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# The mechanism of photosystem-II inactivation during sulphur deprivation-induced H₂ production in *Chlamydomonas reinhardtii*

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## SUMMARY

**Sulphur limitation may restrain cell growth and viability. In the green alga *Chlamydomonas reinhardtii*, sulphur limitation may induce H₂ production lasting for several days, which can be exploited as a renewable energy source. Sulphur limitation causes a large number of physiological changes, including the inactivation of photosystem II (PSII), leading to the establishment of hypoxia, essential for the increase in hydrogenase expression and activity. The inactivation of PSII has long been assumed to be caused by the sulphur-limited turnover of its reaction center protein PsbA. Here we reinvestigated this issue in detail and show that: (i) upon transferring *Chlamydomonas* cells to sulphur-free media, the cellular sulphur content decreases only by about 25%; (ii) as demonstrated by lincomycin treatments, PsbA has a significant turnover, and other photosynthetic subunits, namely RbcL and CP43, are degraded more rapidly than PsbA. On the other hand, sulphur limitation imposes oxidative stress early on, most probably involving the formation of singlet oxygen in PSII, which leads to an increase in the expression of GDP-L-galactose phosphorylase, playing an essential role in ascorbate biosynthesis. When accumulated to the millimolar concentration range, ascorbate may inactivate the oxygen-evolving complex and provide electrons to PSII, albeit at a low rate. In the absence of a functional donor side and sufficient electron transport, PSII reaction centers are inactivated and degraded. We therefore demonstrate that the inactivation of PSII is a complex and multistep process, which may serve to mitigate the damaging effects of sulphur limitation.**

Keywords: *Chlamydomonas reinhardtii*, sulphur, photosystem II, PsbA, hydrogen production, ascorbate.

## INTRODUCTION

Sulphur is an essential element for all living organisms, as it is present in proteins, lipids, carbohydrates, various metabolites, signaling molecules and electron carriers. Microorganisms and plants take up sulphur in the form of sulphate ($SO_4^-$, the most oxidized form of sulphur), by specific transporters, and within the cell $SO_4^-$ is activated by ATP sulphurylase to form adenosine-5′-phosphosulfate (APS), and then reduced to sulfide ($S_2^-$) for incorporation into sulphur-containing amino acids, sulfolipids, polysaccharides and other compounds, such as vitamins, methionine, cysteine, FeS clusters and various enzyme co-factors (Takahashi *et al.*, 2011; Anjum *et al.*, 2015). Sulphur availability can be restricted under certain environmental conditions, resulting in limited growth and development.

Sulphur deprivation in the green alga *Chlamydomonas reinhardtii* may induce H₂ production lasting for several days, which has been proposed to be exploited as a renewable energy source (Melis *et al.*, 2000; reviewed recently by Saroussi *et al.*, 2017). Sulphur limitation results in the early induction of genes responsible for sulphate transport and assimilation, elevated $SO_4^-$ transport activity (Pootakham *et al.*, 2010) and redistribution of internal sulphur reserves. Sulphur deprivation also leads to the cessation of cell growth and division, increased cell size (Zhang *et al.*, 2002) and the alteration of the cell wall structure (Takahashi *et al.*, 2001). Moreover, sulphur deprivation arrests chlorophyll (Chl) biosynthesis, and leads to strong starch and phosphatidylglycerol accumulation, as well as sulfolipid degradation (Sugimoto *et al.*, 2007, 2008). A characteristic

effect is the decline in the expression of a large number of photosynthetic genes, encoding subunits of photosystem I (PSI), photosystem II (PSII) and ATPase, whereas the transcript levels of two particular antenna proteins, LHCBM9 and LHCSR1 are upregulated (Nguyen *et al.*, 2011; Toepel *et al.*, 2013). The decline of photosynthetic activity is manifested first in the loss of Rubisco, decreased PSII activity and the degradation of various photosynthetic complexes (Zhang *et al.*, 2002). On the other hand, respiration is maintained, which, together with the loss of PSII activity, results in the establishment of hypoxia, enabling the expression of the highly $O_2$-sensitive [Fe–Fe] type algal hydrogenases (HYDA1 and HYDA2), located on the acceptor side of PSI (Zhang *et al.*, 2002; Volgusheva *et al.*, 2013).

The electrons feeding $H_2$ production originate mostly from the remaining PSII activity, although starch degradation may also contribute to $H_2$ production significantly, not only by providing reductants, but also by promoting the establishment of hypoxia (Chochois *et al.*, 2009). The $H_2$-producing period typically lasts for 4–6 days; at the end of the $H_2$-producing period, the entire cell system starts to degrade and genes related to apoptosis and protein degradation are upregulated (Toepel *et al.*, 2013).

The reduction of PSII activity is an essential step for the initiation of $H_2$ production. It is generally considered that the loss of PSII activity is caused by the limited turnover of the PsbA (D1) protein (reviewed by Antal *et al.*, 2015), because this protein has the highest turnover among all photosynthetic complexes (Nelson *et al.*, 2014). On the other hand, data from the literature indicate that the decrease of PSII activity is a controlled process it was shown that *sac1* and *snrk2.1* mutants, unable to downregulate their photosynthetic activity under sulphur deprivation, die more rapidly than the wild type (Davies *et al.*, 1996; Gonzalez-Ballester *et al.*, 2008), possibly as a result of the continued production of reactive oxygen species (ROS, González-Ballester *et al.*, 2010).

In our previous paper, we demonstrated that sulphur deprivation also results in a dramatic, about 50-fold, increase in ascorbate (Asc) concentration, and that Asc may inactivate the Mn-cluster of the oxygen-evolving complex (OEC) when present in the mM range (Nagy *et al.*, 2016). Based on these results, we suggested that Asc may contribute to the establishment of hypoxia, and thereby plays a role in the initiation of $H_2$ production. Here, we investigated the inactivation mechanisms of PSII in detail, and demonstrate that upon transferring *Chlamydomonas* cells to sulphur-free media, the cellular sulphur content decreases only moderately and PsbA retains a discernible turnover. Based on our data, we suggest that PSII inactivation is imposed by a complex and multistep process that occurs via oxidative stress, strong Asc accumulation and may involve donor-side-induced photoinhibition.

## RESULTS

### Experimental design and $H_2$ production upon sulphur deprivation

Sulphur deprivation experiments were carried out on the most commonly used CC124 strain of *C. reinhardtii*, which is also a relatively efficient $H_2$ producer (e.g. Kosourov *et al.*, 2005; Lakatos *et al.*, 2014; Oncel and Kose, 2014; Steinbeck *et al.*, 2015). For inducing sulphur limitation, the cells were washed five times with Tris-acetate-phosphate (TAP) media lacking sulphur. The Chl content was set at 20 µg Chl$(a + b)$ ml$^{-1}$, and the cultures were sealed, briefly flushed with $N_2$ to remove $CO_2$ and $O_2$, and subjected to continuous illumination of approximately 100 µmol photons m$^{-2}$ sec$^{-1}$ at 24°C for 96 h. The quantities of accumulated $H_2$ and $O_2$ were determined every 24 h by gas chromatography (GC), followed by $N_2$ flushing in order to prevent any accumulation of $H_2$, which may limit $H_2$ production (Kosourov *et al.*, 2012).

A substantial quantity of $H_2$ was detected at 48 h of sulphur deprivation, when only a small quantity of $O_2$ was present in the headspace of the serum bottles (Figure 1a). In total, about 250 µl $H_2$/ml culture was produced during the 96 h of sulphur deprivation, which is in agreement with earlier results (Scoma *et al.*, 2012). As expected, *HYDA1* gene expression (Figure 1b), and HYDA protein expression (Figure 1c) increased strongly upon sulphur limitation.

### The effects of sulphur deprivation on the elemental composition of *Chlamydomonas* cells

Upon transferring the cells to sulphur-free media, the Chl content remained fairly constant for 96 h (Figure 2a). The initial cell density was about 5.3 million cells/ml culture, which increased moderately, by about 25% within 72 h (Figure 2a). We have also observed a more than twofold increase in cell volume within 24 h, which decreased later (Figure 2B). In parallel with this, a remarkable accumulation of starch took place in the first 24 h (Figure 2c), which was degraded in the following days. These results are in agreement with the literature data, showing that sulphur deprivation results in the cessation of cell division and starch accumulation and its degradation (e.g. Zhang *et al.*, 2002). In addition, we also observed a strong decrease in the cellular protein content just 24 h after transferring the cells to sulphur-free media (Figure 2d).

Next, we investigated the elemental composition of the cells, using inductively coupled plasma atomic emission spectroscopy (ICP-OES; Szentmihályi *et al.*, 2015). We have chosen the 48-h time point for our measurements, at which point $H_2$ production is already substantial (Figure 1a). With the large changes in cell size and starch content upon sulphur deprivation, the quantities of the various elements were determined on dry weight (DW), cell number and Chl bases (Table 1). At 48 h after transferring the cells to
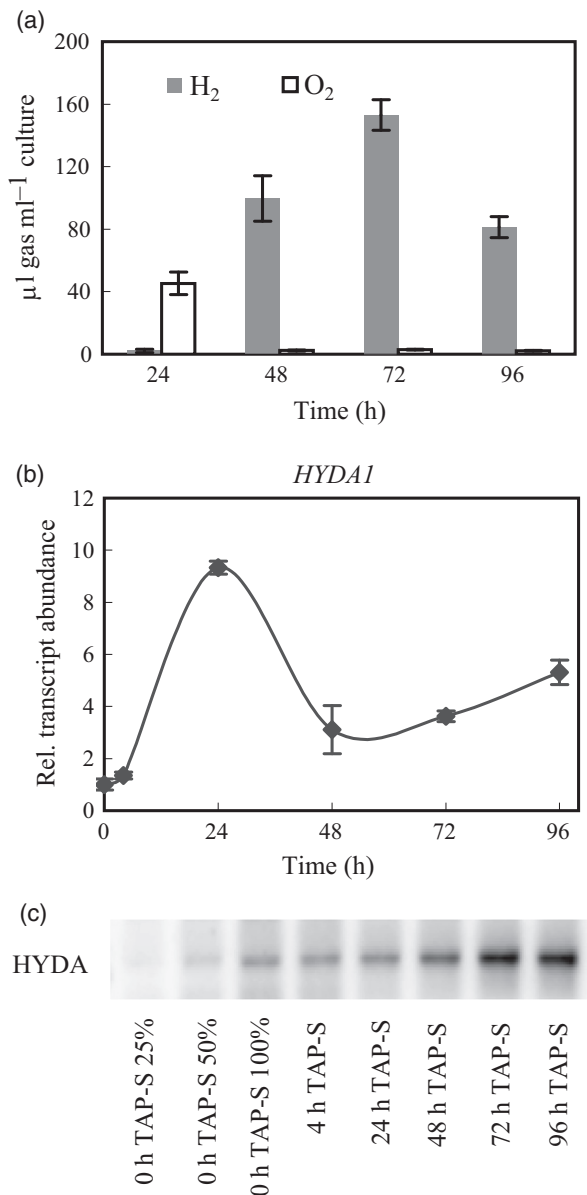
## (a)



## (b)



*HYDA1*

## (c)



HYDA

0 h TAP-S 25%
0 h TAP-S 50%
0 h TAP-S 100%
4 h TAP-S
24 h TAP-S
48 h TAP-S
72 h TAP-S
96 h TAP-S

**Figure 1.** $H_2$ production and hydrogenase expression upon sulphur deprivation in *Chlamydomonas reinhardtii*.
(a) $H_2$ and $O_2$ accumulation, as determined by gas chromatography in the headspaces of the serum bottles. To reach sulphur limitation, the cells were washed five times with TAP media lacking sulphur. The chlorophyll (Chl) content was set at 20 μg Chl($a + b$) ml$^{-1}$, and the cultures were sealed, flushed with $N_2$ and subjected to continuous illumination of approximately 100 μmol photons m$^{-2}$ sec$^{-1}$ at 24°C for 96 h. The quantities of accumulated $H_2$ and $O_2$ were determined every 24 h, followed by $N_2$ flushing.
(b) Relative *HYDA1* transcript level, as determined by qRT-PCR upon sulphur deprivation.
(c) Western blot analysis for HYDA in cultures subjected to sulphur limitation. Samples of 2 μg Chl($a + b$) were loaded and the first to third lanes (25, 50 and 100% of 0-h control) are for the approximate quantitation of the proteins. The 0-h control samples were collected directly after the washing steps with sulphur-free media. Mean values ($\pm$SEMs) are each based on between three and seven biological replicates.

sulphur-free TAP media, the sulphur content, expressed on a DW basis, decreased by about 60%; however, the change was moderate on a cell number and Chl basis (approximately 25% decrease was observed), showing that the cellular sulphur content was only moderately changed.

The concentration of other essential elements, such as Ca and Mn, remained unaltered when expressed on a DW basis, whereas the levels of Fe, K and Zn decreased by approximately 10–25%. Mg and P concentrations show a significant, approximately twofold, increase on a DW basis. When the concentrations of the various elements were expressed on Chl or cell number bases, a very clear increase in their concentration was detected: the most striking increase was observed in the case of Mg and P, both showing an approximately threefold increase in the sulphur-deprived cultures, relative to the controls (Table 1).

Transferring the cells to sulphur-free media resulted in the cessation of cell proliferation (Figure 2a), explaining why the cellular sulphur content did not change drastically. In addition, various adaptive processes are put forward to maintain a steady-state sulphur level in the cell, namely increasing sulphate transporter activity and assimilation (Pootakham *et al.*, 2010; Toepel *et al.*, 2013). On the other hand, the reason for the increase in the concentration of the other elements (Table 1) cannot be easily explained; however, we note that transferring the cells to sulphur-free media results in the increase in the expression of genes encoding phosphate transporters, for instance (Toepel *et al.*, 2013). A similar tendency in concentration changes was observed for Ca, Cu and Fe in the case of maize in a sulfate deprivation experiment (Bouranis *et al.*, 2012).

## The degradation of photosynthetic complexes during sulphur deprivation

The PsbA protein has probably the highest turnover rate among all photosynthetic complexes (reviewed by Järvi *et al.*, 2015), and it is generally supposed that the turnover of PsbA is limited by sulphur availability, responsible for the loss of PSII activity (Zhang *et al.*, 2002; Volgusheva *et al.*, 2013; reviewed by Antal *et al.*, 2015). As shown in Table 1, however, only a moderate, about 25% decrease in sulphur content was observed on a cellular level, which is unlikely to cause substantial PsbA loss upon sulphur deprivation alone. In order to investigate the degradation of PsbA and other photosynthetic complexes in detail, western blot analysis was carried out.

Upon sulphur deprivation, the PsbA content decreased steadily (Figure 3a,b), with about 30% remaining by 96 h of sulphur deprivation. The loss of PsbA occurred in parallel with the loss of PSBO (Figure 3a, c), the major subunit of the OEC. The level of CP43, an inner antenna complex
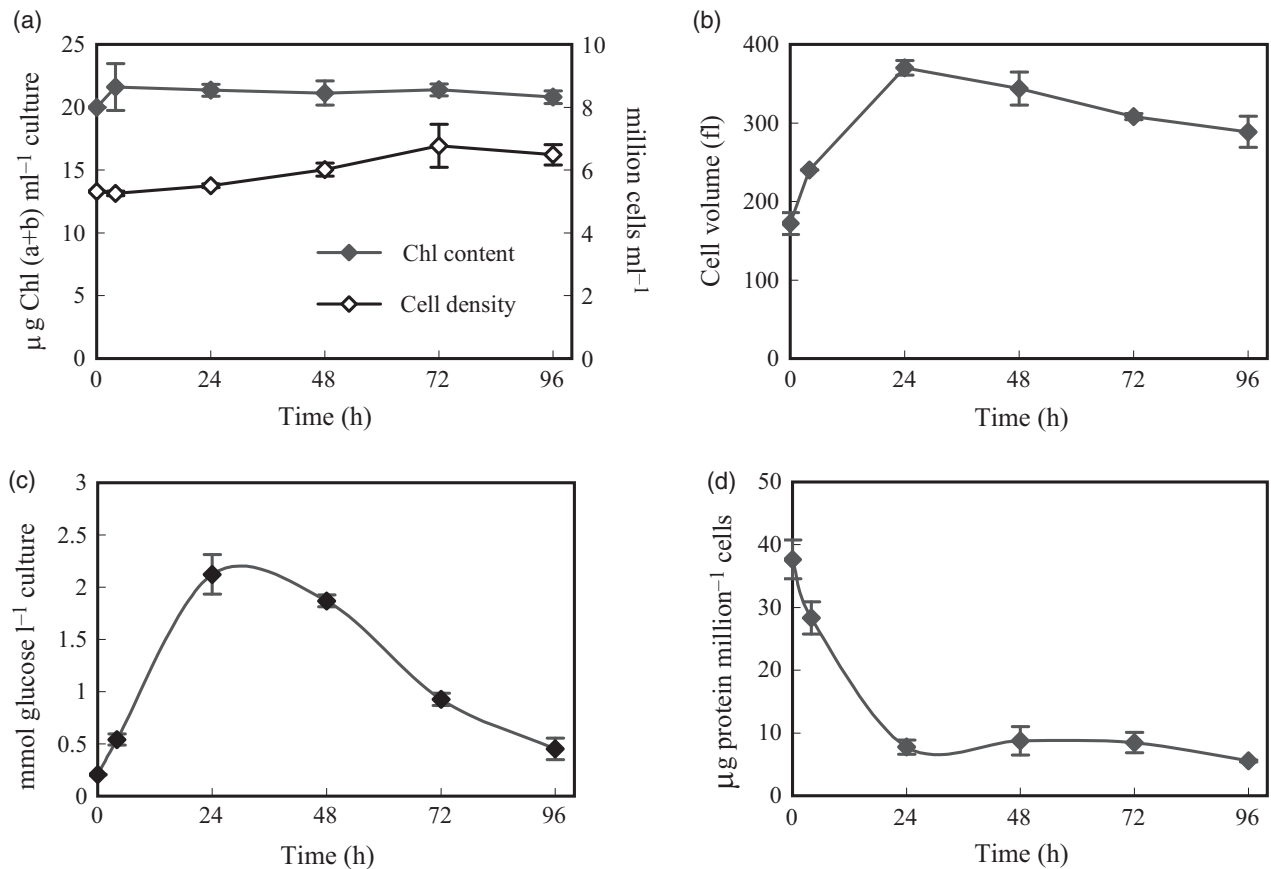
**Figure 2.** The effects of sulphur deprivation on cell density and chlorophyll (Chl) content (a), cell volume (b), starch (c) and protein content (d) in *Chlamydomonas reinhardtii*.
The experimental conditions are identical as described for Figure 1. Mean values (±SEMs) are each based on three biological replicates.

stabilizing PSII (Sugimoto and Takahashi, 2003), decreased rapidly, with a half-life time of less than 24 h (Figure 3a,d).

As expected based on earlier studies, the cytb$_6$f subunit, PetB, and the reaction center core of PSI, PsaA, were better retained (Figure 3a,e,f). The level of RbcL, the large subunit of Rubisco, decreased more rapidly (Figures 3a, g), in line with the literature data indicating that the inactivation of Rubisco is one of the earliest responses to sulphur limitation (Antal *et al.*, 2015).

In the presence of lincomycin (LM), which inhibits the synthesis of chloroplast-encoded proteins, the level of PsbA rapidly dropped (with half-life times of approximately 55 and 17 h in the absence and presence of LM, respectively), whereas the levels of RbcL, PetB, CP43 and PsaA were only moderately decreased upon LM treatment of the sulphur-limited cultures (Figure 3). The variable fluorescence/maximum fluorescence ($F_V/F_M$) parameter, an indicator of PSII activity (reviewed by Schansker *et al.*, 2014), also shows that upon the addition of LM the photosynthetic activity declined more rapidly than in its absence (Figure 3h). These data show that there was a substantial turnover of PsbA upon sulphur deprivation, and it

remained at a relatively high level compared with several other chloroplast-encoded photosynthetic subunits.

PsbA and CP43 were also determined in sulphur-replete cultures (Figure S1). We found that in sulphur-replete cultures approximately 40% of PsbA was retained by 48 h of LM treatment, whereas upon sulphur deprivation, only 12% could be detected at the same time point (Figure 3c). The situation was similar for CP43: in sulphur-replete cultures, approximately 26% of CP43 was retained by 48 h of LM treatment (Figure S1), whereas only 11% was found in the sulphur-limited cultures (Figure 3d). These results show that sulphur limitation leads to an increase in the degradation rates of PsbA and CP43.

### GDP-L-galactose phosphorylase is induced upon sulphur deprivation

In our previous paper, we demonstrated that Asc accumulates drastically upon sulphur deprivation (Nagy *et al.*, 2016). The *VTC2* gene, encoding GDP-L-galactose phosphorylase, plays an essential regulatory role in Asc biosynthesis (Urzica *et al.*, 2012; Vidal-Meireles *et al.*, 2017). Figure 4a shows that there was an approximately

**Table 1** Elemental composition of sulphur-deprived *Chlamydomonas* cultures, as determined by inductively coupled plasma atomic emission spectroscopy (ICP-OES)

| | ng/mg DW | | | $\mu$g/mg Chl($a + b$) | | | ng/million cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | −S (48 h) | P | Control | −S (48 h) | P | Control | −S (48 h) | P |
| S | 3217.83 ± 0.5 | 1236.78 ± 3.4*** | 0.0001 | 576.03 ± 23.9 | 434.86 ± 13.8* | 0.0112 | 2073.67 ± 86.1 | 1530.70 ± 48.6** | 0.0091 |
| Ca | 3649.38 ± 80.0 | 3998.90 ± 458.1 | 0.5979 | 653.86 ± 41.34 | 1407.60 ± 169.2* | 0.0420 | 2353.90 ± 148.8 | 4954.81 ± 595.6* | 0.0441 |
| Cu | 15.44 ± 0.38 | 25.61 ± 1.5* | 0.0138 | 2.77 ± 0.2 | 9.02 ± 0.7** | 0.0078 | 9.96 ± 0.7 | 31.76 ± 2.6** | 0.0076 |
| Fe | 308.85 ± 6.5 | 275.27 ± 6.7* | 0.0429 | 55.3 ± 3.4 | 96.71 ± 2.7** | 0.0025 | 199.20 ± 12.4 | 340.43 ± 9.6** | 0.0028 |
| K | 2607.89 ± 11.7 | 1929.57 ± 138.3* | 0.0321 | 466.75 ± 17.2 | 679.44 ± 58.2 | 0.0684 | 1680.29 ± 62.0 | 2391.61 ± 204.8 | 0.0770 |
| Mg | 2941.29 ± 16.1 | 6448.53 ± 156.3*** | 0.0004 | 526.39 ± 18.9 | 2269.88 ± 119.4** | 0.0015 | 1895.02 ± 68.1 | 7989.99 ± 421.6** | 0.0015 |
| Mn | 239.63 ± 4.1 | 240.50 ± 28.9 | 0.9829 | 42.93 ± 2.5 | 84.04 ± 8.3* | 0.0320 | 154.53 ± 9.0 | 295.81 ± 29.2* | 0.0344 |
| P | 10863.2 ± 203.3 | 20123.51 ± 735.9** | 0.0024 | 1946.10 ± 116.8 | 7088.75 ± 464.1** | 0.0034 | 7005.95 ± 420.7 | 24952.41 ± 1633.6** | 0.0035 |
| Zn | 48.42 ± 0.1 | 37.92 ± 0.7** | 0.0017 | 8.67 ± 0.3 | 13.33 ± 0.5** | 0.0075 | 31.20 ± 1.2 | 46.94 ± 1.8** | 0.0079 |

Averages and standard errors (±SEM) are based on three independent experiments. Statistical significance was analyzed using Student's *t*-test and the significance levels are presented as: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, and the *P* values are also listed.

sevenfold increase in its expression upon sulphur deprivation, correlating well with the increase in Asc content (Figure 4b). Upon stress effects, *VTC2* expression and thereby Asc accumulation are induced both by singlet oxygen ($^1O_2$) and by $H_2O_2$ (Vidal-Meireles *et al.*, 2017), which are produced mostly within PSII and at the acceptor side of PSI, respectively (Asada, 2006; Vass, 2012). To ascertain that oxidative stress occurred upon sulphur deprivation, which may lead to the induction of Asc biosynthesis, we investigated the expression of several genes that are known to respond to $H_2O_2$ and $^1O_2$.

Glutathione peroxidases (GPXs) and transferases are key enzymes in oxidative stress defense: its *Chlamydomonas* homologs GPXH and GSTS1/2 have been shown to respond promptly to $^1O_2$, and on a longer timescale GSTS1 also responds to $H_2O_2$ (Ledford *et al.*, 2007). Figure 5(a,b) shows that *GPXH* and *GSTS1* are strongly induced by 48 h of the experiment, when the *VTC2* transcript abundance is at its maximum (Figure 4a).

The enzymes of the Asc–glutathione cycle play essential roles in ROS detoxification (Asada, 2006). The relative transcript abundance of *APX1* increased significantly (Figure 5c) in parallel with *VTC2* expression. *HSP70A* expression has been shown to respond mainly to $H_2O_2$ stress (Leisinger *et al.*, 2001), and its expression was moderately elevated throughout the experiment (Figure 5d). The relative transcript abundance of the iron-containing superoxide dismutase *FSD1* responds both to $H_2O_2$ and to $^1O_2$ (Leisinger *et al.*, 2001): the 10-fold increase in *FSD1* corroborates the hypothesis that oxidative stress occurs with sulphur deprivation (Figure 5e).

When measuring the level of intracellular $H_2O_2$, we found a steady decrease throughout the experiment (Figure 5f). This can be explained by the reduced photosynthetic electron flow to PSI upon sulphur deprivation (Wykoff *et al.*, 1998), resulting in less superoxide and $H_2O_2$ production.

On the other hand, singlet oxygen is more likely to accumulate upon sulphur deprivation, because initially the rate of PSII excitation exceeds the rate of linear electron transport (Antal *et al.*, 2015). $^1O_2$ is a short-lived species: therefore, quantifying its level *in situ* is a challenging task. The rate of $^1O_2$ production can be measured in cyanobacteria in the presence of histidine, an efficient $^1O_2$ quencher, resulting in $O_2$ uptake (Ur-Rehman *et al.*, 2013); however, this method is not established for green algae, possibly because of insufficient histidine uptake.

### The decline of PSII activity may involve donor-side-induced photoinhibition

In our previous paper we suggested that Asc, at a high concentration reached after sulphur deprivation, is capable of over-reducing the Mn cluster (Nagy *et al.*, 2016). Once the OEC is inactive, Asc may provide electrons to Tyr$_Z^+$ at a slow rate, which cannot fully prevent the accumulation of
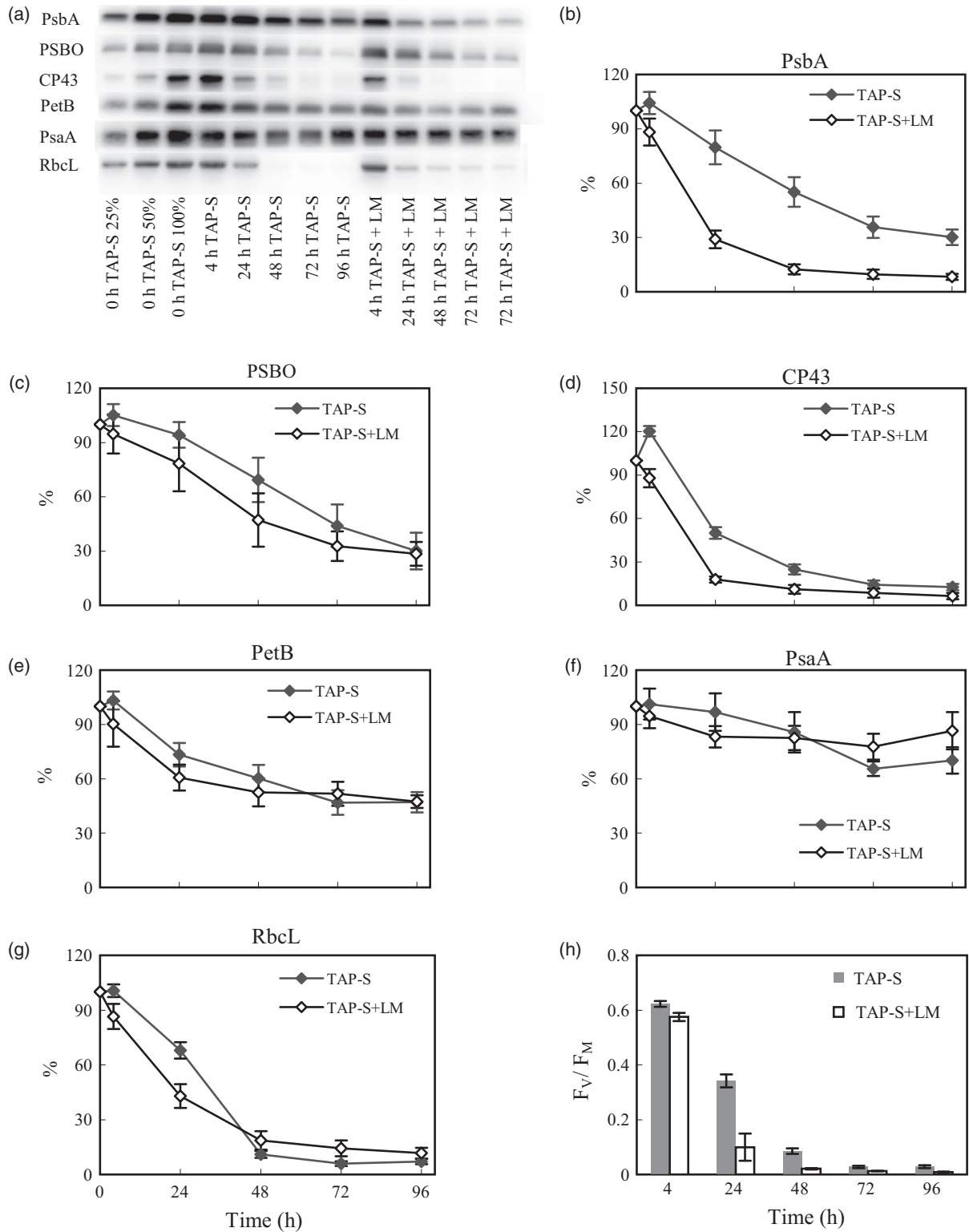
**Figure 3.** Degradation of photosynthetic complexes during sulphur deprivation in *Chlamydomonas reinhardtii*.
(a) Western blot analysis for the semi-quantitative determination of PsbA, PSBO, CP43, PetB, PsaA and RbcL contents in sulphur-limited *C. reinhardtii* cultures, in the presence and absence of lincomycin (LM), added following the transfer of cells to sulphur-free TAP media. Samples of 2 μg Chl(*a* + *b*) were loaded and the first to third lanes (25, 50 and 100% of 0-h control) are for the approximate quantitation of the proteins. The 0-h control samples were collected directly after the transfer of cells to sulphur-free TAP media. Densitometry analysis: (b) PsbA; (c) PSBO; (d) CP43; (e) PetB; (f) PsaA; and (g) RbcL. (h) Changes of $F_V/F_M$ in sulphur-limited cultures in the presence and absence of LM. Mean values (±SEMs) are each based on between three and six biological replicates.
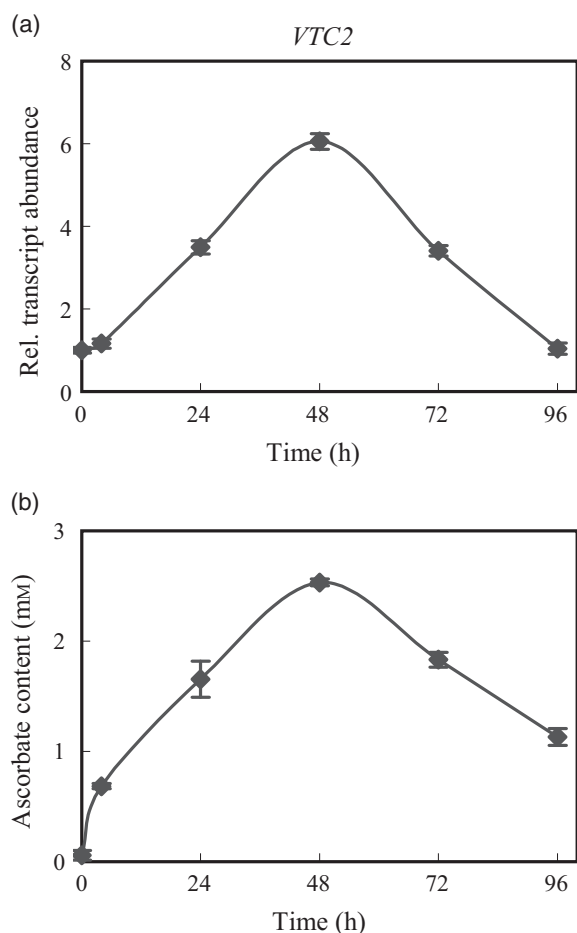
**Figure 4.** Ascorbate (Asc) accumulation upon sulphur deprivation in *Chlamydomonas reinhardtii*.
(a) Transcript level of *VTC2* as determined by real-time qPCR upon sulphur deprivation. (b) Changes in cellular Asc content in *C. reinhardtii* subjected to sulphur limitation. Mean values (±SEMs) are each based on between three and six biological replicates.

strongly oxidizing species in PSII. This so-called donor-side-induced photoinhibition process results in the losses of PsbA, PSBO, CP43 and other components of PSII (Blubaugh *et al.*, 1991; Jegerschöld and Styring, 1996; Arató *et al.*, 2004; Tóth *et al.*, 2011). We note that donor-side-induced photoinhibition has been shown to occur *in vivo* in leaves with heat-inactivated OECs (Tóth *et al.*, 2009, 2011), and it may also occur upon UV-B stress (Mano *et al.*, 2004). The donor-side-induced photoinhibition caused by excessive Asc accumulation was investigated here in detail.

When 20 mM Asc was added to sulphur-replete *Chlamydomonas* cells and the cultures were incubated for 30 min in light, the intensity of the B thermoluminesence band ($S_1S_2$-$Q_B^-$ recombination; Ducruet and Vass, 2009) decreased by approximately 30% (Figure S2a), confirming that Asc, when present in the mM range, over-reduces the

Mn cluster in *Chlamydomonas* (Nagy *et al.*, 2016). As also shown in our previous paper, there is a continuous decrease in the amplitude of the B thermoluminescence band during sulphur deprivation, and within 48 h the B band is lost (Figure S2b).

In order to obtain direct evidence for donor-side-induced photoinhibition, we measured oxygen uptake on isolated thylakoid membranes of *C. reinhardtii* in the presence of methylviologen (MV), as a terminal electron acceptor at PSI (Izawa, 1980) and diphenylcarbazide (DPC), an efficient electron donor to $Tyr_z^+$ (Rashid and Popovic, 1995). As Asc is lost during the thylakoid isolation process (Ivanov and Edwards, 2000), and DPC can supply electrons to PSII without damaging the active OECs, and at a high rate (Rashid and Popovic, 1995), the presence of PSII reaction centers with inactive OECs can be assessed by this approach.

Figure 6(a) shows that upon sulphur deprivation the rate of MV-dependent $O_2$ uptake steadily decreased, and by 48–60 h a minimum was reached, which coincides with the complete loss of the B thermoluminescence band (Figure S2b), suggesting that the number of PSII units with active OECs strongly decreased.

In the presence of DPC, which supplies electrons to PSII without damaging it, the rate of $O_2$ uptake was higher than in its absence, demonstrating the existence of PSII reaction centers, which are capable of charge separations but lack $O_2$-evolving activity. In untreated, sulphur-replete cells, the addition of DPC resulted in a 30% increase in $O_2$ uptake (Figure 6b), which might be caused by a partial loss of OEC activity upon thylakoid isolation, and/or the presence of PSII reaction centers without OECs *in vivo*. Upon sulphur deprivation, DPC-dependent $O_2$ uptake increased considerably relative to the control, by a maximum of about 100% (Figure 6b) at the 48 h time point, when the B band was already absent (Figure S2b). These results demonstrate that the number of PSII reaction centers without active OECs, relative to fully active PSII units, increased upon sulphur deprivation, supporting our hypothesis that upon sulphur deprivation the inactivation of PSII involves donor-side-induced photoinhibition.

## DISCUSSION

It has been assumed for many years that PsbA turnover is limited upon sulphur starvation, and that it is the simple reason for a decline in PSII activity (e.g. Wykoff *et al.*, 1998; Volgusheva *et al.*, 2013; reviewed by Antal *et al.*, 2015); however, several lines of evidence found in the literature, and presented in this paper, show that the decrease in PSII activity is caused by a more complex phenomenon.

When transferring the cells to sulphur-free media, the transcript abundances of a large number of nuclear-encoded photosynthetic complexes decrease within a few hours, including those of the majority of PSI and PSII light-harvesting complexes, subunits of Rubisco, cytb$_6$f complex
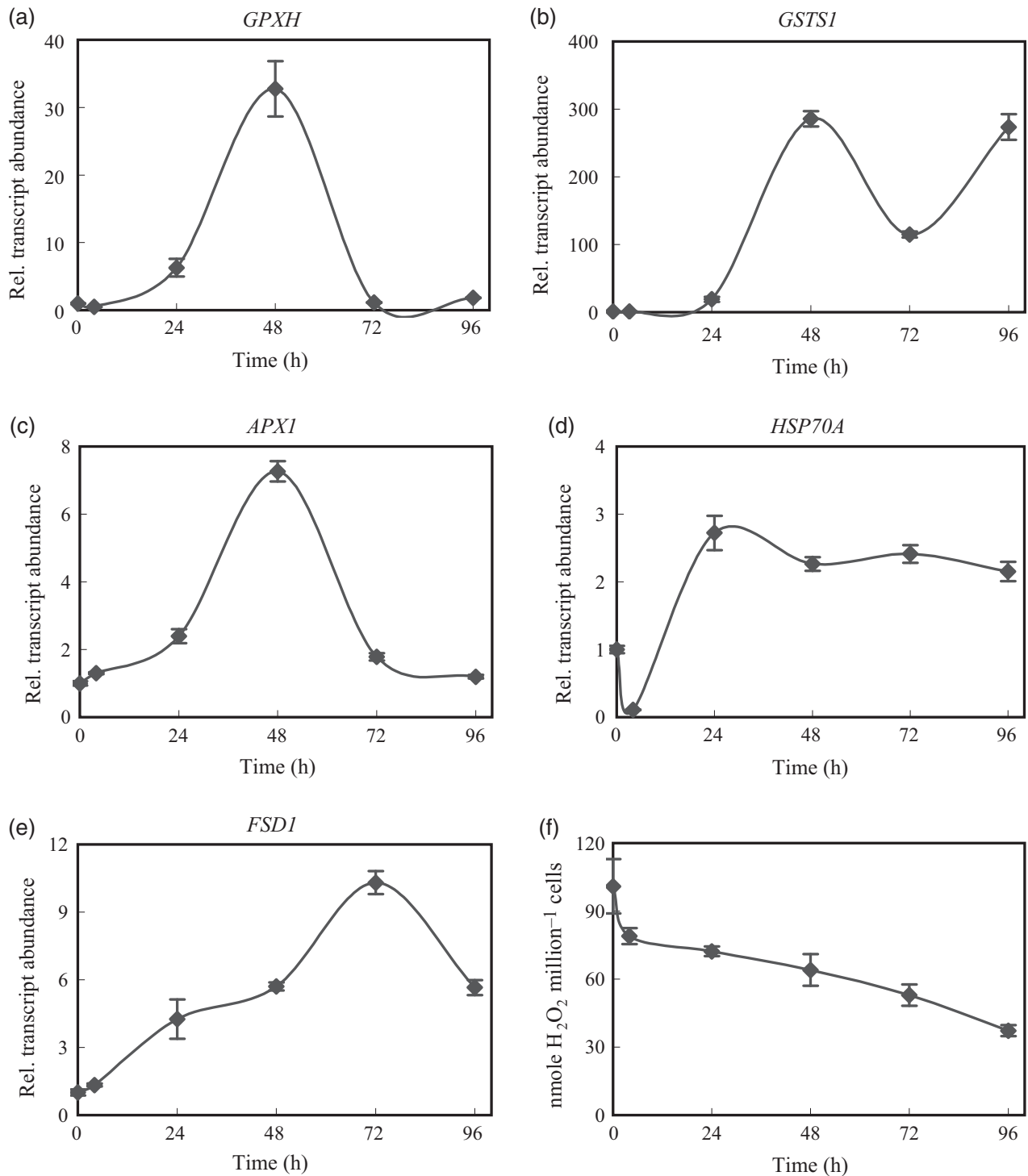
**Figure 5.** Oxidative stress induced by sulphur limitation in *Chlamydomonas reinhardtii*.
(a) Transcript level of the reactive oxygen species-responsive gene *GPXH*, as determined by real-time qPCR upon sulphur deprivation; (b) *GSTS1*; (c) *APX1*; (d) *HSP70A*; and (e) *FSD1*. (f) Changes in $H_2O_2$ content upon sulphur limitation in *C. reinhardtii*. Mean values ($\pm$SEMs) are each based on three biological replicates.

and PSII subunits, including the extrinsic OEC proteins (Nguyen *et al.*, 2011; Toepel *et al.*, 2013). Concomitantly, the transcript abundances of special LHCs, namely LHCSR and LHCBM9, are strongly increased, resulting in the remodeling of light-harvesting supercomplexes (Grewe *et al.*, 2014). Thereupon transferring cells to sulphur-free media, cell division and Chl biosynthesis also cease, and the cell size is doubled, with a concomitant accumulation
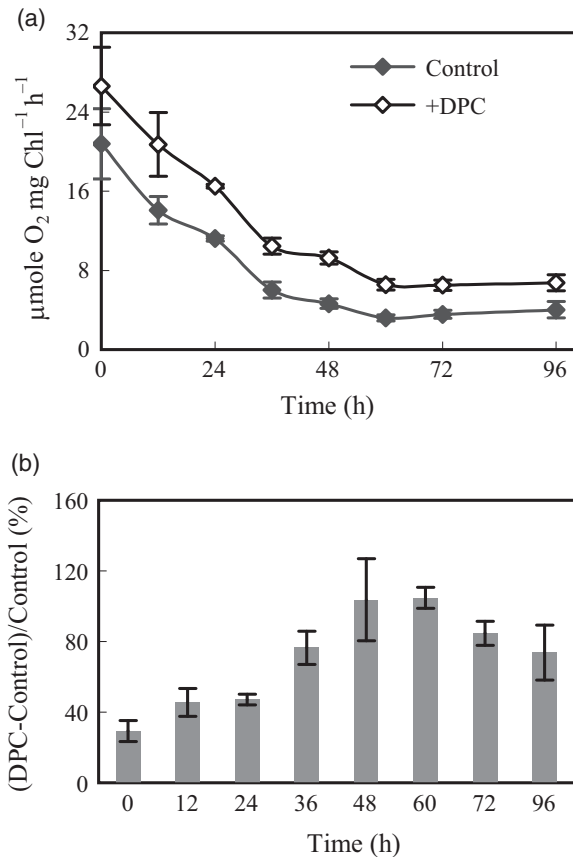
**Figure 6.** Changes in the rate of methylviologen-dependent $O_2$ uptake upon sulphur deprivation of *Chlamydomonas reinhardtii*, as measured in the presence and absence of the artificial photosystem II electron donor, diphenylcarbazide (DPC).
(a) Rates of $O_2$ uptake. (b) Ratio of $O_2$ uptake rates measured in the presence and absence of DPC. Mean values (±SEMs) are each based on three biological replicates.

of starch, which is enabled by the initially active Calvin–Benson–Bassham (CBB) cycle.

As shown by our ICP-OES measurements, 48 h after transferring the cells to sulphur-free media, the sulphur content on a cell number or Chl basis decreases only by about 25% (Table 1). In addition, sulphur reserves, mostly in the form of sulfoquinovosyl diacylglycerol (SQDG), are mobilized upon sulphur deprivation (Sugimoto *et al.*, 2007, 2010). Based on these findings, therefore, it is very likely that the quantity of sulphur per photosynthetic reaction center decreases only moderately, and may not substantially hinder the turnover of PsbA (some minor effect cannot be excluded, however). Our results presented in Figure 3 show that PsbA has a remarkable turnover, and its level decreases more slowly than that of RbcL and CP43, which are both known to possess a lower turnover rate than PsbA under normal conditions (Nelson *et al.*, 2014). In addition, the LM treatments also show that the rate of PsbA degradation is increased upon sulphur

deprivation (Figures 3 and S1a), whereas its resynthesis is not necessarily affected.

The loss of PsbA, therefore, seems to be a complex phenomenon. As mentioned above, gene expression patterns are changed within hours upon transferring *Chlamydomonas* cells to sulphur-free medium, well before the actual damaging effects of sulphur limitation occur. One of the most remarkable effects is the loss of CBB cycle activity, which seem to be a regulated process, because in addition to the remarkable decrease in the expression of the small Rubisco subunits (Toepel *et al.*, 2013), a very rapid loss of RbcL occurs (Figure 3), which, under normal conditions, has a slow turnover (Nelson *et al.*, 2014). During this initial stage of sulphur deprivation (approximately the first 24 h), there is some CBB cycle activity, which enables starch accumulation; however, the decrease of the CBB cycle activity leads to a slowdown of linear electron transport and overexcitation of PSII. We observed a decrease in cellular $H_2O_2$ content instead of an increase, but our gene expression data show that $^1O_2$ is very likely to accumulate (Figure 5), which may damage the photosynthetic apparatus. On the other hand, the transcript abundance of the *VTC2* gene, which plays an important regulatory role in Asc biosynthesis in *C. reinhardtii* (Urzica *et al.*, 2012; Vidal-Meireles *et al.*, 2017), is also increased (Figure 4a), probably in response to $^1O_2$ production, and Asc accumulates to the mm range (Figure 4b). Ascorbate is generally regarded as an ROS scavenger (Foyer and Shigeoka, 2011), and it may also play this role upon sulphur deprivation; however, our data presented in this and in our previous paper (Nagy *et al.*, 2016) show that when present in the mm range, Asc may contribute to the inactivation of PSII by over-reducing the Mn cluster in *C. reinhardtii*.

When considering the physiological roles of Asc, it has to be taken into account that it is a reductant, and therefore its cellular concentration is to be maintained in a certain range and there are substantial differences among species in this respect (Tóth *et al.*, 2017). The basal Asc concentration in green algae is very low compared with higher plants (approximately 60 μm was detected here, whereas the cellular Asc concentration is about 5 mm in non-stressed plants; Zechmann *et al.*, 2011). The regulation of Asc biosynthesis also differs largely: in higher plants, it is induced rather slowly upon stress effects, and it is regulated both by the circadian clock and by the photosynthetic electron transport chain. In contrast, in *Chlamydomonas*, Asc biosynthesis is rapidly induced by light and oxidative stresses, whereas the photosynthetic electron transport chain and the circadian clock do not have a direct regulatory role; additionally, and in contrast to higher plants, Asc biosynthesis is regulated by a feed-forward mechanism in green algae (Tóth *et al.*, 2017; Vidal-Meireles *et al.*, 2017).

As shown in Figure S2 and in Nagy *et al.* (2016) Asc inactivates the OEC in green algae when present in the mm

range; this does not occur in higher plants, in spite the fact that the Asc concentration is about 10 to 20 mM in their chloroplasts (Zechmann *et al.*, 2011). The reason for this important difference is not yet clear, although the structural differences between the extrinsic OEC proteins in higher plants and green algae may be an important factor (Tóth *et al.*, 2017).

Once the OEC is inactivated, Asc may continuously provide electrons to $Tyr_Z^+$. The electron donation by Asc to PSII is rather slow, however: depending on the concentration, it occurs with a half-life time of approximately 20–100 ms (Tóth *et al.*, 2009). For this reason Asc cannot fully prevent the accumulation of $Tyr_Z^+$ and $P680^+$ upon illumination. The presence of these strongly oxidizing species leads to donor-side-induced photoinhibition, resulting in the relatively rapid degradation of PSII reaction center proteins, including PsbA, PSBO, CP43 (Figure 3) and possibly several more subunits. Upon sulphur deprivation, the expression level of the nuclear-encoded PSBO subunit, which normally possesses a relatively long lifetime (Nelson *et al.*, 2014), is also downregulated (Toepel *et al.*, 2013), possibly contributing to the decrease in PSII activity. The rapid loss of CP43 was unexpected; in the absence of this protein, PSII is destabilized (Sugimoto and Takahashi, 2003), which may also contribute to the loss of photosynthetic activity.

The loss of PSII activity could be regarded as a deleterious effect of sulphur deprivation; however, it should be considered that upon the downregulation of PSII activity, overexcitation and further photodamage are minimized. The importance of this process is best demonstrated by the *sac1* and *snrk2.1* mutants, which are unable to downregulate their photosynthetic activity upon sulphur deprivation, resulting in increased $^1O_2$ production and cell death on a timescale of days (Davies *et al.*, 1996; Gonzalez-Ballester *et al.*, 2008). Altogether, the metabolic changes downregulating cell proliferation and photosynthetic activity may serve to maintain the cellular sulphur content at a certain level, and to avoid any more substantial damage.

Excess excitation energy may also be dissipated at the acceptor side of PSI, via various $O_2$-dependent mechanisms, including the Mehler reaction, the malate shuttle, the plastid terminal oxidase and the flavodiiron-dependent photoreduction pathways (reviewed by Erickson *et al.*, 2015; Curien *et al.*, 2016); however, under hypoxic conditions, commonly occurring in the habitats of green algae, electron transport to the hydrogenases may also represent a safety valve (Godaux *et al.*, 2016) instead of the $O_2$-dependent pathways.

In summary, the responses to sulphur limitation in *Chlamydomonas* include: (i) maximizing the uptake of sulphur from the environment; (ii) minimizing the decrease in cellular sulphur content by halting cell division; (iii) with cell division halted, there is an excess of reductants that, in the beginning, will be used for starch biosynthesis; (iv) with the overexcitation of PSII, $^1O_2$ molecules are produced, which trigger Asc biosynthesis; (v) Asc accumulating to the mM concentration range may contribute to the inactivation of the OECs; (vi) thereby, a large proportion of PSII reaction centers also become inactive and are degraded; and (vii) the decrease in $O_2$ evolution contributes to the establishment of hypoxia enabling hydrogenase expression, and $H_2$ production will act as a safety valve of the photosynthetic electron transport. By this means, the damage imposed by sulphur limitation is minimized and the cells may recover if sulphur becomes available again (Kim *et al.*, 2010).

It is important to note that $H_2$ production does not necessarily occur or become significant upon sulphur deprivation: for instance, in the absence of acetate, no or little starch accumulation occurs, which would be necessary for the establishment of hypoxia and hydrogenase expression (Chochois *et al.*, 2009). Under such conditions, it is more likely that other, $O_2$-dependent protective mechanisms are involved in the dissipation of excess energy, and it is not yet known whether the inactivation of PSII occurs in a similar manner as described here.

Sulphur deprivation has been a method of choice for many years to induce $H_2$ production in *C. reinhardtii*, aiming at industrial application (e.g. Zhang *et al.*, 2002); however, as outlined above, this type of $H_2$ production involves the complete remodeling of cellular metabolism, including the degradation of the photosynthetic apparatus and, in addition, it is also largely dependent on acetate as an organic carbon source. For these reasons, other, alternative methods, are preferred that may allow the industrial exploitation of the $H_2$ production capacity of *C. reinhardtii* in the future.

## EXPERIMENTAL PROCEDURES

### Algal growth conditions and sulphur deprivation

*Chlamydomonas reinhardtii* CC124 cultures were grown in Tris-acetate-phosphate (TAP) medium with a light intensity of 100 μmol photons $m^{-2}$ $sec^{-1}$ at 21°C–23°C in an algal growth chamber. The 250-ml flasks containing 50 ml of TAP medium were shaken at 120 rpm, and the cultures were grown for 3 days in sulphur-containing medium.

After 3 days of cultivation, the cells were washed five times with sulphur-free TAP medium (centrifugation at 1000 **g**, at 18°C for 5 min). For the $H_2$ production experiments the Chl content was set at 20 μg Chl (a+b) $ml^{-1}$ (based on Porra *et al.*, 1989), and 30 ml of culture in sulphur-free TAP medium was placed into 125-ml serum vials and sealed off with rubber septa. All steps were carried out under sterile conditions. The gas phase of the bottle was flushed with $N_2$ gas for 10 min and the cultures were kept in the algal growth chamber, under the same conditions as indicated above.

### Determination of $H_2$ and $O_2$ accumulation by gas chromatography (GC)

The net quantities of $H_2$ and $O_2$ produced by the cells were determined by taking a 250-μl aliquot from the gas phase of the

cultures with a gas-tight microsyringe. These samples were injected manually into an Agilent 6890N gas chromatograph (GC) equipped with an HP-PLOT Molesieve 5-Å column (30 m × 0.53 mm × 0.25 μm) and a TCD detector (Agilent, https://www.agilent.com). The oven temperature was 30°C. The carrier gas was argon, and a linear velocity of 115 cm s$^{-1}$ was used. In order to prevent the accumulation of H$_2$ above the critical 5% level in the gas phase (Kosourov *et al.*, 2012), and overpressure, the bottles were flushed with N$_2$ gas every 24 h following the determination of gas production.

### RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) analysis

For RNA isolation, 1 ml of culture was collected and the Direct-Zol RNA kit was used, following the recommendations of the manufacturer (ZymoResearch, https://www.zymoresearch.com). To remove contaminating DNA from the samples, the isolated RNA was treated with DNaseI (ZymoResearch). RNA integrity was checked on a 1% (w/v) 3-(*N*-morpholino)propanesulfonic acid (MOPS) gel. Reverse transcription was primed with oligo dT using 1 μg of total RNA and SuperScript III reverse transcriptase (Life Technologies, now ThermoFisher Scientific, https://www.thermofisher.com). To confirm the absence of DNA contamination, an aliquot of the RNA sample was used without reverse transcriptase.

Real-time qPCR analysis was performed using an Applied Biosystems (now ThermoFisher Scientific, https://www.thermofisher.com) Prism 7900HT Fast Real Time PCR System using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, https://www.sbd.ee). Primers for real-time qPCR analysis were designed using the NCBI Primer Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The melting temperature was set to 60°C, and the amplicon length was set between 100 and 130 bp. To ensure the correct normalization of the transcript levels, we tested the expression levels of several potential reference genes. The three reference genes showing the most stable expression during H$_2$ production were bTUB2 (Cre12.g549550), ACTIN (Cre13.g603700) and UBQ (XP_001694320). The primer pairs for the target genes can be found in Table S1; for the reference genes, see Vidal-Meireles *et al.* (2017). The data are presented as fold-change in mRNA transcript abundance of the target gene, normalized to the average of the three reference genes, and relative to the control sample (cultures grown in TAP medium under normal growth conditions). Real-time qPCR analysis was carried out with three technical replicates for each sample and three biological replicates were measured; the standard error was calculated based on the range of fold-change by calculating the minimum and the maximum of the fold-change using the standard deviations of $\Delta\Delta C_t$.

### Western blot analysis and protein content determination

At each time point, 1 ml of culture was collected, spun-down for removal of the supernatant and frozen in liquid nitrogen. The samples were then solubilized with 370 μl of protein extraction buffer (50 mM Tris/HCl, pH 8.3, 0.25% Triton X-100, 1 mM dithiothreitol and cOmplete Protease Inhibitor Cocktail; Roche, https://www.roche.com), sonicated for 30 sec, incubated in the dark at 4°C for 30 min with vigorous shaking, and then centrifuged at 4700 *g* for 4 min at 4°C. The supernatant was collected into a new Eppendorf tube and the Chl(*a* + *b*) and the protein content of the extract was determined (using the Bio-Rad Protein Assay Kit, according to the recommendations of the manufacturer; Bio-Rad, http://www.bio-rad.com). A quantity equivalent to 2 μg Chl(*a* + *b*) was then mixed with double strength Laemmli buffer and incubated at 75°C for 10 min before loading onto the gel. Proteins separated by SDS-

PAGE (Perfect Blue Twin Gel System; Peqlab, https://www.vwr.com/) were transferred to a polyvinylidene difluoride membrane (Hybond P) using a tank blotting system (Cleaver Scientific Ltd, http://www.cleaverscientific.com). Specific polyclonal antibodies (produced in rabbits) against HydA, PetB, PsbA, PsaA, PSBO, CP43 and RbcL were purchased from Agrisera AB (http://www.agrisera.com). As secondary antibody, a goat anti-rabbit IgG horseradish peroxidase conjugate was used (Bio-Rad). Immunochemical detection was carried out with the ECL Prime System (GE Healthcare, http://www.gehealthcare.com), according to the instructions of the manufacturer.

### Determination of cell size and cell density

The cell density was determined by a Scepter™ 2.0 hand-held cell counter (Millipore, http://www.merckmillipore.com), which also allows a precise determination of cell sizes.

### Determination of starch content

A 2-ml volume of culture containing 40 μg Chl was collected at each time point and spun down (12 000 *g*, 4 min). After re-suspending the pellet twice in 1 ml of methanol (to solubilize the pigments), the samples were washed twice with 1 ml of sodium acetate buffer (0.1 M, pH 4.5), and spun down again. The pellet was then re-suspended in 0.6 ml of sodium acetate buffer and submitted to two sonication cycles of 30 sec at 40% amplitude using a probe with a 3.2-mm tip diameter (EpiShear™ Probe Sonicator; Active Motif, https://www.activemotif.com), after which an additional 0.4 ml of sodium acetate buffer was added. A volume of 1 ml of the sample was incubated in a boiling water bath for 15 min, and after cooling 3 U amyloglucosidase were added and the starch was hydrolysed overnight at 55°C. The glucose content in the sample was measured enzymatically with Fluitest® GLU kit (Analyticon® Biotechnologies AG, https://www.analyticon-diagnostics.com).

### Element determination

An algal sample of 30 mg DW was digested with 10 ml HNO$_3$ of 67% in a microwave sample preparation system (Anton Paar, https://www.anton-paar.com). After digestion, the sample was diluted to 20 ml with high-purity water (1/8.2 MΩ) in which the concentration of various elements (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) was determined by ICP-OES (Spectro Genesis, https://www.spectro.com), according to the method described by Szentmihályi *et al.* (2015). S, P and multi-element standards were used (CPAchem, https://www.cpachem.com) for standardization and the calculation of concentrations.

### Chl *a* fluorescence measurements

Chl *a* fluorescence measurements were carried out at room temperature between 21 and 24°C with a Handy-PEA instrument (Hansatech Instruments Ltd, http://www.hansatech-instruments.com). Cultures of *C. reinhardtii* were dark-adapted for 15 min and then 5 ml of cell suspension (20 μg Chl ml$^{-1}$) was filtered onto a Whatman glass microfibre filter (GF/C) that was placed in a Handy-PEA leaf clip. The algal sample was illuminated with continuous red light (3500 μmol photons m$^{-2}$ sec$^{-1}$, 650-nm peak wavelength; spectral half-width of 22 nm; light emitted by the LEDs is cut off at 700 nm by an NIR short-pass filter). The light was provided by an array of three light-emitting diodes focused on a circle of sample surface of 5 mm in diameter. The first reliably measured point of the fluorescence transient is at 20 μs, which was taken as $F_0$. The length of the measurements was 5 sec.

## Ascorbate content determination

The ascorbate content determination was carried out according to the method described by Kovács *et al.* (2016).

## $H_2O_2$ content determination

For $H_2O_2$ determination, 2 ml of *Chlamydomonas* culture, equivalent to 40 μg Chl($a + b$) was harvested by centrifugation at 12 000 $g$ for 4 min. Cells were resuspended in 200 μl 0.1% trichloroacetic acid solution, and were ground with 200 μl of glass beads (212–300 μm in diamater) at maximum speed for 10 min at 4°C using a Bullet Blender Gold cell distructor (Next Advance, Inc., https://www.nextadvance.com). After homogenisation, 800 μl of 0.1% TCA was added. The cell debris and glass beads were spun down at 21 130 $g$ for 10 min at 4°C. An aliquot of 600 μl was taken from the supernatant and filtered with a 4-mm, 0.22-μm pore sized hydrophilic polytetrafluoroethylene (PTFE) syringe filter. The supernatant was supplemented with 25 mM potassium phosphate buffer (pH 8.0), 0.1 unit $ml^{-1}$ horseradish peroxidase and 50 μM Amplex Red reagent, and incubated at 25°C for 30 min. The accumulation of resorufin was determined fluorometrically at 565/580 nm (Fluoroskan Ascent FL; ThermoFisher Scientific). The cellular $H_2O_2$ content was calculated based on a standard curve, cell numbers and cell sizes.

## Thylakoid isolation

*Chlamydomonas* cell culture equivalent to 1.2 mg Chl($a + b$) were distributed into four 15-ml centrifuge tubes and spun down at 2990 $g$ for 3 min. The pelleted cells were resuspended in 200 μl of homogenisation buffer (50 mM Tricine, pH 7.8, 5 mM $MgCl_2$ and 10 mM NaCl, supplemented with 0.1% BSA prior to isolation). The suspensions were transferred to 1.5 ml microcentrifuge tubes and 200 μl of glass beads, 212–300 μm in diameter, were added into each tube. The cells were ground by Bullet blender Gold cell disruptor (Next Advance, Inc.) at maximum speed for 10 min at 4°C. The glass beads were washed three times by 1 ml homogenisation buffer. The pooled samples were centrifuged at 2990 g for 3 min at 4°C. The supernatant was diluted to 50 ml with homogenisation buffer, and was centrifuged at 30 000 $g$ for 10 min at 4°C. The pellet was resuspended in 250 μl homogenisation buffer without BSA. The isolated thylakoid membranes were frozen in liquid $N_2$ and stored at −80°C until use.

## Measurement of electron transport activity

The photosynthetic electron transport rate of isolated thylakoid membrane was measured as oxygen uptake in the presence of methylviologen (MV), an artificial electon acceptor of PSI (Izawa, 1980), using a Clark-type oxygen electrode (Hansatech Instruments). The isolated thylakoid membranes were diluted to 50 μg Chl $ml^{-1}$ in homogenisation buffer, and 100 μM MV and 2 mM sodium azide was added prior to the measurement. To assess the photosynthetic activity of PSII reaction centers with impaired OECs, besides MV and $NaN_3$, 2 mM of diphenyl carbazide (DPC) was added as an exogenous electron donor. A 1-ml volume of sample was illuminated with white light from a halogen cold-light source (KL2500 LCD; Schott AG, http://www.schott.com) at a photon flux density of 500 μmol $m^{-2}$ $sec^{-1}$ at ambient temperature for 6 min, and the slope of the oxygen uptake curve was determined by linear fitting. Net DPC-dependent photosynthetic activity was calculated based on the difference in oxygen uptake rates measured in the presence or absence of DPC.

## Thermoluminescence (TL) measurements

Thermoluminescence measurements were carried out using a custom-made TL apparatus, similar to the one described in Ducruet and Vass (2009). Cell suspension [300 μl, 20 μg Chl($a + b$) $ml^{-1}$] was placed on a copper sample holder, connected to a cold finger immersed in liquid $N_2$. A heater coil (model SEI 10/50; Thermocoax, http://www.thermocoax.com), placed under the sample holder, ensured the desired temperature of the sample during the measurement. Dark-adapted samples were illuminated at 4°C with a single-turnover flash provided by a stroboslave, and the sample was heated to 70°C in darkness with a heating rate of 20°C $min^{-1}$. The emitted TL was measured with an end-window photomultiplier (model H10721-20; Hamamatsu, http://www.hamamatsu.com) simultaneously with recording the temperature. When applicable, 20 mM Asc was added to the samples 30 min prior to the measurements.

## Statistics

The presented data are based on at least three independent experiments. When applicable, averages and standard errors (±SEM) were calculated. Statistical significance was analysed using Student's *t*-test and the significance levels are presented as: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.
**Figure S1.** Western blot (a) and its densitometry analysis (b) for PsbA and CP43 (c) in sealed, sulphur-replete *C. reinhardtii* cultures, in the presence or absence of lincomycin (LM).
**Figure S2.** Thermoluminescence (TL) emission of *C. reinhardtii* upon excitation with one single turnover flash at 4°C.
**Table S1.** Oligonucleotide primers for RT-qPCR analysis.

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