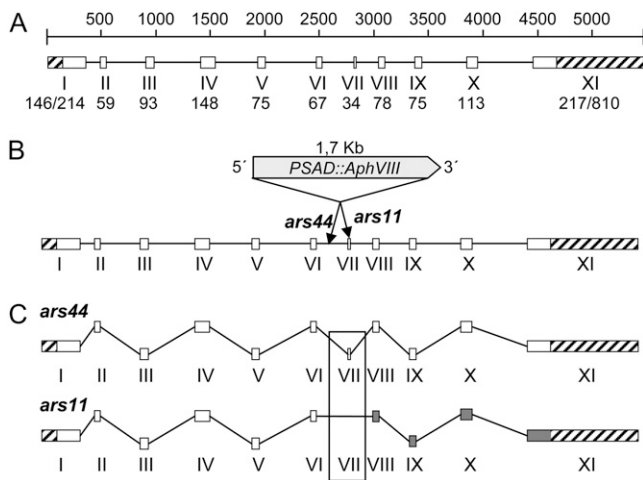
**Figure 2.** qPCR analysis of S-regulated gene expression. The parental strain (A) and mutants (B–E) were grown on TAP medium, washed with TAP-S medium, and then re-suspended in either TAP or TAP-S. Samples were taken for qPCR analysis at 4 and 24 h following the transfer of exponentially growing cells to fresh TAP or TAP-S. Levels of individual transcripts are given as relative fold abundance with respect to the housekeeping control gene (*CBLP*). None of the values obtained was much below 1, and those values that were below 1 are represented as 0 on the graphs. Experiments were performed in triplicate. For the *11-3#16* double mutant, the transcript levels were analyzed only at 4 h (F), and for the *SNRK2.1* complemented strain, *tars11#34*, the *ARS1*, *SULTR2*, and *SLT1* transcript levels were analyzed at 24 h (G). The strains used for these analyses were D66, *ars11* and *ars44* (*snrk2.1*<sup>-</sup>), *tars11#34* (complemented with *SNRK2.1*), *sac1* (*sac1* mutant), *sac3* (*sac3* mutant), and *11-3#16* (*ars11 sac3* double mutant).

#### The cDNAs and Sites of Insertion in *ars11* and *ars44*

There were no EST sequences to help identify the complete coding sequence (CDS) and intron-exon boundaries of the *SNRK2.1* genomic sequence. Therefore, a 2,129-bp cDNA was amplified by reverse transcription (RT)-PCR from the parental strain RNA. Based on the sequence of the cDNA, the gene contains a 1,173-bp CDS with an 810-bp 3' untranslated region (UTR) and a 146-bp 5' UTR. Other cDNA sequences that could represent alternative splice forms of the *SNRK2.1* transcript were also amplified (see below). The cDNA sequence was different from the predicted sequence of the ab initio model for the gene that was generated by the Joint Genome Institute (JGI; <http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>). We recently updated this model to take into account cDNA sequence information. The genomic sequence corresponding to the cDNA was 5.46 kb in length, consisting of 11 exons and 10 introns (Fig. 3A), encoding a predicted polypeptide of 390 amino acids.

Adaptor-mediated PCR was used to identify one of the genomic regions flanking the *AphVIII* marker gene

for both *ars11* and *ars44* (the border close to the 3' end of *AphVIII*; Pollock et al., 2005). In *ars11*, the insertion was positioned within exon VII, whereas in *ars44*, the insertion was within intron 6 (Fig. 3B). To identify the flanking regions on both sides of the insertions, specific primer pairs were designed to anneal to the *SNRK2.1* genomic sequence close to the site of insertion and to the marker gene. Amplifications using these primers and analysis of the flanking sequences showed that neither of the insertion events was accompanied by deletions or by a reorganization of the genome around the site of the insertion (Kindle, 1990). Using RT-PCR, we demonstrated that neither *ars11* nor *ars44* was completely lacking *SNRK2.1* transcripts; however, the mRNA from *SNRK2.1* in *ars11* was missing the sequence encoded by exon VII, while *ars44* retained a full-length *SNRK2.1* transcript that was indistinguishable from that of the parental strain (Fig. 3C). In *ars11*, splicing resulted in a fusion of exon VI to exon VIII, which eliminated exon VII (the site of the insertion) from the processed transcript (Fig. 3C). Furthermore, the translated protein of the *ars11* mutant would



**Figure 3.** A, Genomic structure of *SNRK2.1*. The white blocks and lines represent exons and introns, respectively. Exons are numbered with roman numerals, and the corresponding sizes (bp) are given below. For exons I and XI, the two sizes given represent the 5' UTR/CDS and CDS/3' UTR, respectively. 5' and 3' UTRs are represented with striped blocks. The diagram is drawn to scale. B, Marker insertion in *SNRK2.1* of *ars11* and *ars44*. The *PSAD::AphVIII* marker gene is inserted within exon VII in the *ars11* mutant and within intron 6 in the *ars44* mutant. In both cases, the marker gene has the same orientation as *SNRK2.1*. C, mRNA maturation in the *ars11* and *ars44* mutants. In the *ars44* mutant, all of the introns appear to be properly spliced and a normal protein is synthesized. The *ars11* mRNA lacks exon VII and an aberrant protein is synthesized. The gray blocks represent exons for which the reading frame has changed.

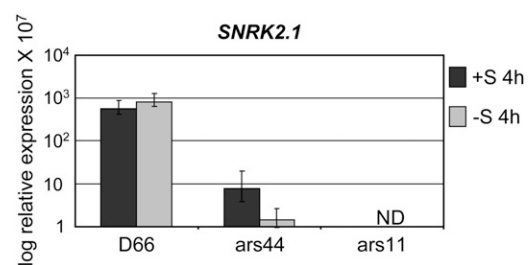
have a shift in the reading frame generated as a consequence of the fusion of exons VI to VIII. When *SNRK2.1* transcript accumulation was determined using primers specific for the 3' UTR, no significant difference between the mutant and wild-type transcript levels was observed (data not shown). However, when *SNRK2.1* transcript accumulation in *ars11* was determined using primers specific for exons VI and VII, no transcript was detected. In contrast, the same primers did reveal the presence of a transcript with the proper fusion of exon VI and VII in *ars44*, although the transcript was reduced by approximately two orders of magnitude in this mutant strain (with the greatest reduction observed in cells exposed to 4 h of  $-S$  conditions) relative to parental cells (Fig. 4). Thus, the *ars44* strain is likely able to synthesize normal *SNRK2.1* protein, but the level of this protein may be significantly lower than in parental cells because of the low-level accumulation of the mRNA. The molecular differences associated with mRNA processing are likely responsible for the different mutant phenotypes. Furthermore, the data demonstrate that splicing of intron 6 of *SNRK2.1* can occur even if the intron is interrupted with the 1.7-kb *PSAD::AphVIII* marker gene.

#### Potential Alternative Splicing of *SNRK2.1*

As mentioned above, *SNRK2.1* cDNA generation led to multiple cDNAs of different lengths. At least five

different *SNRK2.1* cDNAs were identified using the same set of specific primer pairs for RT-PCR (the RNA template was from D66 cells grown in TAP and TAP-S); these cDNAs were designated *SNRK2.1* v1, v2, v3, v4, and v5, and the differences among them are shown in Supplemental Figure S1. *SNRK2.1* v1 was the longest cDNA, encoding a polypeptide of 390 amino acids, and is the isoform discussed above (referred to as *SNRK2.1*). All of these potential transcripts encoded putative polypeptides that used the same start codon, with *SNRK2.1* v1, v2, and v3 maintaining the same coding frame and stop codon. In contrast, the *SNRK2.1* v4 and v5 reading frames changed as a result of the alternative splicing, which also generated a different stop codon. For *SNRK2.1* v2 to v4, the potential alternative 5' splice sites were positioned inside a 54-bp GC-rich region of the transcript. The deduced *SNRK2.1* v2 protein maintained the kinase catalytic domain and the end of the C-terminal domain, whereas v3 lacked 20 amino acids from the kinase domain and part of the C-terminal region. The *SNRK2.1* v4 and v5 predicted polypeptides lacked the kinase domain and the entire C terminus. An alignment of all of the potential splice variants is shown in Supplemental Figure S2. None of the potential splice variants was associated with a specific culture condition (e.g. TAP compared with TAP-S), and the only variant reproducibly observed was *SNRK2.1* v1; the others were less abundant and in some reactions were not detected. Finally, only in *SNRK2.1* v1 did all of the splice junctions match the consensus junctions associated with *Chlamydomonas* genes (Silflow, 1998), which raised the possibility that the variants were a consequence of artifacts associated with cDNA synthesis and amplification. In addition, two alternative 3' polyadenylation sites were identified by 3' RACE from the *SNRK2.1* cDNA sequences (Supplemental Fig. S3); these sites were not associated with specific recombinant libraries or splice variants.

To help establish if the small *SNRK2.1* transcripts were potentially artifacts, we used specific primers to generate PCR products from a pGEM-T clone harboring the *SNRK2.1* v1 cDNA. A multiband amplification



**Figure 4.** *SNRK2.1* expression as determined by qPCR. Primers used were specifically designed to detect the joining of exons VI and VII (see Materials and Methods for details). RNA sample preparation was as described for Figure 2. Levels of individual transcripts are given as relative fold abundance with respect to the housekeeping control gene (*CBLP*). ND, No transcript detected. Experiments were performed in triplicate.

pattern was observed, and at least one of the products had a gap in the sequence starting within the same GC-rich region that was identified as the 5' splice junction for putative splice variants. However, the products generated by PCR from the plasmid did not perfectly match any of the putative *SNRK2.1* splice versions (data not shown). These observations raise the possibility that putative alternative splice forms can arise from aberrant cDNA synthesis and amplification in vitro, and not from in vivo splicing of precursor mRNA.

### Complementation of the *snrk2.1* Mutation

Four of the *SNRK2.1* cDNA potential splice variants (v1, v2, v3, and v4) were introduced into the *ars11* mutant strain. Each cDNA version was inserted between the *PSAD* promoter and the 3' UTR terminator and cloned into a vector containing the *ble* gene as a selectable marker. Fifty *ble*-resistant colonies for each splice variant were tested for ARS activity following exposure to TAP-S. Four colonies transformed with the *SNRK2.1* v1 cDNA restored the wild-type ARS phenotype, based on ARS plate assays. An analysis of ARS activity in liquid for one of the potentially complemented mutant strains (*tars11#34*) showed that the strain was rescued for the *ars*<sup>-</sup> phenotype; it produced high levels of ARS when deprived of S (Fig. 1B), with no ARS activity detectable when grown in TAP (data not shown). No complemented colonies were obtained that harbored v2, v3, or v4 cDNA variants. Analysis of chlorosis of the *tars11#34* strain in TAP-S also revealed restoration of the wild-type phenotype (Fig. 1C); transformants expressing ARS survived S deprivation for an extended period of time, like the D66 parental strain. Finally, the levels of *ARS1*, *SLT1*, and *SULTR2* mRNAs measured in *tars11#34* were similar to those of the parental strain (Fig. 2G). These results clearly demonstrated that the mutant phenotype can be complemented by the full-length *SNRK2.1* gene.

### *ars11 sac3* Double Mutant Analysis

*SNRK2.1* is required for the activation of genes associated with S deprivation responses, while *SAC3* acts as a negative regulator of at least some of these same genes (Davies et al., 1999). To help define the role of the *SAC3* and *SNRK2.1* kinases in the regulation of S deprivation responses, the epistatic relationships between the *sac3* and *ars11* lesions were examined by constructing strains harboring both lesions. Four double mutants were identified, and none exhibited detectable ARS activity when grown on TAP or following transfer of the cells to TAP-S (data not shown), indicating that the *sac3* lesion is hypostatic to the *snrk2.1* lesion; *SNRK2.1* is needed in a *sac3* mutant to enable the constitutive expression of ARS. Expression of *ARS1*, *SLT1*, *SULTR2*, *ECP76*, and *SBDP* was analyzed in the randomly selected double mutant *11-3#16* (Fig. 2F). The double mutant, like the *ars11* single mutant,

exhibited extremely low levels of transcript accumulation for all tested transcripts in both TAP and TAP-S (Fig. 2, compare B with F).

### The SNRK2 Family

*SNRK2.1* belongs to the plant-specific Ser/Thr protein kinase family SNRK2. Like other SNRK2 family members, *SNRK2.1* has an N-terminal conserved catalytic domain similar to those of SNF1/AMP kinases and a short C-terminal regulatory domain that is not highly conserved. *SAC3* (Davies et al., 1999; Irihimovitch and Stern, 2006) is also a member of the SNRK2 family, with 43% sequence identity with *SNRK2.1*. In addition to *SNRK2.1* and *SAC3*, there are six other SNRK2 family members encoded on the *Chlamydomonas* genome. The complete eight-member family, *SNRK2.1* to *SNRK2.8*, with *SAC3* now being designated *SNRK2.2*, is presented in Table I. The deduced proteins for *SNRK2.4*, *SNRK2.5*, *SNRK2.6*, and *SNRK2.8* should be taken as provisional, since the corresponding JGI gene models are impossible to accurately predict because of gaps in the genomic sequences and incomplete EST sequence coverage. Although we have improved on these gene models based on EST and genomic sequences and similarities to other SNRK2 kinases, they are still incomplete, mainly in their C-terminal regions. *SNRK2.1* and *SNRK2.3* are clustered in the same genomic region (scaffold 7, linkage group II) in a tail-to-head orientation and separated by less than 500 bp. *SNRK2.3* and *SNRK2.4* are the most closely related to *SNRK2.1*. These three family members form a *Chlamydomonas* SNRK2 subgroup that is most different from the plant SNRK2 homologs. Members of this subgroup also have unique sequence features that are not present in other SNRK2 proteins; one major feature is the presence of an extra loop of 17 to 22 amino acids immediately preceding the SNF1 kinase activation domain (Hardie and Carling, 1997), which is shown in the alignment in Figure 5 (broken line). Although this extra loop is not conserved at the sequence level among the subfamily members, it does contain a NLH motif (asterisks above the broken line in Fig. 5) and several residues that might serve as phosphorylation sites. An alignment of the entire *Chlamydomonas* SNRK2 family of proteins is shown as Supplemental Figure S4.

### DISCUSSION

In this study, we identified *SNRK2.1*, a member of the plant-specific SNRK2 kinase family and the larger SNF1 superfamily, as a key regulator of the pathway that governs S deprivation responses in *Chlamydomonas*. Two independent mutant strains in which the *SNRK2.1* gene was interrupted, *ars11* and *ars44*, were identified. The insertions did not cause reorganization or deletions of the genomic region at the insertion site, and the mutant phenotypes were linked to the inser-

**Table 1.** *The Chlamydomonas SNRK2 family*

Protein identifiers, scaffold localization, and the presence/absence of associate ESTs are given and can be found on the *Chlamydomonas* JGI Web server. When ESTs are present, they were used to validate the genomic sequences and gene models. The amino acid identities of the deduced N-terminal catalytic sequences with those of Arabidopsis and rice are given as a range of identities (the analysis was performed using MatGAT 2.0; Montclair State University).

Name	Mutant	Identifier	Size	Identity	ESTs	Scaffold	Exons
				%			
SNRK2.1 (ARS11)	<i>ars11/ars44</i>	206379	390	34–40	No	7	11
SNRK2.2 (SAC3)	<i>sac3</i>	185806	357	47–56	Yes, full	78	11
SNRK2.3		53567	408	34–41	Yes, full	7	11
SNRK2.4 <sup>a</sup>		131569	315	35–41	Yes, partial	33	10
SNRK2.5 <sup>a</sup>		132038	312	37–41	Yes, partial	47	6
SNRK2.6 <sup>a</sup>		131583	289	40–46	Yes, partial	33	7
SNRK2.7		153921	372	43–50	Yes, full	69	10
SNRK2.8 <sup>a</sup>		113331	330	60–69	No	4	9

<sup>a</sup>Gene models that are likely inaccurate.

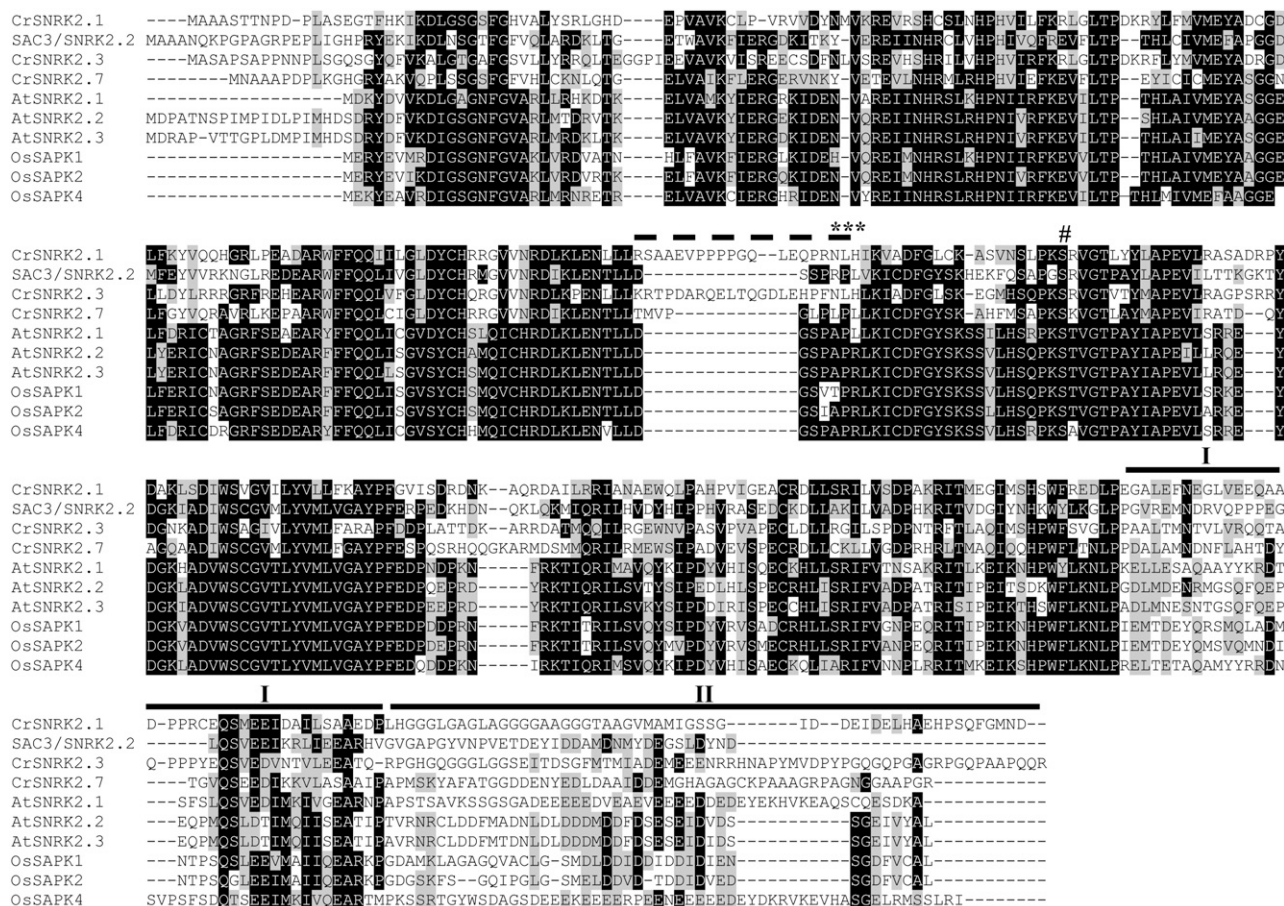
tions. Even though the mutants had a sequence of 1.7 kb (*AphVIII* under the control of the *PSAD* promoter and the 3' UTR) integrated into the *SNRK2.1* gene, the splicing machinery of *Chlamydomonas* was able to excise the inserted sequence and generate a mature mRNA; this mRNA was aberrant in the case of *ars11*. Insertion of the *AphVIII* marker gene into exon VII of *SNRK2.1* in *ars11* caused the loss of this exon in the final splice product and a change in the reading frame of the C-terminal region of the protein. These modifications of the SNRK2.1 protein are likely the reason for the severe mutant phenotype (the lesion probably represents a null mutation). In contrast, in *ars44*, the marker gene is integrated into intron 6. This interrupted intron (with the inserted marker DNA) was spliced out of the nascent transcript, generating a mature transcript that appeared to be identical to the transcript in the parental strain, although the level of accumulation of this mature transcript was more than 10 times lower than in the parental strain (Fig. 4). Therefore, the leaky phenotype of *ars44* is probably a consequence of inefficient splicing of intron 6, which in turn could generate less protein.

Interestingly, in spite of a clear difference in ARS activity between *ars11* and *ars44* mutants based on agar plate assays (Fig. 1A), both strains showed no ARS activity when they were transferred to liquid TAP-S medium for 24 h (Fig. 1B); there was little or no ARS activity measured in either of the mutants, even after several days of S starvation (data not shown). Several hypotheses might explain these findings. (1) There may be a difference in some crucial condition (e.g. oxygen availability) that results in elevated induction/activation of ARS activity on solid medium. This idea is supported by the finding that cells growing under the agar, at lower oxygen levels, exhibit higher ARS activity than cells growing on top of the agar (data not shown). (2) The splicing machinery may be somewhat different under different growth conditions, which might change the efficiency at which some transcripts are spliced. (3) ARS stability could be

lower in cells growing in a liquid environment. Further experimentation should distinguish these possibilities.

The analysis of multiple potential splice variants of the *SNRK2.1* transcript has raised critical issues concerning the in vivo synthesis of these variants and whether the procedure for cDNA synthesis could lead to their artifactual generation. The likelihood of an artifact generated during the synthesis of the cDNA is supported by the finding that the sequences of the splice junction sites for the potential variants identified differ markedly from the *Chlamydomonas* consensus splice junction sequences (Silflow, 1998). Also, the levels of splice variants observed following RT-PCR were highly variable, and truncated transcripts were also observed if the full-length *SNRK2.1* cDNA (as plasmid) was used for PCR amplification. In addition, no complementation of the *ars11* mutant was observed when mutant cells were transformed with the *SNRK2.1* v2 to v4 cDNAs. Others recent works have demonstrated that PCR synthesis of cDNAs could generate artifactual alternative splice forms (Hampl et al., 1998; Oh et al., 2005; Cocquet et al., 2006). In most cases, the shortened transcripts were deleted for part of the internal coding sequence, and it was suggested that these transcripts formed as a consequence of sequence identity between the 3' and 5' splice sites among putative introns, allowing for the formation of heteroduplexes. All of the putative splice variants of *SNRK2.1* have sequence identity between the 3' and 5' splice sites (Supplemental Fig. S1C). During PCR, full-length and partially elongated products would be generated. If the partial products have 3' sequences that can pair with multiple sites within the complementary strand, they can anneal to the complementary full-length strands and serve as primers for elongation. This would lead to the generation of a product that contains internal deletions. Furthermore, since these products would contain the primer sites for both the 5' and 3' PCR primers, they would be efficiently amplified in subsequent PCR cycles. The GC-rich





**Figure 5.** Amino acid sequence alignment. The predicted SNRK2.1, SNRK2.2 (SAC3), SNRK2.3, and SNRK2.7 proteins were aligned with representative Arabidopsis and rice SNRK2 kinases (accession nos. as follows: AtSNRK2.1, NP\_196476; AtSNRK2.2, NP\_190619; AtSNRK2.3, NP\_201489; SAKP1, NP\_001050274; SAKP2, NP\_001060312; SAKP4, NP\_001044930) using BioEdit 7.0.5.3 software. The black and gray boxes indicate identical and similar amino acids, respectively. The C-terminal subdomains are highlighted with heavy black lines above the sequences and roman numerals above the line (Yoshida et al., 2006). The extra loop segment present in CrSNRK2.1 and CrSNRK2.3 (dotted line), the conserved NLH motif (\*\*\*), and the conserved phosphorylated Ser of the activation domain (#) are noted.

region at which all 5' splice sites are localized could elicit the formation of partial elongation products able to form heteroduplexes with the complementary cDNA strand. Although the existence of multiple splice variants cannot be completely excluded based on this work, our results do suggest that caution should be used when considering the generation of splice variants. Additionally, the existence of variability in the placement of the poly(A) tail (Supplemental Fig. S3) suggests some complexity in the regulation of *SNRK2.1* transcripts.

The SNRK2, SNRK1, and SNRK3 kinase families are plant specific and belong to the SNF1 superfamily. SNRK2 kinases constitute a large protein family in plants with an N-terminal conserved catalytic domain and a short regulatory C-terminal region. The regulatory region is not highly conserved but usually has a characteristic stretch of the acidic amino acids Asp and Glu, which define subfamilies SNRK2a and SNRK2b, respectively (Halford and Hardie, 1998). Furthermore,

SNRK2 proteins have multiple phosphorylation states that are critical for activity, but in most cases the specific phosphorylated residues have not been identified (Kobayashi et al., 2004; Boudsocq et al., 2007). Members of the SNRK2 protein family play a role in the acclimation of plants to environmental stresses; a number of the plant enzymes are involved in controlling osmotic stress responses and are activated by abscisic acid (ABA; Li and Assmann, 1996; Mikolajczyk et al., 2000; Boudsocq et al., 2004; Kobayashi et al., 2004). Some downstream target genes that are activated by plant SNRK2 kinases are SSHLP, a phosphatidylinositol transfer-like protein (Monks et al., 2001), AKIP1, a protein regulating RNA stability (Li et al., 2002), and a basic Leu zipper transcription factor required for ABA gene regulation.

Interestingly, some members of the Arabidopsis (*Arabidopsis thaliana*) SNRK2 kinase family may function in controlling S limitation responses. Arabidopsis mutants defective for *SNRK2.3* exhibit a slight decrease



in the level of *SULTR2;2* mRNA (encoding the low-affinity  $\text{SO}_4^{2-}$  transporter) and elevated *O*-acetyl-Ser (precursor to Cys) accumulation (Kimura et al., 2006). However, *snrk2.3* mutant plants did not show alterations in the expression of other S-responsive genes (*APR* and *SAT*), the accumulation of  $\text{SO}_4^{2-}$ , or differences in their growth phenotype relative to the wild-type strain when placed in medium devoid of S. These findings suggest a much more moderate role of AtSNRK2.3 than CrSNRK2.1 in controlling S starvation responses. Curiously, five of the 10 *AtSNRK2* genes showed increased expression following S starvation of plants (Kimura et al., 2006), which raises the possibility that the activity of other SNRK2 proteins from *Arabidopsis* may affect S starvation responses. Overall, these results suggest that the SNRK2 family in plants and algae may have some conserved functionalities with respect to controlling nutrient deprivation responses.

The *Arabidopsis* kinase SNRK2.6 can be activated by two independent mechanisms, one that is ABA dependent and another that is ABA independent and stress dependent (Yoshida et al., 2006). The C-terminal domain, required for both mechanisms, can be functionally divided into domains I and II (Fig. 5). Domain I functions in ABA-independent activation, while domain II, which contains the acidic residues, functions in ABA-dependent activation. *Chlamydomonas* SNRK2 members have the conserved C-terminal domain I and have retained an acidic stretch of amino acids that characterizes domain II, although the number of acidic amino acids in this region is fewer than in most SNRK2 proteins of plants.

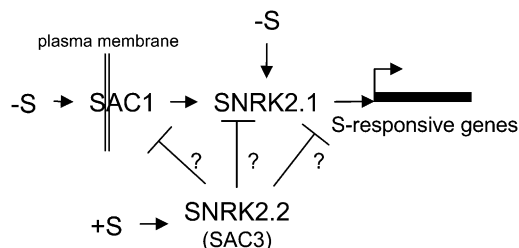
Domain II of *Arabidopsis* SNRK2.6 is needed to elicit full stomatal closure and for the interaction with the PP2C-type phosphatase ABI1, and deletion of the rice (*Oryza sativa*) OSRK1 C terminus destroys the functionality of the protein (Chae et al., 2007). Similarly, the full-length *SNRK2.1* v1 cDNA can fully complement the *ars11* mutant, while the *SNRK2.1* cDNA v2, which lacks 45 amino acids of the C terminus (part of both domains I and II), appears to be unable to rescue the mutant phenotype.

Some of the *Chlamydomonas* SNRK2 proteins (SNRK2.1, SNRK2.3, and SNRK2.4) have a specific feature that is not present in plant SNRK2 proteins: an inserted, nonconserved loop of 17 to 22 amino acids located immediately to the N-terminal side of the activation domain (Fig. 5). The activation domain contains the conserved Ser phosphorylation site (Johnson et al., 1996) that is crucial for kinase activity and/or activation of the enzyme (Kobayashi et al., 2004) upon imposition of a stimulus (Boudsocq et al., 2007). The extra loop present in a subset of the *Chlamydomonas* SNRK2 proteins (1, 3, and 4) has several potential phosphorylation sites that may participate in regulation/activation (Supplemental Fig. S4).

*SNRK2.1*, *SAC1*, and *SAC3* are the three genes that have been shown to have a regulatory role in the S deprivation responses of *Chlamydomonas*. While other

mutants have been isolated that exhibit aberrant S deprivation responses, in most cases the gene responsible for the mutant phenotype has not been identified (Davies et al., 1994; Pollock et al., 2005). It is also intriguing that the regulators encoded by *SNRK2.1* and *SAC3* (*SNRK2.2*) both belong to the plant-specific SNRK2 family. This strongly indicates the importance of phosphorelays in the control of S deprivation-triggered responses. Curiously, SNRK2.1 and SAC3 have opposite regulatory effects, with the former required for activation of the S-responsive genes, including those encoding ARS and the sulfate transporters, and SAC3 required for full suppression of the same genes during growth under S-replete conditions.

Based on the phenotypes of the *sac1* and *ars11* mutants, SAC1 and SNRK2.1 have some similar regulatory features. However, SAC1 resembles animal  $\text{Na}^+/\text{SO}_4^-$  transporters, such as *SLT1* and *SLT2* in *Chlamydomonas* (Davies and Grossman, 1998). Both SAC1 and SNRK2.1 are required for the appearance of ARS activity and the accumulation of transcripts that normally become abundant during S deprivation (Fig. 2, B and D). However, the regulatory aberrations are more extreme in *ars11* than in the *sac1* mutant; this is most evident in a comparison of the levels of transcripts for the sulfate transporters *SULTR2* and *SLT1* in the two strains and in the finding that *ars11* bleaches significantly more rapidly than *sac1* following the imposition of S deprivation. Furthermore, while the *sac1* mutant shows no up-regulation of S deprivation-regulated genes, the levels of transcripts from these genes during S-replete growth are similar to those of the parental D66 strain. In contrast, *ars11* has extremely low levels of the *SLT1* and *SULTR2* transcripts, even under nutrient-replete conditions, relative to the parental strain. As deduced from the mixed phenotype of the *sac1 sac3* double mutant, there is no epistatic relationship between SAC1 and SAC3 (the double mutant exhibits low constitutive ARS activity in TAP and TAP-S media; Davies et al., 1994). In contrast, SNRK2.1 is epistatic to SAC3; the *ars11 sac3* double mutant does not have any detectable ARS activity in TAP-S (Fig. 1B) or TAP (data not shown), and the patterns of *ARS1*, *SLT1*, *SULTR2*, *ECP76*, and *SDBP* transcript accumulation are similar to those of the *ars11* single mutant (Fig. 2F). Hence, SAC3 and SAC1 appear to be dependent on SNRK2.1 for their phenotypic features. These genetic and phenotypic results suggest that SNRK2.1 is central to the pathway regulating S-responsive gene expression in *Chlamydomonas*. SAC1 could be a membrane-bound regulator that senses the S status of the environment and that initiates a signaling cascade that enhances SNRK2.1 activation, which is downstream in this pathway and more directly involved in the activation of a specific transcriptional regulator(s). A possible model that accounts for many of the responses observed in *Chlamydomonas* during S deprivation, and the ways in which the various mutant strains are affected in these responses, is depicted in Figure 6.



**Figure 6.** Model explaining the regulation of the S-responsive genes in *Chlamydomonas*. SAC1 likely acts as a S sensor at the plasma membrane, and under S starvation conditions it initiates a signaling cascade that leads to the activation of SNRK2.1. Additionally, SNRK2.1 may be activated, to a certain level, by an internal S deficiency. SNRK2.2 (SAC3) acts as a negative modulator of the regulatory pathway (at a point that has not been determined) and probably remains active in the presence of sufficient S. Other possible regulatory functions of SNRK2.2, SAC1, and SNRK2.1, such as the control of chloroplast gene expression, are not depicted in this model.

## MATERIALS AND METHODS

### Strains, Culture Conditions, and Mating

*Chlamydomonas reinhardtii* strains used in this work were D66 (*mit2<sup>-</sup> cw15 mt<sup>+</sup>*; Pollock et al., 2003), 21gr (*nit5<sup>-</sup> mt<sup>-</sup>*; Harris, 1989), *sac1* (*sac1<sup>-</sup> mt<sup>+</sup>*), and *sac3* (*sac3<sup>-</sup> mt<sup>+</sup>*; Davies et al., 1994). Cells were cultured under continuous light at 23°C in liquid and solid TAP medium (Harris, 1989). To impose S starvation, cells in midlogarithmic growth were washed twice with liquid TAP-S (Harris, 1989) and then split and resuspended in either TAP or TAP-S. Paromomycin and bleomycin were used at 10 and 3  $\mu\text{g mL}^{-1}$ , respectively. Genetic analyses were performed with the various strains according to a previously described protocol (Harris, 1989).

### Generation of the *ars11 sac3* Double Mutant

The *sac3 mt<sup>+</sup>* mutant was crossed with the parental strain, 21gr (*mt<sup>-</sup>*), to obtain a *sac3 mt<sup>-</sup>* strain. The mating type of the *ars* constitutive progeny (*sac3* phenotype) was determined by a PCR method (Werner and Mergenhagen, 1998), and one of the *sac3 mt<sup>-</sup>* progeny was crossed with *ars11 mt<sup>+</sup>*. The double mutant was identified by first selecting for paromomycin resistance (*ars11* marker) and then determining which of the resistant isolates contained the pBK-*arg2* chimeric sequence (*sac3* marker) using the primers 5'-CGTACAA-GGCCATGCGTGAGTC-3' and 5'-TCGCCGAAAATGACCCAGAGC-3'.

### Transformation of *Chlamydomonas*

Cell wall-less strains D66 and *ars11* were transformed by electroporation (Shimogawara et al., 1998) using a modifications of the procedure reported by Colombo et al. (2002).

### Generation of Insertional Mutants in *Chlamydomonas*

The selectable marker gene used for mutant generation was *AphVIII* under the control of the *PSAD* promoter and terminator; this gene confers paromomycin resistance to transformants (Sizova et al., 2001). A 1.7-kb PCR fragment containing this construction was used to transform *Chlamydomonas* strain D66. After transformation, cells were incubated in TAP for 6 to 12 h under continuous light to allow for the accumulation of the AphVIII protein. Transformants were then selected on TAP supplemented with 5  $\mu\text{g mL}^{-1}$  paromomycin.

### ARS Activity and Chlorophyll Determinations

ARS activity was visualized directly on agar plates (Davies et al., 1994) or quantified from liquid medium (de Hostos et al., 1988) as absorbance units per

cell number or per milligram of chlorophyll. The data presented correspond to mean values of at least three independent experiments. The concentrations of chlorophylls *a* and *b*, extracted from cells in methanol, were estimated using the equations of Porra (2002).

### DNA and RNA Isolation

Genomic DNA and total RNA were isolated according to previously described methods (Schloss et al., 1984; Sambrook et al., 1989).

### Synthesis of cDNA, RT-PCR, and 3' RACE Amplifications

RNA samples were treated with DNase (Qiagen; catalog no. 79254) and further cleaned using RNeasy columns (Qiagen) following the manufacturer's directions. Single-stranded cDNAs were synthesized from total RNA using an N-(polyT)<sub>20mer</sub> primer according to the SuperScript III RNaseH reverse transcriptase manual (Invitrogen). This cDNA population was used as a substrate for real-time PCR with gene-specific primer pairs; the single-stranded cDNAs were diluted 5-fold prior to inclusion in the reaction mixture. The CDS of *SNRK2.1* was obtained by RT-PCR using the specific primers 5'-GCC-TTCTTGGCGACTGCCATACG-3' and 5'-CCTCAGTCGTTTCATGCCGAA-3'. 3' RACE for *SNRK2.1* transcripts was performed using the specific 5' primer 5'-GTTCCGTGAGGACCTACCCGAG-3' and a previously described polyT-Qt primer that allowed semi-nested PCR amplifications (Frohman, 1990). All PCRs were performed in the presence of 2% to 5% dimethyl sulfoxide.

### Complementation of the *ars11* Mutant

The *ble* gene from pSP124S (Lumbreras et al., 1998) was introduced (at the *XhoI-XhoI* site) into the pJM43 plasmid (provided by J. Moseley) containing the *PSAD* promoter; the plasmid generated was designated pBleJM43. *SNRK2.1* single-stranded cDNA was amplified using the modified primers 5'-ATCGATTAACGTTGGGGAACGATTCAATG-3' and 5'-TGGATCCAAC-GTTGGGGAACGATTCAATG-3', which introduced a *Clal* and a *BamHI* site at the 5' and 3' end, respectively, of the amplified sequences. Four different amplicons were obtained: v1, 1,204 bp; v2, 1,068 bp; v3, 952 bp; and v4, 672 bp. These amplification products were introduced between the *PSAD* promoter and terminator in the pBleJM43 vector using the *Clal* and *BamHI* sites. The new plasmids containing the differently sized sequences were designated pDV1, pDV2, pDV3, and pDV4. Sequencing of the insert in each of these vectors confirmed the absence of PCR-introduced sequence errors. The pDV1 to -4 plasmids were introduced into the *ars11* mutant, and transformants were assayed for rescue of the mutant phenotype.

### Quantitative PCR

Real-time PCR was performed using the Chromo4 thermocycler (Bio-Rad). Individual reactions had 25  $\mu\text{L}$  final volume, consisting of 10  $\mu\text{L}$  of DyNAMO SYBR Green quantitative PCR (qPCR) reagent (Finnzymes), 3.7 pmol of each primer, 1 to 2  $\mu\text{L}$  of single-stranded cDNA (5-fold diluted from the reverse transcriptase reaction), and distilled water to 25  $\mu\text{L}$ . The Chromo4 run protocol was as follows: denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 30 s, and amplification at 72°C for 30 s, and fluorescence measurement after 80°C for 15 s. This last step avoids background signals that can result from the formation of primer dimers. The specificity of the PCR amplification was evaluated by a melting curve program (60°C–100°C, with a heating rate of 0.5°C s<sup>-1</sup> and continuous fluorescence measurements) and electrophoretic analysis on 4% agarose gels. We used the *CBLP* gene as a housekeeping gene control (Chang et al., 2005). Threshold cycle (Ct) values were determined in three independent experiments, with three replicates for each experiment. Relative fold differences were calculated based on the relative  $\Delta\text{Ct}$  method [ $2^{-(\text{Ct}_{\text{sample}} - \text{Ct}_{\text{control gene}})}$ ] using the *CBLP* amplification product as an internal standard. The primer pairs used for qPCR were as follows: 5'-CTTCTCGCCCATGACCAC-3' and 5'-CCCACCAGGTTGTTCTTCAG-3' for *CBLP*, 5'-CGCGCCGTCACCTTGTTGTTG-3' and 5'-GCCCACTCTTACCAGCACCTC-3' for *ARS1*, 5'-ACG-ACGCCATGGACAACATGTAC-3' and 5'-ACCCAGTGCCTCCGTTTCAG-3' for *SAC3*, 5'-TCGGTACTTGACGCTGAGGTTAGG-3' and 5'-ACACCGTCC-AGCCCATGTATCTT-3' for the *SNRK2.1* 3' UTR, 5'-TATGGAGCGTGGG-CGTTATCTTG-3' (underlined sequence binds to the beginning of exon VII,

whereas the remaining 5' sequence of the primer binds to the end of exon VI and 5'-CCTCCATGGTATGCGCTT-3' for *SNRK2.1* exons VI and VII, 5'-ACG-TGGCATGCAGCTCAT-3' and 5'-CTTGCCACTTTGCCAGGT-3' for *SULTR2*, 5'-ACGGGTCTTCGAGCGAATTGC-3' and 5'-CGACTGCTTACGCAACAAT-CTGG-3' for *SLT1*, 5'-CCTCGCTCTCCTCGCTGCTG-3' and 5'-CGGCCG-ACCTGGGTAATTGC-3' for *ECP76*, and 5'-GGACGGCAGCATCATGGTG-AGC-3' and 5'-TCCACACGCCCTTGACCTTGAG-3' for *SBDP*. The sizes of the amplification products were between 100 and 300 bp.

## Analysis of Sequences

Sequences were analyzed using DNASTar software version 4.05 (Lasergene 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 Chlre3 (version 3) of the *Chlamydomonas* genome generated by the JGI (<http://genome.jgi-psf.org/Chlre3/Chlre3.home.html>).

Sequence data for *SNRK2.1* cDNA v1 has been deposited with the EMBL database under accession number AM900768.

## Supplemental Data

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

**Supplemental Figure S1.** Putative cDNA splice variants and proposed mechanism that could lead to the PCR generation of artifactual splice variants.

**Supplemental Figure S2.** Alignment of *SNRK2.1* splice variants.

**Supplemental Figure S3.** Detail of exon XI of *SNRK2.1* v1 showing the alternative 5' splice region and the 3' UTR.

**Supplemental Figure S4.** Alignment of the *Chlamydomonas* SNRK2 protein family.

## ACKNOWLEDGMENTS

We thank Dr. Jeffrey Moseley for critical reading of the manuscript and for providing pJM43 vector and the JGI for generating a draft *Chlamydomonas* genome sequence, which has been invaluable for both gene identification and mutant analyses. We would also like to dedicate this manuscript to Winslow Briggs in honor of his 80th birthday; Winslow has been both an enormously supportive colleague and an inspiration to all members of the Grossman laboratory.

Received January 9, 2008; accepted March 2, 2008; published March 7, 2008.

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