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PSR1 Is a Global Transcriptional Regulator of Phosphorus Deficiency Responses and Carbon Storage Metabolism in *Chlamydomonas reinhardtii*¹

Amit K. Bajhaiya², Andrew P. Dean³, Leo A.H. Zeef, Rachel E. Webster⁴, and Jon K. Pittman*
Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

Many eukaryotic microalgae modify their metabolism in response to nutrient stresses such as phosphorus (P) starvation, which substantially induces storage metabolite biosynthesis, but the genetic mechanisms regulating this response are poorly understood. Here, we show that P starvation-induced lipid and starch accumulation is inhibited in a *Chlamydomonas reinhardtii* mutant lacking the transcription factor Pi Starvation Response1 (PSR1). Transcriptomic analysis identified specific metabolism transcripts that are induced by P starvation but misregulated in the psr1 mutant. These include transcripts for starch and triacylglycerol synthesis but also transcripts for photosynthesis-, redox-, and stress signaling-related proteins. To further examine the role of PSR1 in regulating lipid and starch metabolism, PSR1 complementation lines in the psr1 strain and PSR1 overexpression lines in a cell wall-deficient strain were generated. PSR1 expression in the psr1 lines was shown to be functional due to rescue of the psr1 phenotype. PSR1 overexpression lines exhibited increased starch content and number of starch granules per cell, which correlated with a higher expression of specific starch metabolism genes but reduced neutral lipid content. Furthermore, this phenotype was consistent in the presence and absence of acetate. Together, these results identify a key transcriptional regulator in global metabolism and demonstrate transcriptional engineering in microalgae to modulate starch biosynthesis.

The need to mitigate the environmental impacts of industry is prompting the development of sustainable industrial methods, such as the applied use of biological processes (Skjånes et al., 2007). Likewise, the contribution of fossil fuels to greenhouse gas emissions has created significant interest in biofuels (Hill et al., 2006; Georgianna and Mayfield, 2012). Algae, including eukaryotic unicellular microalgae, are an attractive feedstock for sustainable industrial biotechnology. These photosynthetic microorganisms can use solar energy to convert CO₂ into various metabolites that have applications for many industries, including pharmaceuticals, food, health, materials, and energy (Skjånes et al., 2007; Guedes et al., 2011). To harness the full potential of microalgae for industrial biotechnology, an improved understanding of microalgae metabolism is essential, and tools to engineer algae metabolism for the overproduction of desired products are needed.

Microalgae store carbon principally as lipids in the form of triacylglycerol (TAG) and carbohydrate in the form of starch (Johnson and Alric, 2013). In many species, the biosynthesis of these storage metabolites is highly induced under conditions of environmental stress such as nitrogen (N) or phosphorus (P) starvation, high light, or salinity (Hu et al., 2008; Siaut et al., 2011). Nutrient starvation is a particularly reliable means to induce the accumulation of storage metabolites and has been routinely used as a condition to further our understanding of the transcriptional changes that occur at the onset of carbon storage (Merchant et al., 2012), especially for TAG accumulation in response to N starvation in the model species *Chlamydomonas reinhardtii* (Miller et al., 2010; Boyle et al., 2012; Blaby et al., 2013). In particular, these studies have demonstrated that genes encoding acyltransferase enzymes, which are responsible for de novo TAG biosynthesis, including type 1 and type 2 diacylglycerol acyltransferases (DGATs) and a phospholipid diacylglycerol acyltransferase, are highly up-regulated following N starvation. In contrast, very little is known about the transcriptional responses to P starvation or the regulators that control these metabolic changes.
P is an essential macronutrient that is needed for various biochemical and cellular processes (Raghothama, 1999). In the biosphere, P is biologically available to most organisms in the form of inorganic phosphate (Pi), but its concentration is often very limited, particularly due to complexation with metal cations and organic particles (Hudson et al., 2000). To cope with this limited availability, plants and microalgae have evolved adaptive mechanisms to facilitate improved acquisition and conservation of P and to allow survival under P starvation conditions (Moseley and Grossman, 2009; Rouached et al., 2010). In higher plants, the P starvation survival mechanism involves metabolic remodeling, including the replacement of phospholipids with sulfolipids, the use of alternative glycolytic pathways that utilize Pi-independent enzymes, and the accumulation of starch, due in part to activation of the Pi-sensitive ADP-Glc pyrophosphorylase (Plaxton and Tran, 2011). The transcriptional and posttranscriptional regulation of these plant P starvation responses is well understood (Rouached et al., 2010; Chiou and Lin, 2011; Sobkowiak et al., 2012), but it is unclear whether the control mechanisms of such responses are conserved in microalgae. In C. reinhardtii, P starvation responses are known to be controlled by the MYB family transcription factor Pi Starvation Response1 (PSR1), which regulates Pi acquisition through the up-regulation of phosphatases, including the alcaline phosphatase encoded by the PHOX gene, and the up-regulation of Pi transporters, in particular the PO4\(^{3-}\)/Na\(^+\) symporters encoded by members of the Pi Transporter B (PTB) gene family (Shimogawara et al., 1999; Wykoff et al., 1999; Moseley et al., 2006). In addition, PSR1 has been demonstrated to regulate processes to maximize P reallocation, such as through the modification of nucleic acid metabolism (Yehudai-Resheff et al., 2007). However, a potential role of PSR1 in regulating P starvation-induced starch and TAG biosynthesis is unclear and has not been studied.

The use of genetic engineering to enhance carbon storage metabolism in microalgae has so far had mixed success (Radakovits et al., 2010; Driver et al., 2014). For example, down-regulation of lipid catabolism or carbohydrate synthesis through targeted knockdown of a lipase gene or a UDP-Glc pyrophosphorylase gene, respectively, provided an increase in TAG yield (Trentacoste et al., 2013; Daboussi et al., 2014). In contrast, overexpression of DGAT genes, encoding the final enzymatic step of TAG synthesis, failed to increase TAG content (La Russa et al., 2012), demonstrating that direct manipulation of individual genes is not always successful. An alternative approach is the manipulation of transcriptional regulators, as this may provide more substantial metabolic modification by controlling multiple steps in a pathway (Grotewold, 2008). However, few transcriptional regulators of lipid and starch metabolism in microalgae have been described. Furthermore, to date, such transcriptional engineering of microalgae to modulate metabolism has not been explored. Transcriptomic analyses of N starvation responses have begun to identify putative transcriptional regulators (Miller et al., 2010; Boyle et al., 2012; Gargouri et al., 2015; Ngan et al., 2015). This includes Nitrogen Response Regulator1 (NRR1), a putative SQUAMOSA promoter-binding protein-type transcription factor from C. reinhardtii, which was demonstrated, through analysis of the nrr1 knockout mutant, to be a regulator of N-induced TAG biosynthesis (Boyle et al., 2012). More recently, PSR1 has also been identified as a regulator of TAG biosynthesis in response to N starvation (Ngan et al., 2015). However, that study did not begin to understand the mechanism of this PSR1 regulation at the level of individual downstream target genes and did not examine the metabolic control role of PSR1 under P starvation. Recently we observed that, under P starvation, mutation of PSR1 not only inhibits lipid accumulation but also significantly abolishes starvation-induced starch accumulation (Bajhaiya et al., 2016); however, the potential regulation of starch metabolism by PSR1 has not yet been examined.

In order to better understand the transcriptional changes underlying starch induction in response to P starvation, we have performed mRNA transcript abundance analysis of selected transcripts from wild-type and psr1 mutant C. reinhardtii under P-replete and P-starvation conditions and identified specific starch and lipid metabolism genes that are PSR1 regulated. To then further validate the role of PSR1 as a global metabolic regulator, we generated psr1:PSR1 complementation lines and PSR1 overexpression lines and demonstrated that PSR1 is able to modulate metabolism through the transcriptional regulation of specific carbon storage genes, leading to the increased accumulation of starch.

RESULTS

Identification of P Starvation-Induced and psr1-Misregulated Transcripts

To examine the transcriptional response to P starvation in wild-type and PSR1 mutant (psr1) plants, a preliminary RNA sequencing (RNA-Seq) data set of psr1 and the parental wild type (CC125) was generated and used to identify target transcripts to be analyzed further (Supplemental Methods S1). Wild-type cells grown in medium containing 10 \(\mu M\) P (low P) completely exhausted the Pi by day 3 of batch-culture growth, while cells in medium containing 1 mM P (high P) were P replete throughout the entire period of growth (Supplemental Fig. S1, A and B). This starvation of Pi was coincident with the start of lipid and starch accumulation that continued over the next 4 d (Supplemental Fig. S1F). Transcripts were quantified in day-3 cells, at the onset of P starvation, and in day-5 cells, 48 h after the onset of P starvation (Supplemental Fig. S2, A and B), as these were the time points where the transcriptional response was elevated (Supplemental Fig. S1, G and H). Of the 17,737 transcripts identified, at day 3, 1,105 transcripts in the wild type were greater than 2-fold up-regulated by low P,
and of these, only 139 were greater than 2-fold up-regulated in psr1, while 1,042 transcripts in the wild type were greater than 2-fold down-regulated by low P, and of these, only 112 were greater than 2-fold down-regulated in psr1 (Supplemental Data Set S1). A large proportion of the up-regulated but psr1-misregulated transcripts was classified as encoding Pi homeostasis proteins. As anticipated from a previous microarray analysis (Moseley et al., 2006), many of the known Pi homeostasis transcripts, including PSR1, PTB2, PTB4, and most clearly PHOX, were highly up-regulated by low P at day 3 in the wild type, but this up-regulation was abolished in psr1 (Supplemental Fig. S3A).

In addition to Pi homeostasis, the preliminary RNA-Seq data indicated that a high proportion of the P starvation-regulated transcripts were predicted to be involved in primary metabolism, especially starch and lipid metabolism, catabolism, and modification. Clusters of lipid and starch metabolism transcripts were indicated by hierarchical clustering to be down-regulated, up-regulated, or had no change in expression in the wild type in response to low P but were misregulated in psr1 (Supplemental Fig. S2C). A number of these transcripts were further examined in multiple replicate samples by quantitative real-time PCR (qPCR). Many of the down-regulated lipid metabolism transcripts were for fatty acid synthesis, including ACCase subunit transcripts such as BCC2 and fatty acid synthase component transcripts such as ACP2 and KAS1 (Fig. 1). Of particular interest were a group of starch and lipid metabolism transcripts that were highly up-regulated by P starvation and appeared to be down-regulated equally in the wild type and the psr1 mutant, such as PAPI (Fig. 1C). In contrast, a small number of transcripts were up- or down-regulated in the wild type, such as FAD5. Many transcripts that were down-regulated in the wild type but unchanged in psr1 were related to fatty acid synthesis, such as the ACCase subunit BCC2 or the acyl carrier ACP2 (Fig. 1B).

Finally, it was suggested that other gene classes, in addition to phosphate homeostasis and lipid and starch metabolism, are regulated by P starvation in a PSR1-dependent manner (Supplemental Data Set S1). It was determined by qPCR that a subset of photosynthesis- and electron transport/redox-related transcripts were misregulated by the absence of PSR1; for example, a light-harvesting complex II transcript (LHCBM9) was highly up-regulated by P starvation in the psr1 mutant, while the ferredoxin isoform FDX2 and a predicted cupredoxin (CSPI) were highly up-regulated by P starvation in the wild type but not in psr1 (Supplemental Fig. S3B). Nutrient starvation, such as N starvation, can also lead to an onset of oxidative stress, which in turn could lead to the induction of autophagic programmed cell death (Jiménez et al., 2009; Pérez-Pérez et al., 2010). In response to P starvation, there was no evidence from the RNA-Seq data set of any changes in transcripts encoding antioxidant enzymes or those associated with autophagy and programmed cell death in either the wild type or psr1 (Supplemental Data Set S1). However, Ca2+ transporters, which have been implicated in regulating abiotic stress responses, potentially as mediators of cellular Ca2+ signals (McAinsh and Pittman, 2009; Pittman et al., 2009), were substantially up-regulated by P starvation and appeared to be misregulated in psr1. In particular, the Ca2+/H+ exchanger isoforms CAX1 and CAX2 had altered transcriptional responses in the psr1 mutant (Supplemental Fig. S3B).

Overall, these data indicate that a subset of primary metabolism genes, which are induced under P starvation conditions, are transcriptionally regulated by PSR1. In higher plants, there are orthologous P starvation response transcription factors, including Phosphate Starvation Responser1 (PHR1) from Arabidopsis (Arabidopsis thaliana). The PHR1-binding site (P1BS) cis-element sequence (GNATATNC) was determined previously as the binding motif for PHR1 (Rubio et al., 2001), and this element is present within P starvation-induced genes from various higher plants (Sobkowiak and of these, only 139 were greater than 2-fold up-regulated in psr1, while 1,042 transcripts in the wild type were greater than 2-fold down-regulated by low P, and of these, only 112 were greater than 2-fold down-regulated in psr1 (Supplemental Data Set S1). A large proportion of the up-regulated but psr1-misregulated transcripts was classified as encoding Pi homeostasis proteins. As anticipated from a previous microarray analysis (Moseley et al., 2006), many of the known Pi homeostasis transcripts, including PSR1, PTB2, PTB4, and most clearly PHOX, were highly up-regulated by low P at day 3 in the wild type, but this up-regulation was abolished in psr1 (Supplemental Fig. S3A).
et al., 2012). While PSR1 and PHR1 are closely related but distinct MYB-CC family proteins, as shown by phylogenetic analysis, both the MYB-like DNA-binding and the coiled-coil protein dimerization regions are highly conserved within these proteins (Supplemental Fig. S4), increasing the likelihood that PSR1 may also recognize the P1BS element. To identify putative PSR1-binding elements, the GNATATNC sequence was screened in the promoter and noncoding regions of 500 genes, including 200 known and predicted PSR1-regulated genes and 300 genes predicted from the RNA-Seq data to be non-PSR1 regulated. Copies of the P1BS element were identified within the promoter or intron region of many of the validated genes, including within PTB2, PTB4, and PHOX (Supplemental Table S2). In total, 276 of the 500 genes contained the GNATATNC sequence, with 74% of the PSR1-regulated genes containing at least one copy of the P1BS element while only 42% of the non-PSR1-regulated genes contained the sequence (Supplemental Fig. S4, C and D). The GcATATgC sequence was the most frequently identified. Most of the P1BS-containing genes (58%) have a single copy of the element, 30% have two copies, and 12% have more than two copies, while a copy of the element is present within the promoter region in 66% of the genes and within an intron in 34% of the genes.

Figure 1. psr1-dependent misregulation of P starvation-induced starch and lipid metabolism genes. Relative expression is shown for selected starch metabolism genes (A), selected fatty acid synthesis-related genes (B), selected TAG synthesis genes (C), and other selected lipid metabolism genes (D) in high-P-treated (HP) and low-P-treated (LP) wild-type (WT; CC125) and psr1 cells at day 3. Expression of the mRNA transcripts by real-time PCR was determined relative to CBLP expression and is shown relative to the high-P-treated wild-type transcript. Data points are means ± se calculated from at least three biological replicates each with three technical replicates. Asterisks denote significant differences (P < 0.05) from high-P-treated wild-type values as determined by one-way ANOVA.
Generation of PSR1 Expression Lines

The role of PSR1 in regulating starch and lipid metabolism and the selected metabolism genes was further examined through the generation of PSR1 expression lines. A genomic fragment of PSR1 was fused to the tandem Heat Shock Protein70 (HSP70A)-RBCS2 promoter (Fig. 2A), which was shown previously to elicit the strong and stable expression of nucleus-transformed genes in C. reinhardtii (Schroda et al., 1999, 2000). The pCB740-PSR1 plasmid was transformed in the psr1 mutant background, in order to generate complementation lines and confirm that the expressed PSR1 was functional, and in the wild-type background, in order to examine overexpression phenotypes. For the overexpression lines, the cell wall-deficient cw15 strain was used, as this strain has been shown previously to exhibit high levels of carbon storage, especially starch, possibly due to the lack of cell wall glycoproteins that otherwise form a large carbon sink (Siaut et al., 2011). A number of independent psr1:PSR1-OE complementation and cw15:PSR1-OE overexpression lines were generated, of which three psr1:PSR1-OE lines and two cw15:PSR1-OE lines were examined further (Fig. 2B). All five lines showed significant increases in PSR1 expression relative to psr1 or cw15, while all of the psr1:PSR1-OE lines also showed higher PSR1 expression compared with the CC125 wild type, indicating that these lines not only complemented the psr1 mutation but yielded PSR1 overexpression (Fig. 3, A and B). Altered PSR1 expression was not due to the presence of the empty plasmid (Fig. 2D); however, PSR1 expression in all of the psr1:PSR1-OE and cw15:PSR1-OE lines was enhanced by low-P conditions (Fig. 3, A and B), equivalent to the P starvation-induced expression of native PSR1 (Supplemental Fig. S3A). This was unexpected, as the HSP70A-RBCS2 promoter was expected to provide constitutive expression; indeed, the expression of HSP70B driven by the HSP70A-RBCS2 promoter, tested as a control, did not show an increase under low-P conditions (Fig. 2C). Nevertheless, PSR1 expression was increased significantly relative to the wild type in all lines tested under both high-P and low-P conditions. To examine whether PSR1 mRNA stability was affected by P starvation, high-P- and low-P-grown cw15 and cw15:PSR1-OE cells were incubated with the transcriptional inhibitor actinomycin D and the translational inhibitor cycloheximide for 2 and 6 h before RNA was harvested for analysis. Actinomycin D treatment inhibited the transcription of all genes tested, leading to a reduction in mRNA transcript, with the exception of PSR1. The PSR1 mRNA abundance in all lines, including the wild type, was increased in low-P conditions after both 2 and 6 h of actinomycin D treatment (Fig. 4B) and cycloheximide treatment (Fig. 4D), relative to treatment without inhibitor. Under high-P conditions, there was no change in PSR1 abundance following actinomycin D treatment (Fig. 4A) and a smaller relative increase of transcript abundance following cycloheximide treatment that was only apparent after 6 h and only in the cw15:PSR1-OE lines (Fig. 4C).

Increased expression of the known PSR1-regulated PHOX, PTB2, and PTB4 was also observed in all psr1:PSR1-OE and cw15:PSR1-OE lines, compared with psr1 and cw15, respectively, under both high-P and low-P conditions (Fig. 3, A and B). In contrast to the effect on PSR1 abundance, both actinomycin D and cycloheximide treatment caused substantial reductions of PHOX mRNA abundance under low-P conditions in the wild type and cw15:PSR1-OE lines (Fig. 4) compared with PHOX abundance in the absence of inhibitors.
Up-regulation of the PHOX phosphatase and the Pi uptake transporters would be expected to increase Pi content within the cell. At day 3, cellular Pi content was significantly increased in all psr1:PSR1-OE lines compared with psr1 and all cw15:PSR1-OE lines compared with cw15 under both high-P and low-P treatments (Fig. 3C). By day 7 (late exponential growth phase/onset of stationary phase), a significant increase in Pi content was still observable in high-P cells, but in the low-P cells, this was only increased significantly in two of the psr1:PSR1-OE lines, due to the substantial depletion of Pi in the low-P medium by this time (Supplemental Fig. S1B). In all, these findings demonstrate that the expressed PSR1 was functional.

**Increased Cell Volume and Starch Accumulation in PSR1 Overexpression Lines**

Under high-P conditions, there was no difference in cell density, growth rate, biomass yield, or chlorophyll...
content between any of the $psr1$:PSR1-OE lines and the wild type and $psr1$ (Fig. 5, A–C; Supplemental Fig. S5A). Under low P conditions, the growth of $psr1$ cells was inhibited compared with the wild type, as shown previously (Wykoff et al., 1999), and PSR1 expression in $psr1$ fully rescued the reduction in both cell density (Fig. 5A) and biomass (Supplemental Fig. S5A). In the $cw15$ background lines, there was no difference in biomass yield or growth rate under either P condition, although cell density was slightly but significantly reduced in the two $cw15$:PSR1-OE lines in high P (Fig. 5, A and B; Supplemental Fig. S5A). However, there was a large significant increase in total chlorophyll concentration in the $cw15$:PSR1-OE lines particularly under high-P conditions but also in low P (Fig. 5C). The most marked change in morphology of the $PSR1$-expressing cells was with regard to cell size, with many lines showing consistent increases in cell volume compared with the wild type and $psr1$ (Fig. 5D; Supplemental Fig. S6), particularly lines $psr1$:PSR1-OE8 and $psr1$:PSR1-OE14 under low-P conditions but also both $cw15$:PSR1-OE lines under low P.

Increase in cell volume has been linked previously to the accumulation of carbon storage products (Dean et al., 2010; Work et al., 2010). Fourier transform infrared (FT-IR) spectroscopy, which is a powerful method to generate metabolic fingerprints (Ellis et al., 2002; Dean et al., 2010; Bajhaiya et al., 2016), was used to determine a global metabolic profile of each cell line at day 7 when end-point storage metabolites begin to accumulate. Three replicates of FT-IR spectra from each line were analyzed by principal component analysis (Fig. 6). All of the $psr1$:PSR1-OE lines clustered together closely with the wild type but clearly distinct from $psr1$ under low-P conditions, demonstrating that the successful complementation of $psr1$ by the $PSR1$ transgene could be determined at the metabolic level (Fig. 6B). The distinction between $psr1$ and the other cells was largely indicated by principal component 1, with the loading plot demonstrating that the metabolic difference between $psr1$ and the wild type or $psr1$:PSR1-OE cells was due predominantly to an increase in carbohydrates. In contrast, there was a less obvious pattern of clustering in these cells under high-P conditions (Fig. 6A). For the $cw15$ cells, there was a clear separation between the $cw15$:PSR1-OE lines and the wild type under high-P and low-P conditions, in both cases primarily determined by principal component 1. This demonstrates that there were significant metabolic differences within the $cw15$:PSR1-OE lines compared with the wild type, although the loading plots for both P conditions indicated that the metabolic variation was complex and not due to a single metabolite (Fig. 6, C and D). However, a clear change in both P conditions was an increase in a specific undetermined carbohydrate (denoted as band k) in the $cw15$:PSR1-OE cells. In contrast, under P starvation conditions only, the principal component loading plot indicates that the $cw15$:PSR1-OE cells had a marked decrease in the abundance of lipid (denoted as band a) compared with the wild-type cells (Fig. 6D).

Metabolic changes within the cells were further examined by specific metabolite quantification. All $psr1$:PSR1-OE lines showed significant increases in starch compared with $psr1$ in low-P conditions, while two $psr1$:PSR1-OE lines showed significant starch increases in high-P conditions (Fig. 7A). Likewise, both $cw15$:PSR1-OE lines showed large significant increases in starch concentration compared with the wild type in both P conditions, with a mean increase (for the two

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**Figure 4.** Effect of actinomycin D and cycloheximide treatment on transcript abundance in PSR1 overexpression lines. Change in expression is shown for PSR1, PHOX, SSS1, and SP1 in PSR1 overexpression lines and the wild type (WT; $cw15$) at day 3 following treatment for 2 h (solid bars) and 6 h (hatched bars) with the transcription inhibitor actinomycin D during high-P (A) and low-P (B) growth and with the translation inhibitor cycloheximide during high-P (C) and low-P (D) growth. Expression of the mRNA transcripts by real-time PCR was determined relative to $CBLP$ expression, and the increase or decrease in transcript abundance is shown relative to expression without inhibitor treatment. Each data point represents the mean ± se of three biological and technical replicate culture flasks.
lines) of 59% in low-P conditions and 310% in high-P conditions. For the cw15:PSR1-OE lines in low P, this substantial increase in starch was correlated with a 25% reduction in neutral lipid concentration, while in the psr1:PSR1-OE lines, there was no difference in lipid concentration compared with the wild type, but the lipid concentration was increased significantly compared with psr1 (Fig. 7B). These lipid and starch phenotypes were also observed consistently when quantified on the basis of biomass (Supplemental Fig. S5) and when quantified by FT-IR spectroscopy (Supplemental Fig. S7). Protein concentrations per cell did not change markedly, apart from an increase in both cw15:PSR1-OE lines compared with the wild type in high-P conditions (Fig. 7C), but there was no significant difference in protein concentration between lines and in response to P status when normalized on the basis of biomass (Supplemental Fig. S5B).

The quantified increases in starch accumulation were confirmed by imaging of starch granules. Detailed imaging of cell morphology by transmission electron microscopy (TEM; Fig. 8) of line psr1:PSR1-OE8 in comparison with the wild type (CC125) and psr1, and line cw15:PSR1-OE5 in comparison with the wild type (cw15), demonstrated the marked intracellular storage metabolite changes that occur in response to P starvation. The accumulation of many starch granules and lipid bodies was observed in all of the low-P-treated cells except for psr1 cells, which looked equivalent to high-P-grown cells and had a significantly reduced number of starch granules per cell compared with the wild type (Supplemental Fig. S8). This psr1 phenotype was rescued in the psr1:PSR1-OE8 cells, which displayed high numbers of starch granules in the low-P-grown cells (Fig. 8) but also a significant increase in starch granule number when grown in high-P conditions. Mean starch granule number was also significantly higher in cw15:PSR1-OE5 cells compared with wild-type cells in both P conditions, although there was no significant difference in mean starch granule size (Supplemental Fig. S8).

To assess whether the PSR1 overexpression phenotype was dependent on the presence of acetate, cw15 and cw15:PSR1-OE lines were grown without acetate. Cells were harvested and analyzed at days 7 and 14, as growth in the absence of acetate was expected to be slower. Equivalent phenotypes were observed with the acetate-free medium compared with the acetate-containing medium (Fig. 9). At both time points, starch concentration and cell volume were increased significantly in all cw15:PSR1-OE lines compared with the wild type, while there was no significant change in biomass and minor changes in total chlorophyll content.

Altered Expression of Starch and Lipid Metabolism Genes in PSR1 Overexpression Lines

Expression of the starch and lipid metabolism genes shown to be misregulated in psr1 (Fig. 1) was quantified...
Figure 6. FT-IR spectroscopy analysis of PSR1 expression lines. Principal component analysis scores (left) and principal component (PC) loading plots (right) are shown for FT-IR spectra from the wild type (WT; CC125), *psr1*, and *psr1*:*PSR1* complementation lines (A and B) and wild-type (*cw15*) and *PSR1* overexpression lines (C and D) under high-P (A and C) and low-P (B and D) conditions. Analysis was performed on three replicate spectra for each sample and treatment. Selected bands that show strong
in the \textit{psr1}:PSR1-OE and \textit{cw15}:PSR1-OE lines under high-P and low-P conditions. Many of the starch metabolism genes showed altered expression following \textit{PSR1} overexpression both in the \textit{psr1} background lines (Fig. 10A) and in the \textit{cw15} background lines (Fig. 10B). The expression of SSS1, SSS5, and SP1 was increased significantly relative to the wild type in both \textit{psr1}:PSR1-OE and \textit{cw15}:PSR1-OE lines. In contrast, the expression of \textit{ISA3} was reduced slightly compared with the wild type in both sets of lines, although \textit{ISA3} expression was higher than in \textit{psr1} in the complemented lines. Expression of \textit{SP2}, which encodes the PhoB plastidial starch phosphorylase, was repressed significantly in all \textit{PSR1} overexpression lines. The expression of \textit{AMA2} and \textit{AMA3} did not show major changes relative to the wild type, although the expression of both genes was increased relative to \textit{psr1} in all \textit{psr1}:PSR1-OE lines under low-P conditions. Similar to the response of PHOX transcript abundance to transcription inhibition, actinomycin D treatment caused a substantial reduction of SSS1 and SP1 mRNA abundance under both high-P and low-P conditions in the wild type and \textit{cw15}:PSR1-OE lines compared with transcript abundance in the absence of the inhibitor (Fig. 4, A and B). Translation inhibition by cycloheximide treatment had no significant effect on the abundance of both transcripts under high-P conditions, equivalent to the response of PHOX (Fig. 4C), while under low-P conditions, SSS1 abundance was not changed significantly by cycloheximide treatment, but there was a fold reduction in the abundance of SP1 after 6 h in the wild type and the \textit{psr1}: PSR1-OE5 line (Fig. 4D).

Expression changes were also observed for some of the lipid metabolism genes following \textit{PSR1} overexpression. In all of the \textit{psr1} complementation lines under low-P conditions, the presence of \textit{PSR1} induced increased expression of each gene tested relative to \textit{psr1}, and for some of the genes, particularly \textit{PAP2} and \textit{GPD4}, the expression was significantly higher relative to the wild type (Fig. 10C). Likewise, expression of these two genes was increased significantly under low-P conditions relative to the wild type in both \textit{cw15}:PSR1-OE lines (Fig. 10D). Under high-P conditions, the expression changes in the \textit{psr1}:PSR1-OE and \textit{cw15}:PSR1-OE lines were less clear, although \textit{DGTT2} expression was higher relative to \textit{psr1} in all lines.

**DISCUSSION**

In this study, we provide strong evidence that \textit{PSR1} regulates carbon storage metabolism under P starvation conditions through the transcriptional control of specific starch and lipid synthesis genes. In particular, induction or repression of a high number of starch and lipid metabolism genes in response to P starvation was inhibited or abolished in \textit{psr1} knockout lines, while overexpression of \textit{PSR1} mediated changes in the expression of some of these genes, resulting in altered partitioning and storage of starch and TAG. The qPCR analysis showed that an equivalent number of \textit{PSR1}-dependent starch and lipid enzyme genes were modulated by P starvation (Fig. 1), with the \textit{psr1} knockout line showing equivalent inhibition of both starch and lipid accumulation and the \textit{psr1}:PSR1 complementation lines restoring both metabolites to wild-type levels (Fig. 7). However, the most obvious phenotype of the \textit{PSR1} overexpression lines in \textit{cw15} cells was a significant increase in starch under both P-replete and P-starvation conditions, while the neutral lipid concentration in these cells was either unchanged or inhibited. This phenotype may be explained partly by the observation that TAG biosynthesis genes, notably \textit{PAP2} and \textit{GPD4} encoding for the synthesis of DAG and glycerol 3-phosphate, respectively, were only moderately up-regulated in \textit{cw15}:PSR1-OE cells (Fig. 10). In contrast, both of the \textit{SSS} isoforms, particularly \textit{SSS1}, which is most likely required for amylopectin synthesis (Ball and Morell, 2003), was highly up-regulated, as was the PhoA SP encoded by \textit{SP1}, which has been suggested to play a role in starch synthesis (Dauvillée et al., 2006; Fig. 11). The \textit{ISA3} debranching enzyme and \textit{AMA3} were slightly down-regulated and thus are likely to inhibit starch catabolism. The other SP isoform (\textit{SP2}) encoding PhoB was also down-regulated, and although this enzyme has been shown to be involved in starch synthesis (Dauvillée et al., 2006), it is unclear whether PhoB also has a role in starch breakdown.

These data also support the model that starch rather than neutral lipids is the major carbon storage molecule in photosynthetic organisms, including \textit{C. reinhardtii}. Thus, the \textit{PSR1}-dependent component of the P starvation response of remodeling carbon storage metabolism preferentially increases starch accumulation. This antagonism between starch and lipid synthesis is equivalent to the previous observations that lipid accumulation in \textit{C. reinhardtii} can be increased through a reduction in starch synthesis, as seen in starchless mutants, including \textit{sta6} and \textit{sta7} (Work et al., 2010; Blaby et al., 2013; Goodenough et al., 2014), although in each of these cases, under N starvation rather than P starvation conditions. It has been presumed that, in mutant cells that are unable to synthesize starch, during starvation conditions that promote carbon storage, carbon flux instead is directed toward TAG synthesis.

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**Figure 6.** (Continued.)

Changes are highlighted on the loading plots: \(a\), \(\nu C=O\) of ester functional groups from lipids and fatty acids; \(b\), \(\nu C=O\) of amides associated with protein (amide II); \(c\), \(\delta N-H\) of amides associated with protein (amide II); \(d\), \(\delta_\alpha\) CH\(_2\) and \(\delta_\alpha\) CH\(_3\) of lipids and proteins; \(e\), \(\delta_\gamma\) CH\(_2\) and \(\delta_\gamma\) CH\(_3\) of proteins, \(\nu C=O\) of carboxylic groups; \(f\), unknown; \(g\), \(\nu P=O\) of nucleic acids, phosphorlyl group, due to DNA/RNA backbones, phosphorylated proteins, and polyphosphate storage products; \(h\), \(\nu C=O\) of polysaccharides; \(i\), \(\nu C=O\) of carbohydrates; \(j\), \(\nu C=O\) of carbohydrates; \(k\), \(\nu C=O\) of carbohydrates; \(l\), \(\nu C=O\) of carbohydrates; \(m\), \(\nu C=O\) of carbohydrates.
However, it is important to remember that these N starvation-induced TAG accumulation phenotypes, both in wild-type strains and starchless mutants, are pronounced in the presence of acetate, and particularly following an acetate boost, while phototrophically grown N-starved cells in the absence of acetate do not exhibit as substantial a TAG yield (Goodson et al., 2011; Goodenough et al., 2014). The PSR1-induced phenotypes observed in this study were seen in cells grown under both photoheterotrophic conditions and photobiotrophic conditions without acetate, with no obvious difference in the degree of starch accumulation (Fig. 9).

This study also allows us to examine how some of the transcriptional responses to P starvation compare with responses to other nutrient starvation conditions quantified previously, including N and sulfur starvation. It has been stated that global responses to macronutrient limitation are very similar in terms of growth inhibition, protease up-regulation, and lipid and starch accumulation when comparing N, P, and sulfur limitation (Schmollinger et al., 2014). However, gas chromatography-mass spectrometry-based metabolomic analysis has indicated that the metabolic differences between P-starved and nutrient-replete cells are distinct from the metabolic differences observed following N and sulfur starvation (Bölling and Fiehn, 2005). Observation of the sulfur starvation transcriptome indicates that this stress does not elicit

![Figure 7](image1.png)

**Figure 7.** Metabolite contents in PSR1 overexpression lines. Total starch (A), lipid (B), and protein (C) were quantified at day 7 in high-P and low-P growth conditions. Each data point represents the mean ± s.e. of three replicates each with three technical replicates and is representative of three independent experiments. Asterisks denote significant differences (P < 0.05) from wild-type (WT) values and pound signs denote significant differences (P < 0.05) from psr1 values, both as determined by one-way ANOVA.

![Figure 8](image2.png)

**Figure 8.** Changes in the morphology of PSR1-expressing cells in response to P starvation. TEM images (representative of 15–20 images) are shown for wild-type (WT; CC125 and cw15), psr1, psr1:PSR1-OE8, and cw15:PSR1-OE5 cells in high-P and low-P conditions at day 7 of growth. Some subcellular structures are labeled: C, chloroplast; LB, lipid body; N, nucleus; P, pyrenoid; SG, starch granule. Bars = 2 μm.
substantial changes in carbon storage metabolism compared with the marked changes seen here for P starvation (González-Ballester et al., 2010). In particular, although sulfur starvation enhances starch accumulation greatly in microalgae (Brányiková et al., 2011), there appeared to be no significant increase in the abundance of starch metabolism transcripts in response to this stress (González-Ballester et al., 2010). In contrast, N starvation, like P starvation, modifies the expression of many primary metabolism and carbon storage metabolism genes (Miller et al., 2010; Boyle et al., 2012; Blaby et al., 2013; Goodenough et al., 2014; Ngan et al., 2015). Our transcriptomic data generated by qPCR indicate that there are some identical responses between N and P starvation; for example, specific isoforms of starch and TAG synthesis, including SSS5, GPD4, and PAP1, are up-regulated under P starvation (Fig. 1) and N starvation conditions (Miller et al., 2010; Goodenough et al., 2014). However, there are a number of differences between the stresses with regard to fatty acid and TAG synthesis, which is substantially modified under N starvation. Various components of fatty acid synthesis are induced by N starvation, while these are largely down-regulated by P starvation. Variation is seen at the end point of TAG synthesis from diacylglycerol, which is controlled by two acyltransferase pathways: the DGAT and the acyl-CoA-independent phospholipid diacylglycerol acyltransferase pathways. The type 1 DGAT DGAT1 and the type 2 DGAT DGTT1 are both highly up-regulated by N starvation (Boyle et al., 2012), while RNA-Seq analysis indicated that neither of these transcripts appears to be induced either at all or substantially by P starvation, although this needs to be confirmed by qPCR. In contrast, DGTT2 was induced and DGTT3 and DGTT4 were maintained at basal levels under P starvation conditions (Fig. 1). With regard to starch metabolism, both the N and P starvation transcriptomes showed clear starch synthesis gene changes, but these appear to be generally distinct; for example, N starvation mediates significant up-regulation of granule-bound starch synthase genes and starch-branching enzyme genes (Goodenough et al., 2014) that was not seen under P starvation. Recently, Schmollinger et al. (2014) reported a direct comparison of RNA-Seq data derived from N, P, and sulfur limitation treatments and indicated that N and sulfur limitation induces more common transcriptional responses (approximately 23% similarity) compared with N and P limitation (approximately 5% similarity in response). No transcripts related to photosynthesis and tetrapyrrole biosynthesis were reported to be common between P and N limitation, whereas such transcripts showed similar responses between N- and sulfur-limited cells (Schmollinger et al., 2014). Both N and P starvation elicits a reduction in photosynthetic activity, including decreased oxygen evolution and photosynthetic electron transport (Peltier and Schmidt, 1991; Wykoff et al., 1998), but the impact of P starvation on photosynthesis may be less severe. These transcriptomic observations, along with the metabolomic responses to N, P, and sulfur (Bölling and Fiehn, 2005), indicate clear distinctions between different macronutrient limitation conditions.

These PSR1 experiments have also reaffirmed the importance of PSR1 in controlling P homeostasis in C. reinhardtii. We were able to confirm many of the results found in the previous microarray-based transcriptomic analysis that allowed coverage of approximately 3,000 C. reinhardtii transcripts (Moseley et al., 2006). In that study, the microarray data and qPCR validation demonstrated the PSR1-dependent P starvation-induced increase of PHOX, PSR1, and PTB2 to PTB3 and the decrease in transcript abundance of the PTA-type PO₄³⁻/H⁺ symporter isoforms PTA1 and PTA3, many of which are confirmed here. Other P homeostatic and P conservation changes were also apparent at the transcriptional level. Under P limitation, the abundance of the phospholipid phosphatidylycerol decreases,
while the sulfolipid SQDG increases (Riekhof et al., 2003). The SQD1 enzyme that performs the first step of SQDG synthesis is essential for sulfolipid metabolism (Riekhof et al., 2003), and the SQD1 transcript, as well as transcripts for other sulfolipid genes, including SQD3, were found to be highly up-regulated by P starvation in a PSR1-dependent manner (Fig. 1). Another PSR1-dependent transcript up-regulated by P starvation was GDP1, encoding a putative GDP. One assumption could be that this enzyme mediates the scavenging and recycling of P through the hydrolysis of lipid phosphodiester from phospholipids such as phosphatidylcholine. A transcriptomic and proteomic analysis of the marine diatom Thalassiosira pseudonana in response to P limitation also observed an induction of transcripts or proteins for SQDG synthesis and phosphodiesterase activity, in addition to increased alkaline phosphatase and Pi transport (Dyhrman et al., 2012). Our study has gone further and shown that over-expression of PSR1 increases the abundance of the P homeostatic components and, consequently, increases Pi uptake into the cell, including under P-replete conditions (Fig. 3C). This indicates that the P starvation response can be deregulated, allowing excessive P accumulation even when not needed by the cell. Microalgae are able to accumulate and store excess Pi as polyphosphate; thus, increased P accumulation is less likely to be toxic to the cell. Indeed, total biomass yield was unchanged in the cw15:PSR1-OE cells under high-P conditions, although there was a slight but significant

Figure 10. Altered starch and lipid gene expression in PSR1 overexpression lines. Relative expression of selected starch metabolism genes (A and B) and selected lipid metabolism genes (C and D) in psr1:PSR1 complementation lines is shown compared with the wild type (WT; CC125) and psr1 (A and C) and in overexpression lines compared with the wild type (cw15; B and D) under high-P and low-P conditions at day 3. Expression of the mRNA transcripts by real-time PCR was determined relative to CBLP expression and is shown relative to the high-P-treated wild-type transcript. Each data point represents the mean ± s.e. of three biological replicates each with three technical replicates. Asterisks denote significant differences (P < 0.05) from wild-type values and pound signs denote significant differences (P < 0.05) from psr1 values, both as determined by one-way ANOVA.
reduction in cell density and total chlorophyll content was increased significantly. Thus, there may be applications for high-Pi-accumulating microalgae, such as their use in the bioaccumulation and recovery of waste Pi from wastewater (Pittman et al., 2011).

An unexpected result from these experiments was the observation that PSR1 expression in the pCB740-PSR1-transformed cells was significantly higher under low-P conditions compared with high-P conditions (Fig. 3). This response does not appear to be due to the HSP70A-RBCS2 promoter, and it is unclear whether this phenotype is due to the induction of PSR1 by the P-limited status or the repression of PSR1 under P-replete conditions, such as through instability of the PSR1 mRNA transcript. The inhibition of transcription by actinomycin D reduced the abundance of all transcripts tested with the exception of PSR1, for which mRNA transcript was still highly abundant, particularly during P starvation (Fig. 4). Furthermore, treatment with the translational inhibitor cycloheximide did not affect the increase of PSR1 transcript abundance under low-P conditions, and transcript abundance remained high, as mRNA stability is well known to be maintained by the inhibition of protein synthesis (Baker and Liggit, 1993). In fact, cycloheximide treatment caused an elevation in the accumulation of the PSR1 transcript (Fig. 4D), which has been seen previously for other C. reinhardtii transcripts (Aksoy et al., 2013), probably due to the inhibition of turnover or the ribosomal protection of transcript. Interestingly, this cycloheximide-induced elevation of PSR1 accumulation was greater under low-P conditions, suggesting increased stability and/or increased transcription in response to P starvation. Furthermore, this result suggests that protein synthesis was not required for the induction of PSR1. In contrast, a substantial reduction of PHOX and SPI transcript abundance under P starvation following cycloheximide treatment was observed (Fig. 4D), indicating that protein synthesis was required for PHOX and SPI induction and that the inhibition of PSR1 protein synthesis in turn inhibited the transcriptional activation of these genes. Distinct tiers of regulation, either requiring or not requiring protein synthesis, were observed previously with respect to sulfur limitation (Aksoy et al., 2013). One possible explanation for the P-responsive regulation of PSR1 in the absence of the native promoter could be that PSR1 can still be regulated via an intronic cis-regulatory element present within the gene sequence or that other as yet unknown factors are involved in PSR1 regulation. The higher plant homologs of PSR1, including Arabidopsis PHR1, may be regulated in part posttranslationally, through sumoylation (Miura et al., 2005). When PHR1 was overexpressed in Arabidopsis under the control of a strong constitutive promoter, PHR1 transcript abundance was equivalent between P-limiting and P-replete conditions, but even in the wild-type plant, PHR1 expression was not highly induced by P starvation (Nilsson et al., 2007), indicating differences between the regulation of PSR1/PHR1 in plants versus microalgae.

The potential of oil accumulation in microalgae for bioenergy use has been widely discussed (Georgianna and Mayfield, 2012; Merchant et al., 2012; Driver et al., 2014), and likewise, some authors have suggested that starch accumulation in microalgae might also have potential as a feedstock for biofuel production (Brányiková et al., 2011). Furthermore, there are additional potential nutraceutical and industrial applications of metabolites from microalgae (Guedes et al., 2011). However, for such applications to be viable in the future, tools to enhance and modify specific metabolites will be needed. Transcriptional engineering has been proposed as a potentially more efficient approach to engineer metabolic pathways instead of targeting individual enzymes (Grotewold, 2008). Previously, the putative transcription factor NRR1 was identified, which is induced specifically under N starvation and when knocked out causes substantial reduction of N starvation-induced TAG synthesis (Boyle et al., 2012). A

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**Figure 11.** Model of the effects of PSR1 overexpression on starch and lipid metabolism. The schematic diagrams represent starch synthesis and breakdown (A) and fatty acid and TAG synthesis (B). Genes in yellow and blue are those up-regulated and down-regulated by P starvation, respectively, as determined by real-time PCR. For gene definitions, see Supplemental Table S1. Green up arrows and red down arrows indicate genes that are positively or negatively regulated by PSR1 overexpression, respectively.
The study also identified PSR1 as a highly induced transcription factor under N and sulfur starvation, while disruption of PSR1 inhibited nutrient starvation-induced lipid accumulation (Ngan et al., 2015). We have further observed that PSR1 mutation inhibits lipid accumulation in response to P starvation (Bajhaiya et al., 2016; Fig. 7B). Ngan et al. (2015) showed that overexpression of a PSR1 complementary DNA (cDNA) construct in CC125 enhanced lipid accumulation, a function that we have confirmed here, as complementation of the psr1 mutant strain with PSR1 genomic DNA increased lipid content relative to psr1 lipid level (Fig. 7B) and is coincident with an increase in the abundance of various lipid metabolism genes (Fig. 10C). Interestingly, we did not observe a significant increase in lipid concentration when PSR1 was overexpressed in cw15 cells, suggesting that there may be some variation in lipid induction mediated by PSR1 depending on strain background. However, we have clearly shown that overexpression of PSR1 can also markedly enhance starch content, a phenotype not evaluated by Ngan et al. (2015).

Transcriptional regulators for starch metabolism have been identified in higher plants, and manipulation of these transcription factors through overexpression was shown to modify the composition or accumulation of these metabolites (Cernac and Benning, 2004; Fu and Xue, 2010). For example, PSR1 was found to be a negative regulator of starch biosynthesis in rice (Oryza sativa), modulating starch yield and starch granule morphology (Fu and Xue, 2010), while OsbZIP58 was identified as another transcriptional regulator of rice starch metabolism (Wang et al., 2013). However, overexpression of PSR1 did not significantly alter total starch content, while overexpression of OsbZIP58 was not tested. Here, we demonstrated that manipulation of a transcriptional regulator in C. reinhardtii has the potential to increase the content of metabolites such as starch under both nutrient-replete and starvation conditions. However, we also demonstrated that the PSR1 transcription factor is not specific in its modulation of lipid or starch but rather is a global regulator and, therefore, that manipulation with PSR1 would likely alter many processes and thus impact the long-term fitness of the strain. PSR1 is not the only transcriptional regulator of lipid metabolism (Boyle et al., 2012) and is unlikely to be the only starch regulator. Other potential transcription factors induced by nutrient starvation have been identified (Miller et al., 2010; Gargouri et al., 2015; Ngan et al., 2015), some of which are likely to function as metabolic regulators. Therefore, future efforts are needed to identify more metabolism-specific transcriptional regulators.

MATERIALS AND METHODS

Chlamydomonas reinhardtii Strains and Growth Conditions

The C. reinhardtii wild-type strain CC125 (obtained from CCAP; stock no. CCAP11/32C) and the cell-wall-deficient strain cw15 (cw15 arg7-8, referred to throughout as cw15; obtained from the Chlamydomonas Resource Center; stock no. CCAS51) were used as wild-type control strains. The cw15 arg7 Arg auxotrophic strain has an identical metabolic profile under P-starvation and P-replete conditions compared with the cw15 ARG7 strain (CCAP11/32CW15+) lacking the Arg mutation, as determined by FT-IR spectroscopy (Supplemental Fig. S9). The psr1 knockdown strain was kindly provided by Arthur Grossman. CC125 is the isogenic parental strain to psr1. psr1 was originally isolated from a UV light mutagenesis screen (Shimogorwa et al., 1999), although the specific mutation site is unknown. PSR1 expression is severely inhibited in the mutant but not completely abolished (Supplemental Fig. S3A); thus, psr1 is a knockdown mutant rather than a complete null. Furthermore, coverage of RNA-Seq reads indicates that the full-length PSR1 transcript is made and is not truncated in psr1. The PSR1 complementation lines and overexpression lines were created in this study. The strains were grown up to 7 d photobioterotrophically in batch culture in standard Tris-acetate-phosphate (TAP) medium at pH 7 (Harris, 1989; high-P medium) containing 1 mM P in 200-mL glass flasks on an orbital shaker rotator at 2 Hz or in 50-mL Nunc flasks at 25°C under cool-white fluorescent lights (150 μmol m⁻² s⁻¹) with a 16-h/8-h light/dark regime. In some experiments, strains were grown photobioterotrophically in the absence of acetate in Tris-phosphate medium for up to 14 d, with medium pH adjusted to 7 by the addition of HCl. P deficiency was imposed by growing cells in low-P TAP or Tris-phosphate medium containing 10 μM P by reducing the volume of potassium Pi (pH 7) solution (KHP0₄/KH₂PO₄) while maintaining a uniform concentration of potassium by the addition of KCl. Before starting cultivation in low-P medium, low exponential phase P-replete cells were washed twice with low-P medium by centrifugation at 3,000g for 1 min to remove externally bound P. Washed cells were inoculated into fresh low-P medium to give an initial cell count of approximately 65 × 10⁶ cells mL⁻¹. In some experiments, strains were grown in high-P or low-P TAP medium until day 3 and then exposed to the transcription inhibitor cycloheximide in DMSO at a final concentration of approximately 5 mM. PSR1 knockdown strain was kindly provided by Arthur Grossman.

The C. reinhardtii wild-type strain CC125, obtained from CCAP; stock no. CCAP11/32C) and the cell-wall-deficient strain cw15 (cw15 arg7-8, referred to throughout as cw15; obtained from the Chlamydomonas Resource Center; stock no. CCAS51) were used as wild-type control strains. The cw15 arg7 Arg auxotrophic strain has an identical metabolic profile under P-starvation and P-replete conditions compared with the cw15 ARG7 strain (CCAP11/32CW15+) lacking the Arg mutation, as determined by FT-IR spectroscopy (Supplemental Fig. S9). The psr1 knockdown strain was kindly provided by Arthur Grossman. CC125 is the isogenic parental strain to psr1. psr1 was originally isolated from a UV light mutagenesis screen (Shimogorwa et al., 1999), although the specific mutation site is unknown. PSR1 expression is severely inhibited in the mutant but not completely abolished (Supplemental Fig. S3A); thus, psr1 is a knockdown mutant rather than a complete null. Furthermore, coverage of RNA-Seq reads indicates that the full-length PSR1 transcript is made and is not truncated in psr1. The PSR1 complementation lines and overexpression lines were created in this study. The strains were grown up to 7 d photobioterotrophically in batch culture in standard Tris-acetate-phosphate (TAP) medium at pH 7 (Harris, 1989; high-P medium) containing 1 mM P in 200-mL glass flasks on an orbital shaker rotator at 2 Hz or in 50-mL Nunc flasks at 25°C under cool-white fluorescent lights (150 μmol m⁻² s⁻¹) with a 16-h/8-h light/dark regime. In some experiments, strains were grown photobioterotrophically in the absence of acetate in Tris-phosphate medium for up to 14 d, with medium pH adjusted to 7 by the addition of HCl. P deficiency was imposed by growing cells in low-P TAP or Tris-phosphate medium containing 10 μM P by reducing the volume of potassium Pi (pH 7) solution (KHP0₄/KH₂PO₄) while maintaining a uniform concentration of potassium by the addition of KCl. Before starting cultivation in low-P medium, low exponential phase P-replete cells were washed twice with low-P medium by centrifugation at 3,000g for 1 min to remove externally bound P. Washed cells were inoculated into fresh low-P medium to give an initial cell count of approximately 65 × 10⁶ cells mL⁻¹. In some experiments, strains were grown in high-P or low-P TAP medium until day 3 and then exposed to the transcription inhibitor cycloheximide in DMSO at a final concentration of 100 μg mL⁻¹ or the cytoplasmic 80S ribosome translation inhibitor cycloheximide in DMSO at a final concentration of 10 μg mL⁻¹, or a DMSO control, for 2 and 6 h, before cells were harvested and RNA was isolated, as described below.

Generation of PSR1 Complementation and Overexpression Lines

Genomic DNA was isolated from wild-type CC125 using cetyl trimethyl ammonium bromide (CTAB). A dense culture of cells (2 mL) was centrifuged at 3,000g, and the cell pellet was resuspended in 0.5 mL of CTAB buffer containing 2% (w/v) CTAB, 100 mM Tris-HCl (pH 8), 1.4% NaCl, 20 mM EDTA, and 2% (v/v) β-mercaptoethanol, then incubated at 65°C for 1 h. The solution was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and the aqueous phase, obtained following centrifugation (10,000g for 10 min), was incubated with an equal volume of ice-cold isopropanol to precipitate DNA. A 3,764-bp PSR1 genomic DNA fragment, including all exons and introns regions and 5′ and 3′ untranslated region sequences, was amplified from genomic DNA using primers PSR1F (5′-AAACACTTGTCCATGTTGACAGCTTACG-3′; SpeI restriction enzyme site underlined) and PSR1R (5′-AAAGATATCCGGTCTGAGCTACAAATAAA-3′; EcoRV restriction enzyme site underlined). The PCR fragment was digested with SpeI and EcoRV and then ligated into the Nhel and EcoRV sites of pCB740 (Schroda et al., 1999), so that PSR1 was under the control of the HSP70A-RBCS2 tandem promoter and regulated with the native PSR1 terminator (Fig. 2A). The junction site of the HSP70A-RBCS2 tandem promoter fragment with the PSR1 gene at the 5′ end (Nhel site) was 110 bp before the ATG start codon, and the junction site of the PSR1 gene at the 3′ end with the pCB740 plasmid (EcoRV site) was 765 bp after the TAG stop codon. The PSR1 gene within the resulting pCB740-PSR1 plasmid was confirmed by sequencing using the PSR1 primers (Supplemental Table S4). The pCB740 and pCB740-PSR1 plasmids were introduced into cw15 to generate PSR1 overexpression lines and vector control lines and into psr1 to generate psr1:PSR1 complementation lines, using biolistic bombardment as described (Bryon and Gillham, 1993), except using 0.6-μm gold microcarriers and 900-p.s.i. rupture disks (Bio-Rad). cw15-PSR1-GE1 OE lines were selected on TAP medium without Arg. The selection of psr1 transforms (psr1:PSR1-OE lines) was based on tolerance to low-P medium by efficient growth on low-P TAP agar plates. All lines were genotyped using primers AF and BR (Fig. 2, A, and B; Supplemental Table S4). Of the 25 cw15 and 25 psr1 transforms screened, eight to 10 positive transforms were identified for each genotype, of which lines psr1:PSR1-OE3, psr1:PSR1-OE8, and psr1:PSR1-OE14 as well as lines cw15: PSR1-OE3 and cw15:PSR1-OE5 were studied further.
Cell Growth Analysis

Cell density by optical density measurement and exponential phase growth rate was determined as described (Osundeko et al., 2013). Cell counts were determined using a Nexcelom Cellometer T4 (Nexcelom Biosciences). Fresh weight algal biomass was determined following centrifugation of a 50 mL sample at 1,500g for 20 min in a preweighed tube. Cell dimension measurements of 10 to 39 individual cells taken from bright-field light microscopy images were determined using ImageJ (http://rsweb.nih.gov/ij). Cell biovolume was calculated using these measurements by approximating the cells to a sphere using the formula given by Hillebrand et al. (1999).

RNA Isolation, cDNA Synthesis, and qPCR

Total RNA was isolated from replicate samples of liquid N2-frozen day-3, -5, or -7 cultures of high-P and low-P-treated cells using Trizol reagent (Life Technologies) and further purified by phenol/chloroform/isooamyl alcohol (25:24:1) extraction and precipitation with ice-cold isopropanol. RNA quality was checked by NanoDrop UV-Vis spectrophotometer (Thermo Scientific) analysis. First-strand cDNA was synthesized from DNase-treated RNA using BioScript reverse transcriptase (Bioline) and an oligo(dT) primer. The gene expression of selected transcripts was determined by qPCR using a SYBR Green master mix (Roche) and an ABI Prisma 7000 machine (Applied Biosystems) with BioScript reverse transcriptase (Bioline) and an oligo(dT) primer. The gene CBLP was placed in an HTS-XT high-throughput microplate extension, and FT-IR spectra were collected over the wave number range 4,000 to 600 cm⁻¹ in a 96-well silicon microplate and oven dried at 40°C overnight. The plate was submitted to complete amyloglucosidase digestion (Ball et al., 1991), and total starch measurements were performed, using methods by Ball et al. (1991) for starch measurements, day-7 cells (5-mL volume) were harvested and centrifuged as described above, washed with 1 mL EDTA to remove any externally bound Pi and further washed with Milli-Q (Millipore) deionized water, and pelletted by centrifugation at 1,500g for 20 min. Pellets were dried for 24 h at 60°C and then digested with 0.5 mL of 67% (v/v) ultrapure nitric acid at 100°C for 3 h. Digested samples (to determine cellular Pi) and 0.45-μm filtered medium samples (to determine medium Pi) were diluted in Milli-Q water and analyzed using an Autoanalyzer (Seal Analytical) fitted with an X2Y autosampler and AA3 digital colorimeter module HR4. The generated peaks were calibrated against drift and baseline peaks. All starch, lipid, protein, chlorophyll, and Pi measurements were determined on a per cell basis from cell counts or were normalized to fresh weight biomass.

Bright-Field Light Microscopy and TEM

Bright-field differential interference contrast microscopy images of day-7 high-P and low-P-treated cells were observed using a Leica DMR microscope and a 100X oil-immersion objective, and images were taken with a SPOT Xpolorer CCD camera (model 17.4; Diagnostic Instruments). The same cells were used for TEM. The cells were fixed with 4% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M HEPES buffer (pH 7.2) and then postfixed with 1% (v/v) osmium tetroxide and 1.5% (v/v) potassium ferrocyanide in 0.1 M cacodylate buffer (pH 7.2) for 1 h. The cells were then dehydrated in an ethanol series infiltrated with TAAB 812 resin and polymerized for 24 h at 60°C. Sections were cut with a Reichert Ultracut ultramicrotome and observed using a Technai BioTwin microscope (FEI Instruments) at 100-kV accelerating voltage. Images were taken with a Gatan Oris SC1000 CCD camera. From the TEM images, starch granule characteristics were quantified using CellProfiler version 2.1 (http://www.cellprofiler.org).

Bioinformatic Sequence Analysis

C. reinhardtii genomic sequence and gene model information was obtained from Phytozome version 9.1 (http://phytozome.jgi.doe.gov/pz/portal.html) using version 5.3 of the C. reinhardtii genome annotations. Multiple sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic analysis was performed essentially as described previously (Emery et al., 2012) using a ClustalW2 alignment and the maximum likelihood method under the WAG + F model of amino acid substitution and using the fast bootstrap approach to determine tree confidence, using the RAXML version 7.1 program. For bootstrapping, 100 iterations were used. The tree was viewed using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree). For the identification of putative PSR1-binding sites, the PSR1 co-element sequence (GNATATNC) was screened in the noncoding regions, including intronless regions of 500 selected PSR1-regulated genes (Supplemental Table S3), as determined from RNA-Seq data, using the NewPLACE database (http://sogo.dna.affrc.go.jp/cgi-bin/cgi-bin.cgi?page=analysis&lang=eu; Higo et al., 1999).

Statistical Analysis

Differences between treatments and cell lines were assessed using one-way ANOVA performed using IBM SPSS Statistics version 20. When significant differences were detected at the 95% confidence level, the Tukey posthoc test was applied. Sequence data from this article can be found in the EMBL-EBI ArrayExpress data library under accession number E-MTAB-2536.
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Time-course profile of P availability, growth rate, lipid and starch accumulation, and P starvation gene expression in response to P starvation in wild-type cells.

Supplemental Figure S2. Transcriptional responses to P-replete and P-starvation conditions in wild-type and psr1 cells.

Supplemental Figure S3. Expression of selected genes in response to P starvation.

Supplemental Figure S4. Conservation of PSR1 DNA-binding domains and abundance of predicted PSR1-binding cis-elements.

Supplemental Figure S5. Metabolite content in PSR1 overexpression lines normalized by biomass.

Supplemental Figure S6. Changes in morphology of PSR1-expressing cells in response to P starvation.

Supplemental Figure S7. Lipid and carbohydrate contents of PSR1-expressing cells in response to P starvation determined by FT-IR spectroscopy.

Supplemental Figure S8. Starch granule characteristics.

Supplemental Figure S9. Metabolite profile comparison of cw15 ARG7 and cw15 arg7 strains by FT-IR spectroscopy.

Supplemental Table S1. Gene definitions for selected transcripts and gene families described in this study.

Supplemental Table S2. Predicted PSR1-binding cis-elements in PSR1-regulated genes.

Supplemental Table S3. Genes used for P1PB cis-element analysis.

Supplemental Table S4. Primer sequences used for genotyping and sequencing of the pCB740-PSR1 plasmid.

Supplemental Table S5. Primer sequences used for real-time PCR.

Supplemental Data Set S1. Summary of the expression levels and the DESeq normalization for high-P- and low-P-treated wild-type and psr1 mutant cells.

Supplemental Methods S1. RNA-Seq and data analysis.

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Bajhaiya et al.
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