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The mechanism of photosystem II inactivation during sulphur deprivation-induced H_2 production in *Chlamydomonas reinhardtii*

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Abstract *238 words, max 250*

Sulphur limitation may restrain cell growth and viability. In the green alga, *Chlamydomonas reinhardtii*, sulphur limitation is of particular interest, because it may induce H_2 production lasting for several days, to be exploited as a renewable energy source. Sulphur limitation leads to a large number of physiological changes, including the inactivation of the O_2-evolving photosystem II (PSII), leading to the establishment of hypoxia, essential for the induction of hydrogenase expression and activity. The degradation of PSII has been long 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 the *Chlamydomonas* cells to sulphur-free media, the amount of cellular sulphur content decreases only by about 25%, ii) as demonstrated by lincomycin treatments, PsbA has a significant turnover and the photosynthetic subunits RbcL and CP43 are degraded more rapidly than PsbA. On the other hand, sulphur limitation imposes oxidative stress, most probably via the formation of $^1O_2$ 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 inactivates the oxygen-evolving complex and may provide electrons to PSII at a relatively slow rate, and finally, the reaction centers get inactivated and degraded. Our data demonstrate that the inactivation of PSII is a complex, highly regulated process, which may mitigate the damaging effects of sulphur deprivation.

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 by specific transporters in the form of sulphur anion (SO_4^-, the most oxidized form of sulphur), and within the cell, it is activated by ATP sulphurylase to form adenosine 5'phosphosulfate (APS), and 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 cofactors (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*, is particularly relevant from a biotechnological point of view, because it may lead to the induction of H_2 production, lasting for several days (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 alteration in cell wall structure (Takahashi et al., 2001). Besides, sulphur deprivation arrests chlorophyll (Chl) biosynthesis, leads to strong starch accumulation and sulfolipid degradation and phosphatidylglycerol accumulation (Sugimoto et al., 2007; Sugimoto et al., 2008). A characteristic effect is the decline in the expression of a large number of photosynthetic genes, encoding subunits of photosystem II (PSII), photosystem I (PSI) 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 the 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, located at 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; altough starch degradation may also contribute to H_2 production significantly, not only by providing reductants, but also by promoting the establishment of hypoxia (REF). The H_2 producing period typically lasts for 4 to 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 downregulation 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 due to 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 (reviewed by Järvi et al., 2015). However, there is a number of indications in the literature that the decrease of PSII activity is a controlled process. It has been

shown that the *sac1* and *snrk2.1* mutants unable to downregulate their photosynthetic activity die more rapidly than the wild-type (Davies et al., 1996, González-Ballester et al., 2008), which is possibly due to an ongoing 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 have a regulatory role in the initiation of H_2 production. Here we investigate the mechanism of PSII inactivation upon sulphur deprivation in detail and demonstrate that instead of a sulphur-limited PsbA turnover, PSII inactivation is initiated by donor-side induced inhibition imposed by a strong Asc accumulation, most probably in response to increased $^1O_2$ production.

Results

Hydrogenase expression and H_2 production upon sulphur deprivation

The sulphur deprivation experiments were carried out on the most commonly used CC124 strain of *Chlamydomonas reinhardtii*, which is 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 several times with TAP media lacking sulphur. The chlorophyll (Chl) content was set at 20 µg Chl (a+b)/ml, and the cultures were sealed and subjected to continuous illumination of approx. 100 µmole photons $m^{-2}s^{-1}$ at 24°C for 96 hrs. The amounts of accumulated H_2 and O_2 were determined every 24 hrs by gas chromatography (GC), followed by N_2 flushing in to order prevent the accumulation of H_2, which may limit H_2 production (Kosorouv et al., 2012).

A substantial amount of H_2 was detected in the 48th hr of sulphur deprivation, and only a small amount of O_2 was present in the headspace of the serum bottles (Fig. 1A). In total, about 250 µl H_2/ml culture was produced during the 96 hrs of sulphur deprivation, which is in agreement with earlier results (Scoma et al., 2012). As expected, *HYDA1* gene expression (Fig. 1B), and HYDA protein expression (Fig. 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 hrs (Fig. 2A). The initial cell density was about 5.3 million cells/ml culture, which increased moderately, by about 25% within 72 hrs (Fig. 2A). We have also observed a more than two-fold increase in cell volume within 24 h, which decreased later (Fig. 2B). In parallel with this, a remarkable accumulation of starch took place in the first 24 h (Fig. 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, already 24 h after transferring the cells to sulphur-free media (Fig. 2D).

Next, we investigated the elemental composition of the cells, using ICP-OES (Szentmihályi et al., 2015). We have chosen the 48 h time point for our measurements, at which H_2 production is already significant (Kosorouv et al., 2002; Nagy et al., 2016, Fig. 1A). Because of the large changes in cell size and starch content upon sulphur deprivation, the amounts of the various elements were determined on dry weight (DW), cell number and Chl bases as well (Table 1**).** 48 h after transferring the cells to sulphur-free TAP media, the sulphur content, expressed on a dry weight basis, decreased by about 60%; however, the change was moderate on a cell number and Chl basis (approx. 25% decrease was observed, but this change was statistically significant), showing that the cellular sulphur content changed only moderately. The concentration of other essential elements, such as Ca~~, Fe~~ and Mn remained unaltered when expressed on a dry weight basis. The amounts of K and Zn decreased by about 20 to 25% whereas Fe content decreased by 10%. Mg and P concentrations show a significant, approximately two-fold 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 approx. 3-fold increase in the sulphur-deprived cultures relative to the controls. transferring the cells to sulphur-free media (Fig. 1A), it becomes evident 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) is yet unexplored and 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).

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). In the plant, depending on the rate of S deprivation or deficiency implies Fe-deficiency that alters the Fe(III)-chelate reductase activity, the expression of Fe^{2+} transporter (IRT1) and Fe(III) chelate reductase (FRO1) gene (Astolfi et al., 2003, Muneer et al., 2014). In sulphur deprivation by the oxidative stress (Salbitani et al., 2015) signal transduction pathway is activated. In this process Ca plays as a second messenger and transient elevation of Ca level occurs in the cytosol, although in our experiment the Ca concentration expressed in DW didn't change (Table 1). The explanation of this can be that a fast Ca influx becomes from the intracellular Ca strores, e.g. endoplasmic reticulum or Golgi apparatus (Yuan et al., 2017, Tang and Luan, 2017). At the same time Mg concentration increased about twofold. Since Chl content was almost the same at the control and sulphur deprivation groups, most of Mg was presumably used for the forming of MgATP. This is supported by the parallel P concentration elevation (Table 1). Stress involves a higher energy demand e.g. for gene expression and synthesizing enzymes of the antioxidant system,….., for which MgATP is needed.

The degradation of photosynthetic complexes during sulphur deprivation

The PsbA protein has probably the highest turnover rate among all photosynthetic complexes (Järvi et al., 2015), and it is generally thought that the turnover of PsbA may be 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) However, as shown in Table 1, only a moderate, about 25% decrease in sulphur content was observed on a cellular level, which may not explain the loss of PsbA upon sulphur deprivation. In order to investigate the degradation of PsbA and other photosynthetic complexes in detail, western blot analysis was carried out.

Upon sulphur deprivation, as expected, the PsbA content decreased steadily (Figs. 3A, 3B), with about 30% remaining by the 96^{th} h of the sulphur deprivation procedure. The amount of RbcL, the large subunit of Rubisco, decreased more rapidly (Figs. 3A, 6G), and unexpectedly, the amount of CP43, an inner antenna complex of PSII also decreased with a halftime of less than 24 h (Figs. 3A, 3D). The loss of PsbA occurred in parallel with the loss of PSBO (Figs. 3A, 3C), the major subunits of the OEC. As expected, the $cytb_6f$ subunit, PetB, and the reaction center core of PSI,

PsaA were better retained (Figs. 3A, 3E, 3F). In the presence of lincomycin (LM), which inhibits the synthesis of chloroplast-encoded proteins, the degradation rate of PsbA increased strongly (halftimes of 55 and 20 hrs (*FIT!)* in the absence and presence of LM, respectively), whereas the degradation of RbcL, PetB, CP43 and PsaA was only moderately accelerated upon the LM-treatment (Fig. 3). The F_V/F_M parameter, an indicator of PSII activity (Schansker et al., 2014), also shows that upon the addition of LM, the photosynthetic activity declined more rapidly than in its absence (Fig. 3H). Thus, these data show that there was a significant turnover of PsbA upon sulphur deprivation and it remained at a relatively high level compared to the other chloroplast-encoded subunits investigated here.

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). Fig. 4A shows that there was an approx. 7-fold increase in its expression upon sulphur deprivation, correlating well with the increase in Asc content (Fig. 4B). Upon oxidative stress *VTC2* expression and accumulation are induced both by $^1O_2$ and H_2O_2 (Vidal-Meireles et al., 2017), which are produced mostly within PSII and at the acceptor side of PSI, respectively (Vass 2012, Asada 2006). 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 (GPX) and transferases are key enzymes in oxidative stress defense; its Chlamydomonas homologues GPXH and GSTS1/2 have been shown to respond promptly to $^1O_2$ and on a longer time-scale, GSTS1 also responds to H_2O_2 (Ledford et al., 2007). Figures 5A and 5B show that *GPXH* and *GSTS1* are strongly induced by the 48th hr of the experiment, when the *VTC2* transcript abundance is at its maximum (Fig. 4A).

The enzymes of the Asc-glutathione cycle play essential roles in ROS detoxification (Asada 2006). The relative transcript abundance of *APX1* increased significantly (Fig. 5C), in parallel to *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 (Fig. 5D). The relative transcript abundance of the iron-containing superoxide dismutase *FSD1* responds

both to H_2O_2 and $^1O_2$ [REF]; its 10-fold increase corroborate that oxidative stress occurred upon sulphur deprivation (Fig. 5E).

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

Singlet oxygen ($^1O_2$) is likely to get accumulated 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 amount *in situ* is a challenging task. The rate of $^1O_2$ production can be measured in the presence of histidine, an efficient $^1O_2$ quencher, resulting in O_2 uptake (Ur-Rehman et al., 2013). However, in our hands, histidine addition did not result in any O_2 uptake in Chlamydomonas cells (data not shown), therefore, this test could not be carried out.

Photosystem II activity is declined due to donor-side induced photoinhibition

In our previous paper we suggested that Asc, at a high concentration reached upon sulphur deprivation, is capable of overreducing the Mn-cluster (Nagy et al., 2016). Once the OEC is inactivated, Asc may provide electrons to Tyr_Z^+ at a slow rate, which however, cannot fully prevent the accumulation of strongly oxidizing species in PSII. This so-called thus donor-side-induced photoinhibition process results in the loss of PsbA and other components of PSII (Blubaugh et al., 1991; Jegerschöld and Styring, 1996, Arató et al., 2004, Tóth et al., 2011). The occurrence of donor-side induced photoinhibition caused by excessive Asc accumulation is an alternative explanation for the loss of PSII activity upon sulphur deprivation; therefore, it was investigated in more detail.

When 10 mM Asc was added to sulphur-replete Chlamydomonas cells and the cultures were incubated for 1 h in the light (ensuring about 2.6 mM Asc within the cell, i.e. the same concentration as attained upon 48 h of sulphur deprivation, see Fig. 4B), the intensity of the B thermoluminesence band (S_1S_2-Q_B^-recombination, Ducruet and Vass 2009) decreased by approx. X % (Suppl. Fig. 1A), confirming that Asc, when present in the mM range, overreduces 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 (Suppl. Fig. 1B).

In order to obtain direct evidence for inactivation of the OEC by high Asc concentrations, we measured oxygen uptake on isolated thylakoid membranes of *Chlamydomonas reinhardtii* in the presence of methylviologen (MV), as a terminal electron acceptor at PSI (REF on the method –Laci?) and diphenylcarbazide (DPC), an efficient electron donor to Tyr_Z^+ (Rashid and Popovic, 1995). Since 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.

Fig. 6A shows that upon sulphur deprivation, the rate of MV-dependent O_2 uptake steadily decreased, and by the 48^{th} h a minimum was reached, which coincides with the complete loss of the B thermoluminescence band (Suppl. Fig. 1B), suggesting that the amount of PSII units with active OECs 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 (Fig. 6B), which might be due to 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 maximum of about 100% (Fig. 6B). The precise quantification of the number of PSII reaction centers without active OECs may not be possible due to uncertainties regarding the presence of SOD and catalase in our thylakoid preparation; still, these results demonstrate that their amount relative to fully active PSII units increase upon sulphur deprivation thus support our hypothesis that upon sulphur deprivation the inactivation of PSII is initiated at the donor side.

Discussion

It has been assumed for many years that PsbA turnover is limited upon sulphur starvation and that it is the reason for a declined PSII activity (e.g. Wykoff et al., 1998; Volgusheva et al., 2013; reviewed by Antal et al., 2015). However, several lines of evidence presented in this paper and

found in the literature show that the reason for the decreased PSII activity is more complex and it is probably a regulated process to minimize cellular damage upon sulphur limitation.

When transferring the cells to sulphur-free media, the transcript abundance of a large number of nuclear-encoded photosynthetic complexes decrease within a few hours (Nguyen et al., 2011, Toepel et al., 2013), cell division and Chl biosynthesis are ceased, and the cell size is doubled with a concomitant accumulation of starch (Fig. 2, Zhang et al., 2002), which is enabled by the initially active Calvin-Benson-Bassham cycle. As shown by our ICP-OES measurements, the sulphur content per dry weight decreases by about 60% within 48 h of sulphur deprivation, whereas on a cell number or Chl basis, only about 25% decrease is observed (Table 1). In addition, sulphur reserves, mostly in the form of sulfoquinovosyl diacylglycerol (SQDG), are mobilized upon sulphur deprivation (Sugimoto et al., 2007, Sugimoto et al., 2010). Based on these data, therefore, it is very likely that the amount of sulphur per photosynthetic reaction center decreases only moderately and may not directly hinder the turnover of PsbA. Indeed, our results presented in Fig. 3 show that PsbA has a remarkable turnover, and it is degraded more slowly than other photosynthetic subunits (namely RbcL and CP43), which are known to possess a lower turnover rate (Järvi et al. 2015). Still, PSII activity diminishes rapidly upon sulphur deprivation. The data presented in this and in our previous paper (Nagy et al., 2016) show that Asc may play an important role in the decrease of PSII activity. Upon sulphur deprivation, the relative transcript abundance of the *VTC2* gene, which plays an important regulatory role of Asc biosynthesis in Chlamydomonas (Urzica et al., 2012, Vidal-Meireles et al., 2017) is strongly increased (Fig. 4). *VTC2* expression is most probably induced by $^1O_2$, which may be produced upon overexcitation of PSII when the electron transport chain becomes reduced. The basal Asc concentration in green algae is very low compared to higher plants (approx. 0.5 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 rhythm and the photosynthetic electron transport chain. In contrast, in Chlamydomonas, Asc biosynthesis is rapidly induced by light and oxidative stress, 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 (Vidal-Meireles et al., 2017, Tóth et al., 2017).

As shown in Suppl. Fig. 1 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 yet unclear, although the structural differences between the extrinsic 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: in higher plants, it occurs with a halftime of approx. 20 to 50 ms (Tóth et al., 2009), therefore Asc cannot hinder the accumulation of Tyr_Z^+ and $P680^+$ upon illumination. The presence of these strongly oxidizing species leads to donor-side induced photoinhibition, in which PSII reaction center becomes inactivated and degraded (Blubaugh et al., 1991, Tóth et al., 2011). Donor-side induced photoinhibition may occur *in vivo*, upon strong and moderate heat stress (Tóth et al., 2009, Tóth et al., 2011) and possibly upon UV-B stress as well (Mano et al., 2004). Our results presented in this paper strongly suggest that donor-side induced photoinhibition induced by a strong Asc accumulation is the reason for the loss of PSII activity upon sulphur deprivation of Chlamydomonas cells.

The loss of PSII activity decreased 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 also minimized. The importance of this process is best demonstrated by the *sac1* and *snrk2.1* mutants, which are unable to downregulate their photosynthetic activity, resulting in increased $^1O_2$ production and cell death on a timescale of days (Davies et al., 1996, González-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 in order to avoid a 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 reduction 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 represent a more suitable safety valve (Godaux et al., 2016).

In summary, the responses to sulphur limitation in Chlamydomonas include i) maximizing the uptake of sulphur from the environment and ii) minimizing the decrease in cellular sulphur

content by haulting cell division, iii) since cell division is stopped, there is an excess of reductants, which, in the beginning, will be used for starch biosynthesis, iv) due the overexcitation of the photosynthetic apparatus, ROS are produced, which trigger Asc biosynthesis, v) Asc accumulated to the mM concentration range inactivates the OEC, downregulating PSII activity, vi) the decrease in O_2 evolution may lead to the establishment of hypoxia enabling hydrogenase expression, and H_2 production will act as a safety valve of the photosynthetic electron transport (Godaux et al., 2016). By this means, the damage imposed by sulphur limitation is minimized and the cells may recover if sulphur becomes available again (REF).

It is important to note that H_2 production does not necessarily occur or becomes 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 (Fouchard et al., 2005). In aerobic cultures

Sulphur deprivation has been proposed to be exploited to induce hydrogen production, which lasts for several days (REF). However, as outlined above, this type of H2 production involves the complete remodeling of cellular metabolism, including PSII inactivation and degradation of the photosynthetic apparatus. H2 production induced by sulphur-deprivation is also largely dependent on acetate as an organic carbon source, because it enables the accumulation of starch, and at a later stage, starch respiration would provide electrons for H2 production, and it also contributes to the establishment of anoxia.

Materials and Methods

Algal growth conditions

C. reinhardtii CC124 cultures were grown in Tris-acetate-phosphate (TAP) medium at a light intensity of 80-90 μmol photons m^{-2} s^{-1} and 24-25 ºC in an algal growth chamber. The 250 ml flasks containing 50 ml TAP medium were shaken at 120 rpm and the cultures were grown for three days in sulphur-containing medium. After three days of cultivation, the cells were washed five times with sulphur-free TAP medium (centrifugation at 1000 g, at 24 ºC for 5 min). For the H_2 production experiments the Chl content was set at 20 μg chl/ml (based on Porra et al., 1989) and 30 ml 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 cell size and cell density

The cell density was determined either by a Millipore Scepter™ 2.0 hand-held cell counter, which also allows a precise determination of cell sizes.

Determination of starch content

Two mls of culture containing 40 µg chl were collected at each time-point and spun down (12.000 g, 4 min). After re-suspending the pellet twice in 1 ml methanol (to solubilize the pigments), the samples were washed two times with 1 ml 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 s at 40 % amplitude (xxxxx), after which 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).

Determination of protein content: ***André***

Element determination

Alga sample of 30 mg was digested with 10 ml HNO_3 of 67% in an Anton Paar microwave sample preparation system. After digestion, the sample was filled to 20 ml with high purity water water (1/8.2 MΩ) from which the concentration of elements (Ca, Cu, Fe, K, Mg, Mn, P, S, Zn) was determined with Spectro Genesis inductive coupled plasma optical emission spectrometer (ICP-OES, Kleve, Germany) (Szentmihályi et al., 2015). S, P and multi-element standards were used (CPAchem, Stara Zagora, Bulgaria) for standardization and calculation of the results,

RNA isolation and qRT-PCR analysis

For RNA isolation, 1 ml culture, containing approximately 50 µg chl(a+b), was collected and the Direct-Zol RNA kit was used, following the recommendations of the manufacturer (ZymoResearch). To remove contaminating DNA from the samples, the isolated RNA was treated with DNaseI (ZymoResearch). RNA integrity was checked on a 1% (w/v) MOPS gel. Reverse transcription was primed with oligo dT using 1 µg of total RNA and SuperScript III reverse transcriptase (Life Technologies). To confirm the absence of DNA contaminations, an aliquot of the RNA sample was used without reverse transcriptase.

Real-time qPCR analysis was performed using an Applied Biosystems Prism 7900HT Fast Real Time PCR System using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne). 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, the amplicon length was set between 100 and 130 bp. To ensure correct normalization of the *HYDA1* (Cre03.g199800) and *VTC2* (Cre13.g588150) 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 primers for *HYDA1* were GGCGAGTGGGACAATCCAAT and TGCCCGTGAACAGCTCATAG; for the reference genes, see Vidal-Meireles et al. (2017). The data are presented as fold-change in mRNA transcript abundance of *HYDA1*, 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 two or 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 the ΔΔCt.

Ascorbate content determination

The ascorbate content determination was carried out according to Kovács et al., (2016).

H_2O_2 content determination

H_2O_2 content was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, A22188). 20 million *C. reinhardtii* cells were collected by centrifugation and the pellet was homogenized in 0.1% trichloroacetic acid using glass beads and

quartz sand. The broken cells were centrifuged and the supernatant was then used to measure the H_2O_2 content. 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 (Thermo Scientific, Fluoroskan Ascent FL). The cellular H_2O_2 content was calculated based on standard curve, cell numbers and cell sizes.

Thermoluminescence (TL) *measurements*

Thermoluminescence measurements were carried out using a custom-made TL apparatus, similar to the one described in (REF). Cell suspension (300 μl, 20 μg Chl/ml) was placed on a copper sample holder, connected to a cold finger immersed in liquid N_2. A heater coil (Thermocoax, France, model: SEI 10/50) placed under the sample holder, ensured the desired temperature of the sample during the measurement. Dark adapted samples were illuminated at -40 ºC by one saturating single-turnover flash provided by a stroboslave, and the sample was heated to 70 ºC in darkness with a heating rate of 20 ºC/min. The emitted TL was measured with an end-window photomultiplier (Hamamatsu, Japan, model: H10721-20) simultaneously with recording the temperature. When applicable, 10 mM Asc was added to the samples before the measurements. *Exact timing!*

Thylakoid isolation

Chlamydomonas cell culture equivalent to 1200 μg chl were distributed to four 15 ml centrifuge tubes and spun down at 2990 g for 3 min. The pelleted cells were resuspended in 200 μl homogenisation buffer (50 mM Tricine pH 7.8/ 5 mM $MgCl_2$ / 10 mM NaCl supplemented with 0.1% BSA prior to isolation). The suspensions were transferred to 1.5 ml microcentrifuge tubes and 200 μl 212-300 μm glass beads were added into each tube. The cells were grinded by Bullet blender Gold cell disruptor (Next Advance, Inc.) at max RPM for 10 min at 4 °C. The glass beads were washed 3 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 30000 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

Photosynthetic electron transport rate of isolated thylakoid membrane was measured as an oxygen uptake in the presence of methylviologen (MV) an artificial electon acceptor of PSI (REF) using a Clark-type oxygen electrode (Hansatech Instruments, Kings Lynn, UK). 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, beside MV and NaN_3, 2 mM of diphenyl carbazide (DPC) was added as an exogenous electron donor. 1 ml of samples were illuminated with white light from a halogen cold light source (KL 2500 LCD, Schott AG, Germany) at a photon flux density of 500 µmole m^{-2} s^{-1} at ambient temperature for six minutes and the slope of the oxygen uptake curve was determined by linear fitting. The net DPC dependent photosynthetic activity was calculated based on the difference in electron transport rates measured in the presence or absence of DPC.

Western blot analysis

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 1x cOmplete Protease Inhibitor Cocktail [Roche]), sonicated for 30 s, incubated in the dark at 4 ºC for 30 min with vigorous shaking, and then centrifuged at 4700 g for 4 min at 4 ºC. The supernatant was collected into a new Eppendorf tube and the chl(a+b) content of the protein extract determined. An amount equivalent to 2 µg chl(a+b) was then mixed with 2x Laemmli buffer and incubated at 75 ºC for 10 min before loading on the gel. Proteins separated by SDS-PAGE (Perfect Blue Twin Gel System, Peqlab) were transferred to a polyvinylidene difluoride membrane (Hybond P) using a tank blotting system (Cleaver Scientific Ltd). Specific polyclonal antibodies (produced in rabbits) against HydA, PetB, PsbA, PsaA, PSBO, CP43 and RbcL were purchased from Agrisera AB. 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), according to the instructions of the manufacturer.

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

The net amounts of H_2 and O_2 produced by the cells were determined by taking 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 a HP-PLOT Molesieve 5 Å column (30 m*0.53 mm*0.25 µm) and a TCD detector. The oven temperature was 30 ºC. The carrier gas was argon, and a linear velocity of 115 cm/s was used. In order to prevent the accumulation of H_2 above the critical 5% level in the gas phase (Kosorouv et al., 2012) and overpressure, the bottles were flushed with N_2 gas every 24 h following the determination of gas production.

Chl a fluorescence measurements

Chl *a* fluorescence measurements were carried out at room temperature with a Handy-PEA instrument (Hansatech Instruments Ltd, UK). *C. reinhardtii* cultures were dark-adapted for 15 min and then 5 ml of cell suspension (20 µg Chl/ml) was filtered onto a Whatman glass microfibre filter (GF/C) that was placed in a Handy-PEA leaf clip. The alga sample was illuminated with continuous red light (3500 µmol photons m^{-2} s^{-1}, 650 nm peak wavelength; the spectral half-width was 22 nm; the light emitted by the LEDs is cut off at 700 nm by a NIR short-pass filter). The light was provided by an array of three light-emitting diodes focused on a circle of 5 mm diameter of the sample surface. 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 s.

Statistics

The presented data are based on at least three independent experiments. When applicable, averages and standard errors (SE) were calculated. Statistical significance was analysed using Student's t-test and the significance level are presented as: * $p<0.05$; ** $p<0.01$;*** $p<0.001$ (in Fig. 1, analysis between the Asc-treated and untreated samples).

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Table 1

Element content of alga samples (ng/mg dry weight) and the results expressed in …….

	ng/mg DW			µg/ mg Chl			ng/million cells	
	Control	**-S (48 h)**		**Control**	**-S (48 h)**		**Control**	**-S (48 h)**
S	3218±1	1237±6		576.0±33.8	434.9±23.9		2074±122	1531±84
Ca	3649±113	3999±794		653.9±58.5	1408±293		23540±2115	4955±1032
Cu	15.44±0.50	25.61±2.60		2.77±0.30	9.02±1.30		9.96±0.90	31.76±4.50
Fe	308.8±9.2	275.3±11.6		55.30±4.90	96.71±4.70		199.2±17.6	340.4±16.7
K	2608±17	1930±240		466.8±24.4	679.4±100.8		1680±88	2392±355
Mg	2941±23	6449±271		526.4±26.7	2270±207		1895±96	7990±728
Mn	239.6±5.8	240.5±50.0		42.93±3.50	84.04±14.30		154.5±12.8	295.8±50.5
P	10863±288	20124±1275		1946±165	7089±804		7006±595	24952±2829
Zn	48.42±0.20	37.92±1.30		8.67±0.50	13.33±0.90		31.20±1.72	46.94±3.10