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Sac3, an Snf1-like Serine/Threonine Kinase That Positively and Negatively Regulates the Responses of Chlamydomonas to Sulfur Limitation

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The *Sac3* gene product of Chlamydomonas positively and negatively regulates the responses of the cell to sulfur limitation. In wild-type cells, arylsulfatase activity is detected only during sulfur limitation. The *sac3* mutant expresses arylsulfatase activity even when grown in nutrient-replete medium, which suggests that the Sac3 protein has a negative effect on the induction of arylsulfatase activity. In contrast to its effect on arylsulfatase activity, Sac3 positively regulates the high-affinity sulfate transport system—the *sac3* mutant is unable to fully induce high-affinity sulfate transport during sulfur limitation. We have complemented the *sac3* mutant and cloned a cDNA copy of the *Sac3* gene. The deduced amino acid sequence of the *Sac3* gene product is similar to the catalytic domain of the yeast Snf1 family of serine/threonine kinases and is therefore classified as a Snf1-related kinase (SnRK). Specifically, *Sac3* falls within the SnRK2 subfamily of kinases from vascular plants. In addition to the 11 subdomains common to Snf1-like serine/threonine kinases, Sac3 and the plant kinases have two additional subdomains and a highly acidic C-terminal region. The role of Sac3 in the signal transduction system that regulates the responses of Chlamydomonas to sulfur limitation is discussed.

INTRODUCTION

To survive in a dynamic environment, organisms must be able to sense changes in their environment and respond to those changes by altering their metabolism. Signal transduction mechanisms involved in controlling these responses may interact to form a network that links perception of the environment to physiological processes in the cell; this network may be required for survival of organisms in a dynamic environment that is often resource limited. We are using the genetically tractable, unicellular green alga Chlamydomonas (*Chlamydomonas reinhardtii*) as a model system to investigate how photosynthetic organisms acclimate to changes in nutrient availability (de Hostos et al., 1988, 1989; Davies et al., 1994, 1996; Yildiz et al., 1994, 1996; Quisel et al., 1996; Wykoff et al., 1998). Several responses of Chlamydomonas to nutrient limitation are similar to those exhibited by vascular plants and soil-dwelling microbes (Marzluf and Metzenberg, 1968; Harder and Dijkhuizen, 1983; Hawkesford and Belcher, 1991; Tsay et al., 1993; Smith et al., 1995; Trueman et al., 1996; Davies and Grossman, 1998; Wykoff et al., 1998). Much of our work has focused on the ways in which Chlamydomonas adjusts to limiting sulfur levels.

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Sulfur is a macronutrient that is required in relatively high concentrations by all organisms. It is a constituent of proteins, lipids, carbohydrates, electron carriers, and numerous cellular metabolites. For most organisms, the preferred source of sulfur is the sulfate anion (Uria-Nickelsen et al., 1993, 1994; Beil et al., 1996). However, the level of available inorganic sulfate in the soil may be low (David et al., 1982; Autry and Fitzgerald, 1990; Whalen and Warman, 1996). Many soil-dwelling microbes have developed mechanisms to scavenge sulfur from their environment by accessing organic sulfate esters, sulfamates, and sulfonates (Marzluf, 1970; Scott and Metzenberg, 1970; Apte et al., 1974; Lien and Schreiner, 1975; de Hostos et al., 1988; Murooka et al., 1990; Yildiz et al., 1994; Beil et al., 1996), which are abundant in the soil (Fitzgerald et al., 1988; Autry et al., 1990; Dhamala et al., 1990; Dhamala and Mitchell, 1995). In addition, both microbes and plants exhibit an increased efficiency for the import of sulfate when sulfate supplies are limiting (Breton and Surdin-Kerjan, 1977; Clarkson et al., 1983; Biedlingmaier and Schmidt, 1988; Green and Grossman, 1988; Hawkesford et al., 1993; Yildiz et al., 1994; Lappartient and Touraine, 1996).

Chlamydomonas cells exhibit a suite of responses when transferred from sulfate-replete medium to medium lacking sulfur. Cells stop dividing, photosynthetic oxygen evolution declines as a consequence of reduced photosystem II activity (Wykoff et al., 1998), novel proteins, including an

arylsulfatase (Ars polypeptide and *Ars* gene), are synthesized and exported to the periplasm or cell wall (de Hostos et al., 1988), and a high-affinity sulfate transport system accumulates (Yildiz et al., 1994). Extracellular Ars releases the sulfate anion from esterified organic sulfates, allowing Chlamydomonas to access sulfur stores in the soil (Lien and Schreiner, 1975; de Hostos et al., 1988; Yildiz et al., 1994; Davies et al., 1996). To elucidate the mechanisms used to regulate the responses of Chlamydomonas to sulfur limitation, we have developed a colorimetric screen to isolate mutants that display aberrant regulation of *Ars* gene expression; the mutants are designated *sac* (for sulfur acclimation). After transfer to medium devoid of sulfur, the *sac1* and *sac2* mutants do not synthesize Ars or accumulate the high-affinity sulfate transport system to the same extent that wild-type cells do (Davies et al., 1994). In addition, the *sac1* mutant is unable to decrease photosynthetic electron transport during sulfur-limited growth, which results in rapid cell death in the light (Davies et al., 1996). Unlike *sac1* and *sac2*, the *sac3* mutant exhibits constitutive, low-level synthesis of the extracellular Ars in the presence of sulfate. Furthermore, although the *sac3* mutant is unable to produce elevated levels of the high-affinity sulfate transport system, it does accumulate Ars (Davies et al., 1994) and downregulates photosynthetic electron transport (J.P. Davies and A.R. Grossman, unpublished data) during sulfur-limited growth.

To determine the regulatory relationships among the Sac polypeptides, we are cloning, sequencing, and characterizing the *Sac* genes. The *Sac1* gene encodes a polypeptide similar to a class of ion transporters present in cell membranes (Davies et al., 1996; Davies and Grossman, 1998). Despite the similarity of Sac1 to ion transporters, the phenotype of the *sac1* mutant suggests that this protein either functions in sensing the sulfur status of the environment or participates in the signal transduction pathway that controls the responses of Chlamydomonas to sulfur deprivation. The *Sac3* gene product appears to be required for the repression of Ars during sulfur-replete growth and for the full induction/activation of the sulfate transport system during sulfur starvation. Here, we report that the *Sac3* gene encodes a polypeptide that exhibits similarities to Snf1, a protein kinase of *Saccharomyces cerevisiae* known to function in the signal transduction pathway controlling the expression of genes induced during glucose deprivation (Carlson et al., 1981; Carlson and Botstein, 1982). Interestingly, several putative vascular plant Snf1-related kinases have an even higher degree of similarity to Sac3.

RESULTS

Identification of Polymorphic Region in the *sac3* Mutants

Chlamydomonas strain CC425 (*cw15 arg7-8*) was transformed with linearized plasmid pJD67 (containing the *Arg7* gene [Debuchy et al., 1989] in the pBluescript KS+ vector [Stratagene, La Jolla, CA]), and transformants were selected as arginine prototrophs. This procedure generates mutants because the linearized plasmid integrates randomly into the Chlamydomonas genome. Mutants that exhibited constitutive expression of Ars were identified by growing the transformants on nutrient-replete solid medium and then spraying the colonies with the chromogenic Ars substrate 5-bromo-4-chloro-3-indolyl sulfate (Davies et al., 1994). The constitutive Ars-producing strains, designated are9-1, are10-1, and are16-1 (are10-1 was described as the *sac3* mutant in Davies et al. [1994]), were obtained at a frequency of approximately one in 5000 transformants.

To determine whether the mutant phenotype cosegregated with the DNA that was introduced during transformation, we crossed strain are10-1 (*sac3 mt+*) with strain CC2677 (*nit1 mt−*). Twenty random progeny were tested for the mutant phenotype by scoring individuals for constitutive Ars expression and for the presence of the introduced plasmid by using DNA gel blot hybridization with probes specific for both *Arg7* and pBluescript KS+ sequences. Cosegregation of the mutant phenotype and a single copy of the introduced DNA was observed (data not shown), suggesting that the insertion of pJD67 caused the mutant phenotype. The are10-1 strain was backcrossed several times with CC2677 to generate a new mutant strain, are10-12 (*sac3 nit1*), with a homogeneous genetic background. This strain was used for many of the experiments described below.

To identify the gene altered in are10-12, we isolated Chlamydomonas DNA flanking the integrated vector sequences by plasmid rescue. The strategy for plasmid rescue is diagrammed in Figure 1A. Briefly, genomic DNA isolated from are10-12 was digested with BamHI (which cuts between the *Arg7* gene and pBluescript KS+ sequence in linearized pJD67) to generate a >12-kb fragment containing both pBluescript KS+ sequences and flanking Chlamydomonas DNA (based on DNA gel blot hybridizations using pBluescript KS+ as a probe; data not shown). The BamHI fragments were ligated and introduced into *Escherichia coli*, and transformants containing pBluescript KS+ sequences were selected on ampicillin-containing medium. All transformants harbored an identically sized >12-kb plasmid, designated pFY1, that contained both pBluescript KS+ and Chlamydomonas genomic DNA. The insert DNA in pFY1 was mapped, and a 1.7-kb SalI fragment adjacent to the pBluescript KS+ sequence was subcloned into pBluescript KS+ to yield the plasmid designated pFY1a.

Figure 2 shows that hybridization of the 1.7-kb SalI fragment from pFY1a to genomic DNA from the different mutants that constitutively express Ars (i.e., are9-1, are16-1, and are10-12) yielded a polymorphic restriction pattern. A 7.0-kb SalI genomic fragment detected in the untransformed parental strain CC425 (Figure 2, lane 1) was missing in the mutants, and a new 1.7-kb SalI fragment was detected in are10-12 and are9-1 (Figure 2, lanes 2 and 4, respectively), as was a new 1.9-kb SalI fragment in are16-1 (Figure 2, lane

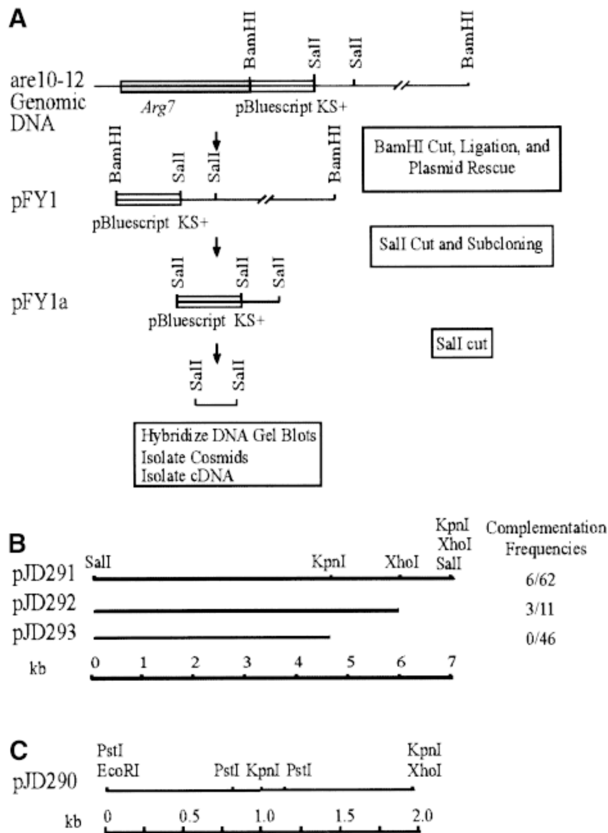

Figure 1. Genomic and cDNA Clones of *Sac3.*

(A) Schematic of the isolation of a genomic DNA adjacent to the introduced *Arg7* gene in the *sac3* mutant are10-12. Genomic DNA from are10-12 was digested with BamHI, ligated, and introduced into *E. coli.* Transformants were selected for growth in the presence of ampicillin, and a plasmid, designated pFY1, containing pBluescript KS+ sequences and a 9-kb fragment of Chlamydomonas genomic DNA, was isolated. A 1.7-kb SalI fragment adjacent to the pBluescript KS+ sequences was purified and cloned into pBluescript KS+ to form pFY1a. This 1.7-kb SalI fragment was used to isolate both the DNA that complemented the *sac3* lesion and the cDNA for the *Sac3* gene.
(B) Maps of fragments used to test complementation. The complementation frequencies for the plasmids pJD291, pJD292, and pJD293 are given as [number complemented]/[number of transformants tested].
(C) Map of the *Sac3* cDNA, designated pJD290.

3). The observed polymorphisms indicate that the three independently isolated mutants that constitutively express Ars are altered in the same region of the Chlamydomonas genome. Furthermore, the 1.7-kb SalI fragment was detected in all of the mutant progeny of the cross of are10-12 with CC2677, whereas the 7-kb fragment was detected in all of the wild-type progeny (data not shown). Together, these results suggest that the three *sac3* mutants are allelic and that the region of the genome defined by the 1.7-kb SalI fragment either contains or is very close to the coding region of the *Sac3* gene.

Complementation of the *sac3* Mutants

Intact copies of the chromosomal region disrupted in are10-12 were isolated by screening a cosmid genomic library of a

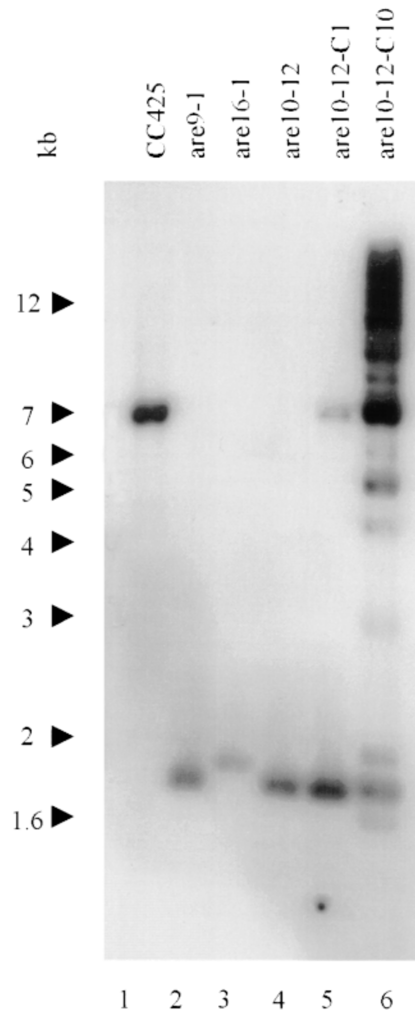

Figure 2. DNA Gel Blot Analysis of Genomic DNA from Wild-Type Cells, *sac3* Mutants, and Complemented *sac3* Mutants.

Genomic DNA from wild-type (CC425), three strains constitutively expressing Ars (are9-1, are16-1, and are10-12), and two complemented strains (are10-12-C1 and are10-12-C10) was digested with SalI, separated by agarose gel electrophoresis, and transferred to nitrocellulose membranes. Hybridization was performed with the 1.7-kb SalI insert of pFY1a. The positions of the molecular length markers are given at left in kilobases.

wild-type Chlamydomonas strain with the 1.7-kb SalI fragment from pFY1a. Three unique clones were isolated and introduced into Chlamydomonas strain are10-12 (*sac3 nit1*) via cotransformation with the pMN24 plasmid (containing the *Nit1* gene; Fernandez et al., 1989) by using particle gun bombardment. To determine whether any of these cosmids complemented the mutant phenotype, we tested transformants for Ars activity on medium containing 5 mM sulfate. Three of 34 cell lines cotransformed by *Nit1* and the cosmid cosFY2 showed no Ars activity on Tris–acetate–phosphate (TAP) medium, which was similar to wild-type cells. Fifty-two cell lines transformed with only pMN24 were tested, and all continued to express Ars activity.

One of the apparently complemented strains, are10-12-C1, was further analyzed to determine whether it contained DNA from cosFY2. Genomic DNA from are10-12-C1 was isolated and digested with SalI, and the resolved genomic fragments were hybridized with the 1.7-kb SalI insert from pFY1a. In addition to the original 1.7-kb SalI genomic fragment present in the mutant strain, are10-12-C1 harbored a second fragment migrating at 7 kb (Figure 2, lane 5). The 7-kb species is identical in size to the wild-type SalI fragment that hybridizes with the pFY1a insert (Figure 2); it was also shown to be present on the cosFY2 DNA (data not shown). To determine whether the complemented phenotype cosegregated with the 7-kb SalI fragment, we crossed are10-12-C1 with are10-11 (*sac3 mt−*), a strain containing the same *sac3* allele but of the opposite mating type. All of the progeny that exhibited the complemented phenotype contained both the 7- and 1.7-kb SalI fragments, whereas the mutant progeny contained only the 1.7-kb SalI fragment (data not shown).

Figure 3A shows that cultures of are10-12 grown in nutrient-replete medium exhibit significant levels of Ars activity, whereas cultures of both wild-type cells and the complemented strain exhibited essentially background levels of Ars activity. In addition, RNA gel blot analysis was used to measure the accumulation of the *Ars* transcript in wild-type cells, are10-12, and are10-12-C1. The loading of the RNA was normalized to the level of ribulose 1,5-bisphosphate carboxylase small subunit (*RbcS1*) transcript. Figure 3B shows that significant levels of the *Ars* transcript accumulated in are10-12 cells grown in nutrient-replete medium, whereas *Ars* mRNA was not detected in either CC125 or are10-12-C1 cells.

When the wild-type strain CC125 was starved for sulfate for 6 hr, the $K_{1/2}$ for sulfate transport (the concentration of sulfate at which import is half-maximal) decreased from ~17 to 2 μM, and the V_{max} increased from 20 to 200 fmol of sulfate sec^{-1} (10^5 cells)$^{-1}$ (Yildiz et al., 1994). Whereas the *sac3* mutant exhibited a decreased $K_{1/2}$ for sulfate transport upon sulfur starvation (similar to wild-type cells), the V_{max} did not increase to the same extent as in wild-type cells (Davies et al., 1994). As shown in Figure 4, the V_{max} for sulfate transport in sulfur-starved, wild-type cells and are10-12 cells was 239 and 70 fmol of sulfate sec^{-1} (10^5 cells)$^{-1}$, respectively. The complemented *sac3* mutant, are10-12-C1, exhibited a V_{max}

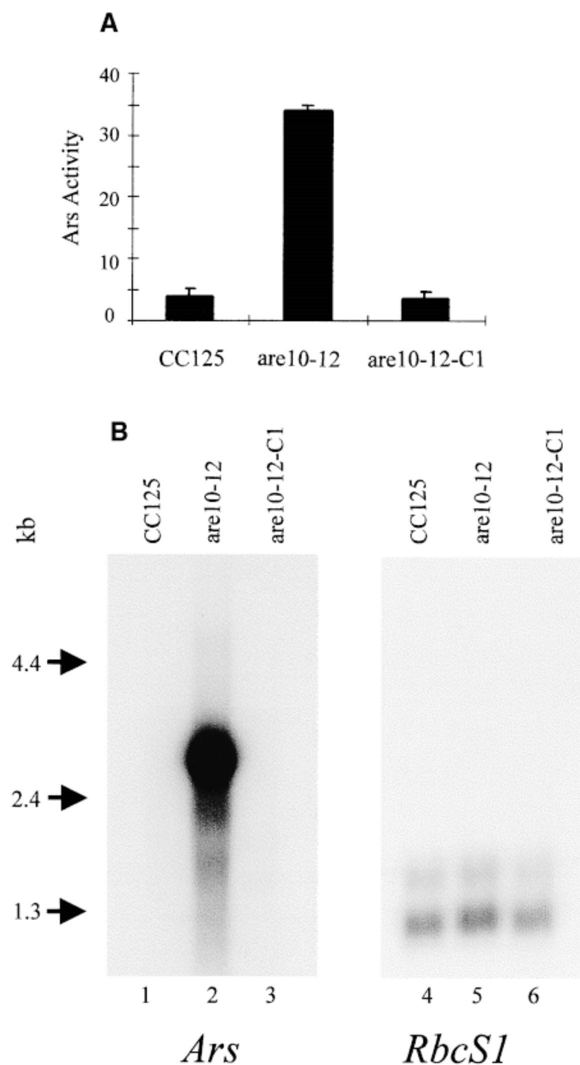

Figure 3. Ars Activity and Transcript Accumulation in Wild-Type Cells, the *sac3* Mutant, and the Complemented Strains.

(A) Strains CC125 (wild type), are10-12 (*sac3*), and are10-12-C1 (complemented *sac3*) were grown to mid-log (2 to 5 × 10^6 cells mL^{-1}) phase, and Ars activity was measured. *p*-Nitrophenyl sulfate was used as the chromogenic substrate, and the activity is expressed as micrograms of *p*-nitrophenol produced by 10^5 cells in 1 hr. The data represent averages of three experiments. The error bars indicate one standard deviation.

(B) Poly(A)$^+$ mRNA from strains CC125 (wild type), are10-12 (*sac3*), and are10-12-C1 (complemented *sac3*) was isolated, resolved on agarose gels by electrophoresis, transferred to nitrocellulose membranes, and hybridized with the 2.4-kb insert from pJD240 (*Ars2* cDNA; J.P. Davies and A.R. Grossman, unpublished data), and the 1.1-kb SalI-BamHI fragment of p2.02 (Goldschmidt-Clermont and Rahire, 1986) containing a portion of the *RbcS1* gene. Positions of the molecular length markers are given at left in kilobases.

for sulfate transport of 224 fmol of sulfate sec^{-1} (10^5 cells)$^{-1}$, which is similar to the value measured in wild-type cells.

Together, the results presented above demonstrate that are10-12-C1 is complemented for the phenotype of the *sac3* mutant and that complementation is linked to the newly introduced 7-kb SalI fragment present on cosFY2.

Complementation of the Mutant Phenotype with the 7.0-kb SalI Fragment

To determine whether the 7-kb SalI fragment (Figure 2) from cosFY2 could complement the phenotype of the *sac3* mutant, we subcloned it into pBluescript KS+, yielding pJD291 (Figure 1B), and introduced it into are10-12 (*sac3 nit1*) via cotransformation with the *Nit1* gene by using particle bombardment (Klein et al., 1987). Random transformants were tested for complementation by assaying for Ars activity in TAP medium. Six of 62 transformants showed no Ars activity, indicating complementation of the mutant phenotype. Subclones of the 7-kb SalI fragment were also generated and introduced into the *sac3* mutant, and transformants were tested for constitutive expression of Ars (Figure 1B). The plasmid pJD292, containing a 6-kb SalI-XhoI fragment of pJD291, was able to complement the *sac3* mutation (three complemented lines in 11 transformants tested), whereas pJD293, containing the 4.7-kb SalI-KpnI fragment of pJD291, was not (zero complemented lines in 46 transformants tested). These data suggest that the *Sac3* gene is located on the 6-kb SalI-XhoI fragment of pJD292 and that it spans the KpnI site located 1.3 kb from the XhoI site.

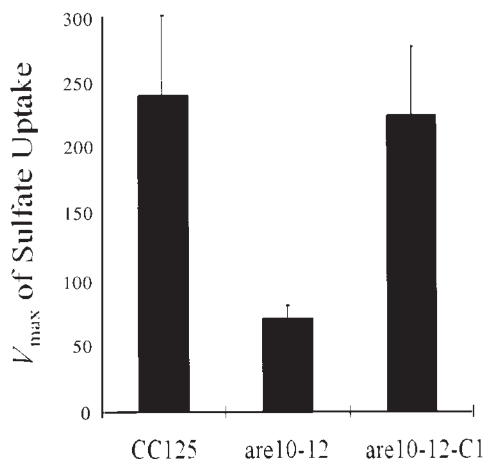

Figure 4. Sulfate Uptake in Wild-Type Cells, the *sac3* Mutant, and a Complemented Strain.

The V_{max} for sulfate uptake in CC125 (wild type), are10-12 (*sac3*), and are10-12-C1 (complemented *sac3*) is given as femtomoles of sulfate per second per 10^5 cells. The values are averages of three experiments, and the bars represent one standard deviation.

DNA gel blot analysis of genomic DNA from one of the transformants complemented with pJD291, are10-12-C10, confirmed that plasmid sequences had integrated into the genome. As shown in Figure 2, are10-12-C10 has, in addition to the 1.7-kb SalI fragment present in the original mutant line, multiple copies of the introduced DNA. Some of these copies are detected as a 7-kb SalI fragment, indicating that the plasmid recombined into the genome within the vector sequences of pJD291, whereas others are either larger or smaller than 7 kb, indicating that the plasmid recombined within the 7-kb insert. To confirm that the introduced 7-kb fragment was responsible for the complemented phenotype, we crossed are10-12-C10 with are10-11 (*sac3 mt−*). All of the progeny analyzed showed cosegregation of the complemented phenotype and this introduced fragment. In addition, none of the progeny with the mutant phenotype contained any of the introduced fragments. These data indicate that the 7-kb SalI fragment in pJD291 contains the *Sac3* gene and includes all the sequences necessary and sufficient to complement the *sac3* lesion.

Isolation of the *Sac3* cDNA and Expression and Sequence Analyses

The 1.7-kb SalI fragment of pFY1a was used to isolate pJD290, a cDNA containing a 1.9-kb insert (Figure 1C). The 0.8-kb PstI fragment of pJD290 containing the 5′ end of the Sac3 cDNA hybridized with pJD291, pJD292 (clones that complement the *sac3* lesion), and pJD293 (a clone that did not complement the *sac3* lesion). The 1.0-kb KpnI fragment of pJD290 containing the 3′ end of the *Sac3* cDNA hybridized with the complementing plasmids pJD291 and pJD292 but not with pJD293, the plasmid that did not complement the *sac3* mutation. These data indicate that the sequences coding for the *Sac3* cDNA are on plasmids pJD291 and pJD292 and that the 3′ portion of the cDNA includes the KpnI site in pJD291 and pJD292. Hence, this cDNA spans the region of the genomic sequence required for complementation.

The cDNA was hybridized with poly(A)$^+$ mRNA from wild-type, mutant, and complemented mutant strains. Figure 5 shows that the 1.9-kb cDNA insert hybridized with a 1.9-kb mRNA from wild-type cells. A 1-kb transcript was detected in the mutant strain are10-12. The complemented mutant, are10-12-C1, had both the truncated and full-length transcripts. Although the 1.9-kb transcript was more abundant in wild-type cells than in are10-12-C1 cells (normalized to either total RNA or the level of *RbcS1* transcript), the low level of transcript in the complemented strain was sufficient to rescue the mutant phenotype.

To determine whether expression of the *Sac3* gene is regulated by sulfate availability, we compared transcript accumulation in wild-type cells grown in complete medium with that of cells exposed for 2 hr to sulfur deprivation, which is sufficient time to observe elevated levels of *Ars* mRNA. Figure 6

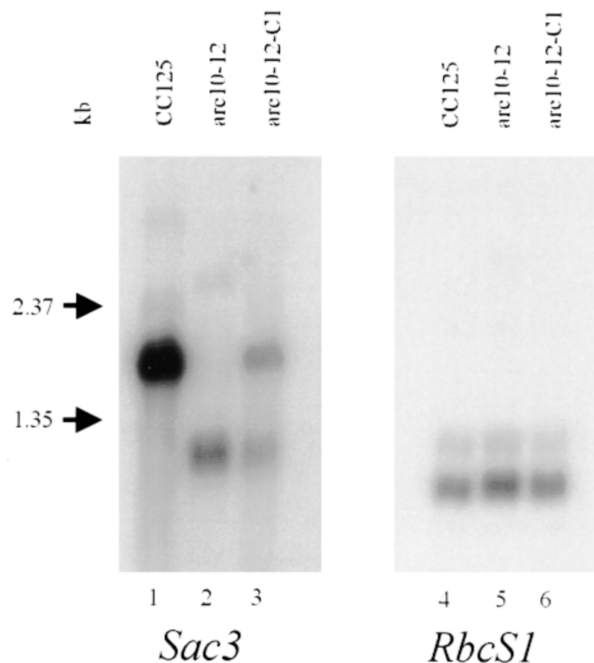

Figure 5. *Sac3* Transcript Accumulation in Wild-Type Cells, the *sac3* Mutant, and the Complemented Strain.

Poly(A)$^+$ RNA from CC125 (wild type), are10-12 (*sac3*), and are10-12-C1 (complemented *sac3*) was isolated, resolved on agarose gels by electrophoresis, blotted onto nitrocellulose, and hybridized with the 1.9-kb insert from pJD290 (*Sac3*) and the 1.1-kb SalI-BamHI fragment of p2.02 (*RbcS1*) (Goldschmidt-Clermont and Rahire, 1986). Positions of the molecular length markers are given at left in kilobases.

shows that there was no significant difference in the level of the *Sac3* transcript in starved and unstarved cells.

The *Sac3* cDNA was sequenced, and an open reading frame of 1064 nucleotides flanked by 5′ and 3′ untranslated regions of 165 and 692 nucleotides, respectively, was detected. The derived amino acid sequence of the open reading frame was compared with sequences in the GenBank database. As shown in Figure 7A, significant similarity was detected between Sac3 and Snf1, an *S. cerevisiae* serine/threonine kinase involved in signaling glucose deprivation. However, Sac3 is also similar to a large number of open reading frames encoding presumed (based on sequence similarity) serine/threonine kinases from plants. All of the plant sequences also have significant identity with the catalytic domain of Snf1 of *S. cerevisiae* and are considered Snf1-related kinases (SnRKs) (Halford and Hardie, 1998). The N-terminal catalytic domain of Sac3 is 36% identical to that of Snf1 and between 50 and 56% identical to that of the plant serine/threonine kinases. All of the sequences presented in Figure 7 have the 11 kinase-specific subdomains (I to XI; Hanks et al., 1988). However, the plant sequences have two additional C-terminal subdomains, which we have designated A and B, and an acidic region, designated Neg, in common with Sac3. The functions of the putative kinases from vascular plants are not known, although many may be involved in the acclimation of plants to various environmental stresses (Halford and Hardie, 1998).

To determine the relationship of Sac3 to other serine/threonine kinases, we performed a phylogenetic analysis of the kinase domain sequences of these proteins (Figure 7B). Sac3 is most closely related to the vascular plant SnRK2 subfamily (Halford and Hardie, 1998). The proteins in this subfamily are characterized by an N-terminal catalytic domain similar to that of the *S. cerevisiae* Snf1 protein and a short C-terminal domain rich in acidic residues. Based on sequence divergence, the SnRK2 subfamily has been divided into SnRK2a and SnRK2b. Although Sac3 clearly groups with the SnRK2 subfamily, it falls outside of both of the SnRK2 subgroupings.

To determine where the *Sac3* gene was interrupted, we sequenced the DNA adjacent to the pBluescript KS+ vector in the plasmid-rescued fragment. This sequence indicated that pJD67 integrated within an intron in the 5′ portion of *Sac3* that separates nucleotides 340 and 341 of the cDNA. This site of integration allowed translation of only the first 58

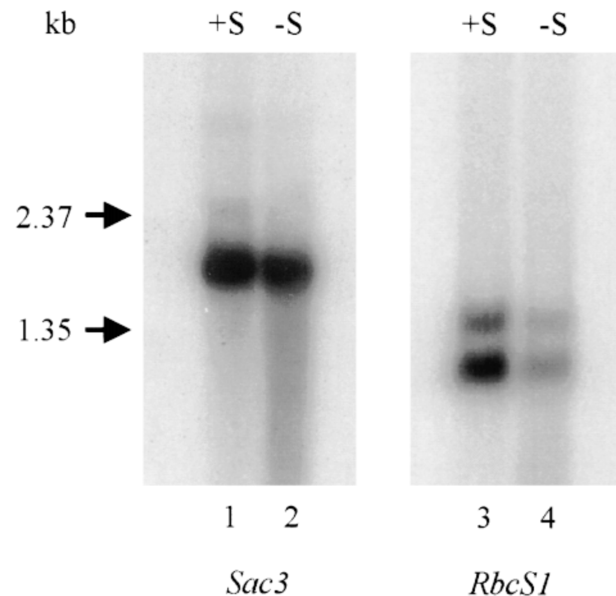

Figure 6. *Sac3* Transcript Accumulation in Wild-Type Cells in Sulfur-Replete and Sulfur-Deficient Media.

Poly(A)$^+$ RNA was isolated from CC125 cells grown in sulfur-replete (+S; lanes 1 and 3) or sulfur-deficient (−S; lanes 2 and 4) medium for 2 hr, resolved by agarose gel electrophoresis, blotted onto nitrocellulose, and hybridized with the 1.9-kb insert from pJD290 (*Sac3*) (lanes 1 and 2) and the 1.1-kb SalI-BamHI fragment of p2.02 (*RbcS1*) (lanes 3 and 4) (Goldschmidt-Clermont and Rahire, 1986). Molecular length markers are given at left in kilobases.

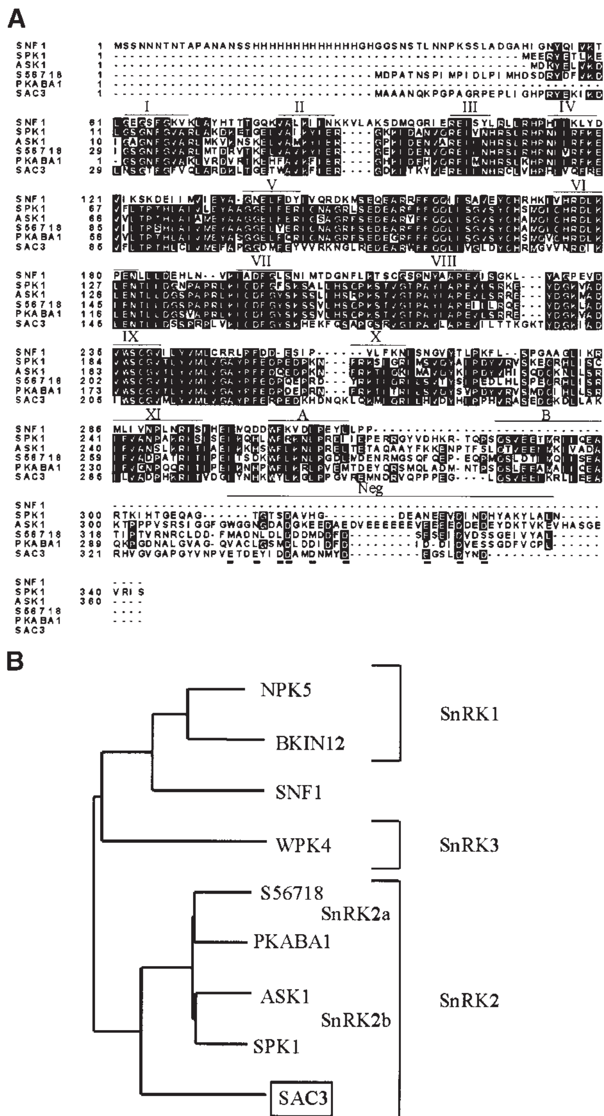

Figure 7. Comparison of Sac3, the Catalytic Domain of Snf1, and Serine/Threonine Kinases of Vascular Plants.

(A) Amino acids 1 to 314 of Snf1 are shown; no similarity between the C-terminal domain of Snf1 and the other proteins was observed. The 11 conserved subdomains of serine/threonine kinases are identified by roman numerals. Additional conserved regions are identified as A, B, and Neg. The dashes underneath the residues in the Neg region designate negatively charged amino acids in Sac3; the locations of negatively charged amino acids in the other proteins may differ. Identical amino acid residues are boxed in black. The name of each protein or the accession number is given at the left border. Dots were introduced to optimize alignment.

(B) Dendogram showing relative evolutionary distances between the catalytic domains of Sac3 and the other SnRKs. The name of each protein or the accession number is given. The alignment was produced using CLUSTALW (Thompson et al., 1994). The dendogram was constructed using Neighbor Joining analysis (Saitou and Nie, 1987) and was midpoint rooted using PAUP* 4.01B1 (Sinauer Press, Sunderland, MA). The GenBank accession numbers of the sequences presented are as follows: NPK5, D26602; BKIN12, X65606; SNF1, M13971; WPK4, D21204; PKABA1, M94726; ASK1, M91548; SPK1, L01453; and SAC3, AF100162. S56718 is the GenBank accession number for a cDNA sequence from Arabidopsis. The same result was obtained when more members of the SnRK2 family were used in the analysis.

amino acids of the Sac3 polypeptide and produced a protein lacking nine of the 11 subdomains important for kinase activity. On the basis of these data and the fact that the lesion in are10 is a recessive mutation, it is likely that the phenotype of this strain is the result of a complete lack of kinase function. This indicates that the Sac3 kinase positively regulates sulfate uptake and negatively regulates Ars activity.

DISCUSSION

Structure of Sac3 and Its Relationship to Other Serine/Threonine Kinases

The deduced amino acid sequence of Sac3 contains a domain that is similar to the catalytic domain of a large family of serine/threonine kinases (Figure 7) related to Snf1 of *S. cerevisiae* and the 5′ AMP-activated protein kinases in mammals. These kinases contain two domains, an N-terminal catalytic domain of 250 to 300 amino acids and a C-terminal regulatory domain of 250 to 380 amino acids (Hanks et al., 1988; Celenza and Carlson, 1989; Jiang and Carlson, 1996; Halford and Hardie, 1998). Two of the Snf1-like serine/threonine kinases in plants, RKIN1 from *Secale cereale* (Alderson et al., 1991) and NPK5 from tobacco (Muranaka et al., 1994), can functionally complement an *snf1* mutation in *S. cerevisiae.*

In plants, there are at least three subfamilies of SnRKs (Halford and Hardie, 1998). The members of all of these subfamilies share significant sequence identity with the N-terminal catalytic domains of Snf1 and the AMP-activated protein kinases. The best-described subfamily, SnRK1, is composed of proteins with significant sequence identity to Snf1 and the AMP-activated protein kinases in both the N- and C-terminal domains. Members of this subfamily, which include the RKINI and NPK5 proteins, have 62 to 64% sequence identity at their N-terminal catalytic domains and 29 to 34% sequence identity at their C-terminal domains (Halford and Hardie, 1998). The C-terminal domain of Snf1 interacts with at least two other proteins and is involved in regulating the kinase activity (Stapleton et al., 1994; Jiang and Carlson, 1996; Hardie and Carling, 1997; Halford and Hardie, 1998). Members of SnRK3 are approximately the same size as Snf1 and the AMP-activated kinases, but the

sequence of their C-terminal domains exhibits no similarity to either (Halford and Hardie, 1998).

Sac3 is a member of the SnRK2 subfamily, as are at least 10 proteins of vascular plants (deduced from cDNA sequences in GenBank). The plant kinases in this subfamily include PKABA1 from wheat (Anderberg and Walker-Simmons, 1992), SPK-3 and SPK-4 from soybean (Yoon et al., 1997), and ASK1 and ASK2 from Arabidopsis (Park et al., 1993). These proteins have a molecular mass of ∼40 kD, with an N-terminal kinase domain linked to a short C-terminal sequence of unknown function. The C-terminal domain has two subdomains, which we have designated A and B, and a patch of acidic amino acids (designated Neg in Figure 7). Of these, only subdomain A is present in Snf1. The proteins in the SnRK2 subfamily have 57 to 61% amino acid identity in their N-terminal catalytic domains and 41 to 51% identity in their C-terminal domains. However, the plant kinases shown in Figure 7 are more similar to each other than to Sac3.

Sac3 regulates the response of Chlamydomonas to sulfur limitation. The functions of the other SnRK2 polypeptides have not been elucidated. However, the accumulation of transcripts encoding specific SnRK2 polypeptides under defined environmental conditions has led to speculation concerning their functions (Anderberg and Walker-Simmons, 1992; Park et al., 1993; Yoon et al., 1997). For example, PKABA1, SPK-3, and SPK-4 (Anderberg and Walker-Simmons, 1992; Yoon et al., 1997) transcripts accumulate in response to dehydration and elevated salt levels, suggesting that these kinases may be involved in the responses of plants to hypoosmotic conditions.

Often, the abundance of transcripts for specific regulators does not change under conditions in which the regulator functions to alter gene expression. Indeed, glucose-deprived yeast cells and sulfur-deprived Chlamydomonas do not exhibit altered levels of the *SNF1* (Celenza and Carlson, 1984) and *Sac3* (Figure 6) transcripts, respectively. The in vitro kinase activity of Snf1, the AMP-activated kinases, and two SnRKs from cauliflower (HRK-A and HRK-B) is controlled by the phosphorylation state of these proteins (Weekes et al., 1994; Woods et al., 1994; Ball et al., 1995). It is not known whether Sac3 kinase activity is regulated by phosphorylation.

Regulation of Sulfur Limitation Responses in Chlamydomonas

The *sac3* mutants of Chlamydomonas exhibit low-level constitutive Ars activity when grown in nutrient-replete medium and do not fully activate sulfate uptake when deprived of sulfur. They respond normally to both phosphorus and nitrogen deprivation. These characteristics indicate that the Sac3 protein is specifically involved in controlling the acclimation of Chlamydomonas to sulfur deprivation.

Sulfur limitation triggers the production of Ars and an increase in the rate of sulfate transport. The inability of *sac3* mutants to fully repress Ars activity during growth in nutrient-replete medium or to fully activate sulfate uptake upon sulfur starvation suggests that Sac3 is part of a phosphorylation-driven signal transduction chain that can positively and negatively regulate the expression of genes associated with the acclimation of Chlamydomonas to sulfur limitation. Several *S. cerevisiae* regulatory proteins can have both positive and negative affects on gene expression. Some of these proteins, such as Paf1, Gal11, Sin4, and Rgr1, form a mediator complex with RNA polymerase and influence gene expression by altering chromatin structure or associating with specific DNA binding proteins (Fassler and Winston, 1989; Jiang and Stillman, 1992; Jiang et al., 1995; Li et al., 1995; Shi et al., 1996). Others, such as Rap1, may modify gene activity by directly binding DNA (Kurtz and Shore, 1991; Shore, 1994).

Mutations in three genes have been shown to affect the responses of Chlamydomonas to sulfur limitation. Two genetic loci, *Sac1* and *Sac2*, positively regulate responses to sulfur limitation, whereas one locus, *Sac3*, appears to both positively and negatively regulate these responses (Davies et al., 1994). Sac1 (Davies et al., 1996) controls the expression of *Ars* and other sulfur stress–induced genes, and it is required for the decrease in photosynthetic activity that accompanies sulfur starvation. It may be involved in sensing the level of sulfate in the medium (Davies and Grossman, 1998). The *Sac1* gene has been cloned, and the derived amino acid sequence has similarity to ion transporters. The *Sac2* gene has not been cloned, but the phenotype of the *sac2* mutant suggests that it is either directly or indirectly involved in the transcriptional regulation of *Ars* genes and possibly other genes induced during sulfur limitation (Davies et al., 1994). In contrast, Sac3 is a protein kinase that appears to have both positive and negative effects on gene expression.

Genetic analysis of the mutants indicates that Sac1 and Sac2 function in a linear pathway to regulate Ars expression and that Sac3 may function in an independent manner. The phenotype of the *sac1* mutant is epistatic to *sac2* (Davies et al., 1994), but no clear epistatic relationship exists between *sac1* and *sac3* or *sac2* and *sac3* (Davies et al., 1994). Both pathways probably control Ars expression and the elevation of sulfate transport.

Distinct signal transduction pathways have been observed for the control of glucose deprivation responses in *S. cerevisiae.* One signaling pathway includes signaling through Snf1 (Trumbly, 1992) and controls the expression of invertase and the ability of the cells to use sugars other than glucose. The other signaling pathway controls expression of the high-affinity hexose transporters and is thought to be initiated by Snf3, which is believed to sense the levels of glucose in the medium (Liang and Gaber, 1996; Ozcan et al., 1996). There are parallels between carbon catabolite repression in *S. cerevisiae* and the responses of Chlamydomonas to sulfur deprivation. First, Sac1 and Snf3 are similar in

sequence to integral membrane proteins involved in transporting nutrients but act as positive regulators (Celenza et al., 1988; Davies et al., 1996; Davies and Grossman, 1998). Second, Sac3 and Snf1 are both serine/threonine kinases (Celenza and Carlson, 1986). However, there are also significant differences between mechanisms involved in the acclimation of Chlamydomonas to sulfur limitation and the acclimation of *S. cerevisiae* to glucose limitation. Sac1 and Sac3 appear to be in distinct signaling pathways that regulate the same responses and genes (i.e., Ars expression and sulfate uptake), whereas Snf1 and Snf3 are components of distinct regulatory pathways that govern different sets of genes. Furthermore, whereas Sac3 negatively regulates Ars expression and positively regulates sulfate uptake, Snf1 appears to exclusively regulate genes in a positive manner. Finally, whereas the *sac3* mutant affects only responses to sulfur deprivation (carbon-, nitrogen-, and phosphorus-deficient conditions were also tested), the *snf1* mutant affects the cell's responses to different types of nutrient stress (Thompson-Jaeger et al., 1991). For example, *snf1* mutants are unable to survive glucose, phosphorus, sulfur, and nitrogen starvation and do not accumulate high levels of glycogen duing glucose or phosphorus deprivation (Thompson-Jaeger et al., 1991).

Continued definition of the factors that regulate sulfur stress acclimation processes in Chlamydomonas will elucidate a control circuit that enables photosynthetic, eukaryotic microbes to interface successfully with a dynamic environment. This control may be similar to systems used by vascular plants. The extensive homology between Sac3 of Chlamydomonas and vascular plant serine/threonine kinases thought to be involved in environmental sensing raises the possibility that the mechanisms for communicating environmental cues to the biosynthetic machinery of the cell are similar in vascular plants and Chlamydomonas.

METHODS

Cell Growth and Mating

Chlamydomonas reinhardtii cells were grown in nutrient-replete or sulfate-deficient Tris–acetate–phosphate (TAP) medium, as described previously (Davies et al., 1994). When appropriate, the medium was supplemented with arginine at 50 μg/mL. SGII/NO_2 and SGII/NO_3 media were identical to Sager-Granick II (SGII; Kindle, 1990), except that they contained 0.43 mM nitrite and 0.35 mM nitrate, respectively, as their sole nitrogen sources. Matings were performed as described by Harris (1989).

Chlamydomonas Mutagenesis and Transformation

Strain CC425 (*cw15 arg7*) was mutagenized by introducing HindIII-digested pJD67 (Davies et al., 1994) by glass bead transformation (Kindle, 1990). Transformants were selected on a nutrient-replete solid medium lacking arginine. Strains constitutively synthesizing arylsulfatase (Ars) were identified by spraying the plates containing colonies of transformants with 300 to 500 μL of 10 mM 5-bromo-4-chloro-3-indolyl sulfate and screening for the formation of a blue halo around colonies.

Strain are10-12 (*sac3 nit1*) was transformed with pMN24, which contains the *Nit1* gene (Fernandez et al., 1989), by using particle bombardment (Klein et al., 1987). The strain was grown in SGII/NO_2 to mid-log phase, and 10^8 cells were collected by centrifugation (5000*g* for 5 min), resuspended in SGII/NO_3, and spread on solid SGII/NO_3 medium (0.8% agarose). Thirty milligrams of tungsten particles (M-17; Bio-Rad) was prepared by vortexing them in 100% ethanol for 3 to 5 min, pelleting them by centrifugation for 1 min in an Eppendorff (Brinkman Instruments, Westbury, NY) microcentrifuge at full speed, removing the supernatant, and resuspending the particles in 0.5 mL of sterile water. The particles were then washed twice by vortexing them for 1 min, pelleting them by centrifugation (as before), and resuspending them in 0.5 mL of sterile water. DNA was precipitated onto the particles by combining 5 μg of DNA, 50 μL of 2.5 M $CaCl_2$, 20 μL of 0.1 M spermidine, and 50 μL of the tungsten particles, while continuously vortexing the mixture. After an additional minute of vortexing, the particles were allowed to settle for 10 min, the supernatant was removed, and the particles were resuspended by vortexing in 250 μL of 100% ethanol for 1 min. The particles were again allowed to settle (3 min) and, after removing the supernatant, were resuspended in 60 μL of 100% ethanol. Particle bombardment was performed with the Bio-Rad Biolistic PDS-1000/He system, with the target plate at 6 cm and using 900 psi rupture disks. After bombardment, the plates were moved to growth conditions, and colonies appeared after 7 to 9 days. Transformants were streaked onto SGII/NO_3 plates and individual colonies, and restreaked twice on SGII/NO_3 before assaying Ars activity.

Ars Activity, Sulfate Uptake, and DNA and RNA Gel Blotting

Ars activity was assayed as described by de Hostos et al. (1988) and Davies et al. (1994). Sulfate uptake was performed as described by Yildiz et al. (1994), except that the V_{max} for sulfate uptake in sulfur-deprived cells was determined by measuring the velocity of sulfate import at 100 μM sulfate. DNA and RNA gel blot analyses were performed as described previously (Davies et al., 1994, 1996).

Isolation of Cosmid Clones

The cosmid library of Purton and Rochaix (1994) was obtained from Saul Purton (University College, London, UK) and screened by colony hybridization (Maniatis et al., 1982). Purified cosmid DNA was obtained using a Qiagen (Valencia, CA) DNA purification kit.

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