1 Trehalose-6-phosphate links singlet oxygen-induced signalling 2 with metabolic signalling in *Chlamydomonas reinhardtii*

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17 Abstract

18 Singlet oxygen (¹O₂) induces retrograde signalling in chloroplasts. Using a novel mutant
19 screen, we identified a mutation in the *TREHALOSE-6-PHOSPHATE PHOSPHATASE 1*
20 (*T6PPI*) gene that results in accumulation of trehalose 6-phosphate, a reprogramming of cell
21 metabolism, and impairment of ¹O₂-induced retrograde signalling in *Chlamydomonas*
22 *reinhardtii*. From transcriptomic analysis and metabolite profiling, we conclude that
23 accumulation or deficiency of certain metabolites directly affect ¹O₂-signalling. ¹O₂-inducible
24 *GLUTATHIONE PEROXIDASE 5* (*GPX5*) gene expression is suppressed by increased
25 content of fumarate, an intermediate in the tricarboxylic acid cycle (TCA cycle) in
26 mitochondria and dicarboxylate metabolism in the cytosol, while it is promoted by another
27 TCA cycle intermediate, aconitate. Furthermore, genes encoding known essential
28 components of chloroplast-to-nucleus ¹O₂-signalling show decreased transcript levels in a
29 *t6ppi* mutant, which can be rescued by exogenous application of aconitate. We demonstrate
30 that chloroplast retrograde signalling involving ¹O₂ depends on mitochondrial and cytosolic
31 processes and that the metabolic status of the cell determines the response to ¹O₂.
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Introduction

Nearly forty years ago, tetrapyrrole biosynthesis (TBS) intermediates were proposed to be involved in chloroplast retrograde (organelle-to-nucleus) signalling. This conclusion was based on the observation that accumulation of chlorophyll precursors negatively affects transcript level of light-harvesting chlorophyll a/b-binding (LHCB) protein¹. Later studies on chloroplast retrograde signalling involved mutants², treatment with a carotenoid biosynthesis inhibitor, norflurazon³, or TBS inhibitors, such as α,α -dipyridyl¹ or thujaplicin⁴. Experiments with norflurazon led to the discovery of the *genomes uncoupled* (*gun*) mutants with a common phenotype of chloroplast status-independent expression of photosynthesis-associated nuclear genes³.

It is noteworthy that treatment with inhibitors causing carotenoid deficiencies results in generation of reactive oxygen species (ROS) and photooxidative damage to chloroplasts. Furthermore, the end-products of TBS, haem and chlorophyll, as well as many of their intermediates (Supplementary Fig. 1) are light-absorbing and redox-reactive molecules, capable to generate ROS. Singlet oxygen ($^1\text{O}_2$) can be produced through interaction of ground (triplet)-state oxygen ($^3\text{O}_2$) with triplet-state chlorophyll or TBS intermediates, e.g. protoporphyrin IX (Proto), which are excited by light⁵. ROS are also metabolic products of other cellular processes in plants, such as photosynthesis and respiration.

Although $^1\text{O}_2$ is not considered to be the most reactive oxygen species, it is thought to be the major ROS involved in photooxidative damage⁶. However, it was shown that production of $^1\text{O}_2$ in the chloroplast induces stress responses that do not result exclusively from physicochemical damage, but also rely on signal transduction triggered by ROS⁷. Additionally, studies on the *fluorescent* (*flu*) mutant of *Arabidopsis thaliana*, which is impaired in regulation of TBS and accumulates protochlorophyllide (Supplementary Fig. 1), demonstrated that $^1\text{O}_2$ -retrograde signalling is integrated into a broader signalling network and affects not only stress responses but also metabolism⁸.

However, $^1\text{O}_2$ has a short half-life (about 200 ns) in the cell⁹. As a result, the distance that it may move was calculated to be approximately 10 nm, based on predicted diffusion rates^{10,11}. Its diffusion range is also limited due to its high reactivity with membrane lipids. Therefore, $^1\text{O}_2$ could play a specific role as an activator of a stress response only if it is detected close to its source, which strongly suggests that other components mediating the $^1\text{O}_2$ signals should exist. Alternatively, altered metabolite contents triggered by $^1\text{O}_2$ may also mediate $^1\text{O}_2$ -retrograde signalling, or as proposed by Pfannschmidt¹², a certain metabolic signature may be required to trigger changes in nuclear gene expression.

Despite the profound effect of $^1\text{O}_2$ on the chloroplast redox state and its apparent involvement in altering nuclear gene expression, little is known about the components involved in $^1\text{O}_2$ -dependent retrograde signalling. The protein factors identified so far include EXECUTER1 (EX1) and EX2 in the *flu* mutant of *A. thaliana*¹³, PSBP DOMAIN-CONTAINING PROTEIN 2 (PSBP2)¹⁴ and SINGLET OXYGEN ACCLIMATION (SAK1)¹⁵ in *Chlamydomonas reinhardtii*, or METHYLENE BLUE SENSITIVITY (MBS1)¹⁶ shown to be involved in $^1\text{O}_2$ -signalling in both *A. thaliana* and *C. reinhardtii*.

In our search for tetrapyrrole-signalling components, we hypothesized that $^1\text{O}_2$ generated via the photosensitizing activity of Proto, rather than porphyrin accumulation itself, triggers signalling cascades that alter nuclear gene expression. Therefore, we used a *C. reinhardtii* mutant *chlD-1*¹⁷ that accumulates Proto due to a dysfunctional Mg-chelatase (MgCh, Supplementary Fig.1). The GENOMES UNCOUPLED 4 (GUN4) protein involved in MgCh function (Supplementary Fig. 1) and signalling degrades upon Proto accumulation¹⁸. However, *chlD-1* with introduced over-expression of GUN4, *chlD-1/GUN4*, showed higher GUN4 content than *chlD-1*, but retained the chlorophyll-free phenotype¹⁸. Additionally, *chlD-1/GUN4* demonstrated higher expression of *GPX5* and higher tolerance to $^1\text{O}_2$ than *chlD-1*¹⁸. Thus, to minimize the GUN4-deficient phenotype and to maintain high and stable $^1\text{O}_2$ -inducibility of *GPX5* expression, *chlD-1/GUN4* was used as a background strain instead of *chlD-1* in the present study to monitor $^1\text{O}_2$ -signalling. For this purpose, expression of the *GLUTATHIONE PEROXIDASE 5* (*GPX5*) gene, which is specifically induced by $^1\text{O}_2$ in *C. reinhardtii*^{19,20} was used as a marker gene. The $^1\text{O}_2$ -inducible promoter region of *GPX5* fused to the promoterless *ARYLSULFATASE 2* gene (*ARS2*) was shown to be an effective reporter to study *GPX5* expression^{14,20,21}.

Mutagenesis of the strain carrying the *GPX5-ARS2* reporter (in a *chlD-1/GUN4* mutant background), followed by a screening for decreased reporter gene expression identified a mutant impaired in $^1\text{O}_2$ -signalling. The lack of the retrograde response to $^1\text{O}_2$ in this mutant was verified to decrease expression of *GPX5*, as well as *SAK1*, *MBS*, and *PSBP2*. The causal mutation was found in a gene annotated as *TREHALOSE 6-PHOSPHATE PHOSPHATASE* (hereafter *T6PPI*). Besides clear impairment in $^1\text{O}_2$ -signalling, mutation in *T6PPI* also resulted in accumulation of trehalose 6-phosphate (Tre6P). Therefore, *T6PPI* is the first confirmed phosphatase acting on Tre6P in *C. reinhardtii* and in fact the first enzyme with a confirmed function in trehalose metabolism in this organism. Our data indicate that accumulation of Tre6P causes changes to the expression of genes involved in several metabolic pathways. However, Tre6P is an intermediate rather than the primary cause of the

impaired $^1\text{O}_2$ -signalling. Here using a combination of genetics, gene expression and metabolic phenotypes, we reveal a complex interaction between chloroplast, mitochondria and cytosol in $^1\text{O}_2$ signal transmission to the nucleus.

Results

Forward genetics screen to identify components of the $^1\text{O}_2$ -signalling

To generate a mutant impaired in $^1\text{O}_2$ -dependent signalling, we first created a reporter strain in a known $^1\text{O}_2$ -generating mutant, which was subsequently subjected to mutagenesis. The *GPX5-ARS2* reporter construct (Supplementary Fig. 2) was introduced into the genome of *chlD-1/GUN4* to express the ARS2 protein in a $^1\text{O}_2$ -dependent manner, and the transformants were tested for the enzymatic activity of ARS2. The reporter strain, which showed the lowest ARS2 activity in darkness and the highest activity in light was named *signalling Reporter* (*sigRep*; Fig. 1a) and was used in further applications. Expression kinetics of the cytosolic (*GPX5_{cyt}*) and chloroplastic (*GPX5_{cp}*) version of *GPX5*²²; Supplementary Fig. 2), as well as the *GPX5-ARS2* construct were examined by quantitative Real-Time PCR (qRT-PCR) upon transfer from dark to light. As expected, higher induction of *GPX5_{cp}* and *GPX5_{cyt}* were observed upon light illumination in *sigRep* compared to WT (Fig. 1b). Induced *GPX5-ARS2* expression in *sigRep* in the light (Fig. 1c) confirmed the results obtained in the ARS activity assay (Fig. 1a).

Subsequently, a random mutagenesis was performed on *sigRep* using bleomycin resistance cassette (*ble^R*) as an insert, followed by screening to isolate strains with lower or undetectable ARS2 activity in the light compared to *sigRep*, and thus, possibly disrupted $^1\text{O}_2$ -dependent signalling. Screen of 804 transformants allowed us to isolate nine, which were named *genomes uncoupled Singlet Oxygen Signalling* (*gunSOS*). The *gunSOS1* mutant (Fig. 1d) was selected for further analysis. Higher sensitivity to light was recorded in *gunSOS1* compared to the *sigRep* background strain (Fig. 1e), in agreement with a higher sensitivity or impaired acclimation to $^1\text{O}_2$. Mutant impaired in $^1\text{O}_2$ -signalling showed similar Proto accumulation in light compared to *sigRep* and *chlD-1/GUN4* (Supplementary Fig. 3), which eliminated the possibility that spurious mutations in *gunSOS1* resulted in reduced content of this photosensitizer.

The *ble^R* insertion in *gunSOS1* was located in the first exon of the gene annotated as *TREHALOSE 6-PHOSPHATE PHOSPHATASE 1* (here *T6PPI*; locus Cre12.g497750) in the JGI portal (Department of Energy Joint Genome Institute, <https://phytozome-next.jgi.doe.gov>, v13, genome v5.6; Fig. 1f). The *T6PPI* transcript abundance was

determined in *gunSOS1* and compared to *sigRep* by qRT-PCR using primers annealing to the coding sequence upstream of the insertion site. At 2 h after transfer from dark to light, *T6PP1* mRNA content increased 2.5-fold in *sigRep* compared to WT, while it was virtually absent in *gunSOS1* (Fig. 1g). Expression of *GPX5_{cyt}* and *GPX5_{cp}* was nearly 10 and 8 times lower in *gunSOS1* compared to *sigRep*, respectively (Fig. 1h). The transcript abundance for *GPX5-ARS2* was 7 times lower in *gunSOS1* than in *sigRep* (Fig. 1i), and explains undetectable ARS2 activity in the $^1\text{O}_2$ -signalling mutant (Fig. 1d).

***C. reinhardtii* T6PP1 is a functional Tre6P phosphatase**

While *gunSOS1* is clearly impaired in $^1\text{O}_2$ -signalling, it was necessary to determine the primary phenotype caused by the mutation in *T6PP1*. The content of Tre6P in *sigRep* and *gunSOS1* was determined by anion-exchange high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Tre6P accumulation increased in *gunSOS1* upon exposure to light in a time-dependent manner, while it decreased in *sigRep* (Fig. 2a). Accumulation of Tre6P in *gunSOS1* indicates that *C. reinhardtii* T6PP1 is a functional phosphatase dephosphorylating Tre6P to trehalose. Subsequently, *T6PP1* expression was found to be induced in the light in *sigRep*, but not in the WT, indicating its inducibility by photooxidative stress rather than light (Fig. 2b). Mutation of the *T6PP1* gene would be expected to lower Tre6P phosphatase activity and dephosphorylation of Tre6P to trehalose, but we also observed accumulation of trehalose in *gunSOS1* (Fig. 2c). This apparent discrepancy can be explained by the existence of another enzyme in *C. reinhardtii* with possible Tre6P phosphatase activity, in combination with higher levels of Tre6P (see Discussion section).

Rescue of the T6PP1 deficiency in *gunSOS1* was performed with the isolated genomic DNA fragment carrying the wild-type *T6PP1* gene. Several independent transformants showed rescued $^1\text{O}_2$ -signalling, which was indicated by the *GPX5-ARS2* expression determined in the ARS assay, and data from three representative strains are shown in Supplementary Fig. 4a. The analysed rescued strains also showed increased *GPX5_{cp}* transcript content compared to *gunSOS1* (Supplementary Fig. 4b). A peptide-specific antibody for T6PP1 was produced to compare the protein content in *gunSOS1* to strains showing $^1\text{O}_2$ -dependent signalling. A faint immune signal of approximately 43 kDa was detected in *gunSOS1* and *chlD-1*, but two narrow bands were seen in *chlD-1/GUN4*, *sigRep* and the *T6PP1*-rescued strains (Fig. 2d). WT and *gunSOS1* showed an additional unspecific immunoreaction with protein of approximately 53 kDa. As expected, GPX5 content was

lower in *gunSOS1* compared to *sigRep* (Fig. 2d), which correlated with qRT-PCR results on *GPX5* transcript levels (Fig. 1 h). Strains with rescued $^1\text{O}_2$ -signalling, *T6PPI-R1*, *-R2*, and *-R3*, showed increased content of GPX5 (Fig. 2d) and higher tolerance to light compared to *gunSOS1* (Fig. 2e).

The *gunSOS1* mutant shows altered metabolism compared to *sigRep*

In *A. thaliana* and other angiosperms, Tre6P functions as a sucrose signal and homeostatic regulator of sucrose metabolism^{23,24}, which links plant growth and development to the availability of sucrose²⁵, reviewed in²⁶. To determine the effect of accumulated Tre6P on metabolism in *C. reinhardtii*, we performed comparative metabolite profiling of *gunSOS1* and *sigRep* (Supplementary Table 1), which revealed a relatively wide range of variation. Aside from Tre6P and trehalose, 11 out of 27 analysed metabolites showed significantly increased content in *gunSOS1* compared to *sigRep* in the light (Figure 3a). By means of the MetaboAnalyst 5.0 portal (<https://www.metaboanalyst.ca>) these metabolites were assigned to the metabolic processes in the cell. A hypergeometric test indicated the most affected metabolic pathways, i.e. the TCA cycle, pyruvate metabolism, and starch and sucrose metabolism (Fig. 3b, Supplementary Table 2). Eight metabolites showed significantly decreased content in *gunSOS1* compared to *sigRep* (Fig. 4a), with the highest impact (MetaboAnalyst 5.0) on fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, and starch and sucrose metabolism (Fig. 4b, Supplementary Table 3). Metabolites, which did not show a significant change in *gunSOS1* compared to *sigRep* are presented in Supplementary Fig. 5.

Starch content was determined in *gunSOS1* and compared to *sigRep*, because of changes in several intermediates of starch and sucrose metabolism (Fig. 3b and Fig. 4b). Indeed, *gunSOS1* accumulated only 60% of the starch content of *sigRep* when the strains were grown in acetate-supplemented media (TAP, Fig. 4c). Both *sigRep* and *gunSOS1* are obligate heterotrophs. *SigRep* contained less starch after transfer from TAP and cultivation in TP for 48 h, whereas the starch content of *gunSOS1* was essentially unchanged (Fig. 4c), indicating that there was net degradation of starch in *sigRep* but not in *gunSOS1*. Starch formation is strongly induced during nitrogen (N) deprivation²⁷. The *sigRep* cells did not increase starch reserves after 3 days in N-deficient medium, suggesting that starch accumulation had already reached its maximum under non-stress conditions. The starch content in *gunSOS1* deprived of N did increase by 50% and was similar to the content in *sigRep* (Fig. 4c). We conclude that *sigRep* accumulates high levels of starch as a

consequence of the *chLD-1/GUN4* genetic background and that the accumulation of Tre6P represses this response in *gunSOS1* leading to a lower accumulation of starch. The *gunSOS1* cells are still capable of responding to the N-deprivation stress and accumulating starch despite disturbed metabolism. Starch degradation on the other hand, is compromised in *gunSOS1*, which could indicate a general impairment of catabolic processes or a defect in sensing carbon limitation. The map-overview of the selected metabolic pathways with depicted metabolites that showed different content, or were not changed, in *gunSOS1* relative to *sigRep* is presented in Figure 5.

Increased Tre6P content alters metabolism in *C. reinhardtii* via alteration of the transcriptome

To determine the extent to which chloroplast retrograde signalling is affected in *gunSOS1*, we performed comparative RNA-sequencing (RNA-seq) of the transcriptomes of *gunSOS1* and *sigRep* upon transfer from dark to light. The analysis showed that 8120 genes were expressed in both strains, but 1377 and 1087 additional genes were uniquely expressed in *gunSOS1* and *sigRep*, respectively (Fig. 6a, detailed listing can be found in Supplementary Data Set 1). Furthermore, differential gene expression analysis showed that the transcript contents of 1606 genes and 1706 genes were lower or higher, respectively, in *gunSOS1* compared to *sigRep* (Supplementary Data Set 2).

Based on the KEGG ontology (<http://www.kegg.jp/>) several genes associated with metabolic pathways were down- (Fig. 6b) or up-regulated (Fig. 6c) in *gunSOS1* compared to *sigRep* (Supplementary Data Set 3). Given the strong overlap between differentially expressed genes and corresponding pathway intermediates, we hypothesized that the altered metabolism can be directly explained by altered gene expression. To decipher a complex correlation between altered signalling, gene expression and metabolism, accumulation or deficiency in metabolites from given pathway(s) was correlated with altered gene expression in *gunSOS1* compared to *sigRep*. Fumarate and aconitate will be described in more detail, as representative examples of the metabolites showing respectively increased or decreased content in *gunSOS1* compared to *sigRep*.

KEGG enrichment of the RNA-seq data indicated increased expression of the gene encoding FUMARATE HYDRATASE CLASS II (FUM2, Enzyme Commission (EC) Number 4.2.1.2, Cre01.g020223) in *gunSOS1* compared to *sigRep*. FUM is responsible for reversible stereospecific interconversion of malate to fumarate. However, specific isoforms of this protein act in defined pathways and may favour one direction over the other depending

on the subcellular localisation and environment²⁸. The mitochondrial isoform catalyses hydration of fumarate to L-malate in the TCA cycle, while the cytosolic form catalyses dehydration of L-malate to fumarate^{29,30}. Two isoforms exist in *C. reinhardtii*, FUM1 encoded in locus Cre06.g254400 and FUM2. Based on the predicted subcellular localisation (PredAlgo; <http://lobosphaera.ibpc.fr/cgi-bin/predalgotdb2.perl?page=main>), FUM1 localizes to the mitochondria, while FUM2 is assigned to the “other” compartment and is likely to be a cytosolic protein. Pathway (KEGG) diagrams for the TCA cycle and pyruvate metabolism can be found in Supplementary Fig. 6, showing integration of metabolite, RNA-seq and qRT-PCR data. Comparison of the transcriptomes of *gunSOS1* and *sigRep* also indicated increased expression of the *FUMARYLACETOACETASE* gene (locus *Cre17.g732802*, EC 3.7.1.2) involved in tyrosine catabolism. FUMARYLACETOACETASE catalyses hydrolysis of 4-fumarylacetoacetate to acetoacetate and fumarate (Supplementary Fig. 7). Increased expression of genes encoding FUM2 and FUMARYLACETOACETASE could explain accumulation of fumarate in *gunSOS1* compared to *sigRep*.

Aconitate deficiency in *gunSOS1* compared to *sigRep* can be explained by transcriptional downregulation of *ACONITATE HYDRATASE (ACH1)*, Cre01.g042750, EC 4.2.1.3). ACH1 interconverts citrate and isocitrate, via cis-aconitate, in the TCA cycle (Supplementary Fig. 8) and is also involved in glyoxylate and dicarboxylate metabolism. At equilibrium, the reactants of the ACH1 reaction are present in the following ratio: 91% citrate, 6% isocitrate and 3% aconitate. With the smallest pool of the three tricarboxylic acids, fluctuations in the level of aconitate are expected to be more pronounced compared to the other metabolites involved, especially citrate, whose content was similar in *gunSOS1* and *sigRep* in the light (Supplementary Fig. 5). Taken together, our data indicate that altered fumarate and aconitate content in *gunSOS1* compared to *sigRep* can be explained by altered expression of genes encoding enzymes involved in key metabolic processes affecting those metabolites.

Impaired ¹O₂-signalling in *gunSOS1* correlates with decreased expression of *PSBP2*, *MBS*, and *SAK1*

The *PSBP2*¹⁴, *MBS*¹⁶, and *SAK1*¹⁵ proteins are required for ¹O₂-induced chloroplast retrograde signalling in *C. reinhardtii*. Therefore, we determined the transcript levels of *PSBP2*, *MBS*, and *SAK1*, which were 8-, 9-, and nearly 41-fold lower, respectively, in *gunSOS1* compared to *sigRep* (Fig. 7a). Decreased expression of *SAK1* in *gunSOS1* prompted us to compare transcript levels of selected ¹O₂-responsive genes between *gunSOS1* and *sak1*,

which lacks acclimation response to $^1\text{O}_2$ ¹⁵. SAK1 is a key regulator of the gene expression response and its involvement in $^1\text{O}_2$ -signalling was determined following treatment with rose bengal¹⁵, which produces $^1\text{O}_2$ in the light. Nevertheless, based on our qRT-PCR analysis, all tested genes had the same reduced inducibility in *gunSOS1* compared to *sigRep* (Fig. 7b), as it was observed in *sak1* compared to WT following $^1\text{O}_2$ -exposure¹⁵. Among the genes that were down-regulated in both *gunSOS1* (Fig. 7b) and *sak1*¹⁵ were two *CYCLOPROPANE FATTY ACID SYNTHASES*, *CFA1* and *CFA2* (*CPLD27*), involved in lipid and sterol metabolism, as well as the gene encoding SOUL1 heme-binding protein (Fig. 7b). It is noteworthy that attenuated expression of both *CFA1* and *CFA2*, as well as *SOUL1*, was also observed in studies on a *gpx5* mutant³¹. Furthermore, in agreement with the *sak1* phenotype presented in Wakao, et al.¹⁵, *gunSOS1* also showed decreases in transcript content of *PEPTIDE METHIONINE SULFOXIDE REDUCTASE* (*MSRA3*), *ALCOHOL DEHYDROGENASE* (*ADH7*), *RETINALDEHYDE BINDING PROTEIN-RELATED* (*RABPR2*), and Cre01.g007300 encoding N-ACETYLPHOSPHATIDYLETHANOLAMINE-HYDROLYZING PHOSPHOLIPASE D (Fig. 7b). Thus, there is an overlap between the phenotypes of *gunSOS1* and *sak1*¹⁵ with respect to their attenuated expression of genes induced during elevated $^1\text{O}_2$ (Fig. 7b).

Altered metabolite content affects $^1\text{O}_2$ -signalling

We hypothesized that at least some of the metabolites showing increased or decreased content in *gunSOS1* compared to *sigRep*, attenuate or propagate $^1\text{O}_2$ -signalling, respectively. We selected fumarate and aconitate as representatives of either group. To test our hypothesis, metabolites were added separately to the liquid cultures at empirically determined sublethal concentrations, followed by incubation in the light to induce $^1\text{O}_2$ generation by Proto. Subsequently, the *GPX5_{cyt}* and *GPX5_{cp}* transcript levels were used as readouts for the $^1\text{O}_2$ signalling efficiency in *gunSOS1* and *sigRep*.

Exogenous application of fumarate significantly ($P < 0.001$, a detailed report from two-way ANOVA analyses is presented in Supplementary Tables 4 and 5) attenuated expression of both *GPX5_{cyt}* and *GPX5_{cp}* in a concentration-dependent manner in *sigRep* (Fig. 8a). As expected, fumarate did not affect *GPX5* expression in *gunSOS1*. Application of 20 μM aconitate significantly ($P < 0.0001$) increased expression of *GPX5* in *gunSOS1* and even further in *sigRep* compared to untreated cells ($P < 0.0001$), but 100 μM had a quenching effect on the *GPX5* expression in *sigRep* (Fig. 8b, Supplementary Tables 6 and 7). This indicates that aconitate up to a certain threshold concentration promotes $^1\text{O}_2$ signalling.

Subsequently, we determined the expression of *SAK1* upon feeding *gunSOS1* and *sigRep* with aconitate. Exogenous application of aconitate at 20 μ M increased *SAK1* transcript abundance in *gunSOS1* more than 120 times compared to the untreated *gunSOS1* control, and exceeded values observed in *sigRep* subjected to the same treatment by a factor of 5 (Fig. 8c). Due to the increased *SAK1* expression in *gunSOS1* upon feeding with aconitate, we also determined the expression of *PSBP2* and *MBS* in the same conditions. The *PSBP2* response to aconitate was similar to *SAK1* in terms of the increased expression (Fig. 8c), but with less pronounced dependence on the metabolite concentration compared to *SAK1*. Although 20 μ M aconitate increased *PSBP2* expression in *gunSOS1* by a factor of 48 compared to the untreated control, it was still 2.7 times lower compared to *sigRep* subjected to the same treatment (Fig. 8c). Similarly to *SAK1* and *PSBP2*, 20 μ M aconitate also increased expression of *MBS* in *gunSOS1*, although values did not exceed those observed in *sigRep* subjected to the same treatment (Fig. 8c). Statistical analyses indicated that the effect of 20 μ M aconitate on *SAK1*, *PSBP2* and *MBS* expression in *sigRep* and *gunSOS1*, interaction between mutants and aconitate, as well as the mutant-dependent expression of these genes were always significant ($P < 0.001$, Supplementary Tables 8-10).

Specificity of the $^1\text{O}_2$ -signalling pathway(s) attenuated in *gunSOS1*

The profound effect of accumulating Tre6P on the transcriptome (Fig. 6a-c) and consequently the metabolism of *gunSOS1* (Fig. 3 and Fig. 4) may have been indicative of a general photooxidative stress response being impaired in *gunSOS1*. To determine the specificity of this response, we compared *gunSOS1* and *sigRep* in terms of the expression of selected genes that are known to be induced by various reactive species or conditions causing photooxidative stress other than $^1\text{O}_2$.

Upon H_2O_2 or organic *tert*-butyl hydroperoxide (*t*-BOOH) treatment, Blaby, et al.³² observed an increase in the expression of the *MSD3* gene, encoding plastid-localised Mn SUPEROXIDE DISMUTASE 3. In our studies, we did not observe an increase in *MSD3* transcript in *sigRep* compared to WT (Fig. 9a), which shows that *MSD3* expression is not inducible by $^1\text{O}_2$ produced by Proto. However, based on qRT-PCR analysis, a 9-fold increase was observed in *gunSOS1* compared to *sigRep* (Fig. 9a, see also RNA-seq in Supplementary Data Set 2). Upon H_2O_2 or *t*-BOOH treatment, Urzica, et al.³³ also observed induced expression of genes involved in the glutathione-ascorbate system, *GDP-L-GALACTOSE PHOSPHORYLASE* (*VTC2*) and *DEHYDROSASCORBATE REDUCTASE* (*DHARI*). Based on our qRT-PCR results, *VTC2* transcript content was not changed in *sigRep* compared to

WT, but an increase was observed in *gunSOS1* (Fig. 9b; Supplementary Data Set 2). *DHAR1* expression was also stimulated in *gunSOS1* compared to *sigRep* (Fig. 9c; Supplementary Data Set 2).

Similarly to *sak1* following treatment with rose bengal¹⁵, we observed increased expression of *GLUTATHIONE S-TRANSFERASE (GSTS1)* in *gunSOS1* compared to *sigRep* (Fig. 9d, Supplementary Data Set 2). However, in another study increased expression of *GSTS1* was shown after treatment with acrolein, which suggests its transcriptional induction by reactive electrophile species (RES)³⁴. Acrolein was also shown to induce *FSD1* encoding Fe SUPEROXIDE DISMUTASE (FeSOD³⁴, which was expressed both in *sigRep* and *gunSOS1* (Supplementary Data Set 1), and no significant difference could be observed in DEG analysis (Supplementary Data Set 2) or qRT-PCR (Fig. 9e). It can be concluded that, despite impaired ¹O₂-signalling, *gunSOS1* retained the ability to respond to photooxidative stress caused by other ROS such as H₂O₂, organic peroxides, or RES.

Discussion

ROS are formed as a by-product of biological redox reactions³⁵ mostly in the mitochondria or chloroplasts^{36,37}. Although excess ROS production can cause oxidative damage to cell components, ROS or the oxidation products play an important role in the signal transduction processes.

Different retrograde signalling pathways have been proposed to involve also TBS-intermediates in plants and green algae (reviewed in^{38,39}). While involvement of Mg-porphyrins in chloroplast retrograde signalling is now excluded^{40,41}, in the present study we have demonstrated that ¹O₂ produced by the photosensitizing activity of Proto in the light, but not Proto itself, triggers signalling cascades that alter nuclear gene expression. Using strains that endogenously accumulate Proto, we identified several mutants that are impaired in ¹O₂-signalling. Endogenous accumulation of Proto in mutants is advantageous for studying ¹O₂-signalling, because it eliminates the need for exogenous application of TBS or carotenoid biosynthesis inhibitors, as well as photosensitizers, such as rose bengal or neutral red (Fischer et al., 2004) to induce ¹O₂ generation. The lack of chlorophyll provides another advantage, because such mutants do not have functional photosynthetic electron transport, so that ¹O₂ production in photosynthesis is avoided. The accumulating Proto is thus the dominant source of generated ¹O₂.

The ¹O₂-induced signalling phenotype in the *gunSOS1* mutant was due to a lesion in the *T6PP1* gene. We demonstrated that T6PP1 is a functional phosphatase capable of

dephosphorylating Tre6P. *T6PP1* is the only representative of the classical plant *TPP* gene family in *C. reinhardtii*, contrasting with the large *TPP* gene families in angiosperms⁴². In addition, *C. reinhardtii* has one representative of the class I *TREHALOSE-6-PHOSPHATE SYNTHASE* (*TPS*) family (Cre16.g662350, hereafter *T6PS1*), and two members of the class II *TPS* family (Cre06.g278221, here *T6PSP1* and Cre16.g686200, here *T6PSP2*). Both class I and class II *TPS* proteins have glucosyltransferase and *TPP*-like domains, but only the class I *TPS* proteins have demonstrated *TPS* activity⁴³⁻⁴⁵. *T6PP1* belongs to the haloacid dehalogenase superfamily of proteins and contains the characteristic DXDX(T/V) active site motif – ₁₀₇DYDGT₁₁₂ – in which the initial Asp residue forms a phospho-acyl intermediate during catalysis⁴⁶. *T6PSP1* also contains the complete active site motif (₅₉₂DYDGT₅₉₇), so we cannot exclude the possibility that the *T6PSP1* protein in *C. reinhardtii* has *TPP* activity, which could explain the increased content of trehalose in *gunSOS1* compared to *sigRep*. Although the intracellular localizations of these proteins have not been determined experimentally in *C. reinhardtii*, PredAlgo analysis indicated possible localization of *T6PP1* and *T6PSP1* in mitochondria, while *T6PS1* and *T6PSP2* are likely to be cytosolic proteins, because they were not assigned to any specific organelle.

Correlation between *T6PP1* (Fig. 2b) and *GPX5* (Fig. 1b, c) expression indicates that low Tre6P content is required for efficient ¹O₂-signalling. However, the apparent negative effect of Tre6P on ¹O₂-signalling is not direct and involves a complex metabolic reprogramming (Fig. 10). Study on the contrasting phenotypes between *A. thaliana* overexpressing bacterial *TPS*, *TPP*, or *TREHALOSE PHOSPHATE HYDROLASE* (*TPH*) pointed to Tre6P, rather than trehalose, playing a signalling function²⁵. Tre6P was shown to be highly correlated with sucrose, leading to the proposal that it functions as a signal of sucrose status²³. Tre6P was also shown to inhibit starch degradation in *A. thaliana*^{47,48}, which is also true in Tre6P-accumulating *gunSOS1* mutant of *C. reinhardtii*.

It was demonstrated that Tre6P acts as an inhibitor of SUCROSE-NON-FERMENTING (SNF)-RELATED PROTEIN KINASE1 (SnRK1) in developing tissues and that this is dependent on a so-far unidentified protein factor⁴⁹⁻⁵¹. Tre6P also inhibits the activation of SnRK1 by SnRK1-activating kinases/geminivirus Rep interacting kinases⁵². SnRK1 belongs to the AMPK-SNF1-SnRK family of protein kinases, which is represented in all eukaryotes⁵³. In plants, SnRK1 plays a central role in energy and metabolic homeostasis, and is activated during energy deficient conditions caused by stresses like nutrient starvation, pathogen attack, or ROS⁵⁴. Baena-González, et al.⁵⁵ established approximately 1000 genes as markers of SnRK1 in *A. thaliana*, which indicates an extensive SnRK1-dependent

transcriptional reprogramming. In *C. reinhardtii*, involvement of various SnRKs in responses to stress was observed during sulphur^{56,57} and nitrogen deprivation⁵⁸, or cold stress⁵⁹. Genome-wide analysis revealed the existence of 21 genes as potential orthologues of the plant SnRK α , β and $\gamma/\beta\gamma$ subunits in *C. reinhardtii*. It was suggested that the proteins encoded by these genes play the same role in cell survival and stress response in *C. reinhardtii* as SnRKs in land plants⁵⁸.

In analogy to *A. thaliana*, altered SnRKs activity was shown to cause metabolic remodelling also in algae⁵⁹. In our study, the majority of changes to gene expression observed in *gunSOS1*, relative to *sigRep*, originates from accumulation of Tre6P and, based on previous studies, this leads to inhibition of one or more SnRKs (Fig. 10). Our analysis showed that altered gene expression in *gunSOS1* relative to *sigRep* (Supplementary Fig. 6-8) is directly responsible for altered fumarate and aconitate content in *gunSOS1* compared to *sigRep*. Thus, Tre6P accumulating in *gunSOS1* is not directly involved in ¹O₂-signalling but controls other processes in the cell which affect ¹O₂-signalling more directly (Fig. 10).

We demonstrated that accumulation of fumarate is responsible for attenuating ¹O₂-signalling (Fig. 8a, Fig. 10). Fumarate is a well-recognized oncometabolite in mammalian cells⁶⁰, associated with development of tumours by competitive inhibition of 2-oxoglutarate-dependent oxygenases, including HYPOXIA INDUCIBLE FACTOR (HIF) hydroxylases, leading to stabilisation of HIF and activation of oncogenic HIF-dependent pathways⁶¹. In fact, accumulation of fumarate in human cells was linked to an aggressive variant of hereditary kidney cancer⁶². In mice, fumarate was also shown to directly modify some proteins by succination of cysteine residues to form 2-succinocysteine derivatives⁶³. Succination of three cysteines crucial for iron-sulphur cluster binding was identified in mitochondrial ACONITASE2 (ACO2) in a *fumarate hydratase 1* knockout (Fh1KO) mouse embryonic fibroblast (MEF) cell line⁶³. Analysis of tryptic peptides derived from ACO2 in Fh1KO MEFs, indicated succination of Cys385. Another tryptic peptide of ACO2 was identified as a mixture of two isomers in Fh1KO MEFs, which showed succination at Cys451 or Cys448⁶³. Succination of these cysteine residues in ACO2 leads to its inhibition⁶³. Equivalent cysteine residues are conserved in *C. reinhardtii* ACH1, which explains the aconitate deficiency in *gunSOS1* (Fig. 4a).

The *gunSOS1* mutant showed decreased levels of aconitate compared to *sigRep*, while exogenous application of this metabolite to *gunSOS1* restored ¹O₂-signalling. Mammalian ACO2 is thought to be a mitochondrial oxidative stress sensor, which requires an active [Fe₄S₄]²⁺ cluster⁶⁴. It is, therefore, proposed that accumulation of fumarate deactivates ACH1

and reduces the aconitate content in *gunSOS1*, thereby impairing oxidative stress sensing and $^1\text{O}_2$ -signalling. Diminished expression of *ACHI* in *gunSOS1* relative to *sigRep* (Supplementary Fig. 8a, b) may be an additional factor adding to the aconitate depletion in *gunSOS1*.

Results obtained in our study indicate that there is a metabolic signature conditioning $^1\text{O}_2$ -signalling. A mechanism in which the chloroplast controls nuclear gene expression via metabolite levels was postulated before^{12,65}. It was shown that altered chloroplast redox conditions result in changes in the metabolome in *A. thaliana*, reflected also by the reallocation of energy resources⁶⁶. Importantly, although our study shows that the metabolic configuration of the cell is essential for $^1\text{O}_2$ -signalling, this does not exclude the involvement of protein components in this signalling network (Fig. 10). On the contrary, our findings support the direct involvement of proteins in $^1\text{O}_2$ -signalling, but we demonstrated that expression of *PSBP2*¹⁴, *MBS*¹⁶, and *SAK1*¹⁵ depends on the metabolic status (Fig. 7a and Fig. 8a, b). The overlap between the *gunSOS1* and the SAK1-dependent transcriptomes (Fig. 7b) as well as other defined ROS or RES-dependent signalling pathways being functional (Fig. 9a-e) indicate specificity of the impaired $^1\text{O}_2$ -signalling in *gunSOS1*. Our results indicate that fumarate and aconitate affect the expression of *PSBP*, *MBS*, or *SAK1*, which subsequently convey information about the metabolic status of the cell to the nucleus and trigger specific responses (Fig. 10). It is not clear yet how the specificity of the signalling is achieved, because the mechanisms by which metabolites affect the expression of these proteins, as well as downstream components of these signalling pathways, remain unknown. Additionally, the protein components involved in $^1\text{O}_2$ -signalling may be subject to additional regulatory mechanisms, such as phosphorylation, as proposed for SAK1 by Wakao, et al.¹⁵.

We have shown that retrograde signalling triggered by $^1\text{O}_2$, which is mostly generated in the chloroplast, strictly depends on the mitochondrial metabolic status. PROLYL-tRNA SYNTHETASE (PRORS1) in *A. thaliana*, a component of the organellar gene expression machinery indicated that mitochondria may contribute to chloroplast retrograde signalling⁶⁷. PRORS1 is targeted both to the plastid and to mitochondria, but downregulation of specific photosynthesis-associated nuclear genes was observed only in a plastidial *prpl1* and mitochondrial *mrpl1* double mutant, but not in *prpl1* or *mrpl1* single mutants⁶⁷. Another study showed that the ROS-dependent signals from chloroplast and mitochondria are integrated at the RADICAL-INDUCED CELL DEATH 1 (RCD1) protein located in the nucleus⁶⁸. RCD1 suppresses transcription factors ANAC013 and ANAC017, which mediate the ROS-signal from mitochondria, while chloroplast regulation of RCD1 takes place through

3'-phosphoadenosine 5'-phosphate. It was proposed that RCD1 may function at the intersection of mitochondrial and chloroplastic retrograde signalling pathways⁶⁸.

The retrograde signalling pathways identified so far assume the existence of separate signalling routes. However, chloroplast retrograde signalling involves a network of different but inter-connected mechanisms, and observation of specific signalling pathways within the network depends on the experimental conditions. Pharmacological or genetic interventions that disrupt specific pathways can be used to characterize alternative pathways. In the manuscript presented here, we report that the metabolic status of the cell, reflecting levels of Tre6P, and components of the mitochondrial TCA cycle are integral factors in the ¹O₂-dependent retrograde signalling network in *C. reinhardtii*.

Methods

Chlamydomonas reinhardtii cultures and genetic manipulations

All strains were cultivated heterotrophically in Tris-acetate-phosphate (TAP) medium in the dark. Additionally, starch measurements were performed in TAP, Tris-phosphate (TP) medium without acetate, or nitrogen-depleted (TAP-N) medium. Experiments were performed in TAP medium in the dark or upon a shift to 20 μmol photons m⁻² s⁻¹ light. Three strains used in this study were described elsewhere, *chlD-1* in von Gromoff, et al.¹⁷, *chlD-1/GUN4* in Brzezowski, et al.¹⁸, and wild type (4A+) in Dent, et al.⁶⁹.

To generate the *GPX5-ARS2* construct (Supplementary Fig. 2), the *GPX5* regulatory region (*GPX5* 5' RR) was amplified by PCR using forward and reverse primers carrying XhoI and EcoRV restrictions sites, respectively (Supplementary Table 11). The obtained fragment was subcloned and ligated between the XhoI/EcoRV sites of the pSL18 vector⁷⁰, replacing the existing promoter region of the gene encoding the PSAD and positioning *GPX5* 5'RR in reverse orientation with respect to the paromomycin resistance cassette (par^R,⁷¹). The DNA fragment carrying par^R and *GPX5* 5' RR was excised by restriction digestion with KpnI and EcoRV and ligated into the corresponding restriction sites of pJD54⁷², which carries a copy of the promoterless version of the gene encoding ARYLSULFATASE 2 (*ARS2*), which allowed *ARS2* expression to be controlled by the *GPX5* 5'RR (Supplementary Fig. 2). The *GPX5-ARS2* construct was verified by sequencing.

The *sigRep* strain was produced in the *chlD-1/GUN4* background by transformation with a *GPX5-ARS2* construct. Transformant selection was performed on TAP agar plates with 10 μg mL⁻¹ paromomycin in dark, followed by a screen for *GPX5-ARS2* inducibility by ¹O₂. The *sigRep* strain showed low *ARS2* activity in the dark and high activity in the light (Fig.

1a) and was selected for further applications. The *gunSOS1* mutant was generated by random insertional mutagenesis performed on *sigRep* using a bleomycin resistance cassette (ble^R) isolated from the pMS188 vector⁷³ using NotI and KpnI. Following mutagenesis, selection was performed on TAP agar plates containing $15 \mu\text{g mL}^{-1}$ zeocin in the dark. More than 800 obtained colonies were screened for decreased or not detectable ARS2 activity in the light and nine mutants that showed impaired inducibility of *GPX5-ARS* expression in light were selected for further analysis. Restriction enzyme site-directed amplification PCR⁷⁴ allowed us to identify the genomic DNA flanking the ble^R insertion sites in five out of the nine mutants. Only analysis of the *gunSOS1* mutant is presented here. Rescue of *gunSOS1* (*t6pp1*) was conducted with 5242 bp fragment of genomic DNA carrying 3241 bp *T6PP1* amplified by PCR (see Supplementary Table 11 for primers) using bacterial artificial chromosome PTQ5987 (Clemson University Genomics Institute, Clemson, SC, USA) as a template. The amplified DNA fragment included 1500 bp upstream and 501 bp downstream of the annotated *T6PP1*. Wild-type *T6PP1* was introduced into *gunSOS1* by co-transformation with a spectinomycin resistance cassette isolated from the pALM32 vector⁷⁵ with *AleI* and *KpnI* endonucleases. Transformant selection was performed on TAP agar plates supplemented with $100 \mu\text{g mL}^{-1}$ spectinomycin in the dark. All genetic transformations were performed by electroporation.

Arylsulfatase activity assay

To assess the level of *GPX5-ARS2* expression in transformed cells, enzymatic assays for arylsulfatase activity⁷⁶ were performed essentially as described before¹⁴. Cells were spotted onto agar-solidified TAP medium plates and cultured in either light or dark conditions. Arylsulfatase is expected to be secreted into the medium if *GPX5-ARS2* is expressed. After removal of the cells, plates were flooded with detection solution containing 0.1 mg mL^{-1} 2-naphthyl sulfate (potassium salt; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) as a chromogenic substrate coupled with 1 mg mL^{-1} tetrazotized-o-dianisidine chloride (Fast Blue B salt, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following 1 h incubation, purple spots appearing on the agar plates identified expressed ARS2.

Analysis of protoporphyrin IX content

The Proto content was analysed by High Pressure Liquid Chromatography (HPLC) essentially as described in Czarnecki, et al. ⁷⁷, with modified sample preparation for *C. reinhardtii*¹⁸. In short, cultures were grown in the dark and transferred to $20 \mu\text{mol photons m}^{-2}$

2 s^{-1} light for 2 h. Samples containing 1.2×10^8 cells were centrifuged at $3000 \times g$ for 5 min at 4°C and the pellets were snap-frozen in liquid N_2 . Proto was extracted in cold (-20°C) acetone/ $0.1 \text{ M NH}_4\text{OH}$ (9/1, v/v) in a three-step cycle of resuspension and centrifugation. Proto analysis was performed using a Nova-Pak C18 column (Waters, $3.9 \times 150 \text{ mm}$, $4 \mu\text{m}$, at 20°C). The results were normalized to $\text{pmol}/10^6$ cells.

RNA isolation, qRT-PCR, and RNA-seq

The total RNA was isolated, after a shift from dark to $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light for 2 h, using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. RNA quality was assessed by electrophoresis on a 1% (w/v) agarose gel, while quantity was determined using a Nanodrop 2000 (Thermo Fischer Scientific, Waltham, MA). Aliquots of $2 \mu\text{g}$ RNA were treated with DNase and RiboLock RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA) and used to synthesize cDNA with RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and oligo(dT)₁₈ primer. Transcript analysis by qRT-PCR were performed using $2\times$ ChamQ Universal SYBR qPCR Master Mix (Viazyme Biotech Co., Ltd., Nanjing, China) and a CFX96-C1000 96-well plate thermocycler (Bio-Rad, Hercules, CA, USA), the 18S rRNA was used as a reference gene²². All primers were designed using Primer3Plus (<http://primer3plus.com>), and are listed in Supplementary Table 11.

RNA-sequencing was performed on total RNA samples isolated from biological triplicates of *sigRep* and *gunSOS1* after the shift from dark to $20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light for 2 h. Library preparation, sequencing, and analysis services were commercially provided by Novogene Europe (Novogene (UK) Company Ltd., Cambridge, UK) and performed using an Illumina NovaSeq 6000 platform operated in 150 bp pair end sequencing mode, with a sequencing depth of 20 million reads per sample. Six gigabases of sequencing data per library was filtered for high-quality reads, which were mapped to the *C. reinhardtii* v5.5 (Department of Energy JGI). Estimated expression was obtained in fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM). Biological replicates were averaged to obtain sample expression estimate. Differential expression analysis between two mutants (three biological replicates per mutant) was performed using DESeq2.v1.20.0, which provides statistical routines for determining differential expression using a model based on the negative binomial distribution. The resulting *p* values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with a $\log_2\text{FoldChange} \geq 2$ and adjusted *p* value (*padj*) < 0.05 found by DESeq2.v1.20.0 were

defined as differentially expressed (DEGs). Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg/pathway.html) pathway analysis were conducted to identify DEGs at the biologically functional level using clusterProfiler R package. GO terms and KEGG pathways with a $\text{padj} < 0.05$ were considered to be significantly enriched.

Metabolite analysis

Cultures were grown in TAP in the dark until they reached mid-log phase of 3×10^6 cells mL^{-1} . Samples were always normalized to contain 6×10^7 cells, all centrifugation steps were carried at $3000 \times g$ at 4°C for 5 min, followed by snap-freezing in liquid N_2 . Samples from dark conditions were collected immediately, while the remainder of each culture was transferred to $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ light. Subsequent sampling took place at 15-min intervals, up to 1 h. Metabolites were extracted using chloroform-methanol as described by Lunn et al. (2006). Tre6P, other phosphorylated intermediates and organic acids were measured by anion-exchange HPLC coupled to tandem mass spectrometry (LC-MS/MS) as described in Lunn et al. (2006) with modifications as described in⁷⁸. Sugars and sugar alcohols were measured by LC-MS/MS as described in⁷⁹. Obtained results were normalized to $\text{pmol}/10^6$ cells.

For starch analysis, cultures were grown in TAP in the dark until they reached mid-log phase of 3×10^6 cells mL^{-1} . For starch determination cultures were either transferred in TAP to the light ($20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 2 h before harvesting, or centrifuged cells were resuspended in Tris-phosphate (TP) medium (without acetate), keeping the same cell concentration, and transferred back to the dark for 24 h. Subsequently, cultures were transferred to $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 2 h before harvesting. A similar protocol was applied for starch analysis in TAP devoid of N (TAP-N), except that cells were cultivated in TAP-N for 3 days in the dark, followed by exposure to $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 2 h. For each sampling, 5×10^6 cells were centrifuged and lyophilized. Starch content was determined by enzymatic degradation and glucose quantification following the protocol of DuBois, et al.⁸⁰.

Protein extraction and immunoblot analysis

Cultures were grown in the dark, followed by a transfer to $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 3 h. Cells were pelleted by centrifugation and total proteins were extracted in 400 μL buffer containing: 56 mM Na_2CO_3 , 56 mM DTT, 2% (w/v) SDS, 12% (w/v) sucrose and 2 mM

EDTA, pH 8.0. Proteins were quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and separated by SDS-PAGE on a 12% polyacrylamide gel, followed by transfer by electroblotting to nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). T6PP1 was detected using a rabbit crude antiserum (dilution 1:500) raised by inoculation with a T6PP1-specific peptide (VEWSKSDSNGWRAKPC) against *C. reinhardtii* T6PP1 (calculated MW 42 kDa). GPX5 was detected with a commercially available antibody (AS15 2882, dilution 1:1000) obtained from Agrisera (Vännäs, Sweden). CHLI1 antibody (PHY5510S, dilution 1:1000) was purchased from PhytoAB (San Jose, CA, USA). For application, all antibodies were diluted in CrossDown buffer (AppliChem, AppliChem GmbH, Darmstadt, Germany). The secondary antibody (AS09 602, goat anti-rabbit IgG, dilution 1:10,000) conjugated to horseradish peroxidase was obtained from Agrisera. The immunoblotting signals were detected using a CCD camera (Intas Biopharmaceuticals, Ahmedabad, India) after application of enhanced chemiluminescence detection kit (Clarity™ Western ECL Substrate; Bio-Rad, Hercules, CA, USA).

Chemical treatments of *C. reinhardtii* cells

Fumarate (sodium fumarate dibasic) and aconitate (*cis*-aconitic acid) were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), dissolved in H₂O to a stock solution of 100 mM and added individually to the mid-log phase (3 x 10⁶ cells mL⁻¹) cultures to final concentrations of 20, 50, and 100 μM, followed by exposure to light for 2 h.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

- Johanningmeier, U. & Howell, S. H. Regulation of light-harvesting chlorophyll-binding protein mRNA accumulation in *Chlamydomonas reinhardtii*. Possible involvement of chlorophyll synthesis precursors. *J. Biol. Chem.* **259**, 3541-3549 (1984).
- Oelmüller, R. Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. *Photochem. Photobiol.* **49**, 229-239, doi:10.1111/j.1751-1097.1989.tb04101.x (1989).
- Susek, R. E., Ausubel, F. M. & Chory, J. Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. *Cell* **74**, 787-799 (1993).

- 649 4 Oster, U., Brunner, H. & Rudiger, W. The greening process in cress seedlings .5. Possible
650 interference of chlorophyll precursors, accumulated after thujaplicin treatment, with light-
651 regulated expression of Lhc genes. *J. Photoch. Photobio. B* **36**, 255-261, doi:10.1016/s1011-
652 1344(96)07388-5 (1996).
- 653 5 Lydon, J. & Duke, S. O. Porphyrin synthesis is required for photobleaching activity of the
654 para-nitrosubstituted diphenyl ether herbicides. *Pestic. Biochem. Physiol.* **31**, 74-83,
655 doi:10.1016/0048-3575(88)90031-4 (1988).
- 656 6 Triantaphylides, C. *et al.* Singlet oxygen is the major reactive oxygen species involved in
657 photooxidative damage to plants. *Plant Physiol.* **148**, 960-968, doi:10.1104/pp.108.125690
658 (2008).
- 659 7 op den Camp, R. G. L. *et al.* Rapid induction of distinct stress responses after the release of
660 singlet oxygen in Arabidopsis. *Plant Cell* **15**, 2320-2332, doi:10.1105/tpc.014662 (2003).
- 661 8 Baruah, A., Simkova, K., Apel, K. & Laloi, C. Arabidopsis mutants reveal multiple singlet
662 oxygen signaling pathways involved in stress response and development. *Plant Mol. Biol.* **70**,
663 547-563, doi:10.1007/s11103-009-9491-0 (2009).
- 664 9 Gorman, A. A. & Rodgers, M. A. J. Current perspectives of singlet oxygen detection in
665 biological environments. *J. Photoch. Photobio. B* **14**, 159-176 (1992).
- 666 10 Beck, C. F. Signaling pathways from the chloroplast to the nucleus. *Planta* **222**, 743-756,
667 doi:10.1007/s00425-005-0021-2 (2005).
- 668 11 Sies, H. & Menck, C. F. M. Singlet oxygen induced DNA damage. *Mutat. Res.* **275**, 367-375
669 (1992).
- 670 12 Pfannschmidt, T. Plastidial retrograde signalling - a true "plastid factor" or just metabolite
671 signatures? *Trends Plant Sci.* **15**, 427-435, doi:10.1016/j.tplants.2010.05.009 (2010).
- 672 13 Kim, C. *et al.* Chloroplasts of Arabidopsis are the source and a primary target of a plant-
673 specific programmed cell death signaling pathway. *Plant Cell* **24**, 3026-3039,
674 doi:10.1105/tpc.112.100479 (2012).
- 675 14 Brzezowski, P., Wilson, K. E. & Gray, G. R. The PSBP2 protein of *Chlamydomonas*
676 *reinhardtii* is required for singlet oxygen-dependent signaling. *Planta* **236**, 1289-1303,
677 doi:10.1007/s00425-012-1683-1 (2012).
- 678 15 Wakao, S. *et al.* Phosphoprotein SAK1 is a regulator of acclimation to singlet oxygen in
679 *Chlamydomonas reinhardtii*. *eLife* **3**, e02286, doi:10.7554/eLife.02286 (2014).
- 680 16 Shao, N., Duan, G. Y. & Bock, R. A mediator of singlet oxygen responses in
681 *Chlamydomonas reinhardtii* and Arabidopsis identified by a luciferase-based genetic screen in
682 algal cells. *Plant Cell* **25**, 4209-4226, doi:10.1105/tpc.113.117390 (2013).
- 683 17 von Gromoff, E. D., Alawady, A., Meinecke, L., Grimm, B. & Beck, C. F. Heme, a plastid-
684 derived regulator of nuclear gene expression in *Chlamydomonas*. *Plant Cell* **20**, 552-567,
685 doi:10.1105/tpc.107.054650 (2008).
- 686 18 Brzezowski, P. *et al.* The GUN4 protein plays a regulatory role in tetrapyrrole biosynthesis
687 and chloroplast-to-nucleus signalling in *Chlamydomonas reinhardtii*. *Plant J.* **79**, 285-298,
688 doi:10.1111/tpj.12560 (2014).
- 689 19 Fischer, B. B., Krieger-Liszka, A. & Eggen, R. I. L. Photosensitizers neutral red (Type I)
690 and rose bengal (Type II) cause light-dependent toxicity in *Chlamydomonas reinhardtii* and
691 induce the Gpxh gene via increased singlet oxygen formation. *Environ. Sci. Technol.* **38**,
692 6307-6313, doi:10.1021/es049673y (2004).
- 693 20 Leisinger, U. *et al.* The glutathione peroxidase homologous gene from *Chlamydomonas*
694 *reinhardtii* is transcriptionally up-regulated by singlet oxygen. *Plant Mol. Biol.* **46**, 395-408
695 (2001).
- 696 21 Fischer, B. B., Il Eggen, R. & Niyogi, K. K. Characterization of singlet oxygen-accumulating
697 mutants isolated in a screen for altered oxidative stress response in *Chlamydomonas*
698 *reinhardtii*. *BMC Plant Biol.* **10**, doi:279 10.1186/1471-2229-10-279 (2010).
- 699 22 Fischer, B. B. *et al.* Function and regulation of the glutathione peroxidase homologous gene
700 GPXH/GPX5 in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* **71**, 569-583,
701 doi:10.1007/s11103-009-9540-8 (2009).

- 702 23 Lunn, J. E. *et al.* Sugar-induced increases in trehalose 6-phosphate are correlated with redox
703 activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in
704 *Arabidopsis thaliana*. *Biochem. J.* **397**, 139-148, doi:10.1042/bj20060083 (2006).
- 705 24 Yadav, U. P. *et al.* The sucrose-trehalose 6-phosphate (Tre6P) nexus: specificity and
706 mechanisms of sucrose signalling by Tre6P. *J. Exp. Bot.* **65**, 1051-1068,
707 doi:10.1093/jxb/ert457 (2014).
- 708 25 Schluepmann, H., Pellny, T., van Dijken, A., Smeekens, S. & Paul, M. Trehalose 6-phosphate
709 is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. *Proc Natl*
710 *Acad Sci U S A* **100**, 6849-6854, doi:10.1073/pnas.1132018100 (2003).
- 711 26 Fichtner, F. & Lunn, J. E. The Role of Trehalose 6-Phosphate (Tre6P) in Plant Metabolism
712 and Development. *Annu. Rev. Plant Biol.* **72**, 737-760, doi:10.1146/annurev-arplant-050718-
713 095929 (2021).
- 714 27 Miller, R. *et al.* Changes in transcript abundance in *Chlamydomonas reinhardtii* following
715 nitrogen deprivation predict diversion of metabolism. *Plant Physiol.* **154**, 1737-1752,
716 doi:10.1104/pp.110.165159 (2010).
- 717 28 Ajalla Aleixo, M. A., Rangel, V. L., Rustiguel, J. K., de Pádua, R. A. P. & Nonato, M. C.
718 Structural, biochemical and biophysical characterization of recombinant human fumarate
719 hydratase. *FEBS J.* **286**, 1925-1940, doi:10.1111/febs.14782 (2019).
- 720 29 Yogeve, O. *et al.* Fumarase: a mitochondrial metabolic enzyme and a cytosolic/nuclear
721 component of the DNA damage response. *PLoS Biol.* **8**, e1000328,
722 doi:10.1371/journal.pbio.1000328 (2010).
- 723 30 Jiang, Y. *et al.* Local generation of fumarate promotes DNA repair through inhibition of
724 histone H3 demethylation. *Nat. Cell Biol.* **17**, 1158-1168, doi:10.1038/ncb3209 (2015).
- 725 31 Ma, X. *et al.* Transcriptomic and Physiological Responses to Oxidative Stress in a
726 *Chlamydomonas reinhardtii* Glutathione Peroxidase Mutant. *Genes* **11**,
727 doi:10.3390/genes11040463 (2020).
- 728 32 Blaby, I. K. *et al.* Genome-wide analysis on *Chlamydomonas reinhardtii* reveals the impact of
729 hydrogen peroxide on protein stress responses and overlap with other stress transcriptomes.
730 *Plant J.* **84**, 974-988, doi:10.1111/tpj.13053 (2015).
- 731 33 Urzica, E. I. *et al.* Impact of oxidative stress on ascorbate biosynthesis in *Chlamydomonas* via
732 regulation of the VTC2 gene encoding a GDP-L-galactose phosphorylase. *J. Biol. Chem.* **287**,
733 14234-14245, doi:10.1074/jbc.M112.341982 (2012).
- 734 34 Roach, T., Stögl, W., Baur, T. & Kranner, I. Distress and eustress of reactive electrophiles
735 and relevance to light stress acclimation via stimulation of thiol/disulphide-based redox
736 defences. *Free radical biology & medicine* **122**, 65-73,
737 doi:10.1016/j.freeradbiomed.2018.03.030 (2018).
- 738 35 Arora, A., Sairam, R. K. & Srivastava, G. C. Oxidative stress and antioxidative system in
739 plants. *Curr. Sci.* **82**, 1227-1238 (2002).
- 740 36 Moller, I. M. Plant mitochondria and oxidative stress: Electron transport, NADPH turnover,
741 and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**,
742 561-591 (2001).
- 743 37 Asada, K. Production and scavenging of reactive oxygen species in chloroplasts and their
744 functions. *Plant Physiol.* **141**, 391-396, doi:10.1104/pp.106.082040 (2006).
- 745 38 Brzezowski, P., Richter, A. S. & Grimm, B. Regulation and function of tetrapyrrole
746 biosynthesis in plants and algae. *Biochimica Et Biophysica Acta-Bioenergetics* **1847**, 968-
747 985, doi:10.1016/j.bbabi.2015.05.007 (2015).
- 748 39 Larkin, R. M. Tetrapyrrole Signaling in Plants. *Front. Plant Sci.* **7**,
749 doi:10.3389/fpls.2016.01586 (2016).
- 750 40 Moulin, M., McCormac, A. C., Terry, M. J. & Smith, A. G. Tetrapyrrole profiling in
751 *Arabidopsis* seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-
752 protoporphyrin IX accumulation. *Proc. Natl. Acad. Sci. USA* **105**, 15178-15183,
753 doi:10.1073/pnas.0803054105 (2008).
- 754 41 Mochizuki, N., Tanaka, R., Tanaka, A., Masuda, T. & Nagatani, A. The steady-state level of
755 Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in *Arabidopsis*.
756 *Proc. Natl. Acad. Sci. USA* **105**, 15184-15189, doi:10.1073/pnas.0803245105 (2008).

757 42 Vandesteene, L. *et al.* Expansive evolution of the trehalose-6-phosphate phosphatase gene
758 family in Arabidopsis. *Plant Physiol.* **160**, 884-896, doi:10.1104/pp.112.201400 (2012).

759 43 Ramon, M. *et al.* Extensive expression regulation and lack of heterologous enzymatic activity
760 of the Class II trehalose metabolism proteins from Arabidopsis thaliana. *Plant Cell Environ.*
761 **32**, 1015-1032, doi:10.1111/j.1365-3040.2009.01985.x (2009).

762 44 Vandesteene, L., Ramon, M., Le Roy, K., Van Dijck, P. & Rolland, F. A single active
763 trehalose-6-P synthase (TPS) and a family of putative regulatory TPS-like proteins in
764 Arabidopsis. *Mol. Plant* **3**, 406-419, doi:10.1093/mp/ssp114 (2010).

765 45 Delorge, I., Figueroa, C. M., Feil, R., Lunn, J. E. & Van Dijck, P. Trehalose-6-phosphate
766 synthase 1 is not the only active TPS in Arabidopsis thaliana. *Biochem. J.* **466**, 283-290,
767 doi:10.1042/bj20141322 (2015).

768 46 Collet, J. F., Stroobant, V., Pirard, M., Delpierre, G. & Van Schaftingen, E. A new class of
769 phosphotransferases phosphorylated on an aspartate residue in an amino-terminal
770 DXDX(T/V) motif. *J. Biol. Chem.* **273**, 14107-14112, doi:10.1074/jbc.273.23.14107 (1998).

771 47 Martins, M. C. *et al.* Feedback inhibition of starch degradation in Arabidopsis leaves
772 mediated by trehalose 6-phosphate. *Plant Physiol.* **163**, 1142-1163,
773 doi:10.1104/pp.113.226787 (2013).

774 48 Dos Anjos, L. *et al.* Feedback regulation by trehalose 6-phosphate slows down starch
775 mobilization below the rate that would exhaust starch reserves at dawn in Arabidopsis leaves.
776 *Plant direct* **2**, e00078, doi:10.1002/pld3.78 (2018).

777 49 Zhang, Y. *et al.* Inhibition of SNF1-related protein kinase1 activity and regulation of
778 metabolic pathways by trehalose-6-phosphate. *Plant Physiol.* **149**, 1860-1871,
779 doi:10.1104/pp.108.133934 (2009).

780 50 Debast, S. *et al.* Altering trehalose-6-phosphate content in transgenic potato tubers affects
781 tuber growth and alters responsiveness to hormones during sprouting. *Plant Physiol.* **156**,
782 1754-1771, doi:10.1104/pp.111.179903 (2011).

783 51 Nunes, C. *et al.* Inhibition of SnRK1 by metabolites: tissue-dependent effects and cooperative
784 inhibition by glucose 1-phosphate in combination with trehalose 6-phosphate. *Plant Physiol.*
785 *Biochem.* **63**, 89-98, doi:10.1016/j.plaphy.2012.11.011 (2013).

786 52 Zhai, Z. *et al.* Trehalose 6-Phosphate Positively Regulates Fatty Acid Synthesis by Stabilizing
787 WRINKLED1. *Plant Cell* **30**, 2616-2627, doi:10.1105/tpc.18.00521 (2018).

788 53 Baena-González, E. & Lunn, J. E. SnRK1 and trehalose 6-phosphate - two ancient pathways
789 converge to regulate plant metabolism and growth. *Curr. Opin. Plant Biol.* **55**, 52-59,
790 doi:10.1016/j.pbi.2020.01.010 (2020).

791 54 Depaepe, T. *et al.* At the Crossroads of Survival and Death: The Reactive Oxygen Species-
792 Ethylene-Sugar Triad and the Unfolded Protein Response. *Trends Plant Sci.* **26**, 338-351,
793 doi:10.1016/j.tplants.2020.12.007 (2021).

794 55 Baena-González, E., Rolland, F., Thevelein, J. M. & Sheen, J. A central integrator of
795 transcription networks in plant stress and energy signalling. *Nature* **448**, 938-942,
796 doi:10.1038/nature06069 (2007).

797 56 Gonzalez-Ballester, D., Pollock, S. V., Pootakham, W. & Grossman, A. R. The central role of
798 a SNRK2 kinase in sulfur deprivation responses. *Plant Physiol.* **147**, 216-227,
799 doi:10.1104/pp.108.116137 (2008).

800 57 González-Ballester, D. *et al.* RNA-seq analysis of sulfur-deprived Chlamydomonas cells
801 reveals aspects of acclimation critical for cell survival. *Plant Cell* **22**, 2058-2084,
802 doi:10.1105/tpc.109.071167 (2010).

803 58 Colina, F. *et al.* Genome-wide identification and characterization of CKIN/SnRK gene family
804 in Chlamydomonas reinhardtii. *Sci. Rep.* **9**, 350, doi:10.1038/s41598-018-35625-8 (2019).

805 59 Valledor, L., Furuhashi, T., Hanak, A. M. & Weckwerth, W. Systemic cold stress adaptation
806 of Chlamydomonas reinhardtii. *Mol. Cell. Proteomics* **12**, 2032-2047,
807 doi:10.1074/mcp.M112.026765 (2013).

808 60 Yang, M., Soga, T., Pollard, P. J. & Adam, J. The emerging role of fumarate as an
809 oncometabolite. *Frontiers in oncology* **2**, 85, doi:10.3389/fonc.2012.00085 (2012).

- 810 61 O'Flaherty, L. *et al.* Dysregulation of hypoxia pathways in fumarate hydratase-deficient cells
811 is independent of defective mitochondrial metabolism. *Hum. Mol. Genet.* **19**, 3844-3851,
812 doi:10.1093/hmg/ddq305 (2010).
- 813 62 Shanmugasundaram, K. *et al.* The oncometabolite fumarate promotes pseudohypoxia through
814 noncanonical activation of NF- κ B signaling. *J. Biol. Chem.* **289**, 24691-24699,
815 doi:10.1074/jbc.M114.568162 (2014).
- 816 63 Ternette, N. *et al.* Inhibition of mitochondrial aconitase by succination in fumarate hydratase
817 deficiency. *Cell reports* **3**, 689-700, doi:10.1016/j.celrep.2013.02.013 (2013).
- 818 64 Lloyd, S. J., Lauble, H., Prasad, G. S. & Stout, C. D. The mechanism of aconitase: 1.8 Å
819 resolution crystal structure of the S642a:citrate complex. *Protein Sci.* **8**, 2655-2662,
820 doi:10.1110/ps.8.12.2655 (1999).
- 821 65 Kleine, T., Voigt, C. & Leister, D. Plastid signalling to the nucleus: messengers still lost in
822 the mists? *Trends Genet.* **25**, 185-190, doi:10.1016/j.tig.2009.02.004 (2009).
- 823 66 Bräutigam, K. *et al.* Dynamic plastid redox signals integrate gene expression and metabolism
824 to induce distinct metabolic states in photosynthetic acclimation in Arabidopsis. *Plant Cell*
825 **21**, 2715-2732, doi:10.1105/tpc.108.062018 (2009).
- 826 67 Pesaresi, P. *et al.* Nuclear photosynthetic gene expression is synergistically modulated by
827 rates of protein synthesis in chloroplasts and mitochondria. *Plant Cell* **18**, 970-991,
828 doi:10.1105/tpc.105.039073 (2006).
- 829 68 Shapiguzov, A. *et al.* Arabidopsis RCD1 coordinates chloroplast and mitochondrial functions
830 through interaction with ANAC transcription factors. *eLife* **8**, doi:10.7554/eLife.43284
831 (2019).
- 832 69 Dent, R. M., Haglund, C. M., Chin, B. L., Kobayashi, M. C. & Niyogi, K. K. Functional
833 genomics of eukaryotic photosynthesis using insertional mutagenesis of *Chlamydomonas*
834 *reinhardtii*. *Plant Physiol.* **137**, 545-556, doi:10.1104/pp.104.055244 (2005).
- 835 70 Depege, N., Bellaïf, S. & Rochaix, J. D. Role of chloroplast protein kinase Stt7 in LHCII
836 phosphorylation and state transition in *Chlamydomonas*. *Science* **299**, 1572-1575 (2003).
- 837 71 Sizova, I., Fuhrmann, M. & Hegemann, P. A *Streptomyces rimosus* aphVIII gene coding for a
838 new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas*
839 *reinhardtii*. *Gene* **277**, 221-229 (2001).
- 840 72 Davies, J. P., Weeks, D. P. & Grossman, A. R. Expression of the arylsulfatase gene from the
841 β 2-tubulin promoter in *Chlamydomonas reinhardtii*. *Nucleic Acids Res.* **20**, 2959-2965
842 (1992).
- 843 73 Schroda, M., Beck, C. F. & Vallon, O. Sequence elements within an HSP70 promoter
844 counteract transcriptional transgene silencing in *Chlamydomonas*. *Plant J.* **31**, 445-455,
845 doi:10.1046/j.1365-3113X.2002.01371.x (2002).
- 846 74 Gonzalez-Ballester, D., de Montaigu, A., Galvan, A. & Fernandez, E. Restriction enzyme
847 site-directed amplification PCR: A tool to identify regions flanking a marker DNA. *Anal.*
848 *Biochem.* **340**, 330-335, doi:10.1016/j.ab.2005.01.031 (2005).
- 849 75 Meslet-Cladière, L. & Vallon, O. Novel shuttle markers for nuclear transformation of the
850 green alga *Chlamydomonas reinhardtii*. *Eukaryot. Cell* **10**, 1670-1678, doi:10.1128/ec.05043-
851 11 (2011).
- 852 76 Ohresser, M., Matagne, R. F. & Loppes, R. Expression of the arylsulphatase reporter gene
853 under the control of the nit1 promoter in *Chlamydomonas reinhardtii*. *Curr. Genet.* **31**, 264-
854 271 (1997).
- 855 77 Czarnecki, O., Peter, E. & Grimm, B. in *Chloroplast research in Arabidopsis: Methods and*
856 *Protocols, Vol II* Vol. 775 357-385 (2011).
- 857 78 Figueroa, C. M. *et al.* Trehalose 6-phosphate coordinates organic and amino acid metabolism
858 with carbon availability. *Plant J.* **85**, 410-423, doi:10.1111/tpj.13114 (2016).
- 859 79 Fichtner, F. *et al.* Trehalose 6-phosphate is involved in triggering axillary bud outgrowth in
860 garden pea (*Pisum sativum* L.). *Plant J.* **92**, 611-623, doi:10.1111/tpj.13705 (2017).
- 861 80 DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. Colorimetric Method
862 for Determination of Sugars and Related Substances. *Analytical chemistry* **28**, 350-356,
863 doi:10.1021/ac60111a017 (1956).

- 81 Johnson, X. & Alric, J. Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii*: metabolic constraints for carbon partitioning between oil and starch. *Eukaryot. Cell* **12**, 776-793, doi:10.1128/ec.00318-12 (2013).
- 82 Findinier, J. *et al.* The *Chlamydomonas mex1* mutant shows impaired starch mobilization without maltose accumulation. *J. Exp. Bot.* **68**, 5177-5189, doi:10.1093/jxb/erx343 (2017).

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Author contributions

P.B. designed the research. P.B. and W.A.Y. performed most of the experiments. R.F. and J.E.L. performed the LC-MS/MS measurements. M.S.-S. and X.J. performed the starch quantification. P.B., B.G., and J.E.L. analysed the data. P.B. wrote the manuscript. P.B., B.G., J.E.L., and X.J. revised the manuscript. All authors discussed the results and commented upon the manuscript.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information accompanies this manuscript:

Supplementary Figure 1. Schematic representation of tetrapyrrole biosynthesis pathway.

Supplementary Figure 2. Schematic representation of the *GPX5-ARS2* reporter gene construct.

Supplementary Figure 3. Analysis of Proto content in *gunSOS1*.

Supplementary Figure 4. Rescue of the $^1\text{O}_2$ -signalling in *gunSOS1* by introduction of the wild-type *T6PPI*.

Supplementary Figure 5. Metabolites content in *gunSOS1* compared to *sigRep*.

Supplementary Figure 6. Accumulation of fumarate in *gunSOS1* compared to *sigRep* could be explained by increased expression of *FUMARATE HYDRATASE 2*.

Supplementary Figure 7. Accumulation of fumarate in *gunSOS1* compared to *sigRep* could be explained by increased expression of *FUMARYLACETOACETASE*.

Supplementary Figure 8. Deficiency in aconitate in *gunSOS1* compared to *sigRep* could be explained by decreased expression of *ACONITATE HYDRATASE 1*.

Supplementary Table 1. Metabolites analyzed in *gunSOS1* compared to *sigRep* (LC-MS/MS).

Supplementary Table 2. Impact of accumulating metabolites on cell metabolism.

Supplementary Table 3. Impact of deficient metabolites on cell metabolism.

Supplementary Table 4. Statistical analyses for *GPX5_{cyt}* expression following treatment with fumarate, presented in Figure 7a.

Supplementary Table 5. Statistical analyses for *GPX5_{cp}* expression following treatment with fumarate, presented in Figure 7a.

Supplementary Table 6. Statistical analyses for *GPX5_{cyt}* expression following treatment with aconitate, presented in Figure 7b.

Supplementary Table 7. Statistical analyses for *GPX5_{cp}* expression following treatment with aconitate, presented in Figure 7b.

Supplementary Table 8. Statistical analyses for *SAK1* expression following treatment with aconitate, presented in Figure 7c.

Supplementary Table 9. Statistical analyses for *PSBP2* expression following treatment with aconitate, presented in Figure 7c.

Supplementary Table 10. Statistical analyses for *MBS* expression following treatment with aconitate, presented in Figure 7c.

Supplementary Table 11. Primers used in this study.

Supplementary Data Set 1. Co-expression analysis

Supplementary Data Set 2. DEG analysis

Supplementary Data Set 3. KEGG analysis

Figure 1. Generation and initial characterization of the mutant impaired in $^1\text{O}_2$ -signalling. **a** Arylsulfatase (ARS2) assay for selection of the strain expressing *GPX5-ARS2* reporter gene in a $^1\text{O}_2$ -inducible manner. The mutant selected for further applications was named *signalling Reporter* (*sigRep*). WT and *chlD-1/GUN4* do not carry *GPX5-ARS2* and were used as negative controls in ARS2 assay. **b** Expression kinetics of cytosolic (*GPX5_{cyt}*) and chloroplast *GPX5* (*GPX5_{cp}*) in *sigRep* compared to WT upon exposure to light; the values were normalized to WT in dark. **c** Expression of *GPX5-ARS2* in *sigRep* positively correlates with time of exposure to light. WT was used as a negative control. **d** ARS2 activity assay following mutagenesis of *sigRep*. Screen was performed to identify mutants not expressing *GPX5-ARS2* compared to *sigRep* in the same conditions. The mutant selected for further applications was named *genomes uncoupled Singlet Oxygen Signalling 1* (*gunSOS1*). WT and *chlD-1/GUN4* were used as negative controls. **e** Strains with impaired $^1\text{O}_2$ -signalling demonstrated increased sensitivity to light compared to *sigRep* and *chlD-1/GUN4*. Higher light tolerance of *chlD-1/GUN4* and *sigRep* compared to *chlD-1* is also visible. **f** *T6PP1* (Cre12.g497750) gene model. The insertion (*ble^R*) was identified in the first exon of 3241 bp *T6PP1*. **g** Expression of *T6PP1* in *gunSOS1* compared to *sigRep*. **h** Expression of *GPX5_{cyt}* and *GPX5_{cp}* in *gunSOS1* compared to *sigRep*. **i** Expression of *GPX5-ARS2* in *gunSOS1* compared to *sigRep*. WT was used as a negative control. Transcript analyses were performed by qRT-PCR on biological triplicates, values were calculated either as a normalized fold expression ($2^{-\Delta\Delta\text{Ct}}$; WT=1) or relative expression ($2^{\Delta\text{Ct}}$). For b and c, horizontal bars represent the calculated mean ($n = 3$), vertical error bars represent calculated $\pm\text{SD}$; significant differences were calculated using two-tailed Student's *t*-test and are indicated by asterisks (non-significant not shown), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. For g-i, error bars indicate calculated $\pm\text{SD}$; one-way ANOVA, pair-wise comparison with the Tukey's post-hoc test (non-significant not shown), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 2. *C. reinhardtii* T6PP1 is a functional phosphatase induced during photooxidative stress. **a** Kinetics of Tre6P accumulation in *gunSOS1* compared to *sigRep* upon transfer from dark to light. **b** Kinetics of *T6PP1* expression upon transfer from dark to light in *gunSOS1* compared to WT indicate inducibility by photooxidative stress rather than light; values were calculated as a normalized fold expression ($2^{-\Delta\Delta C_t}$; WT=1). **c** Higher content of trehalose in *gunSOS1* compared to *sigRep* upon transfer from dark to light may be resulting from the function of T6PS/T6PP1. **d** Immuno-blot analysis of T6PP1 (calculated MW of 42 kDa) and GPX5 content in *gunSOS1* compared to *sigRep* and strains rescued with wild-type copy of *T6PP1*. The CHLI1 content was used as a loading control. The unspecific immunoreaction is indicated by an asterisk. **e** Light sensitivity examination showed that the strains with rescued ¹O₂-signalling have an increased tolerance to light compared to *gunSOS1*. Experiments in a, b and c were performed in biological triplicates ($n = 3$) horizontal bars represent the calculated mean, vertical error bars represent $\pm$ SD; significant differences were calculated using two-tailed Student's *t*-test and are indicated by asterisks (non-significant not shown), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 3. Metabolites with significantly increased content in *gunSOS1* compared to *sigRep* in the light. a LC-MS/MS analysis; fumaric acid (fumarate), oxoglutaric acid (2-OG), myo-Inositol, ADP-glucose (ADPGlc), galactose 1-phosphate (Gal1P), fructose 1-phosphate (Fru1P), alpha-D-Glucose 1,6-bisphosphate (Glc1, 6BP), glycerol 3-phosphate (Gly3P), phosphoenolpyruvic acid (PEP), L-Malic acid (malate). Measurements were performed in biological triplicates and are presented as mean ($n = 3$), horizontal bars represent the calculated mean, vertical error bars represent calculated $\pm$ SD; significant differences were calculated using two-tailed Student's *t*-test and are indicated by asterisks (non-significant not shown), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. **b** Impact of accumulating metabolites on cell metabolism analysed using MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>), sorted by the *P* value; 1. Tricarboxylic acid cycle (TCA cycle; $P = 4.41\text{E-}05$), 2. Pyruvate metabolism ($P = 0.00136$), 3. Starch and sucrose metabolism ($P = 0.00136$), 4. Glyoxylate and dicarboxylate metabolism ($P = 0.00429$), 5. Arginine biosynthesis ($P = 0.01473$), 6. Alanine, aspartate and glutamate metabolism ($P = 0.02019$), 7. Glycerolipid metabolism ($P = 0.02019$), 8. Carbon fixation in photosynthetic organisms ($P = 0.02217$), 9. Galactose metabolism ($P = 0.03566$), 10. Amino sugar and nucleotide sugar metabolism ($P = 0.07616$), 11. Fructose and mannose metabolism ($P = 0.13874$), 12. Tyrosine metabolism ($P = 0.1872$), 13. Phenylalanine, tyrosine and tryptophan biosynthesis ($P = 0.22414$), 14. Inositol phosphate metabolism ($P = 0.24202$), 15. Glycolysis / Gluconeogenesis ($P = 0.24202$), 16. Phosphatidylinositol signaling system ($P = 0.25082$), 17. Glycine, serine and threonine metabolism ($P = 0.27665$), 18. Glycerophospholipid metabolism ($P = 0.34157$); detailed results are presented in Supplementary Table 2.

Figure 4. Metabolites with significantly decreased content in *gunSOS1* compared to *sigRep* in the light. a LC-MS/MS analysis; cis-Aconitic acid (aconitate), mannose 6-phosphate (Man6P), Glucose 6-phosphate (Glc6P), D-fructose (fructose), Fructose 6-phosphate (Fru6P), Glucose 1-phosphate (Glc1P), Fructose 1,6-bisphosphate (Fru1, 6BP), sorbitol. Measurements were performed in biological triplicates and are presented as mean ($n = 3$), horizontal bars represent the calculated mean, vertical error bars represent calculated $\pm$ SD; significant differences were calculated using two-tailed Student's *t*-test and are indicated by asterisks (non-significant not shown), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. **b** Impact of deficient metabolites on cell metabolism analysed using MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>), sorted by the *P* value; 1. Fructose and mannose metabolism ($P = 6.23E-05$), 2. Amino sugar and nucleotide sugar metabolism ($P = 8.99E-05$), 3. Starch and sucrose metabolism ($P = 0.000282$), 4. Glycolysis / Gluconeogenesis ($P = 0.01094$), 5. Galactose metabolism ($P = 0.013767$), 6. Pentose and glucuronate interconversions ($P = 0.081139$), 7. Pentose phosphate pathway ($P = 0.1195$), 8. Tricarboxylic acid cycle (TCA cycle; $P = 0.13197$), 9. Glycerolipid metabolism ($P = 0.13197$), 10. Carbon fixation in photosynthetic organisms ($P = 0.13815$), 11. Inositol phosphate metabolism ($P = 0.15646$), 12. Glyoxylate and dicarboxylate metabolism ($P = 0.19785$); detailed results are presented in Supplementary Table 3. **c** Starch accumulation in *gunSOS1* compared to *sigRep*. Measurements were performed in biological triplicates ($n = 3$); median is shown as a center line; upper and lower quartiles are shown as dotted lines. Significant differences were calculated using two-tailed Student's *t*-test and are indicated by asterisks (non-significant not shown), $*P < 0.05$, $**P < 0.01$.

Figure 5. The overview of the selected metabolic pathways in *gunSOS1* compared to *sigRep*. The map was constructed based on the measured metabolites presented in Figures 3, 4, and Supplementary Figure 5. Metabolites increased in *gunSOS1* compared to *sigRep*: fumaric acid (fumarate), oxoglutaric acid (2-OG), myo-Inositol, ADP-glucose (ADPGlc), galactose 1-phosphate (Gal1P), fructose 1-phosphate (Fru1P), alpha-D-Glucose 1,6-bisphosphate (Glc1, 6BP), glycerol 3-phosphate (Gly3P), phosphoenolpyruvic acid (PEP), L-Malic acid (malate). Metabolites decreased in *gunSOS1* compared to *sigRep*: cis-Aconitic acid (aconitate), mannose 6-phosphate (Man6P), Glucose 6-phosphate (Glc6P), D-fructose (fructose), Fructose 6-phosphate (Fru6P), Glucose 1-phosphate (Glc1P), Fructose 1,6-bisphosphate (Fru1, 6BP), and sorbitol. Metabolites that did not show significant difference in *gunSOS1* relative to *sigRep*: D-Glucose (glucose), D-Maltose (maltose), maltotriose, citric acid (citrate), isocitric acid (isocitrate), pyruvic acid (pyruvate), succinic acid (succinate), 3-phosphoglyceric acid (3PGA). The colour-coding: blue = increased, red = decreased, black = unchanged, grey = not measured or ^abelow the limit of detection. Significant differences were calculated using two-tailed Student's *t*-test. The maximal significant differences from any time point in light (Fig. 4 and 5) are indicated by asterisks, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. The metabolic map was constructed based on the review by⁸¹ and⁸².

Figure 6. Transcriptome analysis based on RNA-seq. a Venn diagram showing number of genes expressed in *gunSOS1* and *sigRep* (8120), or uniquely expressed in *gunSOS1* (1377) or in *sigRep* (1087). **b** Pathway enrichment analysis (KEGG, <http://www.kegg.jp/>) based on the differentially expressed genes (RNA-seq); genes down-regulated in *gunSOS1* compared to *sigRep*. **c** KEGG analysis showing genes up-regulated in *gunSOS1* compared to *sigRep*. Effect on metabolic processes is sorted by the *p* value. Asterisk indicates the core (significant) enrichment.

Figure 7. The qRT-PCR analysis of gene expression in *gunSOS1* compared to *sigRep*. **a** Expression of genes encoding proteinaceous components previously associated with the chloroplast retrograde signalling involving $^1\text{O}_2$, *PSBP2*¹⁴, *SAK1*¹⁵, and *MBS*¹⁶. **b** transcript levels of the selected genes showing attenuated expression in *gunSOS1* compared to *sigRep* in the context of the SAK1-transcriptome¹⁵, genes description in the text. Experiments were performed in biological replications; results are presented as normalized fold expression ($2^{-\Delta\Delta\text{Ct}}$, WT=1); the error bars represent calculated $\pm$ SD. Significant differences were calculated using one-way ANOVA, pair-wise comparison with the Tukey's post-hoc test (non-significant not shown), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 8. Effect of selected metabolites applied exogenously on ¹O₂-signalling inducing *GPX5_{cyt}* or *GPX5_{cp}* expression. **a** Fumarate (Fum) significantly ($P < 0.0001$) decreased *GPX5* expression in *sigRep* in concentration-dependent manner. Interaction between mutants and fumarate, as well as the mutant-dependent expression of *GPX5* were also significant, $P < 0.0001$ and $P < 0.001$, respectively. Fumarate had no effect on *GPX5_{cyt}* expression in *gunSOS1* or WT, but attenuating effect was observed for *GPX5_{cp}* expression in WT ($P \leq 0.0001$). Detailed reports from statistical analyses are presented in Supplementary Tables 4 and 5. **b** Aconitate (Acon) at 20 μ M concentration rescues *GPX5* expression in *gunSOS1* and increases *GPX5* expression in *sigRep*. Statistical analysis showed that 20 μ M aconitate significantly ($P \leq 0.0001$) affect *GPX5_{cyt}* expression in *gunSOS1* and *sigRep*, but 100 μ M concentration ($P = 0.0001$) decreased this expression in *sigRep*. An increase in *GPX5_{cp}* expression ($P < 0.05$) was also observed in WT upon treatment with 50 μ M aconitate (Supplementary Table 6 and 7). **c** Transcripts content for proteins necessary for the ¹O₂-signalling, *SAK1*, *PSBP2*, and *MBS* significantly ($P < 0.01$) increased upon treatment with 20 μ M aconitate. An increase in *PSBP2* and *MBS* expression was also observed in WT treated with 50 μ M aconitate. Detailed statistical analyses are presented in Supplementary Tables 8-10. The qRT-PCR experiments were performed in biological replications ($n = 3$); results are presented as normalized fold expression ($2^{-\Delta\Delta C_t}$, untreated WT=1); the error bars represent calculated $\pm$ SD. Significant differences were calculated using two-way ANOVA with Dunnett's multiple comparison test and are indicated by asterisks (non-significant changes are not shown on the graph for clarity), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Figure 9. Expression of genes, which induction was previously associated with H₂O₂ or organic peroxides, but not with ¹O₂. **a** *Mn superoxide dismutase 3 (MSD3)*. **b** *ASCORBATE PEROXIDASE 1 (APX1)*. **c** *Mono-functional catalase (CAT1)*. **d** *GDP-L-galactose Phosphorylase (VTC2)*. **e** *monodehydroascorbate reductase (MDAR1)*. **f** *dehydroascorbate reductase (DHAR1)* **g** *GLUTATHIONE REDUCTASE (GSHR1)*. **h** *γ-glutamylcysteine synthetase (GSH1)*. **i** *Glutathione S-transferase (GSTS1)*. **j** *Fe superoxide dismutase (FSD1)*. Experiments were performed in biological replications ($n = 3$); results are presented as normalized fold expression ($2^{-\Delta\Delta C_t}$, WT=1); the error bars represent calculated $\pm$ SD. Significant differences were calculated using one-way ANOVA, pair-wise comparison with the Tukey's post-hoc test (non-significant not shown), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.