The Central Role of a SNRK2 Kinase in Sulfur Deprivation Responses

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ABSTRACT

In the absence of sulfur, *Chlamydomonas reinhardtii* increases the abundance of several transcripts encoding proteins associated with sulfur acquisition and assimilation, conserves sulfur amino acids and acclimates to suboptimal growth conditions. A positive regulator, SAC1, 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 kinase designated SNRK2.1. Like the *sac1* mutant, both *snrk2.1* mutants were deficient in expression of sulfur-responsive genes. Furthermore, the mutant cells bleached more rapidly than wild-type cells during sulfur 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 non-epistatic 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 sulfur-deprivation responses in Chlamydomonas. The phylogenetic relationships and structures of the eight members of the SNRK2 family in Chlamydomonas are discussed.
INTRODUCTION

Sulfur (S) is an essential element present in proteins, lipids, carbohydrates, electron carriers, various metabolites (some involved in detoxification of heavy metals and xenobiotics) and signaling molecules (Meister and Anderson, 1983; Gupta et al., 1990; Schultze et al., 1992; Marrs, 1996; Grossman and Takahashi, 2001). The preferred S source for most organisms is sulfate (SO$_4^{2-}$) which can be limiting in the environment; limitations for SO$_4^{2-}$ may result in reduced quality and yield of seeds and cause stunted plant growth (Mahler and Maples, 1986; Mahler and Maples, 1987; Warman and Sampson, 1994). Most organisms are unable to efficiently store S, and 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 synthesizes periplasmic arylsulfatases (ARS) that catalyze the hydrolysis of organic SO$_4^{2-}$ esters (de Hostos et al., 1989), develops more efficient 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, accumulation of storage carbohydrates and 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 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 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 upregulated 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 photosystem II (PS II), suggesting that modification of photosynthetic electron transport during S deprivation is critical for cell survival.

SAC1 has significant sequence similarity to the animal S transporters (Na⁺/SO₄²⁻ transporters). Three additional genes that encode proteins with strong sequence similarity to mammalian Na⁺/SO₄²⁻ transporters (SLT1-3 genes) 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 raises 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 serine-threonine kinase in the plant-specific SNF1-related protein kinase 2 (SNRK2) family. The \textit{sac3} mutants exhibit low-level constitutive ARS activity in S-replete medium, but like wild-type cells, accumulate high levels of ARS following exposure of the cells to S deprivation. Other S-responsive genes (in addition to \textit{ARS}) are also negatively regulated by SAC3 (Ravina et al., 2002). Furthermore, the \textit{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 non-epistatic since the \textit{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, \textit{ars11} and \textit{ars44}, that harbor an interruption in the same gene. This gene, designated \textit{SNRK2.1}, encodes a SNRK2 kinase with sequence similarity to SAC3. In the present work, we demonstrate that SNRK2.1 plays a crucial role in the control of S-deprivation responses. The \textit{ars11} lesion dramatically affects S-deprivation responsive gene expression, and the mutant cells rapidly bleach following transfer to medium lacking S. Furthermore, unlike \textit{sac1}, \textit{ars11} is epistatic to \textit{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. Two kinds of mutants were identified: those with no or low ARS activity (ars⁻ mutants), and those that exhibited more ARS activity than the parental strain (ars⁺ mutants). Approximately 30,000 transformants were screened, and from those, 50 strains exhibited an ars⁻ phenotype while 2 exhibited an ars⁺ phenotype (Pollock et al., 2005).

An adaptor-mediated PCR technique (Padegirnas and Reichert, 1998) was successfully used to determine the genomics 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 previously reported (Pollock et al., 2005). Among the ars⁻ 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 serine/threonine protein kinase belonging to the SNRK2 plant kinase family (Halford and Hardie, 1998). This gene has been designated SNRK2.1.

**Co-segregation of paromomycin resistance with ars phenotype:** To determine whether the ars⁻ 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⁺ progeny (~30 of a total of 60 analyzed) co-segregated with the ars⁻ 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 time (Fig. 1A). In liquid cultures ars44 showed essentially no ARS activity after 24h 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, 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 responses of the ars11 and ars44 strains to S deprivation, 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+/SO$_4^{2-}$-like transporter (Zhang et al., 2004), SULTR2 for the H$^+$/SO$_4^{2-}$- like transporter (Pootakham and Grossman, unpublished), 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. 2A, B, C). Both the ars11 and ars44 mutants had a lower basal level 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. 1A-C) 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). Like in ars11, the sac1 mutant showed no or 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.

**The cDNAs and sites of insertion in ars11 and ars44.** There were no EST sequences to help identify the CDS and intron-exon boundaries of the SNRK2.1 genomic sequence. Therefore, a 2,129 bp cDNA was amplified by 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‘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 than the predicted sequence of the \textit{ab initio} model for the gene that was generated by the JGI (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). We have 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 \textit{AphVIII} marker gene for both \textit{ars11} and \textit{ars44} (the border close to the 3’end of \textit{AphVIII}) (Pollock et al., 2005). In \textit{ars11} the insertion was positioned within exon VII whereas in \textit{ars44} the insertion was within intron 6 (Fig. 3B). To identify the flanking regions on both sides of insertions, specific primer pairs were designed to anneal to the \textit{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 a deletions or a reorganization of the genome around the site of the insertion (Kindle, 1990). Using RT-PCR we demonstrated that neither \textit{ars11} nor \textit{ars44} was completely lacking \textit{SNRK2.1} transcripts; however, the mRNA from \textit{SNRK2.1} in \textit{ars11} was missing the sequence encoded by exon VII, while \textit{ars44} retained a full-length \textit{SNRK2.1} transcript that was indistinguishable from that of the parental strain (Fig. 3C). In \textit{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 \textit{ars11} mutant would have a shift in the reading frame generated as a consequence of the fusion of exons VI-VIII. When \textit{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 \textit{SNRK2.1} transcript accumulation in \textit{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 \textit{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 \textit{–S} conditions) relative to parental cells (Fig. 4). Thus, the \textit{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 low level accumulation of the mRNA. The molecular differences associated with mRNA processing are likely responsible for the different mutant phenotypes. Furthermore, the data demonstrates that splicing of intron 6 of \textit{SNRK2.1} can occur even if the intron is interrupted with the 1.7 Kb \textit{PSAD::AphVIII} marker gene.

**Potential alternative splicing of \textit{SNRK2.1}:** As mentioned above, \textit{SNRK2.1} cDNA generation led to multiple cDNAs of different lengths. At least five different \textit{SNRK2.1} cDNAs were identified using the same set of specific primer pairs for RT-PCR (RNA template was from D66 cells grown in TAP and TAP-S); these cDNAs were designated \textit{SNRK2.1} v1, v2, v3, v4 and v5 and the differences among them are shown in **Supplemental Figure 1.** \textit{SNRK2.1} v1 was the longest cDNA, encoding a polypeptide of 390 amino acids, and is the isoform discussed above (referred to as \textit{SNRK2.1}). All of these potential transcripts encoded putative polypeptides that used the same start codon, with \textit{SNRK2.1} v1, v2, and v3 maintaining the same coding frame and stop codon. In contrast, the \textit{SNRK2.1} v4 and v5 reading frames changed as a result of the alternative splicing, which also generated a different stop codon. For \textit{SNRK2.1} v2-4, the potential alternative 5´ splice sites were positioned inside a 54 bp GC-rich region of the transcript. The deduced \textit{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 \textit{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 \textbf{Supplemental Figure 2}. None of the potential splice variants were associated with specific culture condition (e.g. TAP compared with TAP-S), and the only variant reproducibly observed was \textit{SNRK2.1} v1; the others were less abundant and in some reactions were not detected. Finally, only in \textit{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 \textit{SNRK2.1} cDNA sequences (\textbf{Supplemental Fig. 3}); these sites were not associated with specific recombinant libraries or splice variants.

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

\textbf{Complementation of the snrk2.1 mutation:} Four of the \textit{SNRK2.1} cDNA potential splice variants (v1, v2, v3 and v4) were introduced into the \textit{ars11} mutant strain. Each cDNA version was inserted between the \textit{PSAD} promoter and 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 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 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 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 medium (compare Fig. 2F and Fig. 2B).

**The SNRK2 family:** SNRK2.1 belongs to the plant-specific serine/threonine protein kinase family SNRK2. Like others 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 SNRK2 family, with 43% sequence identity with SNRK2.1. In addition to SNRK2.1 and SAC3, there are six others SNRK2 family members encoded on the Chlamydomonas genome. The complete 8-membered family, SNRK2.1-8, 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 others SNRK2 proteins; one major feature is the presence of an extra loop of 17-22 amino acids immediately preceding the SNF-1 kinase activation domain (Hardie and Carling, 1997), which is shown in the alignments of Fig. 5 (broken line). Although this extra loop is not conserved at the
sequence level among the subfamily members, it does contain a NLH motif (asterisk above 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 4.

DISCUSSION

In this study we identified SNRK2.1, a member of the plant-specific SNRK2 kinase family and the larger SNF-1 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 insertions. Even though the mutants had a sequence of 1.7 kb (AphVIII gene under PSAD promoter and 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 that of 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: a) 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 the top of the agar (data not shown). b) The splicing machinery may be somewhat different under different growth conditions, which might change the efficiency at which some transcripts are spliced. c) 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 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 junctions sites for the potential variants identified markedly differ from the Chlamydomonas consensus splice junction sequences (Silflow, 1998). Also, 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-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 3´ and 5´ splice sites (**Supplemental Fig. 1 C**). 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 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 can not be completely excluded based on this work, our results do suggest that caution should be taken when considering the generation of splice variants. Additionally, the existence of variability in the placement of the poly(A) tail (**Supplemental Fig. 3**) suggests some complexity in the regulation of *SNRK2.1* transcripts.

SNRK2, SNRK1 and SNRK3 kinase families are plant specific and belong to the SNF-1 superfamily. SNRK2 kinases comprise 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 aspartate or glutamate, which defines 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 involve 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 phosphatidyl inositol transfer-like protein (Monks et al., 2001), AKIP1, a protein regulating RNA stability (Li et al., 2002), and a basic leucine zipper (bZIP) transcription factor required for ABA gene regulation.

Interestingly, some members of the Arabidopsis SNRK2 kinase family may function in controlling S-limitation responses. Arabidopsis mutants defective for \textit{SNRK2.3} exhibit a slight decrease in the level of \textit{SULTR2;2} mRNA (encoding the low affinity SO$_4^{2-}$ transporter) and elevated O-acetyl-serine (precursor to cysteine) accumulation (Kimura et al., 2006). However, \textit{snrk2.3} mutant plants did not show alterations in expression of other S-responsive genes (\textit{APR} and \textit{SAT}), the accumulation of 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 ten \textit{AtSNRK2} genes showed increased expression following S starvation of plants (Kimura et al., 2006), which raises the possibility that activity of other SNRK2 proteins from Arabidopsis may impact S-starvation responses. Overall, the 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 other 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-terminus 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 interaction with the PP2C-type phosphatase ABI1, and deletion of the rice OSRK1 C-terminus destroys 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, .3 and .4) have a specific feature that is not present in plant SNRK2 proteins; this distinguishing feature is an inserted, non-conserved loop of 17-22 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 & 4) has several potential phosphorylation sites that may participate in regulation/activation (Supplemental Fig. 4).
SNRK2.1, SAC1 and SAC3 are the three genes that have been shown to have a regulatory role in 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 phospho-relays 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, while SAC3 appears to be 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 Na⁺/SO₄⁻ 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. 2B, 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 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 SULRT2 transcripts, even under nutrient-replete conditions, relative to the parental strain. As deduce 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 medium (data not shown), and the patterns of ARS1, SLT1 and 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 for 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 enhance SNRK2.1 activation which is downstream in this pathway and more directly involved in 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.

METHODS

Strains, culture conditions and mating: Chlamydomonas reinhardtii strains used in this work were D66 (nit2− cw15 mt+) (Pollock et al., 2003), 21gr (nit5− mt−) (Harris, 1989), sac1 (sac1− mt+), and sac3 (sac3− mt+) (Davies et al., 1994). Cells were cultured under continuous light at 23°C in liquid and solid Tris-Acetate-Phosphate (TAP) medium (Harris, 1989). To impose S starvation, cells in mid-logarithmic growth were washed twice with liquid TAP medium without S (TAP-S) (Harris, 1989) and then split and resuspended in either TAP or TAP-S. Paromomycin and bleomycin were used at 10 and 3 μg/mL,
respectively. Genetic analyses were performed with the various strains according to a previously described protocol (Harris, 1989).

**Generation of ars11 sac3 double mutant:** The sac3 mt+ mutant was crossed with the parental strain, 21gr (mt-), to obtain a sac3 mt- 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- progeny was crossed with ars11 mt+. 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’CGTACAAGGCCCATGCGTGAGTC and 5’TCGCCGAAAATGACCCAGGC.

**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. (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 6-12 h under continuous light to allow for the accumulation of the AphVIII protein. Transformants were then selected on TAP supplemented with 5 µg/ml of 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 mg of chlorophyll. The data presented corresponds to mean values of at least three independent experiments. The concentrations
of chlorophylls $a + b$, extracted from cells in methanol, were estimated using the equations of Porra (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, Valencia, CA; Cat. No. 79254) and further cleaned using RNeasy columns (Qiagen, Valencia, CA) following the manufacturer’s directions. Single-stranded cDNAs were synthesized from total RNA using a N-(polyT)$_{20}$mer primer according to the SuperScript III RNaseH-Reverse Transcriptase manual (Invitrogen, Carlsbad, CA). This cDNA population was used as substrate for real time PCR with gene-specific primer pairs; the single-stranded cDNAs were diluted five-fold prior to inclusion in the reaction mixture. The complete coding sequence (CDS) of $SNRK2.1$ was obtained by RT-PCR using the specific primers 5´GCCTTCTTGCGACTGACTG and 5´CCTCAGTCGTTCATGCCA. 3´RACE for $SNRK2.1$ transcripts was performed using the specific 5´ primer GTTCCGTGAGGACCTACCG and a previously described polyT-Qt primer that allowed semi-nested PCR amplifications (Frohman, 1990). All PCRs were performed in the presence of 2–5% DMSO.

**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 and 5´TGGATCCAGTGTTGGGAACGATTCAATG, which introduced a ClaI and BamHI site at the 5´ and 3´ ends, respectively, of the amplified sequences. Four different amplicons...
were obtained, v1, 1204 bp; v2, 1068; v3, 952; v4, 672. 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 pDGV1-4 plasmids were introduced into the ars11 mutant and transformants were assayed for rescue of the mutant phenotype.

**qPCR:** Real time PCR was performed using the Chromo4 thermocycler (BioRad). Individual reactions were 25 µl final vol, consisting of 10 µl of DyNAMO SYBR Green qPCR reagent (Finnzymes, Espoo Finland), 3.7 pmoles of each primer, 1–2 µl of single-strand cDNA (5-fold diluted from the reverse transcriptase reaction) and distilled water to 25 µl. The Chromo4 run protocol was: denaturation at 95ºC for 15 min followed by 40 cycles of denaturation at 94ºC, 10 s; annealing at 60ºC, 30 s; amplification at 72ºC, 30 s; and fluorescence measurement after 80ºC 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 (60–100ºC with a heating rate of 0.5ºC/s and continuous fluorescent measurements) and electrophoretic analysis on 4% agarose gels. We used the CBLP gene as a housekeeping gene control (Chang et al., 2005). Ct values were determined in three independent experiments, with three replicates for each experiment. Relative fold differences were calculated based on the relative ∆Ct method ($2^{-(C_{\text{sample}} - C_{\text{control gene}})}$) using the CBLP amplification product as an internal standard. The primer pairs used for qPCR were: 5′CTTCTCGCCCATGACCAC and 5′CCCACCAGGTGTCTTCAG for CBLP, 5′CGCGCCGTCACTTTGTTGTTG and
5’GCCCACTCTTTACCCAGCACCTC for ARS1,
5’ACGACGCATGGACAACATGTAC and 5’ACCCAGTGCGCTCCTCCGACG for SAC3,
5’TCCGTACTTGCAGCTGAGTTAGG and
5’ACACCGTCCAGCCCATGTATCTT for the SNRK2.1 3’UTR,
5’TATGGAGCGTGGGCGTATCTTG (underline 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’CCTCCATGGTGATGCGCCTT for SNRK2.1 exons VI-VII,
5’ACGTGGCATGCAGCTCAT and 5’CTTGCCACCTTTGCAGGT for SULTR2,
5’ACGGGTCTTCGAGCGAATTGC and 5’CGACTGCTTACGCAACAATCTTGG for SLT1, 5’CCTCGCTCTCCTGCTGCTG and 5’CGGCCGACTTGGGTAATTGC for ECP76,
and 5’GGACGGCCCATCATGCTTGCAG and
5’TCCACACGCCCCTTGACCTT for SBDP. The sizes of the amplification products were between 100 and 300 bp.

Analysis of sequences: Sequences were analyzed using DNAstar software v.4.05 (Lasergene Navigator), Bioedit Sequence Alignment Editor v. 5.0.9 (Department of Microbiology North Carolina State University, USA), NCBI Blast server (http://www.ncbi.nlm.nih.gov/BLAST/) and Chlre3 (version 3) of the Chlamydomonas genome generated by the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). Sequence data from SNRK2.1 cDNA v1 (see text below for SNRK2.1 cDNA v1 characterization) has been deposited with the EMBL database under accession number AM900768.
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Table and Figure Legends

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 + b* content was normalize to the value on day 0 for each strain. Values represent the mean of three independent experiments. Strains used are D66 (parental strain), *ars11* and *ars44 (snrk2.1); tars11#34 (ars11 complemented with SNRK2.1), sac1 (sac1 mutant), sac3 (sac3 mutant), 11-3#16 (ars11 sac3 double mutant).

Figure 2. **qPCR analysis of sulfur-regulated gene expression.** The parental strain (A) and mutants (B-E) were grown on TAP medium, washed with TAP-S medium and then resuspended in either TAP or TAP-S. Samples were taken for qPCR analysis 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 were much below 1, and those values that were below 1 are represented as 0 on the graph. 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), tars11#34 (complemented with SNRK2.1), sac1 (sac1 mutant), sac3 (sac3 mutant), and 11-3#16 (ars11 sac3 double mutant).

**Figure 3.** A) **Genomic structure of SNRK2.1.** The white blocks and the lines represent exons and introns respectively. Exons are numbered with roman characters 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 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 grey blocks represent exons for which the reading frame has changed.

**Figure 4.** **SNRK2.1 expression (qPCR).** Primers used were specifically designed to detect the joining of exons VI and VII (see METHODS for details). RNA sample preparation was as described in 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.

**Figure 5.** **Amino Acid Sequence Alignment.** The predicted SNRK2.1, 2 (SAC3), 3 and 7 proteins were aligned with representative Arabidopsis thaliana and Oryza sativa SNRK2 kinases (accession numbers: AtSNRK2.1, NP_196476; AtSNRK2.2, NP_190619;
AtSNRK2.3, NP_201489; SAPK1, NP_001050274; SAPK2, NP_001060312; SAPK4, NP_001044930) using BioEdit 7.0.5.3 software. The black and grey boxes indicate identical and similar amino acids, respectively. The C-terminal subdomains are highlighted with heavy black lines above the sequence 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 serine of the activation domain (#) are noted.

**Figure 6. Model explaining regulation of the S-responsive genes in Chlamydomonas.** SAC1 is likely acting 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. SNRK.2.2 (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.

**Table I. Chlamydomonas SNRK2 family.** Protein IDs, 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 identity of the deduced N-terminal catalytic sequence with those of *Arabidopsis thaliana* and *Oryza sativa* are given as a range of identities (the
analysis was performed using MatGAT 2.0, Montclair State University). Gene models that are likely inaccurate.

Table I. Chlamydomonas SNRK2 family

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

1Gene models that are likely inaccurate.
SUPPLEMENTAL FIGURES

Supplemental Figure 1. Putative cDNA splice variants and proposed mechanism that could lead to artifactual splice variants generated by PCR

Supplemental Figure 2. Alignment of \textit{SNRK2.1} splice versions

Supplemental Figure 3. Detail of exon XI of \textit{SNRK2.1} v1 containing the alternative 5´splice region and the 3´UTR

Supplemental Figure 4. Alignment of SNRK2 protein family in Chlamydomonas
Figure 1. Accumulation of ARS activity and chlorophyll in *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* + *b* content was normalized to the value on day 0 for each strain. Values represent the mean of three independent experiments. Strains used are D66 (parental strain), *ars11* and *ars44* (*snrk2.1*); *tars11*#34 (*ars11* complemented with *SNRK2*), *sac1* (*sac1* mutant), *sac3* (*sac3* mutant), 11-3#16 (*ars11 sac3* double mutant).
Figure 2. qPCR analysis of sulfur-regulated gene expression. The parental strain (A) and mutants (B-E) were grown on TAP medium, washed with TAP-S medium and then resuspended in either TAP or TAP-S. Samples were taken for qPCR analysis 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 were much below 1, and those values that were below 1 are represented as 0 on the graph. 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-), tars11#34 (complemented with SNRK2.1), sac1 (sac1 mutant), sac3 (sac3 mutant), and 11-3#16 (ars11 sac3 double mutant).
Figure 3

A) Genomic structure of **SNRK2.1**. The white blocks and the lines represent exons and introns respectively. Exons are numbered with roman characters 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 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 grey blocks represent exons for which the reading frame has changed.
Figure 4. *SNRK2.1* expression (qPCR). Primers used were specifically designed to detect the joining of exons VI and VII (see METHODS for details). RNA sample preparation was as described in 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.
Figure 5. Amino Acid Sequence Alignment. The predicted SNRK2.1, 2 (SAC3), 3 and 7 proteins were aligned with representative Arabidopsis thaliana and Oryza sativa SNRK2 kinases (accession numbers: AtSNRK2.1, NP_196476; AtSNRK2.2, NP_190619; AtSNRK2.3, NP_201489; SAPK1, NP_001050274; SAPK2, NP_001060312; SAPK4, NP_001044930) using BioEdit 7.0.5.3 software. The black and grey boxes indicate identical and similar amino acids, respectively. The C-terminal subdomains are highlighted with heavy black lines above the sequence 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 serine of the activation domain (#) are noted.
Figure 6. Model explaining regulation of the S-responsive genes in Chlamydomonas. SAC1 is likely acting 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.