

Photorespiratory 2-phosphoglycolate metabolism and photoreduction of O₂ cooperate in high-light acclimation of *Synechocystis* sp. strain PCC 6803

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Abstract In cyanobacteria, photorespiratory 2-phosphoglycolate (2PG) metabolism is mediated by three different routes, including one route involving the glycine decarboxylase complex (Gcv). It has been suggested that, in addition to conversion of 2PG into non-toxic intermediates, this pathway is important for acclimation to high-light. The photoreduction of O₂ (Mehler reaction), which is mediated by two flavoproteins Flv1 and Flv3 in cyanobacteria, dissipates excess reductants under high-light by the four electron-reduction of oxygen to water. Single and double mutants defective in these processes were constructed to investigate the relation between photorespiratory 2PG-metabolism and the photoreduction of O₂ in the cyanobacterium *Synechocystis* sp. PCC 6803. The single mutants

$\Delta flv1$, $\Delta flv3$, and $\Delta gcvT$, as well as the double mutant $\Delta flv1/\Delta gcvT$, were completely segregated but not the double mutant $\Delta flv3/\Delta gcvT$, suggesting that the T-protein subunit of the Gcv (GcvT) and Flv3 proteins cooperate in an essential process. This assumption is supported by the following results: (1) The mutant $\Delta flv3/\Delta gcvT$ showed a considerable longer lag phase and sometimes bleached after shifts from slow (low light, air CO₂) to rapid (standard light, 5% CO₂) growing conditions. (2) Photoinhibition experiments indicated a decreased ability of the mutant $\Delta flv3/\Delta gcvT$ to cope with high-light. (3) Fluorescence measurements showed that the photosynthetic electron chain is reduced in this mutant. Our data suggest that the photorespiratory 2PG-metabolism and the photoreduction of O₂, particularly that catalyzed by Flv3, cooperate during acclimation to high-light stress in cyanobacteria.

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Abbreviations

2PG	2-Phosphoglycolate
AL	Actinic light
Car	Carotenoids
Chl	Chlorophyll a
F_0	Minimal fluorescence of dark-adapted cells
Flv	Flavoprotein
F_m/F_m'	Maximal fluorescence
F_s	Fluorescence of actinic light adapted cells
F_v	Variable fluorescence
F_v/F_m	Maximal PSII yield
Gcv	Glycine decarboxylase complex
GcvT	T-protein subunit of the glycine decarboxylase complex

ML	Measuring light
NPQ	Non-photochemical quenching
OCP	Orange carotenoid protein
OD	Optical density
PC	Phycocyanin
PSI	Photosystem I
PSII	Photosystem II
ROS	Reactive oxygen species
RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SD	Standard deviation
WT	Wild type

Introduction

Cyanobacteria evolved about 3.5 billion years ago and were the first to perform oxygenic photosynthesis. These organisms are considered the ancestors of plant chloroplast (e.g., Deusch et al. 2008). The oxygen produced is, in fact, toxic for photosynthetic organisms, particularly under high-light conditions, because oxygen can serve as an acceptor of excess electrons generating reactive oxygen species (ROS). In addition to their general damaging effect, in photosynthetic organisms the reaction center protein D1 of photosystem II (PSII) is the preferential target of ROS. A higher rate of D1 protein destruction than repair at high-light leads to photoinhibition (Aro et al. 1993; Nishiyama et al. 2001; Takahashi et al. 2007). In addition, molecular oxygen competes with CO₂ as a substrate for RubisCO and thereby lowers the carboxylation reaction and forms the toxic intermediate 2-phosphoglycolate (2PG), which inhibits Calvin–Benson cycle enzyme activities. 2PG is rapidly metabolized by the photorespiratory 2PG-metabolism. For scavenging of 2PG and other toxic compounds, it employs at least ten different enzymes in higher plants (Ogren 1984; Tolbert 1997; Bauwe and Kolukisaoglu 2003).

During their evolution, photosynthetic organisms adapted to the oxygen-containing environment and developed several strategies for acclimation to high-light. Over-reduction of the electron chain is initially avoided by the dissipation of excess absorbed light energy from the chlorophylls, mainly via carotenoids and other non-photochemical quenching (NPQ) mechanisms (Havaux et al. 2005; Kirilovsky 2007). In addition, a substantial part of electrons can be transferred from photosystem I (PSI) to molecular oxygen, which results in photoreduction of O₂ via superoxide anion to H₂O₂ in plant chloroplasts, i.e., the Mehler reaction (Mehler 1951; Asada 1999). The produced ROS are quickly detoxified by the combined action of superoxide dismutase and peroxidases. Thereby, the photoreduction of O₂ acts as electron sink under certain conditions, where up to 30% of the electrons from the light reactions can be directed to oxygen (Helman et al. 2005). Accordingly, it helps to prevent PSII

from photodamage and is regarded as an important protection system in all photosynthetic organisms (Asada 1999; Badger et al. 2000; Helman et al. 2003).

Intriguingly, the photoreduction of O₂ in cyanobacteria is quite different from that of plants. For the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), it was shown that O₂ is reduced directly to water in one reaction mediated by A-type flavoproteins (Vicente et al. 2002; Helman et al. 2003). A-type flavoproteins, also referred to as flavodiiron proteins, are module proteins consisting of an N-terminal flavodoxin-like module (binding FMN) and a beta-lactamase module (harboring the non-heme diiron active site) as core modules (Wasserfallen et al. 1998; Frazão et al. 2000; Vicente et al. 2002). Cyanobacterial flavoproteins contain an additional C-terminal NAD(P)H:flavin oxidoreductase domain and are able to couple the NAD(P)H oxidation with the substrate reduction without an additional redox partner (reviewed in Vicente et al. 2008). The genome of *Synechocystis* encodes four putative A-type flavoproteins, but only two of them, Flv1 (Sll1521) and Flv3 (Sll0550), are apparently involved in light-dependent O₂ reduction activity (Helman et al. 2003). Accordingly, the mutants $\Delta flv1$ and $\Delta flv3$ lack light-enhanced O₂ consumption and hence the flavoproteins Flv1 and Flv3 are suggested to catalyze the cyanobacterial photoreduction of O₂ (Helman et al. 2003). Recently, a role in the photoprotection of PSII has been shown for the two other *Synechocystis* flavoproteins, Flv2 and Flv4 (Zhang et al. 2009).

Photosynthetic CO₂ assimilation represents the main acceptor for electrons from the photosynthetic water cleavage system. However, under CO₂-limiting conditions the Calvin–Benson cycle activity is strongly reduced and oxygenase activity of RubisCO increases. The photorespiratory 2PG-metabolism recycles 75% of the organic carbon from 2PG and hence helps to avoid depletion of Calvin–Benson cycle intermediates (Osmond 1981; Wingler et al. 2000). Due to the operation of the efficient inorganic carbon concentrating mechanism (as reviewed in Kaplan and Reinhold 1999; Giordano et al. 2005; Badger et al. 2006), it was assumed that cyanobacteria do not possess a photorespiratory 2PG-metabolism (reviewed in Colman 1989). In contrast to this earlier view, we could recently show that an active photorespiratory 2PG-metabolism exists in *Synechocystis*, employing a plant-like 2PG-cycle, a bacterial-like glycerate pathway, and complete decarboxylation of glyoxylate *via* formate (Eisenhut et al. 2008). While defects in one or two of these metabolic branches only cause reduced growth under low-CO₂ (0.035% CO₂) conditions (Hagemann et al. 2005; Eisenhut et al. 2006), the complete loss of all three pathways in such *Synechocystis* mutants leads to a high-CO₂-requiring-phenotype and highlights the essential function of photorespiratory 2PG-metabolism for

cyanobacteria despite the carbon concentrating mechanism (Eisenhut et al. 2008).

For higher plants, it has been demonstrated that the photorespiratory 2PG-metabolism also plays a crucial role in high-light acclimation, since it helps to regenerate the acceptors, NADP⁺ and ADP, for ongoing reduction and energy storage, respectively, under excess light energy and/or lack of CO₂ (Kozaki and Takeba 1996). Accordingly, mutants affected in the photorespiratory 2PG-metabolism showed depletion of Calvin–Benson cycle intermediates, which resulted in decreased consumption of ATP and NADPH (Wingler et al. 2000; Takahashi et al. 2007). While the cooperation of photorespiratory 2PG-metabolism and photoreduction of O₂ in acclimation to high-light has been investigated in plants, the relation between these two oxygen-consuming mechanisms has not been investigated in cyanobacteria. In this work, we used *Synechocystis* mutants impaired in the photorespiratory 2PG-metabolism and in the photoreduction of O₂, respectively, to address this question. Our results indicate that in cyanobacteria also these two pathways cooperate in the acclimation to high-light.

Materials and methods

Strains and culture conditions

The glucose-tolerant strain of *Synechocystis* sp. PCC 6803 was obtained from Prof. N. Murata (National Institute for Basic Biology, Okazaki, Japan) and served as the wild type (WT). Axenic cultures were grown on agar-solidified BG11 medium (Rippka et al. 1979) plates buffered with 20 mM TES–KOH to pH 8.0 at 30°C, under constant illumination (30 μmol photons m⁻² s⁻¹). Transformants were initially selected on media containing either 10 mg l⁻¹ kanamycin, 4 mg l⁻¹ spectinomycin or 5 mg l⁻¹ chloramphenicol, but the segregation of clones and cultivation of mutants were performed either at 50 mg l⁻¹ kanamycin, 20 mg l⁻¹ spectinomycin, or 15 mg l⁻¹ chloramphenicol. For the physiological characterization under standard conditions, axenic cultures (OD₇₅₀ 0.8–1.0, about 10⁷ cells ml⁻¹) were grown photoautotrophically in batch cultures (3 cm glass vessels with 5 mm glass tubes for aeration) at 29°C under continuous illumination at 165 μmol photons m⁻² s⁻¹ (warm light, Osram L58 W32/3, Munich, Germany) with bubbling of air enriched with CO₂ (5% CO₂ in air designated HC) in BG11 medium at pH 8.0. Pre-cultivation under slow growing conditions was performed in shaken Erlenmeyer flasks at low light of 50 μmol photons m⁻² s⁻¹ and at air level of CO₂ (designated LC). For microarray analyses, cells were grown photoautotrophically in BG11 medium at pH 7.0 with bubbling of air enriched with CO₂ (5% CO₂ in air designated HC).

Growth was monitored by measurements of the OD at 750 nm. Photosynthetic pigment concentrations were measured and corrected as described by Huckauf et al. (2000). Absence of contamination by heterotrophic bacteria was checked by spreading 0.2 ml of culture on LB plates.

Generation of mutants

In order to generate mutations in the selected genes, interposon mutagenesis was applied by insertion of drug resistance cartridges against antibiotics into the coding sequences at selected restriction sites. The construction of the single mutants $\Delta flv1$ (*sll1521::Cm*), $\Delta flv3$ (*sll0550::Sp*) and $\Delta gcvT$ (*sll0171::Km*) was already described by Helman et al. (2003) and Hagemann et al. (2005), respectively. The double mutants $\Delta flv1/\Delta gcvT$, $\Delta flv3/\Delta gcvT$, and $\Delta gcvT/\Delta flv3$ were raised using the original constructs for a second transformation of the single mutants $\Delta flv1$, $\Delta flv3$ or $\Delta gcvT$. The genotype of the mutants was confirmed by PCR using total chromosomal DNA isolated from mutant clones and gene-specific primers (Table 1). Total DNA from *Synechocystis* strains was isolated according to Hagemann et al. (1997).

Conditions for photodamage

Strains were pre-cultivated for 2–5 days under standard conditions. For the photoinhibition experiments, concentrated cells at 10 μg Chl ml⁻¹ were incubated for 30 min under high-light (1,400 μmol photons m⁻² s⁻¹) to induce photodamage. Subsequently, cells were transferred back to standard light (165 μmol photons m⁻² s⁻¹) and the repair was followed for 30 min. The high-light was provided by six standard fluorescence lamps (warm light, Osram L18 W32 and W76) and one strong light source (SOL 500/III Nr. 930044, Dr. Hoene AG, Munich, Germany). For the photodamaging experiments, cells were incubated in smaller culture tubes (1.5 cm diameter with 5 mm glass tubes for aeration) at 29°C. In some experiments the de novo synthesis of proteins was blocked by 250 μg ml⁻¹ lincomycin (Fluka, Sigma-Aldrich Chemie, Munich, Germany), which was added 1 min before the incubation under strong light. At defined time points, cells from 700 μl culture suspension were harvested by centrifugation (60 s at 2,000g at room temperature). The pellets were suspended in 350 μl BG11 to obtain 20 μg Chl ml⁻¹ and used immediately for PAM measurements.

PAM measurements

Fluorescence measurements were performed with a modulated fluorometer (PAM-210, Walz, Effeltrich, Germany) using the saturation pulse method (Schreiber et al. 1995;

Table 1 Strains and primers used in this work

Strains and primer	Genotype or sequence (5' → 3')	Reference
<i>Synechocystis</i> strains		
<i>Synechocystis</i> sp. PCC 6803 wild type		
$\Delta gcvT$ mutant	PCC 6803 <i>sll0171::Km</i>	Hagemann et al. (2005)
$\Delta flv1$ mutant	PCC 6803 <i>sll1521::Cm</i>	Helman et al. (2003)
$\Delta flv3$ mutant	PCC 6803 <i>sll0550::Sp</i>	Helman et al. (2003)
$\Delta flv1/\Delta gcvT$ double mutant	PCC 6803 <i>sll1521::Cm/sll0171::Km</i>	This work
$\Delta flv3/\Delta gcvT$ double mutant	PCC 6803 <i>sll0550::Sp/sll0171::Km</i>	This work
$\Delta gcvT/\Delta flv3$ double mutant	PCC 6803 <i>sll0171::Km/sll0550::Sp</i>	This work
Primer		
<i>sll0171</i> -fw	AGA CCT GAA GGA AGC TGT AG	
<i>sll0171</i> -rev	GAG GAA GTG GTG CAC AGG TT	
<i>sll1521</i> -fw	CCG TTG TTG GTC AGT TG	
<i>sll1521</i> -rev	CTC CAG CCG TTG TTG TA	
<i>sll0550</i> -fw	ACG GCA TGT TCA CTA CC	
<i>sll0550</i> -rev	GAT TCG GAG CAC TGA CA	

Schreiber 1997). Strains were pre-cultivated for 2–5 days under standard conditions. For PAM measurements, cell suspensions were adjusted to 20 $\mu\text{g Chl ml}^{-1}$ and incubated in the dark for a minimum of 30 min. Dark-adapted cells were illuminated with measuring red light (665 nm) at 0.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 65 s (ML; dark) followed by red actinic light (650 nm) at 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 150 s (AL; light) to measure F_0 (ML) and F_s (AL), respectively (for nomenclature see van Kooten and Snel 1990). At defined time points, saturating pulses (3 μs , 3,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were applied to measure F_m (ML) and F_m' (AL) (Schreiber 1997). F_0 and F_s values were used to calculate the increase of fluorescence ($F_s - F_0$) from dark to light in WT and mutant cells.

In order to estimate the maximum PSII yield [$F_v/F_m = (F_m - F_0)/F_m$] after strong light the cells were adjusted to 20 $\mu\text{g Chl ml}^{-1}$ in the dark (about 2 min) and subsequently illuminated for 70 s with measuring red light (665 nm) at 0.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to measure F_0 . F_m was monitored during repeated saturating pulses (3 μs , 3,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at intervals of 10 s. The average F_m of five saturating pulses was used to calculate the maximal PSII yield (Schreiber 1997; Takahashi et al. 2007).

RNA-isolation and DNA-microarray analyses

Cells from 10 ml of culture were harvested by centrifugation at 2,860g for 5 min at 4°C and were immediately frozen at -80°C. Total RNA was extracted after pre-treatment with hot phenol and chloroform using the High-Pure RNA isolation kit (Roche Diagnostics, Mannheim,

Germany). Direct cDNA labeling was done using the fluorescent dye either Cy3 or Cy5 (Amersham, GE Healthcare, Munich, Germany). Labeled cDNA was hybridized to 60-mer oligonucleotide DNA microarrays (Agilent, Amstelveen, The Netherlands) designed from the complete *Synechocystis* genome sequence. The whole procedure is described in detail by Eisenhut et al. (2007). Given values are the means and standard deviations of at least two independent experiments using RNA isolated from separate cultures. Inductions of 1.75-fold and repressions of 0.5-fold represented significant expression changes and were taken into consideration. The complete data set of microarray experiments is given as Supplementary Material.

Protein isolation and immuno-blotting

Cells from 50 ml of culture were harvested by centrifugation at 5,300g for 2 min at 4°C and were immediately frozen at -80°C. For protein isolation the pellets were re-suspended in 500 μl of 0.01 M HEPES buffer (pH 7.3) supplemented with 10 mM phenylmethylsulfonyl fluoride and sonicated (2 \times 1 min, 35 W) under ice cooling. Total protein extracts (3 μg each) were separated in denaturing gels and used for immuno-blotting analyses (Eisenhut et al. 2007). A specific antibody was used against the orange carotenoid protein (OCP) from *Synechocystis* (dilution 1:1,250; received from Dr D. Kirilovsky, CNRS, France). Horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Bio-Rad, Munich, Germany) was used as the secondary antibody.

Results

Generation and characterization of double mutants

In order to examine whether the photorespiratory 2PG-metabolism is linked to high-light acclimation in cyanobacteria like in plants, we generated *Synechocystis* double mutants with an impaired photoreduction of O₂ as well as an impaired photorespiratory 2PG-metabolism. For this purpose, the mutants $\Delta flv1$ and $\Delta flv3$ (Helman et al. 2003) defective in the photoreduction of O₂ were transformed with a DNA construct bearing an inactivated *gcvT* gene encoding the T-protein subunit of the glycine decarboxylase complex (Gcv, Hagemann et al. 2005). The T-protein subunit produces NH₄⁺ and methylene-tetrahydrofolate from the aminomethyl-group of glycine bound to the H-protein subunit of Gcv (Bauwe and Kolukisaoglu 2003). Putative $\Delta flv1/\Delta gcvT$ and $\Delta flv3/\Delta gcvT$ double mutants were selected as chloramphenicol/kanamycin- and spectinomycin/kanamycin-resistant clones, respectively. Characterization of their genotypes by PCR analyses showed complete segregation of the three single mutants and of the double mutant $\Delta flv1/\Delta gcvT$. All the WT copies of these genes were inactivated by the relevant cartridges leading to larger PCR fragments (Fig. 1). In contrast, the double mutant $\Delta flv3/\Delta gcvT$ still retained WT copies of *gcvT* that were amplified in addition to the mutated gene fragment. Incomplete segregation of the double mutant $\Delta flv3/\Delta gcvT$ was detected in several independently obtained clones, even after many generations of growth under selective conditions. This unexpected behavior was also observed when we used the single mutant $\Delta gcvT$ as the parental strain and

transformed it with an inactivated *flv3* gene; in this case the cells maintained WT copies of *flv3* (data not shown).

The fact that it was not possible to combine the two mutations in *gcvT* and *flv3* in one cell provided the first indication of a functional relationship between photorespiratory 2PG-metabolism and the photoreduction of O₂ in a cyanobacterium. This result was unexpected since in previous studies the single mutant $\Delta gcvT$ showed only a little reduction of growth under low-CO₂ conditions (Hagemann et al. 2005; Eisenhut et al. 2006) and the single mutant $\Delta flv3$ behaved also similar to WT cells despite the defect in the photoreduction of O₂ (Helman et al. 2003). Moreover, these results indicated that the Flv3 protein seems to be more important than the Flv1 protein, at least when combined with a mutation in glycine decarboxylase. This view is also supported by expression analyses, in which only *flv3*, but not *flv1*, was found to be strongly up-regulated after transfer of the cells from a high to a low level of CO₂ (Wang et al. 2004; Eisenhut et al. 2007).

Microarray analyses

We performed these analyses in order to characterize the effect of a mutated *flv3* gene on global gene expression in *Synechocystis*. Similar experiments were performed previously with the single mutant $\Delta gcvT$ (Eisenhut et al. 2007). The complete data set of microarray experiments is given as supplementary material. Despite the observed changes in its phenotype (see below), only a few genes showed significant expression changes in mutant $\Delta flv3$ as compared to WT. In general, the up-regulated genes encoded for three groups of proteins (Table 2). The first group comprises

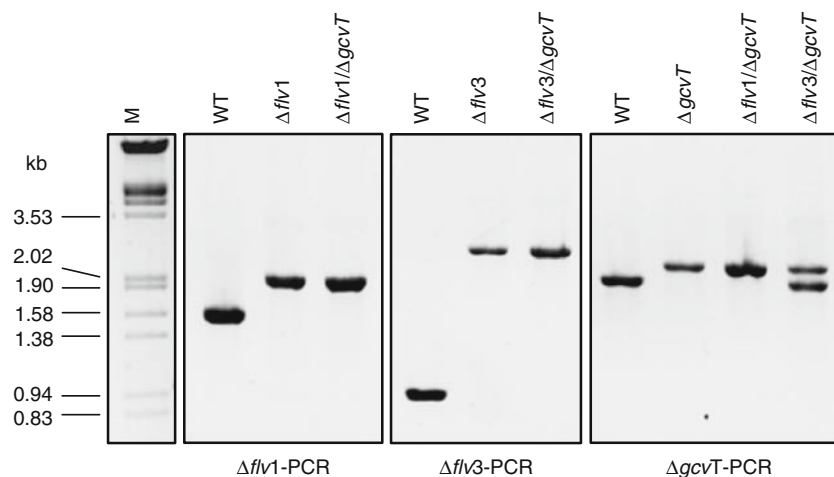


Fig. 1 Genotypic characterization of the *Synechocystis* single mutants $\Delta flv1$ and $\Delta flv3$ defective in the photoreduction of O₂, $\Delta gcvT$ blocked in the photorespiratory 2PG-metabolism, as well as double mutants $\Delta flv1/\Delta gcvT$ and $\Delta flv3/\Delta gcvT$ defective in both processes by PCR. For the PCR reactions total DNA of the mentioned strains (upper line) and the gene-specific primers (lower line) were used as given in Table 1.

(Abbreviations and expected fragment sizes: *M*, length marker λ -DNA *EcoRI/HindIII*; WT: 1.6 kb for *flv1*, 0.9 kb for *flv3* and 1.9 kb for *gcvT*. The sizes of the mutated genes after insertions of drug resistance cartridges are: 2.0 kb for *flv1::Cm*, 2.6 kb for *flv3::Sp* and 2.2 kb for *gcvT::Km*)

Table 2 Complete list of genes significantly up-regulated in cells of the mutant $\Delta flv3$ compared to WT cells grown under standard conditions (165 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 5% CO_2 ; pH 7; 29°C; OD_{750} 0.8–1.0, about 10^7 cells ml^{-1})

Gene ID	Mean (fold)	SD	Annotation
sll1862 ¹	5.30	0.26	Unknown protein—salt-induced
sll1532	4.20	0.08	Hypothetical protein, periplasmic, putative Zn-binding motif
sll1863 ¹	3.87	0.26	Unknown protein—salt-induced
ssl2982	3.72	0.50	<i>ycf61</i> , probable DNA-directed RNA polymerase omega subunit
slr0798 ²	3.61	0.60	<i>ziaA</i> , Zinc exporter ZiaA
slr0967	3.41	0.20	Hypothetical protein—TPR-motif
sll1696 ³	3.41	0.52	Hypothetical protein
sll1695 ³	3.36	0.17	<i>pilA2</i> , Pilin polypeptide PilA2
slr1291	3.15	0.17	<i>ndhD2</i> , NADH dehydrogenase subunit 4—salt-induced
slr1164	3.14	0.35	<i>nrdA</i> , <i>dnaF</i> , Ribonucleotide reductase subunit alpha
slr0797 ²	2.97	0.26	<i>coaT</i> , <i>corT</i> , Cobalt transporter CoaT
sll1694 ³	2.83	0.25	<i>pilA1</i> , Pilin polypeptide PilA1
sll0858 ⁴	2.80	0.67	Hypothetical protein—CpX protein family, periplasmic
slr1204	2.62	0.68	<i>htrA</i> , Protease
slr2048	2.53	0.24	Unknown protein—TPR-motif, periplasmic
sll0857 ⁴	2.49	0.10	Unknown protein
sll0378	2.46	1.83	<i>cysG</i> , <i>cobA</i> , Uroporphyrin-III C-methyltransferase
sll1514	2.46	0.16	<i>hspA</i> , <i>hsp1</i> , 16.6 kDa small heat shock protein
sll0856 ⁴	2.34	0.60	<i>sigH</i> , <i>rpoE</i> , Group3 RNA polymerase sigma factor
sll0680	2.26	0.17	<i>pstS</i> , <i>phoS</i> , Phosphate-binding periplasmic protein
slr0076 ⁵	2.25	0.13	SufB, FeS assembly protein
slr0075 ⁵	2.16	0.20	<i>ycf16</i> , <i>sufC</i> , ABC transporter ATP-binding protein
slr0772	2.15	0.12	<i>chlB</i> , Light-independent protochlorophyllide reductase subunit
sll0684	2.11	0.17	<i>pstB</i> , <i>phoT</i> , Phosphate transport ATP-binding protein
slr0074 ⁵	2.07	0.46	<i>ycf24</i> , ABC transporter subunit—SufB
slr0554	2.06	0.46	Hypothetical protein—RepA-like domain
sll0792	2.05	0.16	<i>ziaR</i> , <i>smtB</i> , Zinc-responsive repressor ZiaR
ssl0452	2.04	0.22	<i>nblA1</i> , Phycobilisome degradation protein NblA
ssl3177	2.03	0.20	<i>repA</i> , Hypothetical protein—rare lipoprotein A
sll0381 ⁶	2.03	0.46	Hypothetical protein—salt-induced
sll0382 ⁶	2.02	0.09	Hypothetical protein—salt-induced
sll1621	2.01	0.14	<i>ahpC</i> , TSA family protein—thioredoxin like
sll0789 ⁷	1.96	0.06	<i>copR</i> , <i>rre34</i> , Two-component response regulator OmpR family
sll0846	1.95	0.10	Hypothetical protein
sll0681	1.95	0.28	<i>pstC</i> , <i>phoW</i> , Phosphate transport system permease
slr1084 ⁸	1.91	0.19	Unknown protein
slr0756	1.90	0.36	<i>kaiA</i> , Circadian clock protein KaiA homolog
sll0788 ⁷	1.90	0.10	Hypothetical protein
sll0794	1.90	0.12	<i>coaR</i> , <i>corR</i> , Cobalt-responsive regulator CoaR
slr1612	1.87	0.03	Hypothetical protein
slr0077 ⁵	1.86	0.49	<i>nifS</i> , <i>sufS</i> , Cysteine desulfurase
sll1878	1.83	0.42	<i>futC</i> , Iron(III)-transport ATP-binding protein
ssr1766	1.82	0.09	Hypothetical protein
slr0626	1.76	0.23	Probable glycosyltransferase
slr1083 ⁸	1.75	0.05	Hypothetical protein
slr1963	1.75	0.09	<i>ocp</i> , Water-soluble carotenoid protein OCP

1–8: co-regulated genes forming an operon

Table 3 Complete list of genes significantly down-regulated in cells of the mutant $\Delta flv3$ compared to WT cells grown under standard conditions (165 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 5% CO_2 ; pH 7; 29°C; OD_{750} 0.8–1.0, about 10^7 cells ml^{-1})

Gene ID	Mean (fold)	SD	Annotation
sll1580 ¹	0.20	0.01	<i>cpcC1</i> , Phycobilisome rod linker polypeptide
sll1579 ¹	0.21	0.05	<i>cpcC2</i> , Phycobilisome rod linker polypeptide
sll1578 ¹	0.22	0.02	<i>cpcA</i> , Phycocyanin alpha subunit
sll1577 ¹	0.22	0.00	<i>cpcB</i> , Phycocyanin beta subunit
sll0550	0.24	0.14	<i>flv3</i> , Flavoprotein 3
sll0784 ²	0.33	0.05	<i>merR</i> , Possible nitrilase
ssl3093	0.33	0.07	<i>cpcD</i> , Phycobilisome small rod linker polypeptide
sll0783 ²	0.34	0.04	Unknown protein
ssl3803	0.41	0.03	<i>petL</i> , Hypothetical protein
slr1834	0.42	0.02	<i>psaA</i> , P700 apoprotein subunit Ia
ssr3383	0.43	0.05	<i>apcC</i> , Phycobilisome small core linker polypeptide
slr1655	0.43	0.02	<i>psaL</i> , Photosystem I subunit XI
slr1986	0.44	0.03	<i>apcB</i> , Allophycocyanin beta subunit
sll1316	0.44	0.13	<i>petC1</i> , Cytochrome b6-f complex iron-sulfur subunit
ssl1263	0.44	0.01	Hypothetical protein
slr0737	0.45	0.03	<i>psaD</i> , Photosystem I subunit II
sll1304 ³	0.46	0.03	Unknown protein
ssr2831	0.47	0.02	<i>psaE</i> , Photosystem I subunit IV
sll1472	0.47	0.00	Unknown protein
slr1544	0.48	0.03	Unknown protein
slr1841	0.48	0.03	Probable porin; major outer membrane protein
sll1305 ³	0.49	0.04	Probable hydrolase
sll0662	0.49	0.03	Hypothetical protein
ssl0483	0.49	0.02	Hypothetical protein
slr0906	0.49	0.09	<i>psbB</i> , Photosystem II core light harvesting protein
sll0785 ²	0.52	0.14	Unknown protein
sml0008	0.52	0.01	<i>psaJ</i> , Photosystem I subunit IX
ssl0563	0.52	0.03	<i>psaC</i> , Photosystem I subunit VII

1–3: co-regulated genes forming an operon

proteins known to respond to various stress treatments and can perhaps be regarded as general stress responsive (e.g., Sll1862–1863, HtrA, HspA, SigH, RepA) (Los et al. 2008). The second group is involved in the homeostasis of metals such as iron, zinc, and cobalt (ZiaA, CoaT, SufBCS, FutC). The third group of proteins might be necessary to cope with the alterations in electron flow such as NdhD2, OCP, and the thioredoxin-like AhpC (Ohkawa et al. 2000; Kobayashi et al. 2004; Wilson et al. 2006). It is noteworthy that none of the genes encoding proteins involved in 2PG-metabolism (e.g., *gcvT*) or in the acclimation to low- CO_2 displayed significant changes in its expression level in the mutant $\Delta flv3$, as compared to WT, under standard conditions. The expression of the second flavoprotein *flv1* involved in the photoreduction of O_2 was also not affected in the mutant $\Delta flv3$.

Among the down-regulated genes (Table 3) we found genes encoding for subunits of the phycobilisome (*cpcA*, *B*, *C1*, *C2*, *D*; *apcA*, *B*), which is in accordance with the up-regulation of *nblA1* gene for the protein responsible for

their degradation (Baier et al. 2001). Moreover, many genes for subunits of PSI (*psaA*, *B*, *C*, *D*, *E*, *J*, *L*) decreased about twofold in mutant $\Delta flv3$ as compared to WT (Table 3), while no or only slight changes were observed in the abundance of transcripts for PSII subunits. In addition, many genes for proteins of unknown function were down-regulated in mutant $\Delta flv3$.

Effects of light intensity and CO_2 level on growth

While characterizing changes in the phenotypes of the single and double mutants we observed that particularly the mutants affected in *flv3* were extremely sensitive when transferred from slow (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 0.035% CO_2 , routinely used for pre-cultivation) to rapid growth conditions (165 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 5% CO_2). In order to investigate this behavior in more detail, cells were transferred from the pre-culture into the CO_2 -gassed culture system under defined conditions. The ability of the various strains to acclimate to the new environment was strongly

affected by the initial cell density (equivalent to different actual light intensities for the cell suspension). When applying standard growth conditions, i.e., inoculum densities of $OD_{750} = 0.8\text{--}1.0$, both the WT and mutants were able to acclimate to the new conditions. In contrast, when the initial cell density was reduced ($OD_{750} = 0.2$), mutants $\Delta gcvT$, $\Delta flv3$, and $\Delta flv3/\Delta gcvT$ showed a significantly longer lag phase as compared to WT, appeared yellowish and sometimes even bleached after 46 h of cultivation (Fig. 2a, b). Under these conditions, mutants $\Delta flv1$ and even $\Delta flv1/\Delta gcvT$ behaved similarly to WT.

Furthermore, the differences observed under transient situations were also apparent under steady state conditions. Growth rates of the single mutants $\Delta gcvT$ and $\Delta flv3$ and particularly of the double mutant $\Delta flv3/\Delta gcvT$ were reduced under high- or low- CO_2 level as compared to the WT and the single mutant $\Delta flv1$ (Table 4). The strains exhibiting reduced growth appeared yellowish due to a reduction in the Chl content and an increased carotenoid level (Table 4). The latter findings are in agreement with our microarray data, where genes for PSI binding the majority of Chl were strongly reduced and the *ocp* gene was up-regulated (Tables 2, 3).

Characterization of Chl fluorescence parameters

Results presented in Fig. 2 suggested higher sensitivity to high-light conditions in mutants bearing a combined defect in the photorespiratory 2PG-metabolism and the photore-

duction of O_2 , which could be linked to the activity of the PSII. Fluorescence parameters are often used to assess PSII activity and photoinhibition in plants and cyanobacteria (e.g., Krause and Weis 1991; Campbell et al. 1998). We applied a defined PAM measuring protocol which consisted of three illumination stages and several flashes with saturating light to obtain the fluorescence parameters (Fig. 3; for nomenclature see van Kooten and Snel 1990; Campbell et al. 1998).

As observed previously (Helman et al. 2003), the maximal PSII yield (F_v/F_m) did not differ significantly between WT and mutants $\Delta flv1$, $\Delta flv3$, and $\Delta gcvT$ (Table 4). However, comparison of the rise in fluorescence from F_0 (ML) to F_s (AL) revealed interesting differences (Fig. 3c). The single mutant $\Delta gcvT$ showed no alterations in F_s as compared with the WT. In contrast, a considerably elevated F_s (65 to 72% as compared to WT) was observed in the single mutants $\Delta flv1$ and $\Delta flv3$ (consistent with the results from Helman et al. 2003) and even more so in the double mutants $\Delta flv1/\Delta gcvT$ and $\Delta flv3/\Delta gcvT$ (115–160%) indicating a lower ability to oxidize the reduced PQ pool. The further increase of F_s in the combined mutants defective in photorespiratory 2PG-metabolism and in photoreduction of O_2 could indicate that both mechanisms operate as effective electron acceptors for the linear electron transport chain (Fig. 3c) in agreement with results obtained with higher plants (Kozaki and Takeba 1996; Takahashi et al. 2007).

Recently, the water-soluble OCP was implicated in quenching of phycobilisome excitation in high-light-treated

Fig. 2 Acclimation of cells of the *Synechocystis* WT and defined mutants to a shift from slow to rapid growth conditions. **a** The representative growth curves are shown (increase in OD at 750 nm) of cells observed after the transfer of cells from shaken Erlenmeyer cultures at $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and air level of CO_2 into the standard cultivation system with $165 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ and high CO_2 (5% CO_2). All strains were inoculated with a relatively low OD_{750} of 0.2. Each point represents the average from at least three independent experiments with standard deviations. **b** Optical appearance of the cultures after incubation of the cells under the new growth conditions for 46 h. Please note that the pictures were taken from one typical experiment. The average pigment changes are displayed in Table 4

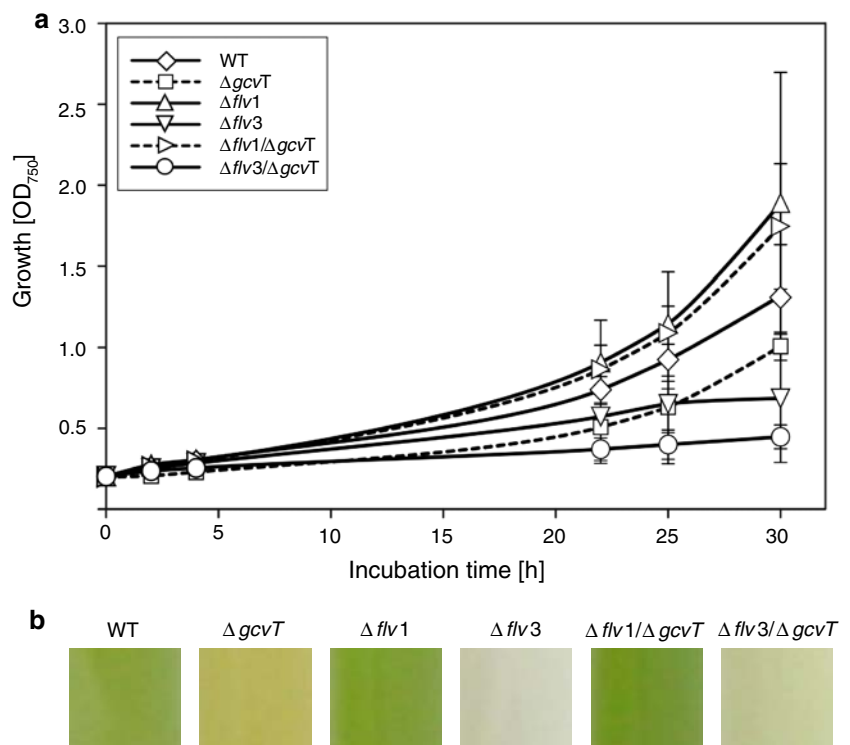


Table 4 Physiological parameters from cells of the wild type and of *Synechocystis* mutants affected in subunit T of glycine decarboxylase complex GcvT and the flavoproteins Flv1 and Flv3, respectively

Condition		Strain					
		WT	$\Delta gcvT$	$\Delta flv1$	$\Delta flv3$	$\Delta flv1/\Delta gcvT$	$\Delta flv3/\Delta gcvT$
(1) LC and low light	Growth rate (h^{-1})	0.0123 ± 0.0002	0.0110 ± 0.0022	0.0122 ± 0.0007	0.0103 ± 0.0003	0.0106 ± 0.002	0.0066 ± 0.0013
	PC/Chl	0.38 ± 0.02	0.31 ± 0.03	0.31 ± 0.02	0.4 ± 0.01	0.42 ± 0.04	0.48 ± 0.01
	Car/Chl	2.32 ± 0.26	1.93 ± 0.41	1.88 ± 0.38	2.65 ± 0.21	2.69 ± 0.47	3.44 ± 0.18
(2) HC and standard light	Growth rate (h^{-1})	0.057 ± 0.003	0.054 ± 0.005	0.065 ± 0.008	0.050 ± 0.003	0.061 ± 0.007	0.048 ± 0.005
	PC/Chl	0.35 ± 0.01	0.37 ± 0.07	0.38 ± 0.01	0.47 ± 0.01	0.38 ± 0.01	0.47 ± 0.02
	Car/Chl	1.49 ± 0.07	2.05 ± 0.22	1.30 ± 0.11	1.84 ± 0.09	1.34 ± 0.25	1.91 ± 0.12
	Maximal PSII yield (F_v/F_m)	0.456 ± 0.019	0.497 ± 0.048	0.459 ± 0.031	0.489 ± 0.031	0.478 ± 0.009	0.527 ± 0.036
(3) High–light	Recovery rate PSII [F_v/F_m (%)]	100 ± 29.34	74.89 ± 5.72	74.24 ± 14.56	44.6 ± 12.08	64.47 ± 28.44	39.19 ± 8.46

Cells were grown in BG11 medium pH 8.0 under (1) low- CO_2 (0.035% CO_2 , LC) and low light ($50 \mu mol photons m^{-2} s^{-1}$) and (2 and 3) high CO_2 (5% CO_2 , HC) and standard light ($165 \mu mol photons m^{-2} s^{-1}$). (3) Repair of PSII was analyzed under standard light (30 min, $165 \mu mol photons m^{-2} s^{-1}$) after high-light treatment (30 min, $1,400 \mu mol photons m^{-2} s^{-1}$). Mean values and standard deviations from at least three (growth and pigment under conditions 1 and 2) and four (recovery rate after high-light treatment) are given. Details of fluorescence measurements are given in legends to the Figs. 3 and 5

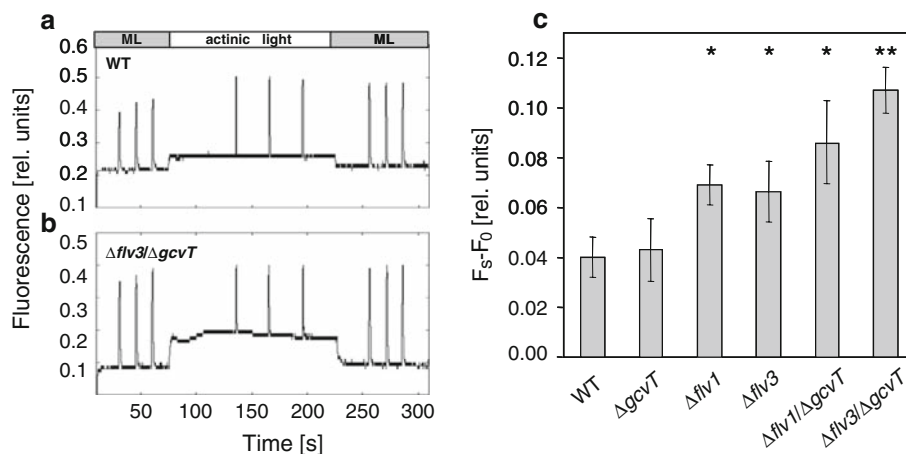


Fig. 3 Changes in fluorescence levels induced by different light levels measured in a PAM fluorometer using the saturation pulse method. Dark-adapted cell suspensions of wild type (a) and double mutant $\Delta flv3/\Delta gcvT$ (b) at the same Chl contents ($20 \mu g Chl ml^{-1}$) were illuminated with ML (dark) for 65 s followed by AL (light) for 150 s to measure the minimal fluorescence F_0 (ML) and the steady state fluorescence F_s (AL). To estimate the maximal fluorescence in the dark (F_m) and actinic light (F_m'), saturating pulses of 3 μs were applied. The minimal fluorescence F_0 was calculated from values obtained after

10–12 s (before the first saturating pulse), while the steady state fluorescence F_s was calculated from values around 210–212 s (after the last saturating pulse during actinic light). F_m values are the average from all maximal fluorescence values measured after saturating light pulses in the presence of measuring light. c The increase in fluorescence from F_0 to F_s is shown. Each column and bar represents the average of five independent experiments. Statistically significant differences in the fluorescence increase compared to WT (asterisk) and corresponding single-mutant (double asterisk) cells

cyanobacteria (Wilson et al. 2006). Our microarray analyses suggested a rise in the transcript abundance of the respective gene (Table 2). Using immuno-blot analyses, we investigated whether this effect is translated to the protein level. Corresponding to the transcriptional data, an increase in the quantity of OCP was detectable in all single and double mutants with interrupted *flv3*, whereas the mutant $\Delta gcvT$ exhibited a lower amount of OCP (Fig. 4) corresponding to the reduced *ocp* mRNA level (Eisenhut et al.

2007). We also examined the amount of the D1 and RubisCO proteins but did not observe significant alterations in their levels under the standard growth conditions (data not shown).

Recovery of maximal PSII yield after high-light treatment

In order to investigate the response of the various strains to excess light and recovery, we analyzed the level of

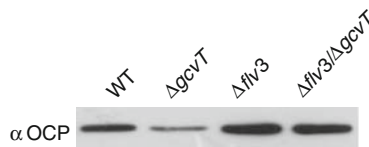
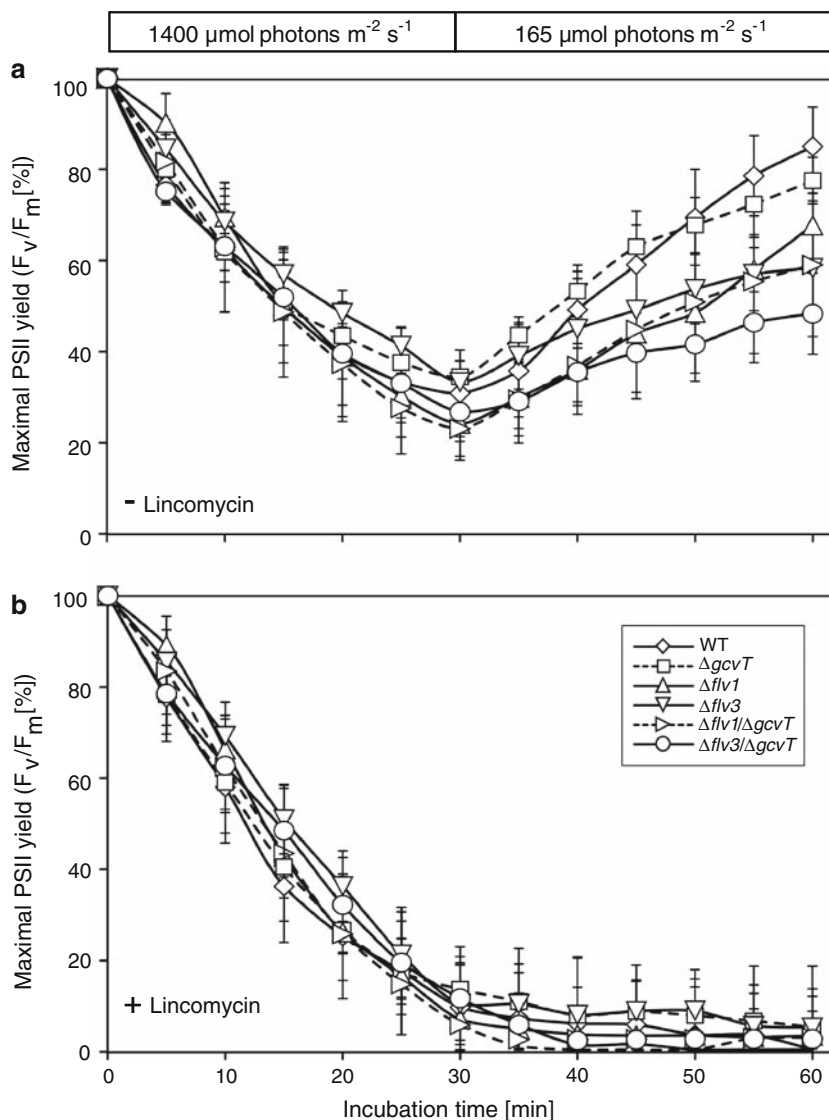


Fig. 4 Immuno-blotting analyses with protein extracts from cells of the WT and mutants $\Delta gcvT$, $\Delta flv3$ and $\Delta flv3/\Delta gcvT$ of *Synechocystis*, respectively, to determine the amount of the orange carotenoid protein OCP. The cells were cultivated under standard growth conditions. Three micrograms of total soluble protein was applied per lane on a SDS-PAGE gel. OCP was detected by a specific antibody via chemiluminescence

maximal PSII yield F_v/F_m in WT and mutants (Fig. 5a). During exposure to high-light of $1,400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 30 min the maximal photochemical efficiency decreased to 25–35% (without lincomycin) and 5–15% (with lincomycin), respectively, in all strains used here as compared

with the control, i.e., dark-incubated cells (Table 4). The rate of decline in maximal PSII yield reflects the balance between damage to the photochemical machinery and its repair. In order to eliminate repair processes we used lincomycin, which inhibits protein synthesis-dependent recovery. As expected, the rate of decline in the photochemical activity was faster in the presence of lincomycin but did not differ between the various strains used (Fig. 5b). We conclude that the drainage of electrons by the photoreduction of O_2 and photorespiratory 2PG-metabolism (inhibited in the Δflv and $\Delta gcvT$ mutants, respectively) did not alter the damage caused by excess light. In contrast, while the WT and mutant $\Delta gcvT$ regained about 80% of the initial activity after 30 min recovery at standard light intensity, mutants impaired in the photoreduction of O_2 recovered significantly slower (Fig. 5a). Here, too, the mutation in $flv3$ led to stronger effects than in $flv1$. In addition, the double mutant $\Delta flv3/\Delta gcvT$ could only reach 48% of the

Fig. 5 Photoinhibition experiments with cells of the *Synechocystis* WT and mutants $\Delta gcvT$, $\Delta flv3$, $\Delta flv1$, $\Delta gcvT/\Delta flv1$ and $\Delta flv3/\Delta gcvT$. Maximal PSII yield expressed as the percentage of $F_v/F_m [(F_m - F_0)/F_m]$ of dark controls was measured using cells exposed to high-light ($1,400 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 30 min and then incubated 30 min under standard light ($165 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) to allow the recovery of PSII **a** without and **b** with lincomycin treatment ($250 \mu\text{g ml}^{-1}$), respectively. The average F_m of five repeated saturating pulses (660 nm , $3 \mu\text{s}$, $3,500 \mu\text{mol m}^{-2} \text{s}^{-1}$) in intervals of 10 s was used to calculate F_v . Each point and bar represents the average from four independent cultivation experiments. The maximal PSII yields (F_v/F_m) at time point 0 were: WT— 0.492 ± 0.039 , $\Delta gcvT$ — 0.548 ± 0.018 , $\Delta flv1$ — 0.497 ± 0.035 , $\Delta flv3$ — 0.562 ± 0.028 , $\Delta flv1/\Delta gcvT$ — 0.504 ± 0.044 , $\Delta flv3/\Delta gcvT$ — 0.547 ± 0.033



photochemical activity in the control cells within that time (Fig. 5a).

Discussion

We examined a possible cooperation between the photoreduction of O₂ and the photorespiratory 2PG-metabolism in the acclimation of *Synechocystis* to high-light. Despite the fact that inactivation of both *flv1* and *flv3* resulted in complete arrest of the light-dependent O₂ reduction, Flv3 is more important for light acclimation than Flv1. This is indicated by the severe inhibition of $\Delta flv3$ growth, but not $\Delta flv1$, after transfer to excess light. Our conclusion is also supported by the differential expression of *flv1* and *flv3*: transcription of *flv3* but not of *flv1* increased under high-light and low-CO₂ conditions (Hihara et al. 2001; Wang et al. 2004; Eisenhut et al. 2007).

Due to the clear inability to photoreduce O₂ in both $\Delta flv1$ and $\Delta flv3$ mutants, it was proposed that the photoreduction of O₂ is catalyzed by an Flv1–Flv3-heterodimer in vivo (Helman et al. 2003). The observed clear change in the phenotype of mutant $\Delta flv3$, in contrast to the missing effects of the mutation in *flv1* (Fig. 2) raises the possibility that under these conditions, and in particular increased light stress, a homodimer of Flv3 could function in mutant $\Delta flv1$ as is the case with isolated Flv3, which exhibits NADPH-dependent O₂ reduction in vitro (Vicente et al. 2002; Helman et al. 2003).

Our microarray analyses revealed that inactivation of *flv3* resulted in an increased effect of excess light on the expression of genes known to be affected by such conditions in the WT (Hihara et al. 2001). The stressed status of mutant $\Delta flv3$ is indicated by the elevated expression of several stress proteins such as *ndhD2* and *ocp*, which are normally induced by different environmental stresses (Los et al. 2008) and by the changes in expression of various genes encoding proteins involved in the metal homeostasis (Singh et al. 2003). On the other hand, expression of genes for phycobilisome and PSI subunits was considerably depressed in $\Delta flv3$ as compared with the WT (Tables 2, 3). Hihara et al. (2001) proposed that down-regulation of phycobilisome genes is likely to reduce the effective light-harvesting cross-section and, thus, helps minimizing the damage to PSII. Taken together, the transcript abundance data suggest that the $\Delta flv3$ mutant experiences a more severe stress at standard growth conditions than the WT.

The loss of the protein Flv3, which is responsible for the major activity in photoreduction of O₂, caused a high-light-phenotype. This is not only illustrated by the transcriptional changes but also demonstrated by the physiological characteristics, particularly under transient conditions. Earlier studies (Helman et al. 2003) did not reveal a significant

effect of the inactivation of the *flv* genes on the steady state growth parameters. In contrast, we found changes in the light acclimation of the $\Delta flv3$ mutant, but not in $\Delta flv1$, particularly following transitions in the growth conditions or initiation of growth at a low level of inoculum size. The $\Delta flv3$ mutant showed impaired ability to cope with these conditions (Fig. 2).

An important outcome from this study is that the various mechanisms that help dissipate excess light energy, such as CO₂ cycling (Tchernov et al. 2003) or NPQ (Campbell et al. 1998; Wilson et al. 2006) or the action of Flv2 and Flv4 (Zhang et al. 2009) could not compensate for the loss of the photoreduction of O₂ (Fig. 2). It was, therefore, surprising that the rate of decline in maximal PSII yield following exposure to excess light, often ascribed to PSII activity, was not affected by the mutations introduced here, including $\Delta flv3$, in either the absence or presence of a protein synthesis inhibitor (Fig. 5). This is in agreement with the results of Helman et al. (2003) who also reported that, despite the very large flux of electrons via the photoreduction of O₂, *flv3* inactivation hardly affected the extent of photoinhibition (assessed by the decline in F_v). Naturally, in the absence of a protein synthesis inhibitor the decline in photosynthetic ability with time (Fig. 5) mirrors the balance between the damage to PSII and its repair. This is also reflected in the difference in the slopes obtained in the absence or presence of lincomycin (Fig. 5a, b, respectively).

In a recent study, Takahashi et al. (2007) suggested that photorespiratory 2PG-metabolism is involved in the repair of photoinhibitory damage in *Arabidopsis*. The fact that the rate of repair in a single $\Delta gcvT$ mutant was identical to that of the WT (Fig. 5a) did not lend support to this possibility in *Synechocystis*. On the other hand, the rate of repair was significantly reduced in mutants $\Delta flv3$ and $\Delta flv3/\Delta gcvT$, suggesting that removal of electrons from the linear photosynthetic electron chain by the photoreduction of O₂ may be involved in the repair.

Unexpectedly, the most severe phenotype in all the aspects examined here was observed in the double mutant $\Delta flv3/\Delta gcvT$, despite the fact that it was not completely segregated and that a single mutation in *gcvT* did not produce a clear phenotype (Figs. 2, 5), since other routes for the photorespiratory 2PG-metabolism are intact (Eisenhut et al. 2006, 2008). Obviously, the plant-like route for 2PG-metabolism involving the Gcv is of highest importance for 2PG-metabolism and here for the dissipation of excess reductants, since the Gcv step releases CO₂ and NH₃ from glycine, which are re-assimilated using high amounts of NADPH₂ and ATP. Phenotypic differences in non-segregated *Synechocystis* mutants have already been observed (e.g., Wang et al. 2002; Gutekunst et al. 2005; Oliveira and Lindblad 2008), suggesting that the reduced gene dosage certainly results in a lower content of the corresponding

protein. Furthermore, growth inhibition was observed when the cells were transferred from a low-light intensity and air level of CO₂ to standard-light intensity and 5% CO₂. The elevated CO₂ level would be expected to inhibit the oxygenase activity of RubisCO, but we previously found glycolate accumulation under such conditions in *Synechocystis* (Eisenhut et al. 2006, 2008). Nevertheless, the fact that mutant $\Delta flv3/\Delta gcvT$ exhibits the most severe phenotype supports the notion that the two processes, photoreduction of O₂ and photorespiratory 2PG-metabolism, seem to cooperate in the dissipation of excess reducing equivalents and in the prevention of a low redox poise possibly by a mechanism with mutual functional replacement in the cells.

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