Genetic interactions between regulators of Chlamydomonas phosphorus and sulfur deprivation responses.

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Running title: Integration of regulatory pathways controlling the responses to phosphorus and sulfur deprivation in Chlamydomonas.
ABSTRACT

The *Chlamydomonas reinhardtii* *PSR1* gene is required for proper acclimation of the cells to phosphorus (P) deficiency. P-starved *psr1* mutants show signs of secondary sulfur (S) starvation, exemplified by the synthesis of extracellular arylsulfatase and the accumulation of transcripts encoding proteins involved in S scavenging and assimilation. Epistasis analysis reveals that induction of the S-starvation responses in P-limited *psr1* cells requires the regulatory protein kinase SNRK2.1, but bypasses the membrane-targeted activator, SAC1. The inhibitory kinase SNRK2.2 is necessary for repression of S-starvation responses during both nutrient-replete growth and P-limitation; arylsulfatase activity and S deficiency-responsive genes are partially induced in the P-deficient *snrk2.2* mutant and become fully activated in the P-deficient *psr1snrk2.2* double mutant. During P-starvation, the *sac1snrk2.2* double mutants or the *psr1sac1snrk2.2* triple mutants exhibit reduced arylsulfatase activity compared to *snrk2.2* or *psr1snrk2.2*, respectively, but the *sac1* mutation has little effect on the abundance of S deficiency-responsive transcripts in these strains, suggesting a post-transcriptional role for SAC1 in elicitation of S starvation responses. Interestingly, P-starved *psr1snrk2.2* cells bleach and die more rapidly than wild-type or *psr1* strains, suggesting that activation of S-starvation responses during P deprivation is deleterious to the cell. From these results we infer that i) P-deficient growth causes some internal S-limitation, but the S-deficiency responses are normally inhibited during acclimation to P-deprivation, ii) the S-deficiency responses are not completely suppressed in P-deficient *psr1* cells and consequently these cells synthesize some arylsulfatase and exhibit elevated levels of transcripts for S deprivation genes, and iii) this increased expression is controlled by regulators that alter the
transcription of S responsive genes during S-deprivation conditions. Overall, the work strongly suggests integration of the different circuits that control nutrient-deprivation responses in Chlamydomonas.
INTRODUCTION

The elements phosphorus (P), and sulfur (S) are essential macronutrients for sustaining life. P is a structural component of nucleic acids and phospholipids, and is a ubiquitous modifier of carbohydrates and proteins, while S is incorporated into sulfolipids, polysaccharides, proteins, cofactors, and a wide variety of important metabolites including S-adenosyl-methionine, glutathione and phytochelatins. The preferred forms of P and S that are assimilated by plants and microbes are the orthophosphate ion, \( \text{PO}_4^{3-} \) (Pi), and the sulfate ion, \( \text{SO}_4^{2-} \). The available pools of these anions can vary significantly as environmental conditions change. Most organisms have a limited capacity to store S, and thus require a continual supply of S-containing nutrients for survival. In contrast, cells often have considerable reserves of P, which are bound in polymers of DNA, RNA and polyphosphate. The ability of microbes to acclimate to periods of nutrient insufficiency is essential to their survival in the natural environment (reviewed by (GROSSMAN and TAKAHASHI 2001).

The green, unicellular alga *Chlamydomonas reinhardtii* (Chlamydomonas throughout) exhibits both specific and general responses when experiencing P or S deprivation. The general responses are common to a number of different stress conditions, while the specific responses enable processes that are advantageous during particular nutrient deficiencies, often allowing for better scavenging of the limiting nutrient from internal and external stores. P- and S-limitation elicit qualitatively similar effects on growth and photosynthesis, differing only in that the responses to S-starvation occur more quickly following exposure of cells to medium devoid of S. General responses to nutrient limitation that have been analyzed include the cessation of growth at low cell
densities (Shimogawara et al. 1999; Zhang et al. 2002) and a reduction in photosynthetic O$_2$ evolution, which is mostly a consequence of reduced photosystem II (PS II) activity (Wykoff et al. 1998). The specific S-deprivation responses include an elevated rate of extracellular SO$_4^{2-}$ uptake (Yildiz et al. 1994), secretion of extracellular arylsulfatases (De Hostos et al. 1988; Lein and Schreiner 1975) and an increased cellular capacity to assimilate SO$_4^{2-}$ by increasing levels of enzymes required for cysteine biosynthesis (Ravina et al. 2002). Mechanisms for conserving S during limiting conditions include the rapid turnover of sulfolipids and their replacement with phospholipids (Sugimoto et al. 2008; Sugimoto et al. 2007), the synthesis of putative cell-wall proteins with a very low abundance of S-containing amino acids (Takahashi et al. 2001), and a potential change in the polypeptide composition of light harvesting complexes, favoring the synthesis of complexes with polypeptides containing low levels of sulfur amino acid (D. Gonzalez-Ballester and A. Grossman, unpublished). Many S starvation-elicited responses appear to be controlled, at least in part, at the level of gene expression. Genes encoding the arylsulfatases (De Hostos et al. 1989; Ravina et al. 2002), extracellular proteins (Takahashi et al. 2001), ATP sulfurylase (Yildiz et al. 1996), adenylylphosphosulfate reductase, serine O-acetyl transferase, OAS (thiol)-lyase (Ravina et al. 2002), sulfite reductases and putative SO$_4^{2-}$ transporters (Zhang et al. 2004) are upregulated in cells deprived of S.

A suite of specific responses also enables Chlamydomonas to acclimate to P-limitation. P-starved cells induce high-affinity Pi uptake (Shimogawara et al. 1999) and synthesize extracellular phosphatases (Quisel et al. 1996) that enhance Pi scavenging from the environment. Polyphosphate stores (Hebeler et al. 1992; Werner et al. 2007)
and chloroplast DNA (Yehudai-Resheff et al. 2007) are also mobilized, and phospholipids are replaced by galactolipids and sulfolipids (Riekhof et al. 2003) as cells redistribute their internal P resources. P-starved cells also conserve Pi by inhibiting the turnover of chloroplast transcripts by the phosphorylytic chloroplast polynucleotide phosphorylase (Yehudai-Resheff et al. 2007). Like S-deprivation responses, P deficiency-specific responses are often regulated at the level of gene expression, as transcripts encoding extracellular phosphatases and Pi transporters increase markedly in abundance following elimination of P from the growth medium (Chang et al. 2005; Kobayashi et al. 2003; Moseley et al. 2006).

Screens for mutants that fail to acclimate properly to S-limitation have enabled identification of three key regulators of the S-deficiency responses (Davies et al. 1994; Pollock et al. 2005). The Sulfur Acclimation 1 (SAC1) gene encodes an integral membrane protein that is similar to the SLC13 family of transporters (Davies et al. 1996). SAC1 is a positive regulator critical for the activation of genes involved in scavenging and assimilating S from the environment (Davies et al. 1996; Gonzalez-Ballester et al. 2008; Ravina et al. 2002; Zhang et al. 2004). The light-sensitivity of S-deficient sac1 strains probably reflects their inability to decrease photosynthetic electron transport (Davies et al. 1994; Wykoff et al. 1998), although the role of SAC1 in the regulation of photosynthesis is not well understood. The SNF1-related protein kinase 2.2 (SNRK2.2) gene, previously known as SAC3 (for Sulfur Acclimation 3), encodes a serine/threonine kinase that acts as a negative regulator of S deficiency-responsive gene expression (Davies et al. 1999). The snrk2.2 mutants display low, constitutive arylsulfatase activity and express elevated basal levels of S deficiency-responsive genes in S-replete medium.
SNRK2.2 is also required for the proper down-regulation of chloroplast transcription in S-starved cells (IRIHMIOVITCH and STERN 2006). There is no clear epistasis relationship between SAC1 and SNRK2.2. The sac1snrk2.2 double mutants have lower constitutive arylsulfatase activity than snrk2.2 cells when grown under nutrient-replete conditions, but in contrast to the sac1 single mutants, the double mutants can still partially up-regulate ARS gene expression in response to S-deficiency, (DAVIES et al. 1994). A related serine-threonine kinase, SNRK2.1, has recently been shown to perform a central regulatory function in controlling S-starvation responses (GONZALEZ-BALLESTER et al. 2008). The snrk2.1 mutants are unable to activate expression of most S starvation-responsive genes and cannot maintain normal basal levels of expression for some genes during S-replete growth. A snrk2.1 null allele is even more light sensitive during S-starvation than sac1, suggesting that SNRK2.1 also plays a role in modulating photosynthetic activity during S-starvation (GONZALEZ-BALLESTER et al. 2008). In contrast to the non-epistatic relationship between SAC1 and SNRK2.2, the phenotype of a snrk2.1snrk2.2 double mutant is indistinguishable from that of the snrk2.1 single mutant, indicating that SNRK2.1 is epistatic to SNRK2.2 (GONZALEZ-BALLESTER et al. 2008). Models based on genetic data propose that SAC1, SNRK2.1 and SNRK2.2 interact to form a signaling cascade that senses the cellular S-status, maintains basal levels of the transcripts encoded by the S-responsive genes in S-replete cells, and activates (or represses) the expression of target genes during S-limitation (GONZALEZ-BALLESTER et al. 2008; POLLOCK et al. 2005).
To date, a single regulator of P-deficiency responsive gene expression, encoded by the *Phosphate Starvation Response 1 (PSR1)* gene, has been identified (Wykoff et al. 1999). Two independent genetic screens resulted in the generation of multiple *psr1* alleles (Shimogawara et al. 1999). The *psr1* strains fail to accumulate extracellular phosphatases or activate high-affinity Pi-uptake during P-starvation, and consequently grow poorly on a hydrolysable P source such as glucose-1-phosphate (Shimogawara et al. 1999). In contrast to the phenotype of S-deprived *sac1* strains, P-starved *psr1* cells down-regulate photosynthesis more rapidly than wild-type cells (Shimogawara et al. 1999). Nevertheless, when *psr1* cells are deprived of P they become more sensitive to high light intensities than wild-type cells (Moseley et al. 2006).

PSR1 is a complex protein with characteristics of transcriptional regulators, including a DNA-binding MYB domain (reviewed by Lipsick 1996), a coiled-coil domain that may be involved in protein-protein interactions (reviewed by Burkhard et al. 2001) and a helix-loop-helix motif (reviewed by Jones 2004). The C-terminal region of *PSR1* is rich in glutamine, a common feature of transcriptional activators (reviewed by Pabo and Sauer 1992). More than 95% of PSR1 is located in the nucleus, irrespective of cellular P-status (Wykoff et al. 1999). Although sequence-specific DNA-binding has not been demonstrated for PSR1, the *Arabidopsis thaliana* homolog, PHR1, has been shown to bind regulatory motifs in the promoters of P-starvation inducible genes (Rubio et al. 2001). The vascular plant homologs of PSR1 contain similar DNA-binding and protein-protein interaction domains, but lack the glutamine-rich transcriptional activation domains of the algal protein (Rubio et al. 2001; Wykoff et al. 1999). Candidate gene targets under PSR1 control have been identified on the basis of altered transcript
accumulation in P-deficient versus P-replete conditions, and the loss of differential gene expression in psr1 mutants (MOSELEY et al. 2006). PSR1 controls expression of genes encoding enzymes with roles in P-scavenging, including the major secreted phosphatase, PHOX, putative high-affinity Pi transporters, and proteins potentially involved in mobilizing polyphosphate from internal stores. It has also been implicated in the activation of genes whose products may reduce the accumulation of excitation energy in the photosynthetic complexes during P-starvation (MOSELEY et al. 2006).

Studies of fungal and plant model systems have demonstrated that various regulatory pathways linked to nutrient deprivation responses have a set of common target genes (reviewed by (GASCH and WERNER-WASHBURN 2002). Many genes that allow cells to cope with stress (e.g. a decreased potential for growth because of suboptimal conditions) are regulated similarly in S. cerevisiae during P and S deprivation (SALDANHA et al. 2004). A more specific intersection between the regulation of P and S metabolism in S. cerevisiae was demonstrated with the finding that Pho4p, a P-deprivation-responsive transcription factor, could functionally substitute for the sequence specific DNA-binding protein Cp1p, which is required for activation of methionine biosynthetic genes. Activation of Pho4p by P-deprivation, overexpression of Pho4p and the use of constitutive alleles all resulted in suppression of methionine auxotrophy in the S. cerevisiae cep1 mutant, which lacks the Cp1p factor (O'CONNELL and BAKER 1992). In this study we explore genetic interactions between the regulators of P- and S-starvation responses in Chlamydomonas, and provide evidence supporting the integration of these pathways.
MATERIALS AND METHODS

Media and growth conditions. Tris-acetate-phosphate (TAP) medium was made according to the standard recipe (GORMAN and LEVINE 1966). To make Pi-free (TA) medium, potassium phosphate (1.8 mM potassium, 1.1 mM Pi) was replaced with 1.8 mM potassium chloride. SO$_4^{2-}$-free (TAP–S) medium was prepared by substituting chloride salts for the SO$_4^{2-}$ salts. Solid medium contained 0.75% agarose; TA plates were always supplemented with 100 µM glucose-1-phosphate while TAP–S plates always contained 2 mM sodium or potassium thiocyanate (cells grew identically with either cation). TA liquid medium was not supplemented with glucose-1-phosphate, and TAP–S was not supplemented with thiocyanate. Liquid cultures were grown in Erlenmeyer flasks at 25°C, with agitation at 200 rpm and under fluorescent bulbs at intensities of 30-40 µEm$^{-2}$s$^{-1}$. Dark-grown liquid cultures were grown similarly except that the flasks were securely wrapped in aluminium foil, and were only exposed to light for ~5 min during sampling. Cells on plates were grown at 25°C at 40 to 70 µEm$^{-2}$s$^{-1}$.

Chlorophyll measurements and viability staining. To determine the chlorophyll content of liquid cultures, 0.75 mL of cell culture was transferred to a 1.5 mL eppendorf tube and centrifuged for 5 min at 13,800xg to pellet the cells. The supernatant was discarded and the chlorophyll in the pellet was extracted with 0.75 mL of a mixture of 80% acetone, 20% methanol. The chlorophyll concentration per mL of cultured cells was estimated from the absorbance at 652 nm divided by 34.5 (ARNON 1949). Viability staining with Evans blue dye was performed as described previously (YEHUDAI-RESHEFF et al. 2007).
**Phosphatase and sulfatase assays.** Colorimetric assays for alkaline phosphatase (Shimogawa et al. 1999) and arylsulfatase (Davies et al. 1994) activities were performed. Cells were grown on solid medium for 4-5 d and the color reactions were allowed to develop for 20-24 h before the cells were washed from the surface of the medium with de-ionized water to allow examination of the colored precipitate embedded in the medium. Liquid assays for alkaline phosphatase and arylsulfatase activities were performed as described previously (De Hostos et al. 1988; Lein and Schreiner 1975; Quisel et al. 1996).

**Strains and genetic analysis.** A comprehensive list of strains used in these studies is presented in Table I. Wild-type strains were CC-125 (nit\(^{-}\) mt\(^{+}\)) and CC-1690 (nit\(^{+}\) mt\(^{+}\)). The psr1 strains were psr1-1nit\(^{-}\)mt\(^{+}\) which had been back-crossed 5-6 times with CC-124 and CC-125 and psr1-2 (psr1-2::ARG7 cw15nit\(^{-}\)) (Shimogawa et al. 1999). The psr1-1 mutant was complemented with plasmid pKS1, containing a genomic PSR1 sequence (Wykoff et al. 1999), to generate the psr1-complemented strain. A psr1-1 nit\(^{-}\)mt\(^{-}\) strain was backcrossed three times to CC-1690 to generate the psr1-1 nit\(^{+}\) mt\(^{-}\) strain, which was used in subsequent experiments and for a number of crosses. psr1 progeny were scored as growing poorly on TA + glucose-1-phosphate plates and failing to express abundant alkaline phosphatase based on the colorimetric assay for this activity (Shimogawa et al. 1999). The sac1 mutant (ars5-3nit\(^{-}\)mt\(^{-}\)) (Davies et al. 1994) was backcrossed three times to CC-1690 to produce a sac1(ars5-3) nit\(^{+}\) mt\(^{+}\) strain, while a snrk2.2 strain (sac3(are10)nit\(^{-}\)mt\(^{-}\)) (Davies et al. 1994) was crossed once to CC-1690 to produce a snrk2.2(are10)nit\(^{+}\) strain. The psr1-1nit\(^{+}\)mt\(^{-}\) strain was crossed to sac1(ars5-3)nit\(^{+}\)mt\(^{+}\), sac1(ars4-15)nit\(^{-}\)mt\(^{+}\) and sac1(ars4-55)nit\(^{-}\)mt\(^{+}\). The sac1
progeny were identified by their inability to synthesize arylsulfatase on TAP–S + 2 mM thiocyanate plates. A representative sac1(ars5-3) mutant and a psr1sac1(ars5-3) double mutant from a tetratype tetrad were used for gene expression studies and enzyme activity assays. The snrk2.2(are10)nit+mt+ and snrk2.2(are16)cw15mt+ strains (DAVIES et al. 1999) were crossed to psr1-1nit+mt−. The snrk2.2 progeny were recognized based on their constitutive arylsulfatase activity on TAP medium. Representative snrk2.2(are10) and psr1snrk2.2(are10) strains were chosen from a tetratype tetrad for further analyses. Representative snrk2.1(ars11) and psr1snrk2.1(ars11) strains were selected by crossing psr1-1nit+mt− to the snrk2.1::aphVIII(ars11)nit−cw15mt+ strain (GONZALEZ-BALLESTER et al. 2008; POLLOCK et al. 2005). The snrk2.1 mutants, which are paromomycin resistant due to integration of the aphVIII gene, grew poorly and did not induce arylsulfatase on TAP–S + thiocyanate (GONZALEZ-BALLESTER et al. 2008; POLLOCK et al. 2005). Triple mutants were generated by crossing the appropriate double mutant strains, and a representative sac1snrk2.2(are10) strain was selected from the progeny of a backcross of the psr1sac1snrk2.2(are10) strain to CC-1690. The presence of the sac1(ars5-3) and snrk2.2(are10) alleles in the respective triple mutant strains was verified by amplification of the pJD76 and pJD67 insertional markers from isolated genomic DNA (see (DAVIES et al. 1994) and supplemental data). cw+nit+ representative mutant strains were selected for phenotypic and epistasis analysis to eliminate any potential contributions to the observed phenotypes by the cw15, nit1 or nit2 mutations. Although for some genotypes the phenotypic expressivity varied significantly between individual isolates, all of the representative strains used in the experiments described in
the RESULTS section were selected because they displayed average responses within an observed phenotypic range.

**RNA isolation.** Total RNA was isolated using a protocol modified from Schloss et al (Schloss et al. 1984). Briefly, cells from 50 mL of culture were pelleted by centrifugation, frozen in liquid N$_2$ and stored at -80°C. The pelleted cells were kept frozen until they were lysed in 3 mL of a solution containing 50 mM Tris-HCL pH 7.5, 150 mM sodium chloride, 15 mM EDTA pH 8, 2% SDS and 40 µg/mL proteinase K (Invitrogen, Carlsbad, CA). The aqueous emulsion of lysed cells was extracted 4 times with 3 mL of a 50:50 mixture of acidic (pH 4.5) phenol (US Biochemicals, Cleveland, OH) and chloroform (Fisher, Pittsburg, PA), followed by 2 extractions with 3 mL chloroform (Fisher, Pittsburg, PA). Nucleic acids were precipitated by adding 3 mL (vol equal to the original cell suspension vol) of 8 M lithium chloride to the extracted solution, incubated at 4°C for at least 4 h and then centrifuged at 14,500xg for 30 min. Nucleic acid pellets were washed with 70% ethanol, vacuum-dried and dissolved in water. Trace DNA contamination was removed by digestion with TurboDNase (Ambion, Austin, TX) according to the manufacturer’s instructions.

**Real-time quantitative RT-PCR.** Real-time quantitative RT-PCR was performed using the iScript One-Step RT-PCR kit with SYBR green and the Chromo4 Real-Time System (Bio-Rad Laboratories, Hercules, CA) according to the protocol described previously by (Moseley et al. 2006). The primers for amplification were: CBLP, forward (5’-CTTCTCGCCCATGACCAC-3’), reverse (5’-CCCACCAGGTTGTTCTCAG-3’); PHOX, forward (5’-TTCCGTTTCCGTTCTCTGAC-3’), reverse (5’-CCCTGCATCTTTGTCTCAG-3’); ARSI, forward (5’-
CGCGCCGTCACCCGTTGGTGTG-3’), reverse (5’-GCCCACCCGACCGCCTC-3’); ARS2, forward (5’-GTACTGCGTCGCTGATTCC-3’), reverse (5’-CCTAAACATTTGGCTCCGCAGTCC-3’); SLT1, forward (5’-ACGGGTCTTCGGACGAATTGC -3’), reverse (5’-ACGGGTCTTCGGACGAATTGC -3’); SLT2, forward (5’-GTACTGCCGCGTGCGTGATTCC-3’), reverse (5’-TTCTTCGCCACCAGATGAGC -3’); SULTR2, forward (5’-ACGTTGCATTGCAGCTCAT -3’), reverse (5’-CTTGCCAATTTCAGCCAGT -3’); SAT1, forward (5’-TGCGGCACCCTTTCCCAGAG-3’), reverse (5’-CACCCGATCACACGCAAACTTCA-3’); OASTL4, forward (5’-GGCCCTCCTGGAAGCTGAATCA-3’), reverse (5’-CCCCACCCCACCATTAGATGGC-3’); ECP76, forward (5’-CCTCGCTCTCCTCGCTGCTG -3’), reverse (5’-CGGCCGACTTGGGTAATTGC -3’); SBDP, forward (5’-GGACGGCAGCATCAGTGGT -3’), reverse (5’-TCCACACGCCCTGGCAGCTTGAG -3’). The sizes of the amplification products range from 100-300 bp. The purity of PCR amplification products was assessed by melting curve analysis and by gel electrophoresis.

RESULTS

Arylsulfatase is induced in P-starved psr1 cells. The psr1 mutants, represented by psr1-1 in this study, are unable to acclimate properly to P starvation and consequently bleach and exhibit much less growth than wild-type strains on TA medium supplemented with glucose-1-phosphate as the sole P source (Shimogawa et al. 1999) (Figure 1A,
growth, −P +S). The P-starved psr1-1 strain also displays very low phosphatase activity compared with the wild-type strain CC-125 or the psr1-1 mutant that was complemented with the PSR1 gene (Shimogawara et al. 1999; Wykoff et al. 1999) (Figure 1A, phosphatase activity, −P +S, Figure 1B). However, we noted that psr1-1 cells exhibit significant arylsulfatase activity in Pi-free medium, and that this activity is not observed in the wild-type or the psr1-complemented strains (Figure 1A, sulfatase activity, −P +S, Figure 1C). Similar induction of arylsulfatase is observed when the psr1-2 strain is starved for Pi (see Supplemental Figure S1). The activation of arylsulfatase in P-deficient psr1 cells is not a consequence of secondary light stress since similar arylsulfatase activity is produced by psr1 cells grown without Pi in the light or in the dark (compare Figure 1C and 1D). The wild-type, psr1-1 and psr1-complemented strains all show copious arylsulfatase activity after growth in SO$_4^{2-}$-free medium (Figure 1A, sulfatase activity, +P −S, Figure 1E), indicating that the psr1 mutation shows no apparent alteration in normal acclimation responses to S-deprivation. The arylsulfatase activity measured for psr1-1 cells after 7 d of P-starvation is approximately 7% of the peak activity measured after 24 h of S-starvation (compare Figure 1C and 1E).

Activation of SO$_4^{2-}$-assimilation pathway genes in P-deficient psr1 cells. A previous study analyzed global patterns of P deficiency-responsive gene expression in the parental wild-type strain and the psr1-1 mutant, demonstrating that a number of genes encoding proteins associated with S assimilation were upregulated in P-starved psr1-1 cells (Moseley et al. 2006). To validate these results we used quantitative real-time PCR to compare the relative abundance of transcripts from a cross-section of genes involved in S scavenging and assimilation. After 24 h of P-deprivation in wild-type cells, the transcript
abundance of PHOX, which encodes the major, derepressible secreted phosphatase, increased by ~5000-fold, whereas expression of the S-assimilation genes either remained constant or declined slightly (Figure 2). In contrast, psr1-1 cells grown for 24 h in P-free medium failed to upregulate PHOX, but exhibited ~30- to 200-fold increases in the levels of the ARS1 and ARS2 transcripts, which encode the major, secreted arylsulfatase enzymes (Figure 2). Although arylsulfatase activity steadily increased in P-deficient psr1 cultures over a 7 d period (Figure 1C), the peak abundance of ARS2 transcripts was observed at 24 h after the cells were transferred to medium devoid of Pi (data not shown). The SLT1 and SLT2 transcripts, encoding putative Na+/SO$_4^{2-}$ transporters, increase in abundance by ~2000-fold and ~50-fold, respectively, compared to the transcript levels in P-replete cells (Figure 2). The amount of the SULTR2 transcript, which encodes a putative H$^+$/SO$_4^{2-}$ transporter, increased in the psr1-1 strain by ~40-fold after 24 h of P-starvation. Smaller increases of between 2.5- and 20-fold were observed for SAT1, OASTL4, ECP76 and SBDP mRNAs, encoding, respectively, a serine acetyl-transferase, an O-acetyl-serine(thiol)lyase (cysteine synthase), an extracellular protein potentially associated with the cell wall and a putative selenium binding protein. It should be noted that the maximum levels of all of these transcripts in psr1-1 cells starved for P were 1 to 2 orders of magnitude below peak levels attained for wild-type cells starved for S (Gonzalez-Ballester et al. 2008) and D. Gonzalez-Ballester and W. Pookhatam, unpublished). The results shown in Figures 1 and 2 clearly demonstrate that the regulatory pathway that controls S deficiency-responsive gene expression is partially activated in P-starved psr1-1 cells.
Epistasis analysis of PSRI and regulators of the S-deficiency response. Are components of the signal transduction pathway that elicit changes in gene expression during S-starvation also involved in the aberrant activation of S-assimilation genes in P-starved psrl cells? To investigate the relationship between PSRI and genes that encode regulators of S-deficiency acclimation responses, the psrl-1 mutant was crossed to sac1, snrk2.2 and snrk2.1 strains. As mentioned in the introduction, SAC1 and SNRK2.1 encode a putative sensor and serine/threonine kinase, respectively, that are required for activation of S deficiency-responsive gene expression (DAVIES et al. 1996; GONZALEZ-BALLESTER et al. 2008). SNRK2.2 encodes a serine/threonine kinase that acts as a negative regulator of S deficiency-responsive gene expression (DAVIES et al. 1999; RAVINA et al. 2002). Assays for growth, alkaline phosphatase activity and arylsulfatase activity were used to compare the acclimation response phenotypes of wild-type, psrl, sac1, snrk2.2, snrk2.1 and combinations of double and triple mutants during P- and S-starvation (Figure 3). In control experiments, strains with wild-type PSRI exhibited normal induction of phosphatase activity in –P medium, but only low, basal levels of activity were observed for strains carrying the psrl-1 allele (Figure 3A, column 7; Figure 3B). Conversely, after 24 h of S-starvation arylsulfatase expression was blocked in all strains containing the snrk2.1 allele, and in sac1 strains that did not also contain the snrk2.2 mutation (Figure 3A, column 6; Figure 3D). As has been reported previously, snrk2.2 strains have low, constitutive arylsulfatase activity in S-replete medium, and accumulate approximately one third as much arylsulfatase activity in S-deficient medium as wild-type cells (DAVIES et al. 1994; DAVIES et al. 1999) (Figure 3A, column 4, Figure 3C, Figure 3D). The epistasis relationship between SAC1 and SNRK2.2 is unclear. Strains
containing both the \textit{sac1} and \textit{snrk2.2} alleles have slightly lower constitutive arylsulfatase activity in S-replete medium and express less arylsulfatase in S-deficient medium than the \textit{snrk2.2} single mutant (DAVIES \textit{et al.} 1994) (Figure 3A, column 6, Figure 3D).

The level of arylsulfatase activity in P-starved \textit{psr1-1} and \textit{psr1-1sac1} cells was similar, suggesting a hypostatic relationship between \textit{SAC1} and \textit{PSR1} with respect to this phenotype (Figure 3A, column 5; Figure 3C). However, we cannot eliminate the possibility that the \textit{sac1} \textit{(ars5-3)} allele may produce some partially functional \textit{SAC1} protein since in this allele the \textit{ARG7} marker is integrated into the 13^{th} intron of the \textit{SAC1} gene without generating a deletion (see Supplemental Figure S3) and \textit{SAC1} transcript levels are normal (data not shown). However, double mutants of \textit{psr1} with either of two additional \textit{sac1} alleles \textit{(ars4-15} and \textit{ars4-55}) also produced arylsulfatase during P-starvation (data not shown). The \textit{sac1} \textit{(ars4-55)} allele is likely to be null since the \textit{SAC1} transcript could not be detected by quantitative real-time PCR (data not shown), supporting the conclusion that \textit{SAC1} is not required for the accumulation of arylsulfatase activity observed in the \textit{psr1} mutant during P-starvation.

In contrast to the results discussed for the \textit{sac1} alleles, \textit{SNRK2.1} is epistatic to \textit{PSR1}; no P deficiency-inducible arylsulfatase is expressed in any of the \textit{psr1} strains that also harbor a \textit{snrk2.1} allele (Figure 3A, column 5; Figure 3C). The \textit{SAC1} and \textit{SNRK2.1} genes are both essential for normal activation of arylsulfatase when cells are exposed to –S conditions, but only the wild type \textit{SNRK2.1} allele is required for low level arylsulfatase activity in P-starved \textit{psr1} cells. Conversely, a synergistic epistasis relationship is observed between \textit{PSR1} and \textit{SNRK2.2} since the arylsulfatase activity produced by P-starved \textit{psr1snrk2.2(are10)} double mutants is far greater than the sum of the activities of
either single mutant (compare Figure 3A, column 5; Figure 3C). Similar results were obtained with psr1snrk2.2(are16) double mutants (see Supplemental Figure S1). The level of arylsulfatase activity associated with psr1snrk2.2 cells deprived of P for 72 h is comparable to the level attained by wild-type cells after 24 h of S-starvation (compare Figure 3C, Figure 3D). This result implies that SNRK2.2 inhibits full activation of arylsulfatase in P-starved psrl cells.

As expected, the triple mutants psrlsac1snrk2.1 and psrlsnrk2.2snrk2.1 do not exhibit arylsulfatase on –P medium (Figure 3A, column 5), demonstrating that SNRK2.1 is essential for arylsulfatase induction during P starvation. Interestingly, the psrlsac1snrk2.2 triple mutant does not display the high level of ARS in P-deficient cells that is observed in the psrlsnrk2.2 double mutant; rather the level of ARS activity resembles that of the psrl single mutant. Consequently we conclude that: 1) SNRK2.1 is absolutely essential for all arylsulfatase activity observed during P-deprivation of the psrl mutant, 2) neither SAC1 nor SNRK2.2 is essential for the low level of arylsulfatase activity that occurs when psrl cells are starved for P, and 3) SAC1 is required for the high arylsulfatase activity measured in P-deprived psrlsnrk2.2 mutant cells.

S deficiency-responsive transcripts in psrl and S-assimilation regulatory mutants. Comparison of ARSl transcript abundance in P-replete versus P-starved wild-type, psrl, sac1, snrk2.2, snrk2.1 and the respective double and triple mutants (Figure 4A) is mostly consistent with the level of ARS activity observed in these strains (Figure 3), with the exception of psrlsnrk2.2 relative to psrlsac1snrk2.2 (see below). As shown in Figure 2, wild-type cells display no significant activation of ARSl after 24 h of P-deficiency, but ARSl is upregulated 100-200 fold in P-deprived psrl cells. Like the situation in wild-type
cells, very limited \textit{ARS1} induction occurs in the \textit{sac1} and \textit{snrk2.1} strains (although there are differences in the basal level of \textit{ARS1} transcripts between the tested strains). An average \(~200\)-fold increase in \textit{ARS1} mRNA is observed in P-deficient \textit{psr1sac1} cells, consistent with the hypostatic relationship of \textit{SAC1} to \textit{PSR1} reported above; on the other hand, \textit{ARS1} mRNA does not accumulate in P-starved \textit{psr1snrk2.1}, \textit{psr1sac1snrk2.1} or \textit{psr1snrk2.2snrk2.1} mutant cells, confirming that \textit{SNRK2.1} is absolutely required for activation of S deficiency-responsive gene expression in \textit{psr1} cells that are starved for P.

The \textit{snrk2.2} mutant expresses \textit{ARS1} at a high basal level (Figure 4A) (DAVIES et al. 1999), consistent with the constitutive arylsulfatase activity observed in P-replete medium (Figure 3A, column 4, 3C) (DAVIES et al. 1994). However, we were surprised to observe that after 24 h of P-starvation, \textit{ARS1} transcript abundance in the \textit{snrk2.2} single mutant increased by \(~30\)-fold and reached an even higher level than that observed in \textit{psr1-1} (Figure 4A). This increase in \textit{ARS1} mRNA does not yield a proportional increase in arylsulfatase activity (Figure 3C), suggesting that post-transcriptional mechanisms may regulate arylsulfatase synthesis. In fact, as with P-deficient \textit{psr1} cells, a general derepression of S deficiency-responsive genes is observed in P-starved \textit{snrk2.2} cells (data not shown). Like the \textit{snrk2.2} single mutant, P-replete \textit{psr1snrk2.2} cells have high basal levels of \textit{ARS1} and other \textit{–S} transcripts (Figure 4A, 4C), but in this case the transcript levels that are produced in P-deficient cells are comparable with wild-type induction in \textit{–S} (GONZALEZ-BALLESTER et al. 2008). This high level of expression is consistent with the high arylsulfatase activity observed in P-deficient \textit{psr1snrk2.2} cells (Figure 3A, 3C). Taken together, these results suggest that activators of the S-assimilation pathway are stimulated both in wild-type and in \textit{psr1} under P-starvation conditions, but the negative
regulatory effect of SNRK2.2 normally dominates, suppressing full expression of S deficiency-responsive genes in cells starved for P.

Surprisingly, SAC1 appears to have only a limited effect on the abundance of –S transcripts in P-deficient snrk2.2 or psr1snrk2.2 cells. Although P-starved sac1snrk2.2 cells have slightly less arylsulfatase activity compared to snrk2.2 cells, and psr1sac1snrk2.2 cells have significantly less arylsulfatase activity than psr1snrk2.2 strains (Figure 3A, 3C), the abundance of ARS1 and other S deficiency-responsive transcripts is minimally affected in any of the strains containing the sac1 mutation (Figure 4A, 4C). The discrepancy between the abundance of ARS transcripts and arylsulfatase activity in the psr1snrk2.2 and psr1snrk2.2sac1 strains suggests that SAC1 is essential for post-transcriptional regulation of arylsulfatase synthesis, activity or both.

**psr1snrk2.2 cells bleach during P-limitation.** We observed, on a per volume basis, that psr1snrk2.2 cultures from two independent psr1snrk2.2(are10) isolates began bleaching significantly after 3 d of growth in medium with no added Pi, whereas P-deficient wild-type and psr1 cultures exhibited no decline or less of a decline, respectively, during the same time period (Figure 5A). The cell density increased over the 7 d time course for all of the cultures with the exception of psr1snrk2.2snrk2.2 (wild-type cells divided ~2-3 times, psr1 and psr1snrk2.2 cells divided ~1-2 times) (Figure 5B), indicating that chlorophyll declined on a per cell basis in the psr1 and psr1snrk2.2 strains. Examination of cell staining with Evan’s Blue dye during growth in P-deficient medium revealed that psr1snrk2.2 cells began to lose membrane integrity following the third day in medium devoid of P, in parallel with the bleaching (Figure 5C). Essentially no loss of viability was observed for wild-type cells over the same time period (Figure 5C). Viability of
psr1 cells also declined after 3 d of P-starvation, but at a slower rate than in psr1snrk2.2 cultures. Interestingly, P-deficient psr1snrk2.1snrk2.2 cells did not bleach and maintained significantly higher cell viability than psr1 or psr1snrk2.2 cultures (Figure 5A, 5C). Furthermore, the bleaching of P-deficient psr1 and psr1snrk2.2 cells is partially rescued by growth in the dark (data not shown), suggesting that to some extent the loss of cell viability may be caused by photodamage. These results indicate that the high level of activation of the S-deficiency responses is deleterious to the survival of P-deficient psr1snrk2.2 cells, and that the cells can be rescued to a significant extent by the snrk2.1 lesion, which blocks expression of the S-responsive genes.

**DISCUSSION**

We have demonstrated that the psr1 mutant activates S deficiency-responsive genes during growth in P-deficient medium, and have investigated the role of the regulatory elements SAC1, SNRK2.1 and SNRK2.2, which normally control S-deprivation responses, in this activation response. Epistasis analysis indicates that activation of S deficiency-responsive genes in P-starved psr1 cells does not require a significant positive regulatory input from the SAC1 gene product. On the other hand, the central regulatory kinase, SNRK2.1, is absolutely required for S-assimilation gene activation in P-deficient psr1 cells. The synergistically epistatic relationship between PSR1 and SNRK2.2 shows that the SNRK2.2 kinase has an inhibitory effect on the expression of S-deficiency response target genes in psr1 cells deprived of P, similar to its role in S-replete wild-type cells. However, unlike the situation for nutrient-replete cells, where S deficiency-responsive gene expression is only partially induced, the absence of
SNRK2.2 in the psr1snrk2.2 double mutant enables close to full activation of the –S target genes during P deprivation. The phenotype of P-starved psr1snrk2.2 strains points to the existence of an internal sensor of cellular S-status that is capable of almost fully activating S deficiency-responsive gene expression even when high levels of SO$_4^{2-}$ are present in the medium. The psr1sac1 and the psr1sac1snrk2.2 mutants reveal that although SAC1 is not required for the induction of S-deficiency responsive genes in P-deficient psr1 cells, it is required for the high activity of arylsulfatase observed in P-starved psr1snrk2.2 cells, suggesting a role for SAC1 in post-transcriptional regulation of some S deficiency-inducible proteins.

**Regulation of S deficiency-response genes.** What is the mechanism of –S gene activation in P-starved psr1 cells? In Figure 6 we present a speculative model to describe the interactions between the regulatory factors that control expression of the S-assimilation genes and activation of the S-responsive genes in the psr1 mutant. Under nutrient-replete conditions where the external concentration of SO$_4^{2-}$ ion is high, the SNRK2.2 kinase represses S deficiency-responsive gene expression (Figure 6A). Since the cell is both internally and externally replete for S, the positive regulators SAC1 and SNRK2.1 are not activated and only basal levels of transcripts for the S-assimilation genes accumulate. However, exclusion of SO$_4^{2-}$ from the medium leads to activation of SAC1 and SNRK2.1 (Figure 6B). From the phenotype of sac1 mutants, in which the induction of S deficiency-responsive transcripts is attenuated (DAVIES et al. 1994; GONZALEZ-BALLESTER et al. 2008; ZHANG et al. 2004), we infer that a major function of SAC1 is to inactivate SNRK2.2 in S-starved cells, allowing for full, high-level induction of S deficiency-responsive gene targets by the activator SNRK2.1 (Figure 6B). In –S
sac1 mutant strains, repression by SNRK2.2 is never released and consequently there is only low-level activation by SNRK2.1. We have noted that many of the sac1 progeny from genetic crosses have low and variable levels of arylsulfatase activity when deprived of S (see Supplemental Figure S1). This may reflect strain-to-strain variation in the balance between repression and activation of S deficiency-responsive gene expression by SNRK2.2 and SNRK2.1, respectively.

The phenotypes of snrk2.2 strains are very informative with respect to our understanding of the roles of the S-deficiency response regulators. Nutrient-replete snrk2.2 mutants display elevated basal expression of most S-assimilation genes compared to wild-type cells (Figure 4A) (DAVIES et al. 1994; GONZALEZ-BALLESTER et al. 2008; RAVINA et al. 2002), but the loss of SNRK2.2 is not sufficient by itself to cause expression of S-assimilation genes at the levels observed in S-starved cells. S-deficiency-responsive transcripts are induced similarly in S-starved wild-type and snrk2.2 cells (DAVIES et al. 1994; GONZALEZ-BALLESTER et al. 2008; RAVINA et al. 2002), suggesting that activation of the –S acclimation response is not achieved simply through repression of SNRK2.2; rather, the SAC1-dependent inactivation of SNRK2.2 is coordinated with the S deprivation-dependent activation of the SNRK2.1 kinase (Figure 6B). Although the absence of SNRK2.2 does not significantly affect induction of S deficiency-responsive genes in S-starved cells, like SAC1 the SNRK2.2 kinase also may play a role in post-transcriptional regulatory processes that affect protein expression and activity. S-starved snrk2.2 strains have only 40-50% of the wild-type level of arylsulfatase activity (Figure 3D) (DAVIES et al. 1994) and fail to accumulate the normal complement of extracellular proteins that are expressed in –S cells (DAVIES et al. 1994).
The partial de-repression of S deficiency-responsive genes that is observed in nutrient-replete and P-deficient snrk2.2 mutants also occurs in sac1snrk2.2 double mutants (Figure 4A) (DAVIES et al. 1994), and ARS transcript abundance increases in S-starved sac1snrk2.2 cells, albeit to a somewhat lesser extent than in wild-type or in snrk2.2 single mutants (DAVIES et al. 1994), indicating that activation of the S deficiency-responsive genes does not absolutely require SAC1 (Figure 3) (DAVIES et al. 1994).

In contrast to the situation in wild-type cells, SNRK2.2 does not completely inhibit expression of S deficiency-responsive genes in P-starved psrl cells (Figure 6D). This result is consistent with the hypothesis that the metabolic state of P-starved cells leads to a secondary, internal S deficiency (discussed below) that results in partial gene activation. Since the psrlsac1 double and psrl single mutants under –P conditions have similar levels of transcripts associated with the S deficiency-response, the stimulatory signal that overrides SNRK2.2 repression is not transmitted via SAC1. Therefore, accumulation of S deprivation-associated transcripts in P-starved, psrl cells must depend either on a positive regulator that interacts with SNRK2.1 or on promiscuous modification/activation of SNRK2.1. Unlike the situation during nutrient-sufficiency, elimination of SNRK2.2 repression in P-deficient psrlsnrk2.2 mutant cells leads to full activation of the signaling cascade and high S deficiency-responsive gene expression (Figure 4A, 4C). Therefore, while low internal SO$_4^{2-}$ can lead to partial activation of SNRK2.1 in the psrl strain, additional relief of SNRK2.2 repression can cause full activation. Furthermore, under these conditions the activation of –S responsive genes is
uncoupled from SAC1 since similar transcript levels are attained in P-starved *psr1snrk2.2* and *psr1sac1snrk2.2* cells (**Figure 4A, 4C**).

While SAC1 does not influence the level of transcripts from S deprivation-responsive genes in P-starved, *psr1snrk2.2* cells, it does have a significant stimulatory effect on the level of arylsulfatase activity in this strain (**Figure 3A, 3C**). SAC1 is an integral membrane protein similar to SO$_4^{2-}$ transporters of the SLC13 family, and it has been hypothesized that the protein functions as a sensor of external SO$_4^{2-}$ (DAVIES *et al.* 1996). In situations where the external SO$_4^{2-}$ concentration is high and the abundance of ARS transcripts is comparable, such as nutrient-replete *snrk2.2* and *sac1snrk2.2* cells, or P-starved *psr1snrk2.2* versus *psr1sac1snrk2.2*, the presence of SAC1 affects the amount of active arylsulfatase. In addition, preliminary data indicates that SAC1 is also required for the accumulation of SO$_4^{2-}$ transporter polypeptides in *psr1snrk2.2* cells starved for P (W. Pootakham, unpublished), supporting a role for SAC1 in posttranscriptional regulatory processes.

The partial induction of arylsulfatase and high level expression of the S deficiency-responsive genes in S-starved *sac1snrk2.2* strains and P-starved *psr1sac1snrk2.2* strains (**Figure 3, Figure 4**) confirm that gene activation during S deficiency can be largely uncoupled from SAC1 and SNRK2.2, providing us with new insight into the regulation of the S-responsive pathway. A simple hypothesis is that the activation state of the SNRK2.1 kinase can be directly affected by the intra-cellular S-status (**Figure 6**). Alternatively, an unknown regulator may interact with SNRK2.1 and stimulate its activity when intracellular S is low. In all cases tested, SNRK2.1 is
absolutely required for induction of S-assimilation genes, confirming the central regulatory role of this kinase (Figure 6) (GONZALEZ-BALLESTER et al. 2008).

**P-limitation affects intracellular S-metabolism.** What leads to the activation of SNRK2.1 in P-starved psrl cells? A hypothesis that is consistent with the experimental evidence is that P-limitation causes a secondary, internal S deficiency, despite the abundance of SO$_4^{2-}$ in the –P medium (Figure 6C). P-deficient cells could experience internal S-limitation as a consequence of at least two acclimation responses: i) cellular S is redirected towards sulfolipid synthesis for replacement of thylakoid membrane phospholipids (RIEKHOF et al. 2003), and ii) SO$_4^{2-}$-assimilation is inhibited, possibly the consequence of a Pi conservation regime in which cell redirects ATP away from SO$_4^{2-}$ assimilation and towards other essential processes. Recently, it was shown that P-starved cells accumulate 25 times more cysteine than nutrient-replete cells (BOLLING and FIEHN 2005), supporting the idea that P-deficiency causes significant changes in the intracellular S-status. In psrl cells starved for P, the degree of internal P-deficiency may be even more extreme than in wild-type cells. While psrl mutants cannot scavenge Pi from the medium (SHIMOGAWARA et al. 1999) and may be defective in mobilization of internal P stores (MOSELEY et al. 2006), they arrest growth and down-regulate photosynthesis more quickly than wild-type cells, indicating that they are to some extent able to adjust to their abnormal physiological state (SHIMOGAWARA et al. 1999). In this respect the phenotype of P-deficient psrl mutants contrasts with the phenotypes of S-starved sacl and snrk2.1 strains, which fail both to upregulate S-scavenging and assimilation, and to down-regulate photosynthetic electron transport, resulting in photodamage and cell death at moderate light intensities (GONZALEZ-BALLESTER et al. 2008; SHIMOGAWARA et al.
A possible consequence of the extreme P-deficiency experienced by P-starved \textit{psr1} cells may be that the level of secondary S-stress reaches a threshold that activates SNRK2.1 beyond the point at which it can be fully repressed by SNRK2.2, resulting in low level induction of the S-assimilation genes (Figure 6D). A constitutively inhibitory allele of \textit{SNRK2.2} might produce a similar effect in S-starved cells. These hypotheses could be tested by comparing the levels of S-containing metabolites in P-starved wild-type and \textit{psr1} cells and by analyzing the phosphorylation state of SNRK2.1.

\textbf{Alternative models.} While a preponderence of circumstantial evidence suggests that S-metabolism is affected by P-deficiency, alternative models can be invoked to explain the mutant phenotypes. For example, there are six additional members of the SNRK2 family of protein kinases besides SNRK2.1 and SNRK2.2 in Chlamydomonas (GONZALEZ-BALLESTER \textit{et al.} 2008). If one or more of these function in a regulatory cascade that activates PSR1 during P-starvation, in \textit{psr1} mutants where the regulatory pathway is blocked the kinase(s) might interact with SNRK2.1 and cause aberrant induction of S deficiency-responsive gene expression. Other stress signals could also contribute to the activation of S-starvation responses during P-limitation. In Arabidopsis, cysteine biosynthesis is enhanced during light and oxidative stress through the activity of the cyclophilin CYP20-3, which assists in the folding of chloroplast serine acetyltransferase (DOMINGUEZ-SOLIS \textit{et al.} 2008). We have ruled out light stress as the causative agent in the activation of –S responses in the \textit{psr1} mutant since similar arylsulfatase activity was observed in P-deficient cultures in the light or in the dark (Figure 1D). Nevertheless, it is
possible that other “general” stress signals are responsible for the aberrant –S responses in P-starved \( psr1 \) cells.

**Proximal versus secondary phenotypes of \( psr1 \).** Genetic analysis of the interactions between regulators of the S deficiency-responsive genes and \( PSR1 \) provides a cautionary tale that illustrates the difficulty of deciphering the wild-type function of a gene from its mutant phenotype. Some aspects of a mutant phenotype may be caused by acclimation to a novel physiological state that is caused by the lesion, rather than resulting directly from the loss-of-function of a particular gene. For example, a preponderance of evidence indicates that \( PSR1 \) encodes a nuclear-localized transcriptional activator (RUBIO et al. 2001; WYKOFF et al. 1999). A possible model that would account for the expression of the S deficiency-responsive genes in P-starved \( psr1 \) cells is that PSR1 itself negatively regulates their expression, either by participating directly in the S deprivation-responsive signaling cascade, interacting with the promoters of the target genes of this cascade, or by inducing expression of a gene encoding a repressor of S-responsive genes during P-deficiency. While we cannot fully rule out a role for PSR1 in repressing the S-deficiency responses, this hypothesis does not explain why cells that acclimate properly to P-starvation would find it necessary to repress the S deficiency-responsive genes when plenty of \( \text{SO}_4^{2-} \) is present in –P growth medium, or why expression of S-assimilation genes should increase in P-starved \( snrk2.2 \) cells (Figure 4), even though PSR1-dependent responses occur normally in this strain (Figure 3, Figure 4). Given the evidence that P-starvation affects the internal S-status of the cell, regulation of the S-deficiency responses can be understood without invoking any direct involvement of PSR1 in the signal transduction pathway. Instead, the metabolic state of P-starved \( psr1 \) cells provides an
“artificial” intracellular environment in which interactions between the P- and S-stress responses are unmasked.

Similar care should be taken in ascribing control of aspects of a wild-type acclimation response to a particular regulatory protein. For example, part of the response of wild-type cells to P-limitation is to stabilize chloroplast RNA transcripts (cpRNAs), and this stabilization correlates with down-regulation of the chloroplast polynucleotide phosphorylase (PNPase) (Yehudai-Resheff et al. 2007). PSRI has been implicated in this process, since cpRNA abundance declines drastically in psr1 cells that are starved for P and the expression of the PNP1 gene is not downregulated as it is in P-starved wild-type cells (Yehudai-Resheff et al. 2007). However, reduced cpRNA abundance is also characteristic of S-starved cells (Irihimovitch and Stern 2006), making it unclear whether the decline in cpRNAs in P-starved psr1 cells is a consequence of their inability to reduce the level of PNPase activity, the partial activation of the S-deficiency response or a combination of the two. It should be possible to distinguish between these possibilities by analyzing cpRNA stability and transcription rates in P-deficient psr1 cells.

Why would it be important for wild-type cells to repress S deficiency-responsive genes during P-limitation? This would be a logical strategy for a cell that perceives internal P- and S-deprivation but that can sense that SO$_4^{2-}$ is not limiting in the external environment. Upregulation of S-deficiency responses would be both energetically costly and futile in this situation since, because of P-insufficiency, the cell would be unable to grow even if it acquired more S. In this context, it is unclear whether partial activation of S-deficiency responses provides any benefit to P-starved psr1 cells. Along this vein, we wondered whether the more rapid downregulation of photosynthesis in P-deficient psr1
cells compared to wild-type cells (SHIMOGAWARA et al. 1999) was a byproduct of the partial activation of the S-deficiency response, since photosynthesis is downregulated more quickly during S-starvation than in P-starvation (WYKOFF et al. 1999). Comparison of PAR curves for P-deficient wild-type, psrl and psrlsnrk2.1 cells revealed that the S-deficiency responses were not likely to be involved in the downregulation of photosynthesis; both the psrl and psrlsnrk2.1 strains showed a similar, rapid reductions in photosynthetic electron transfer rates after 24 h of P-starvation, while wild-type cells maintained relatively high rates of photosynthesis (data not shown). Cell viability during P-starvation is actually improved in a strain that harbors both the psrl and the snrk2.1 mutations, compared to psrl (Figure 5C). Furthermore, the rapid loss of chlorophyll and cell viability in psrlsnrk2.2 cultures during P deprivation (Figure 5) indicates that full activation of the S-deficiency response is harmful rather than beneficial to the cell.

While P- and S-limitation have qualitatively similar effects on growth and photosynthesis (WYKOFF et al. 1998), some specific responses to these two limitations are diametrically opposed. For example, while P-starved cells turn over phospholipids and increase synthesis of sulfolipids (RIEKHOF et al. 2003), the opposite occurs in S-starved cells; sulfolipids are degraded and phospholipid abundance increases (SUGIMOTO et al. 2008; SUGIMOTO et al. 2007). It is conceivable that opposing responses prevent maintenance of proper phospho- to sulfolipid ratios in the thylakoid membrane, contributing to the bleaching of –P psrlsnrk2.2 cells. Examination of the lipid profiles of P-deficient psrlsnrk2.2 cells should be informative in this regard.

Conclusion. It has been somewhat surprising to find that analysis of the phenotype of the psrl mutant, which does not acclimate normally to P-deprivation, provides insight
into regulatory mechanisms controlling S-starvation responses. However, there are still many questions about the SAC1, SNRK2.1 and SNRK2.2 regulatory proteins that remain unanswered. What are the internal signals that trigger S-starvation responses? What are the targets of the kinases? Do the phosphorylation states of the regulatory proteins change during S-starvation? Do the regulatory proteins physically interact, and does S-starvation change these interactions? Detailed biochemical analyses will be required to achieve a more comprehensive understanding how cells acclimate to P- and S-limiting conditions and the integration between the two responses.

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LEGENDS TO THE FIGURES

Figure 1. *psr1* growth and hydrolytic activity. A. Growth, phosphatase and sulfatase activities of wild-type (CC-125), *psr1* and and *psr1*-complemented strains on solid agar media. +P +S plates were made with TAP medium, which contains 1.1 mM Pi and 0.4 mM SO$_4^{2-}$. –P +S plates were made with TA medium, containing no added Pi, but supplemented with 100 µM glucose-1-phosphate. +P –S plates contained 2 mM potassium thiocyanate, but no added SO$_4^{2-}$. Cells were grown for 5 d, and then assayed for phosphatase and sulfatase activity using the colorimetric assays described in the MATERIALS AND METHODS.
period. **B.** Quantitative analysis of phosphatase activity over 7 d of growth in TA liquid medium (no added Pi, no glucose-1-phosphate) for wild-type, psr1 and the psr1-complemented strain. Alkaline phosphatase activity is represented as absorbance at 410 nm of p-nitrophenol produced per mg of chlorophyll. Data points are the average of duplicate measurements, plus or minus the standard deviation. **C.** Arylsulfatase activity in the same cultures was measured using a similar method to that described in (B) and as reported by Davies *et al.* (Davies *et al.* 1994). **D.** Alkaline phosphatase (AP) and arylsulfatase activity (ARS) in wild-type and psr1 cultures grown for 3 d in the dark in TAP (+P) vs TA (−P) medium. Enzyme activities were normalized to equal cell number ($A_{410}$ per 4 x 10^8 cells) rather than equal chlorophyll in this experiment, since dark-grown cells accumulate less chlorophyll than in the light (Ford *et al.* 1981). However, for these strains, this number of cells (in the light) is approximately equivalent to 1 mg of chlorophyll (Moseley *et al.* 2002) so the enzyme activities are roughly comparable between (B - E). **E.** Arylsulfatase activity in TAP and after 24 h of growth in TAP –S medium.

**Figure 2. Expression of S deficiency-responsive genes in P-starved wild-type and psr1 mutant cells. A.** Relative abundance of P deficiency- and S-deficiency-responsive transcripts compared to the the CBLP control transcript in wild-type (CC-1690) and psr1 cells grown in TAP, and after 24 h of growth in TA. Relative transcript abundance is calculated from the formula $2^{-\Delta C(t)} \times 10^5$. PHOX encodes alkaline phosphatase, ARS1 encodes arylsulfatase 1, ARS2 encodes arylsulfatase 2, SLT1 encodes SAC1-like $\text{SO}_4^{2-}$-transporter 1, SLT2 encodes SAC1-like $\text{SO}_4^{2-}$-transporter 2, SULTR2 encodes a putative $\text{H}^+/$$\text{SO}_4^{2-}$ transporter, SAT1 encodes serine acetyl transferase, OASTL4 encodes O-acetyl
serine (thiol)lyase 4, *ECP76* encodes a 76 kDa extracellular protein, and *SBDP* encodes a putative selenium binding protein. Results from two independent experiments are shown.

**Figure 3. Epistasis analysis of *PSR1, SAC1, SNRK2.2* and *SNRK2.1*.**

A. Phenotypic analysis of growth, alkaline phosphatase and arylsulfatase activities for single, double and triple mutants on TAP (+P +S), TA + glucose-1-phosphate (–P +S) and TAP–S + thiocyanate (+P –S) solid medium. All strains were spotted onto plates from TAP medium, grown for 4 d and then assayed for phosphatase or arylsulfatase activities. B. Liquid culture assays for alkaline phosphatase activities in TAP and after 24 and 72 h of growth in TA medium for wild-type cells (CC-1690) and various mutants, including *psr1, sac1, snrk2.2, snrk2.1* and double and triple mutant strains, described in the text and in Table I. Data points are the average of duplicate measurements (+/− standard deviation). C. Arylsulfatase activity assay of cells from the same strains as in (B). D. Comparison of arylsulfatase activity in TAP versus after 24 h of growth in TAP–S medium. Data for A, B, C and D are from one representative experiment; comparable results were obtained from experimental replicates.

**Figure 4. *ARS1* and *PHOX* gene expression in *psr1* and the various S-deficiency response mutants.** Relative abundance of A. *ARS1* transcripts and B. *PHOX* transcripts in wild-type (CC-1690), *psr1, sac1, snrk2.2, snrk2.1* and double and triple mutant strains grown in TAP (+P), and after 24 h of growth in TA (−P). The data for the wild-type and *psr1* strains is reproduced from Figure 2 to facilitate comparison with the other strains. C. Comparison of the relative abundance of the indicated transcripts in the *psr1snrk2.2* and *psr1sac1snrk2.2* strains. Results from one to four experimental replicates are shown for each strain and each condition.
Figure 5. Chlorophyll content and cell viability in –P cultures. A. Time course of chlorophyll concentration per culture volume in wild-type cells and various mutants grown in TA (Pi-free) medium for 7 d. Chlorophyll concentrations are the average of duplicate measurements, plus or minus the standard deviation. The data are from one representative experiment; similar patterns were observed in two additional, independent experiments (see Supplemental Figure S2). B. Change in cell number for wild-type cells and the different mutant strains at various times following the transferring of cells to TA (Pi-free) medium. The change in cell number are the average of 2-4 technical replicates, plus or minus the standard deviation. C. Time course of viability of strains grown in TA (Pi-free) medium. Non-viable cells were distinguished from viable cells by staining with 0.25% Evans Blue dye. Percentage viability is calculated from the average of 2-4 measurements, plus or minus the standard deviation. The data are from one representative experiment; similar results were obtained in an additional, independent experiment (see Supplemental Figure S2). For all experiments the wild-type strain used was CC-1690 while the mutants were *psr1, psr1snrk2.2* and *psr1snrk2.1snrk2.2*.

Figure 6. Models of S deficiency-responsive gene regulation. A. Wild-type, nutrient-replete conditions. (i) SO$_4^{2-}$ ion is bound to SAC1 which is in an inactive state. (ii) SNRK2.2 makes the central kinase, SNRK2.1, ineffective in activation of gene expression. (iii) The cell is internally replete for S, so (iv) SNRK2.1 is maintained in a basal activation state. (v) S deficiency-responsive genes are expressed at basal levels. B. Regulation of S-starvation responses in wild-type cells under S-starvation conditions. (vi) SAC1 no longer binds SO$_4^{2-}$ ion and becomes activated. SAC1 causes inactivation of (vii) the SNRK2.2 repressor, which in turn allows SNRK2.1 to function as a
transcriptional activator. (viii) Internal S-deficiency further activates (ix) SNRK2.1, causing (x) full expression of S deficiency-responsive genes. (xi) SAC1 and SNRK2.2 also function in post-translation regulation of arylsulfatase and other sulfur-responsive proteins. C. Regulation of S-starvation responses in wild-type cells under P-starvation conditions. (xii) The SAC1 protein is bound to SO$_4^{2-}$ and is unable to block repression of gene expression by SNRK2.2. Sulfolipid biosynthesis increases and SO$_4^{2-}$ uptake and assimilation may be limited during P-stress, potentially causing (xiii) moderate internal S-deficiency. Nevertheless, (xiv) SNRK2.2 suppresses activation of the S-starvation responsive genes by (xv) SNRK2.1, so (xvi) expression of S-starvation responsive genes is maintained at the low basal level. (xvii) PSR1 becomes active and elicits (xviii) full expression of the P-starvation responsive genes. The cells fully acclimate to P-starvation conditions. D. Regulation of S-starvation genes in psr1 mutant cells under P-starvation conditions. Since SO$_4^{2-}$ is plentiful in the medium, (xix) SAC1 is in the inactive state, and (xx) SNRK2.2 suppresses activation of the S-starvation responsive genes by SNRK2.1. psr1 cells may experience (xxi) severe internal S-deprivation as a result of (xxii) their inability to acclimate properly to P-starvation. An equilibrium between repression by SNRK2.2 and (xxiii) activation of SNRK2.1 by the internal signal is achieved, resulting in (xiv) a low level of induction of S deficiency-responsive genes.
<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Parental strains</th>
<th>Figure(s)</th>
<th>Reference</th>
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<tr>
<td>CC-125</td>
<td>wild-type, <em>nit1nit2</em></td>
<td>na</td>
<td>1 &amp; S1</td>
<td>(SHIMOGAW ARA et al. 1999)</td>
</tr>
<tr>
<td>CC-1690</td>
<td>wild-type, <em>nit+</em></td>
<td>na</td>
<td>2-5, S1 &amp; S2</td>
<td></td>
</tr>
<tr>
<td><em>psr1-1</em></td>
<td><em>psr1-1cw+nit−mt−</em></td>
<td><em>psr1-1cw15</em> x CC-124, CC-125, backcrossed x5</td>
<td>1B-C</td>
<td>(SHIMOGAW ARA et al. 1999)</td>
</tr>
<tr>
<td><em>psr1-1</em> (66)</td>
<td><em>psr1-1cw+nit−mt+</em></td>
<td><em>psr1-1</em> x CC-125</td>
<td>1A, 1D-E</td>
<td>this study</td>
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<tr>
<td>pKS1 #19</td>
<td><em>psr1-1cw+nit−mt−</em> complemented with PSR1 genomic clone</td>
<td><em>psr1-1cw+nit−mt</em></td>
<td>1A-C, 1E</td>
<td>this study</td>
</tr>
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<td><em>psr1(A4)</em></td>
<td><em>psr1-1cw+nit+mt−</em></td>
<td>CC-1690 x <em>psr1-1cw+nit−mt+</em>, backcrossed x3</td>
<td>2-5, S1 &amp; S2</td>
<td>this study</td>
</tr>
<tr>
<td><em>psr1-2</em></td>
<td><em>psr1-2cw15</em></td>
<td><em>psr1-2cw15</em> x CC-124, CC-125, backcrossed x4</td>
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<td>(SHIMOGAW ARA et al. 1999)</td>
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<td><em>sac1(ars5-3)</em></td>
<td><em>sac1(ars5-3)cw15nit1mt−</em></td>
<td>CC-2267 x <em>ars5-1</em></td>
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<td>(DAVIES et al. 1996)</td>
</tr>
<tr>
<td><em>sac1(B2)</em></td>
<td><em>sac1(ars5-3)cw+nit+mt+</em></td>
<td>CC-1690 x <em>sac1(ars5-3)cw15nit1</em>, backcrossed x3</td>
<td>S1</td>
<td>this study</td>
</tr>
<tr>
<td><em>sac1(E3)</em></td>
<td><em>sac1(ars5-3)cw+nit+</em></td>
<td><em>psr1(A4)</em> x <em>sac1(B2)</em></td>
<td>3-4 &amp; S1</td>
<td>this study</td>
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<td><em>sac1(ars4-15)nit−</em></td>
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<td>(DAVIES et al. 1999)</td>
</tr>
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<td><em>psr1(A4)</em> x CC-3800</td>
<td>S1</td>
<td>this study</td>
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<td><em>snrk2.1(ars11)</em></td>
<td><em>snrk2.1(ars11)cw15nit2mt+</em></td>
<td>D66</td>
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<td>(GONZALEZ-BALLESTER et al. 2008; POLLOCK et al. 2005)</td>
</tr>
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<td><em>snrk2.1(ars11)cw+nit+</em></td>
<td><em>psr1(A4)</em> x <em>ars11</em></td>
<td>3-4 &amp; S1</td>
<td>this study</td>
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<tr>
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<td><em>snrk2.1(ars11)cw+nit+</em></td>
<td><em>psr1(A4)</em> x <em>ars11</em></td>
<td>S1</td>
<td>this study</td>
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<tr>
<td><em>sac1snrk2.2(F3)</em></td>
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<td><em>psr1sac1snrk2.2(A1)</em></td>
<td>3-4 &amp; S1</td>
<td>this study</td>
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<tr>
<td><em>psr1sac1(E1)</em></td>
<td><em>psr1-1sac1(ars5-3)cw+nit+</em></td>
<td><em>psr1(A4)</em> x <em>sac1(B2)</em></td>
<td>3-4 &amp; S1</td>
<td>this study</td>
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</tbody>
</table>

1 Strains used for enzyme and transcript assays presented in this study are listed in bold type.

2 na = not applicable
| **psrlsac1**(H3) | **psrl-1sac1(ars5-3) cw+nit+** | **psrl(A4) x sac1(B2)** | S1 | this study |
| **psrlsac1**(49) | **psrl-1sac1(ars5-3) cw+nit−** | **psrl-1(66) x sac1(ars5-3)** | S1 | this study |
| **psrlars4-15** | **psrl-1sac1(ars4-15) nit+** | **psrl(A4) x CC-3801** | this study |
| **psrlars4-55** | **psrl-1sac1(ars4-55) nit+** | **psrl(A4) x CC-3802** | this study |
| **psrlsnrk2.2**(D3) | **psrl-1snrk2.2(are10) cw+nit+** | **psrl(A4) x snrk2.2(are10)** | 3-5, S1 & S2 | this study |
| **psrlsnrk2.2**(N1) | **psrl-1snrk2.2(are10) cw+nit+** | **psrl(A4) x snrk2.2(are10)** | S1 | this study |
| **psrlsnrk2.2(#28)** | **psrl-1snrk2.2(are16) cw+** | **psrl(A4) x CC-3800** | S1 | this study |
| **psrlsnrk2.1**(B1) | **psrl-1snrk2.1(ars11) cw+nit+** | **psrl(A4) x ars11** | 3-4 & S1 | this study |
| **psrlsnrk2.1**(A3) | **psrl-1snrk2.1(ars11) cw+nit+** | **psrl(A4) x ars11** | S1 | this study |
| **psrlsac1snrk2.1**(A3) | **psrl-1sac1(ars5-3) snrk2.1(ars11) cw+nit+** | **psrl1sac1mt− x snrk2.1(ars11)** | 3-4 & S1 | this study |
| **psrlsnrk2.2snrk2.1**(A2) | **psrl-1snrk2.2(are10) snrk2.1(ars11) cw+nit+** | **psrl1snrk2.2mt− x snrk2.1(ars11)** | 3-4 & S1 | this study |
| **psrlsac1snrk2.2**(A1) | **psrl-1sac1(ars5-3) snrk2.2(are10) cw+nit+** | **psrl1sac1mt− x snrk2.2(are10)** | 3-5, S1 & S2 | this study |
| **psrlsac1snrk2.2**(D1) | **psrl-1sac1(ars5-3) snrk2.2(are10) cw+nit+** | **psrl1sac1mt− x snrk2.2(are10)** | S1 | this study |
Table II. Summary of arylsulfatase (ARS) activity and S deficiency-responsive gene expression phenotypes in psr1 and S-deficiency acclimation regulatory mutants.

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>+P−S</th>
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<tr>
<td></td>
<td>ARS activity</td>
<td>−S expression</td>
<td>ARS activity</td>
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<tr>
<td>wild-type</td>
<td>−³</td>
<td>−</td>
<td>−</td>
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<tr>
<td>psr1</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>sac1</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>psr1/sac1</td>
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<td>−</td>
<td>+</td>
</tr>
<tr>
<td>snrk2.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>psr1/snrk2.2</td>
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<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>sac1/snrk2.2</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>snrk2.1</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>psr1/snrk2.1</td>
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<td>−</td>
<td>−</td>
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<tr>
<td>psr1/sac1/snrk2.1</td>
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<tr>
<td>psr1/snrk2.1/snrk2.2</td>
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<td>−</td>
<td>−</td>
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<td>psr1/sac1/snrk2.2⁸</td>
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</table>

³ arylsulfatase activity and −S transcript abundance are represented on an arbitrary scale from − to ++++, where − represents basal levels and ++++ represents maximum levels.
⁴ Some sac1 strains produce limited amounts of arylsulfatase and activate the expression of −S genes slightly. This variation is represented by +/−.
⁵ Epistatic lesions are indicated with bold type.
⁶ nd= not done
⁷ The only data on −S gene expression for this genotype is from RNA blot analysis of ARS2 by Davies et al (1994).
⁸ The synergistic epistasis relationship between PSR1 and SNRK2.2 is maintained with respect to gene expression, but not with respect to arylsulfatase activity.
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Figure 1

A

wild-type
psr1-1
psr1-complemented

growth
sulfatase activity
phosphatase activity

B

C

D

E
Figure 4
Figure 5

A

![Graph showing chlorophyll levels over time for different genotypes: wild-type, psr1, psr1snrk2.2 (1), psr1snrk2.2 (2), psr1snrk2.1snrk2.2.](image)

B

![Graph showing cell counts over time for different genotypes: wild-type, psr1, psr1snrk2.2 (1), psr1snrk2.2 (2), psr1snrk2.1snrk2.2.](image)

C

![Graph showing proportion of unstained (live) cells over time for different genotypes: wild-type, psr1, psr1snrk2.2 (1), psr1snrk2.2 (2), psr1snrk2.1snrk2.2.](image)
Figure 6

A. **wild-type, nutrient replete**

B. **wild-type, –S**

C. **wild-type, –P**

D. **psr1, –P**