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Physiological role of reactive oxygen species scavenging enzymes and methyl viologen resistance in the cyanobacterium *Synechocystis* sp. PCC 6803

Ph.D. Thesis

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Ceské Budejovice
2010

Prosecká J., 2010: Physiological role of reactive oxygen species scavenging enzymes and methyl viologen resistance in the cyanobacterium *Synechocystis* sp. PCC 6803. Ph.D. Thesis, in English. – p.66, Department of Molecular Biology and Biochemistry, The University of South Bohemia, České Budějovice, Czech Republic

Supervisor: RNDr. Martin Tichý, Ph.D.

Annotation:
Reactive oxygen species (ROS) are normal by-products of photosynthesis and respiration. Many environmental conditions lead to increased ROS production causing oxidative stress. To overcome oxidative damages the cells have to employ various anti oxidative processes, as ROS detoxification by scavenging enzymes. Cyanobacteria were the first organisms that supplied dioxygen to the atmosphere and were also the first organisms dealing with reactive oxygen species. To better understand how the cyanobacterium *Synechocystis* sp. PCC 6803 responds to oxidative stress, we characterized several mutants lacking individual scavenging enzymes (superoxide dismutase, catalase-peroxidase, thioredoxin peroxidase, alkyl-hydroperoxide reductase), determined expression profiles of genes participating in protection against oxidative stress under several stress conditions and generated spontaneous mutants resistant to superoxide generating agent - methyl viologen, to identify genes responsible for the resistance.

The work was supported by the Ministry of Education, Youth and Sports of the Czech Republic (projects no. MSM6007665808 and ME 881) and by the Czech Academy of Sciences (AV0Z50200510).

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V Českých Budějovicích, 12. 1. 2010 podpis:

This dissertation was accomplished at the Laboratory of Photosynthesis, Department of Autotrophic Microorganisms, Institute of Microbiology, Academy of Sciences of the Czech Republic, Opatovický mlýn, Třeboň, Czech Republic

List of enclosed publications

This thesis is based on the following publications:

I. **Jana Prosecka, Artem Vadimovich Orlov, Yuri Sergeyevich Fantin, Vladislav Vladimirovich Zinchenko, Michael Michaylovich Babykin, Martin Tichy** (2009) A novel ATP-binding cassette transporter is responsible for resistance to viologen herbicides in the cyanobacterium *Synechocystis* sp. PCC 6803. *FEBS J* **276**, 4001-4011

II. **Jana Prosecká, Martin Tichý** (2009) *In vivo* role of the type II peroxiredoxin (AhpC) in the cyanobacterium *Synechocystis* sp. PCC 6803; gene expression during stress acclimation. *Manuscript*

These publications will be referred in the text by the above Roman numerals.

Declaration
I declare that my role in preparation of publications was following:

Paper I. Main author – preparations of spontaneous methyl viologen resistant mutants of S*ynechocystis* and localization of mutations; methyl viologen sensitivity determination; expression data; preparation of the manuscript

Paper II. Main author – preparation and characterization of deletion mutants and their characterization; RT-PCR expression study of estimated genes during stress treatments; preparation of the manuscript

On behalf of the co-authors, I confirm that the contribution of Jana Prosecká is as stated above.

RNDr. Martin Tichý, Ph.D. ……..
coresponding author of the papers I and II

In České Budějovice, January 12, 2010

Ing. Jana Prosecká …….

Acknowledgements

I would like to thank to my supervisor Martin Tichý for his support and advices during my study, for his help and mostly for his patience with preparation of titles and of this work.

I wish to thank to all my colleagues from laboratory in Třeboň for their help and friendship during all years, especially to Eva Prachová for her cooperation and friendship not only in the scientific field; to Roman Sobotka for his helpful attitude and to Jiři Masojídek for his energizing mind and interesting discussions about life.

Finally, I would like to thank to my family, especially to my husband for support and encouragement to finish this work and for his love.

Abbreviations

ABC-type	ATP-binding cassette multidrug transporter
AhpC	alkyl-hydroperoxide reductase, thiol specific antioxidant protein
Apx	ascorbate peroxidase
ATP	adenosine triphosphate
t-BuOOH	tertiary butyl hydroperoxide
CAT	catalase
CyanoBase	genome databases of cyanobacteria
Cys	amino acid residue, cysteine
Dps	DNA-binding proteins
Fd	ferredoxin
FOX	ferrous oxidation/xylenol orange assay
Fur	ferric uptake regulation
Gpx	glutathione peroxidase
GSH	glutathione
Hik	histidine kinase, part of two-component regulation system
His	amino acid residue, histidine
H_2O_2	hydrogen peroxide
MATE	multidrug and toxic compound extrusion family transporters
MV	methyl viologen, herbicide (superoxide generator)
$NADP^+$	nicotianamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotianamide adenine dinucleotide phosphate, reduced form
NBD	nucleotide binding-domain
•OH	hydroxyl radical
OxyR	transcriptional regulator
O_2^-	superoxide anion
Per	peroxidase
PerR	peroxide sensing repressor, transcriptional regulator
PrqR	tanscriptional regulator of TetR family
Prx	peroxiredoxin
PS I; PS II	photosysthem I; photosysthem II
Rre	cognate response regulator, part of two-component regulation system
ROOH	lipid hydroperoxides
ROS	reactive oxygen species
RT-PCR	real time polynucleotide chain reaction
SOD	superoxide dismutase
SodB	Fe-containing superoxide dismutase B
SoxR	transcriptional regulator
SoxS	transcriptional regulator
Synechocystis	*Synechocystis* sp. strain PCC 6803
16S rRNA	small subunit of ribosomal RNA
TetR	tetracycline resistance regulator
TMD	transmembrane domain
Tpx	thioredoxin peroxidase
WT	wild type of cyanobacterium *Synechocystis* sp. strain PCC 6803

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1. INTRODUCTION

1.1. Cyanobacteria and oxygen

It is widely believed that cyanobacteria were the first organisms that supplied dioxygen to the atmosphere by a system using the oxidation of water for electron donation to the reaction center. The present-day cyanobacteria, also called blue-green algae, with algae and higher plants are expected to be responsible for a major part of oxygen production on the Earth and they share striking similarity in the machinery of photosynthesis (Falkowski, 2006). Like plants, cyanobacteria carry out photosynthesis, but their cellular organization closely resembles that of bacteria. They are found almost everywhere: from the tropics to the poles, in lakes and in the oceans, on rocks and in soils (Stainer and Cohen-Bazire, 1977). From evolutionary viewpoint it is generally accepted that progenitors of present-day unicellular cyanobacteria were also the progenitors of plant plastids through endosymbiotic events.

Cyanobacteria have long been used as model organism for the study of oxygenic photosynthesis in higher plants. Although cyanobacteria are one of the largest groups of Gram-positive bacteria, only a few strains for which genetic system is available have been used for physiological and genetic studies. Among them the cyanobacterium *Synechocystis sp*. strain PCC 6803 (*Synechocystis*) is most extensively studied one. It is one of the most popular organisms for genetic and physiological studies of photosynthesis for two main reasons; it is naturally transformable by exogenous DNA with efficient homology recombination and grows heterotrophically in the presence of glucose. This unicellular non-nitrogen fixing cyanobacterium was the first phototrophic organism to be fully sequenced (Kaneko et al., 1996). The genomic sequence has revealed the structure of the genome and its gene constituents (3167 genes), as well as the relative map positions of each gene. The functions of nearly half of the genes have been deduced using similarity searches. The genome sequence has also allowed for the implementation of systematic strategies to study gene function and the mechanisms of gene regulation on a genome-wide level. The genome database CyanoBase (http://genome.kazusa.or.jp/cyanobase) has been established and acts as a central repository for information on gene structure and gene function. As a result of the genome sequencing and the establishment of this database, *Synechocystis* provides an extremely versatile and easy model to study the genetic systems of photosynthetic organisms (Fig. 1.1).

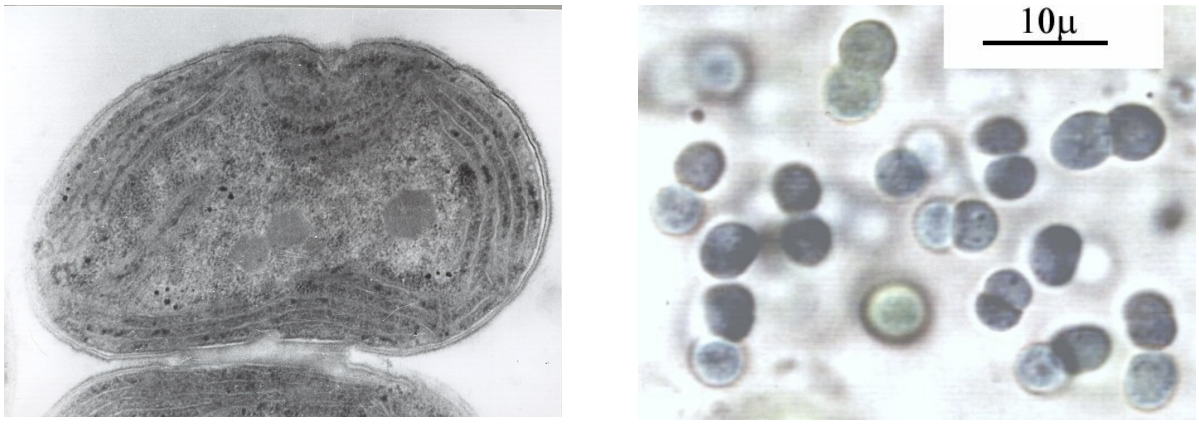


Fig. 1.1 Model organism: Synechocystis sp. strain PCC 6803

1.2. Oxidative stress and reactive oxygen species

Although, molecular oxygen is essential for most organisms at the same time its metabolic by-products are toxic. These by-products called reactive oxygen species (ROS) are formed during normal aerobic metabolism and many environmental conditions are leading to increased ROS production causing an oxidative stress (see Imlay, 2003 for review). Due to the overlap between plants and cyanobacteria on the mechanisms of ROS production and scavenging, studies in cyanobacteria would contribute to better understanding of oxidative stress responses in phototrophic organisms in general. This chapter deals with the origin and impact of ROS on photosynthetic apparatus.

1.2.1. ROS

Although molecular oxygen by itself is not very active, it can be activated by partial reduction forming ROS superoxide radical anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) (Imlay, 2003). In photosynthetic organisms ROS are produced during normal photosynthetic and respiration electron transport and their generation augments upon exposure to illumination, UV irradiation, pollutants, pathogens or other stress conditions (Fig. 1.2) (see Latifi et al., 2009 for review).

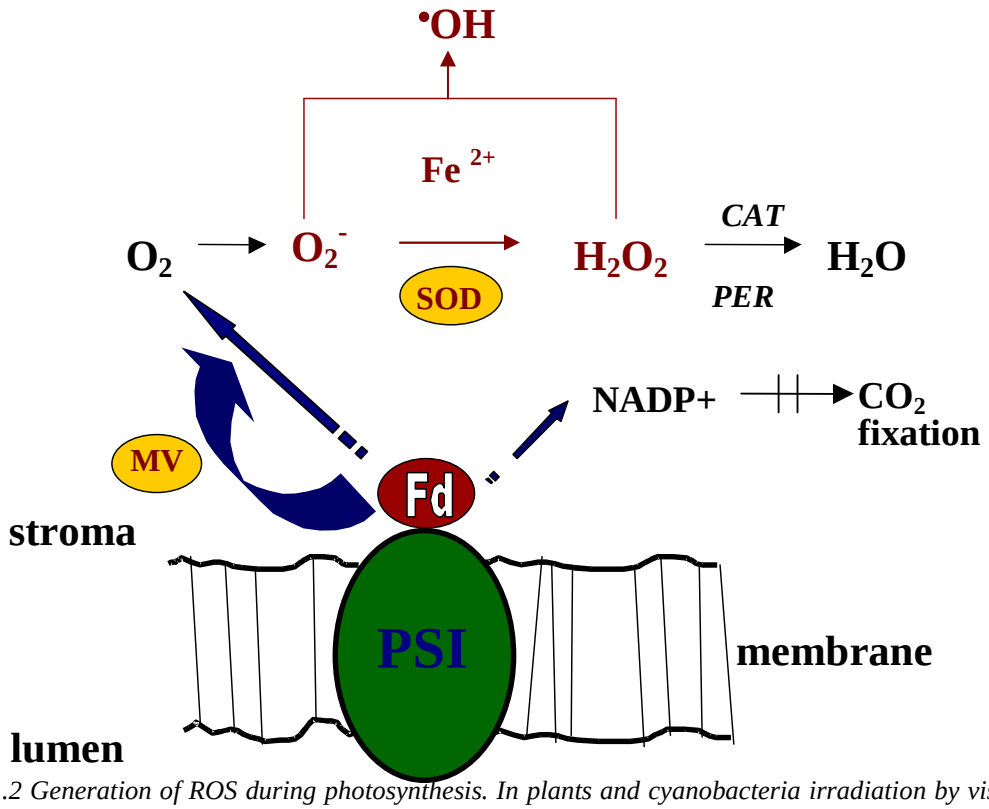


Fig. 1.2 Generation of ROS during photosynthesis. In plants and cyanobacteria irradiation by visible light may lead to the production of ROS. In cyanobacteria the major site of superoxide ion production in photosynthetic electron transport chain is at the acceptor side of Photosystem I. Normally, electrons are transported from ferredoxin reductase (Fd) via NADP+ to Calvin cycle. Under condition when NADPH utilization is suboptimal and NADP+ levels are low, oxygen rather than NADP+ may occasionally accept an electron from Photosystem I forming superoxide. This Mehler reaction (Mehler, 1951) is greatly amplified in the presence of herbicide methyl viologen. Tolerance of photosynthetic organisms to oxidative stress is enhanced by defense responses that prevent oxidative damage to cells (Okamoto et al., 2001). Superoxide dismutase (SOD) is further reducing superoxide to another ROS, hydrogen peroxide that is detoxified by catalase (CAT) or peroxidase (PER) to water. In presence of iron, the most reactive hydroxyl radical (•OH) can be formed via Fenton reaction.

1.2.2. Superoxide (O_2^-)

Superoxide is produced in photosynthetic organisms by photoreduction of oxygen by electron transport at the acceptor site of Photosystem I (PSI) (Asada, 2000). This photoreduction, called Mehler reaction, is a common feature of photosynthetic electron flow in chloroplasts and cyanobacteria, particularly when photosynthesis is limited by lack of carbon dioxide (Fig. 1.2) (Herbert et al., 1992; Mehler, 1951). Generation of superoxide is highly increased in the presence of herbicide, called paraquat or methyl viologen (MV), because MV accepts an electron from PS I and reduces oxygen to superoxide (Fig. 1.2). For this reason MV is frequently used as a stressor.

Superoxide can act as either oxidant or reductant; it can oxidize sulfur, ascorbic acid or NADPH; it can reduce cytochrome *c* and metal ions. Superoxide has high reactivity with thiol groups and iron-sulphur clusters causing inactivation of reaction centers of very important enzymes and release of iron (Flint et al., 1993; Hassan and Fridovich, 1978; Halliwell and Gutteridge, 1984). Resulting free iron can than catalyze formation of very reactive ROS, hydroxyl radical (see below). Superoxide radicals usually occur in their anionic form and are unable to permeate membranes. Therefore, it is expected that locally generated superoxide will have mostly local effect.

1.2.3. Hydrogen peroxide (H_2O_2)

Until the discovery of the production of superoxide in the 1970s, the primary product of oxygen reduction was believed to be hydrogen peroxide. Hydrogen peroxide and alkyl hydroperoxides are produced by reduction of molecular oxygen during photosynthesis, in metabolic processes catalyzed by oxidases, dioxygenases and other oxidative enzymes, or in metal-catalyzed reactions. Most of hydrogen peroxide arises from one-electron reduction of superoxide catalyzed by superoxide dismutases (Asada, 1994).

Hydrogen peroxide is an uncharged compound. It can easily diffuse through membranes and is more stable than superoxide. Hydrogen peroxide and alkyl hydroperoxides react with transition metal reductants or catalysts and can form reactive radicals. Peroxides cause oxidative damage over long distance and defense mechanisms are essential for the cells to degrade the peroxide intermediates (Klughamer et al., 1998). Hydrogen peroxide also participates in transduction pathways activating and coordinating plant stress responses. It has been also found to be signaling molecule affecting the intracellular activity of key signaling components including protein kinases and protein phosphatases.

1.2.4. Hydroxyl radical (•OH)

Hydroxyl radical is the most dangerous ROS because of its high reactivity and resulting absence of scavenging mechanism. The production of hydroxyl radicals was demonstrated in illuminated thylakoids and algal cells, and may occur via transition metal-catalyzed reduction of hydrogen peroxide, called *Fenton reaction* (Halliwell and Gutteridge, 1984):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH^-$$

The oxidized metal ions may be re-reduced by superoxide. Therefore, in presence of trace amount of Fe, the reaction of superoxide and hydrogen peroxide will form destructive hydroxyl radical and initiate oxidation of organic substrates. For that reason, superoxide and hydrogen peroxide have to be efficiently scavenged.

1.2.5. Oxidative damage

ROS will interact with almost any cell component including DNA, proteins and lipid membranes (for review see Imlay, 2003).

Characterization of damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation, causing degradation, single strand breakage, and cross-linking to protein (Imlay and Linn, 1986). DNA is very sensitive to oxidative stress. As DNA is effective in binding metals that are involved in Fenton reactions, less damages to DNA than to other macromolecules can be tolerated. As a consequence, the cell has a number of DNA repair enzymes. To prevent oxidative damage to DNA can be one of the reasons, why eukaryotic organisms have compartmentalized DNA in the nucleus away from sites of redox cycling.

Oxidative attack on proteins results in specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction product, altered electrical charge and increased susceptibility to proteolysis (Stadtman, 1986). Especially, thiol groups are very susceptible. Oxidative degradation of proteins is enhanced in the presence of metal cofactors that are capable of redox cycling. In *E. coli*, it has been demonstrated that twofold increase in superoxide concentration substantially diminished the activity of labile dehydratases, fourfold increase measurably impaired growth, and a fivefold increase sensitized cells to DNA damage in *E. coli* (Gort and Imlay, 1998). For example, oxidation of iron-sulfur clusters by superoxide destroys enzymatic function and leads to release of free iron that can catalyze generation of hydroxyl radical. In photosynthesis the D1 protein of Photosystem II is noted for its high rate of turnover, and it is assumed that this is a consequence of oxidative attack at specific sites on the proteins (Barber and Andersson, 1992).

The ROS can cause lipid peroxidation to forming lipid hydroperoxides (ROOHs) which are unstable and can undergo Fenton reaction in the presence of iron (Frankel, 1984). Peroxidation leads to destabilization of lipids membranes and endangering of membrane processes such photosynthesis and respiration.

1.3. Scavenging mechanisms

Because of high reactivity of ROS with almost of any cell component, ROS have to be efficiently scavenged. The mechanism of protection against ROS is particularly important in photosynthetic organisms including plants and cyanobacteria because they generate O_2 during photosynthesis. Therefore, photosynthetic cells have to be equipped with efficient defense system. Organisms possess different mechanisms to sense and scavenge ROS, or to repair ROS-caused damage.

1.3.1. Superoxide dismutases

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (McCord and Fridovich, 1969) (Fig. 1.3).

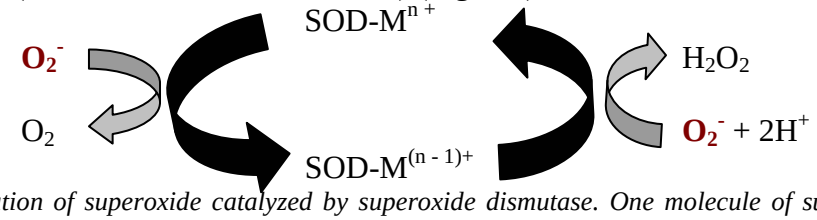


Fig. 1.3 Disproportionation of superoxide catalyzed by superoxide dismutase. One molecule of superoxide is reduced to hydrogen peroxide with electron derived from oxidation of the second molecule of superoxide to oxygen; M^{n+} metallic cation, $M^{(n-1)}$ reduced metallic cation.

Because superoxide anion is the precursor of several other ROS, control over the low steady-state superoxide levels by superoxide dismutase is critical. SODs are metalloenzymes and although they differ with the respect to their metal cofactor, all isoenzymes catalyze dismutation of superoxide anion to water and hydrogen peroxide. In photosynthetic eukaryotes three types of SODs exist: CuZn-SOD is usually located in the cytosol and plastids. Mn-SOD and Fe-SOD are found within the mitochondria and chloroplast, respectively (Okamoto et al., 2001). In almost all plants, chloroplasts contain CuZn-SOD as the major isoform of SOD.

Cyanobacteria are known to use Fe- and Mn-containing SOD (Fe- and Mn-SOD), which are believed to be more ancient forms, to scavenge superoxide radicals (Grace, 1990). The *sod* genes of a number of cyanobacteria have been characterized. The genome of *Synechocystis* contains single *sod* gene coding for Fe-SOD (*sodB; slr*1516). In contrast, *Plectonema boryanum* UTEX 485 contains 4 *sod* genes (Campbell and Laudenbach, 1995). *Synechococcus sp*. strain PCC 7942 contains two SODs thylakoid bound Mg-SOD and cytosolic Fe-SOD. Inactivation of FeSOD in this organism led to conclusion that in this cyanobacterium Fe-SOD did not protect PS II during oxidative stress but that it did protect PS I. The enzyme was also able to protect cells from the effects of chilling in the light (Thomas et al., 1999; Samson et al., 1994). Deletion mutant exhibited significantly greater damage to its photosynthetic system when grown under increased oxygen tension or with MV. Results also indicated interruption of cyclic electron flow around PSI (Herbert et al., 1992). The results of many studies indicated that SOD has a central role in the defense against oxidative stress (Tsang et al., 1992; Scandalios, 1993).

1.3.2. Catalases and peroxidases

Catalases and peroxidases are responsible for reduction of hydrogen peroxide and organic hydroperoxides. Scavenging of hydrogen peroxide by catalase occurs by disproportionation reaction, where one molecule of hydrogen peroxide is reduced to water and the second is oxidized to oxygen. Peroxidases are using external electron donor for peroxide reduction (Fig. 1.4). Common reductants are thioredoxin, glutathion or ascorbate. The reduction power of these compounds is restored by reduction equivalents originating from photosynthesis or respiration. In plants and some algae the main pathway of hydrogen peroxide inactivation is mediated by thylakoid-bound and stromal ascorbate peroxidase (Miyake et al., 1991). Here we will discuss scavenging enzymes present in cyanobacteria.

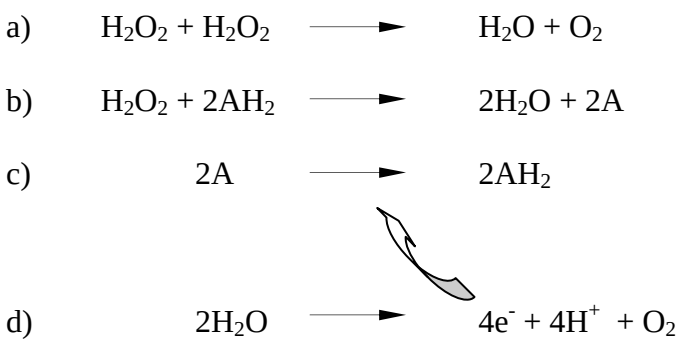


Fig. 1.4 Catalase and peroxidase scavenging mechanisms of hydrogen peroxide; a) disproportionation of hydrogen peroxide by catalase; b) reduction of hydrogen peroxide by peroxidase using photoreductant AH_2 ; c) oxidized photoreductant A is rereduced by d) reduction power originated from photosynthesis; A is glutathione, ascorbate or thioredoxins.

1.3.2.1. Catalase

Catalases have been classified into three groups: monofunctional heme-containing catalases, bifunctional heme-containing catalase-peroxidases and nonheme-manganese catalase (Zamocky and Koller, 1999; Chelikani et al., 2004). Both types of heme-catalases have a high cyanide-sensitive catalytic activity but exhibit significant differences in their sequences, structures and mechanisms (Passardi et al., 2007). Catalase-encoding genes are present in a number of cyanobacterial genomes and catalases have been reported for ten cyanobacterial species (Myiake et al., 1991). Several recent studies have shown catalytic mechanism of the bifunctional catalase-peroxidase, encoded by *katG* gene from *Synechocystis* (see Smulevich et al., 2006 for review). Protective role of this enzyme against exogenous hydrogen peroxide has been suggested (Tichy and Vermaas, 1999).

1.3.2.2. Ascorbate peroxidase

Ascorbate peroxidases play a crucial role in H_2O_2 detoxification in plants (Asada, 2000). These enzymes use ascorbate as a specific electron donor. Cyanobacteria exhibit low levels of ascorbate and no gene coding for ascorbate peroxidase has been found in sequenced cyanobacterial genomes (CyanoBase).

1.3.2.3. Glutathione peroxidase (Gpx)

Glutathione peroxidase (Gpx) catalyzes the reduction of hydrogen peroxide or organic hydroperoxides to water or alcohols by oxidation of glutathione (GSH), which is than reduced by glutathione reductase by electrons derived from NADPH (Ursini et al., 1995). The initially characterized mammalian cytosolic Gpxs contain conserved selenocysteine residue in its active site whereas multiple Gpxs homologues identified in higher plants carry a cysteine residue. It has been shown that Gpxs are important in the defense against oxidative stress in many organisms although conserved cysteine resulted in lower activity in comparison to mammalian selenocysteine residue (Maiorio et al., 1995).

Gpx-like activities have not only been measured in mammals but also in yeast (Inoue et al., 1999), algae, plants and cyanobacteria (Leisinger et al., 2001). In *Synechocystis* two genes *slr1171* and *slr1992* have been detected coding for Gpx-like proteins. Whereas both recombinant proteins were able to reduce alkyl hydroperoxides and utilized NADPH they were not able to use glutathione as their electron donor. It has been shown that most of amino acid residues involved in binding of GSH are missing in these proteins (Gaber et al., 2001, 2004).

1.3.2.4. Peroxiredoxins (Prxs)

During the last years, various genes have been identified in bacteria, yeast, plants or animals and defined as a new ubiquitous TSA/AhpC family of thiol-specific antioxidant proteins which catalyze the reduction of H_2O_2 and alkyl-hydroperoxides using thioredoxin and other thiol containing reducing agents as electron donors (Wood et al., 2003). Prxs are able to reduce ROS by oxidation of N-terminal catalytic Cys (-SH), called peroxidatic cysteine, to sulfenic acid (-SOH). In the second step sulfenic acid undergoes regeneration to thiol group by different mechanisms. Based of the number of conserved cysteines (Cys), their location and different regeneration mechanism, Prxs can be divided into four groups (Dietz et al., 2003; Stork et al., 2005):

(i) The first includes the 2-Cys Prxs, which form homodimers. The sulfenic acid on N-terminal of one subunit reacts with Cys on the C-terminal on second subunit to

form disulfide bond, which is then reduced by thioredoxins or glutaredoxins (Baier and Dietz, 1997; Poole et al., 2000).

(ii) The second group 1-Cys Prxs, contains only peroxidatic cysteine, their sulfenic acid is regenerated by a thiol-containing partner via mechanism which has not been elucidated (Stacy et al., 1996; Kang et al., 1998).

(iii) The third category includes atypical 2-Cys, they can use thioredoxins and glutaredoxins as the reductant and can exist in multiple isoforms, which are monomeric enzymes and its peroxidatic and resolving cysteine are on the same subunit (Seo et al., 2000).

(iv) The fourth category contains atypical 2-Cys Prx (PrxQ) which are homologues of the *E. coli* bacterioferritin co-migratory protein (Bcp) (Kong et al., 2000; Dietz et al., 2006).

Prxs play protective and detoxification role of ROS in all organisms. Prxs together with thioredoxins and glutaredoxins are important for holding intracellular redox state. In photosynthetic organisms, Prxs offer a powerful mechanism directing reducing power from the photosynthetic electron transport chain via ferredoxin, ferredoxin reductase and thioredoxin, into the chemical reaction of peroxide reduction. Interestingly, a cyanobacterial 2-cys peroxiredoxin has been referred as a progenitor of chloroplast Prx (Baier and Dietz, 1997) that implicates the importance of Prx for protection from ROS in the photosynthetic machinery.

The cyanobacterium *Synechocystis* contains five genes encoding probable Prxs with representing each of the above mentioned groups: 1x 1-Cys Prx (Slr1198); 1x 2-Cys Prx (Sll0755, Tpx); 1x typeII Prx (Sll1621, AhpC) and 3x PrxQ (Sll0221; Slr0242). Most of these Prxs have been recently studied (Kobayashi et al., 2004; Hosoya-Matsuda et al., 2005; Li et al., 2004; Hihara et al., 2003; Klughammer et al., 1998; Yamamoto et al., 1999).

1.3.3. DNA-binding proteins, bacterioferritins

In addition to the above mentioned scavenging enzymes new family of proteins with anti-oxidative protection of DNA was reported. The DNA-binding proteins (Dps) belong to a group of bacterial stress-inducible polypeptides that bind DNA and confer resistance to peroxide damage during periods of oxidative stress and long-term nutrient limitation (Almiro et al., 1992). Dps proteins are divergent members of the bacterioferritin/bacterioferritin superfamily. By oxidizing and mineralizing iron, Dps contributes to the storage of the Fe(III) form in the enzyme core and quenching of hydrogen peroxide, avoiding oxidative damage mediated by the Fenton reaction (Zhao et al., 2002). Dps homologues have been reported in several cyanobacteria species (Shcolnick et al., 2006; Castruita et al., 2006; Xiong et al., 2007). The *Synechococcus sp.* PCC 7942 Dps, named DpsA, is a DNA-binding hemoprotein having hem-dependent catalytic activity and has been localized in the thylokoid membranes, indicating that it might exist in two fractions: a chromosome-bound involved in DNA-protection and a soluble fraction required for the proper function of photosynthetic apparatus (Dwivedi et al., 1997).

1.3.4. Other scavenging mechanisms (non-enzymatic)

Cell protection against ROS can be provided not only by enzymatic reactions but also by non-enzymatic scavenging mechanisms. The best known group of antioxidants belongs to carotenoids. In chloroplasts they function as accessory pigments in light harvesting, but more important role is their ability to detoxify various forms of activated oxygen and triplet chlorophyll that are produced as a result of excitation of the photosynthetic complexes by light. Their antioxidant properties protect the photosystems in four ways: by reacting with

lipid peroxidation products to terminate peroxidation chain reaction (Burton and Ingold, 1984); by scavenging ROS and dissipating the energy as heat (Mathis and Kleo, 1973); by reacting with triplet or excited chlorophyll molecules to prevent formation of singlet oxygen; or by dissipation of excess excitation energy through the xanthophylls cycle. The anti-oxidative properties and their involvement in protection of the photosynthetic apparatus against oxidative stress have been shown by many studies (Young and Frank, 1996; Gotz et al., 1999; Schäfer et al., 2005).

Other efficient non-enzymatic scavengers are tocopherols, known as vitamins E (see for review Munné-Bosch, 2005). Their antioxidant ability is related to the fully substituted benzoquinone ring and reduced phytyl chain. Because of hydrophobic character, they are located in cell membranes. The antioxidant properties of tocopherol are the result of its ability to quench both singlet oxygen and peroxides. Tocopherols are less efficient scavengers of singlet oxygen than carotenoids, but are able to terminate the peroxidation chain reaction making them an effective free radical trap. The sensitivity of tocopherol-deficient mutants of several cyanobacteria under stress conditions indicate that the antioxidant function is conserved among oxygen-evolving phototrophs (Maeda et al., 2005).

1.4. Response to oxidative stress and its regulation

Response to environmental stresses can be provided by many different regulatory mechanisms. These mechanisms coordinate the cell effort to keep homeostasis. The coordination is realized by compartmentation of enzyme activities, but mostly on the level of DNA transcription, posttranscriptional modification or by sensing and signal transduction. As many regulatory mechanisms of defense against oxidative stress have been reported on the molecular level, it is apparent, that these mechanisms are highly interconnected. Recently developed microarrays techniques helped to reveal some of these mechanisms in cyanobacteria.

1.4.1. Oxidative stress induction

In photosynthetic organisms, environmental stresses like drought, salt stress, dormancy and pathogen infection or abiotic stress conditions like changes in temperature, light intensity, drought, salinity or chemical agents like herbicides, hydrogen peroxide and ozone generate oxidative stress that is associated with increased concentration of ROS and subsequent damage caused by ROS (Fig. 1.5). The activation of various regulators in response to an increase in ROS concentration frequently modulates the transcription of a subset of genes coding for enzymes involved in the defense against oxidative stress, allowing appropriate answer to the perceived stress.

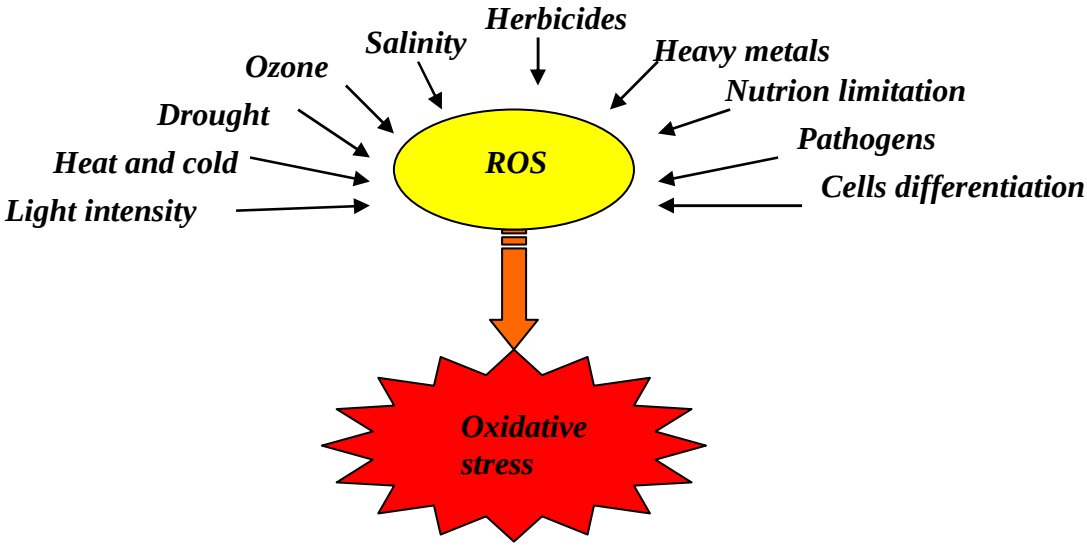


Fig. 1.5 Induction of oxidative stress by different environmental stress conditions

1.4.2. SoxRS and OxyR regulation

In *E. coli* and some other prokaryotes the adaptive response to superoxide anion and hydrogen peroxide is regulated by transcriptional regulators SoxRS and OxyR (Gaudu and Weiss, 1996; Gaudu et al., 1997; Carmel-Harel and Storz, 2000) cooperating with other regulatory mechanisms like is Fur-mediated regulation (see for review Storz and Imlay, 1999; Zheng et al., 1999).

SoxRS regulon mediates an oxidative stress response that protects the cell against the superoxide anion by oxidation of the [2Fe-2S] clusters in SoxR. The oxidized SoxR activates SoxS expression (Gaudu et al., 1997; Carmel-Harel and Storz, 2000) leading to elevated expression of antioxidant genes. *SoxRS* regulon consists of over 10 genes including detoxification enzymes as catalase, peroxidase or DNA repair proteins (se for review Storz and Imlay, 1999).

Several hydrogen peroxide inducible genes in *E. coli* are regulated by OxyR a redox-sensing LysR-type positive regulator. OxyR activation is based on Cys residues, hydrogen peroxide activates OxyR through the formation of an intramolecular disulfide bond directly inducing transcription of several antioxidant genes (Zheng et al., 2001; Toledano et al., 1994). OxyR regulon regulates genes coding for KatG, alkyl hydroperoxide reductase, glutathion reductase and some Dps (Storz and Imlay, 1999; Dwivedi et al., 1997).

1.4.3. Fur regulation and iron stress

In other bacteria without SoxRS or OxyR homologues another, Fur-like peroxide sensing repressor PerR, was found. This PerR is a Zinc-metalloprotein with ligated Fe^{2+} or Mn^{2+} (Lee and Helman, 2006). When oxidized, PerR binds iron and is easily dissociated from DNA by low levels of hydrogen peroxide, thus releasing repression of PerR controlled genes (Li et al., 2004; Herbing and Helman, 2001; Singh et al., 2004) including genes for Dps, catalase, alkyl hydroperoxidase (Mongkolsuk and Helmann, 2002).

There is evidence of the relationship between iron assimilation and oxidative stress. It is assumed, that precise regulation of iron assimilation prevents excess of free iron inside the cell which would lead to oxidative stress. On the other hand the iron deficiency can limit the activity of iron containing enzymes as SOD and catalases and lead to oxidative stress, too (Rodriguez and Smith, 2003; Touati et al., 1995).

1.4.4. Regulation in Synechocystis

Until now, neither SoxRS nor OxyR regulation has been found in cyanobacteria. However, peroxide inducible PerR homologue slr1738 has been identified in Synechocystis by Li et al. (2004). It negatively regulates expression of 9 hydrogen peroxide inducible genes including *ahpC*, where PerR binds the promoter region (Kobayashi et al., 2004; Kanesaki et al., 2007). Although PerR is involved in response to oxidative stress in *Synechocystis* (Houot et al., 2007; Singh et al., 2004) it does not work as a major regulator. The main regulatory role in *Synechocystis* seems to be accomplished by histidine kinases (Hiks).

1.4.5. Histidine kinase sensing and regulation

Two-component systems of histidine kinase (Hik) and cognate response regulator (Rre) have been found in many organisms including cyanobacteria, fungi, yeast and lower animals (Koretke et al., 2000) but did not in higher animals. Hiks perceive changes from environment and undergo autophosporylation with ATP as a donor of the phosphate group, which is then transferred to cognate Rre. Rres change their conformation and then bind to promoter regions of the regulated genes.

The Hik-Rre system can regulate gene expression in both positive and negative ways. In the positive regulation the regulated genes are silent due to inactive forms of Hik and Rre. When the cell is exposed to stress, the proper Hik is activated followed by Rre activation and enhanced expression of the regulated genes. In the negative regulation the regulated genes are repressed due to active Hik and Rre under non-stress condition. After stress exposure the Hik become inactive resulting in repressor release, and expression of the regulated genes.

In last years some two component regulation systems have been revealed to regulate the responses of cyanobacteria to different stress conditions, which can lead to oxidative stress. From 47 putative genes coding for Hiks and 45 genes coding for Rres in *Synechocystis* (Kaneko et al., 1996; Kaneko et al., 2003; Mizuno et al., 1996), Hik33-Rre31 and Hik34-Rre1 systems have been identified using microarrays and characterized. These systems are activated under different stress conditions and regulate expression of several ROS scavenging enzymes. Hik33-Rre31 regulates the expression of *LilA* in salt and hyperosmotic stress, Hik34-Rre1 is involved in *sodB* gene regulation (Shoumskaya et al., 2005; Paithoonrangsarid et al., 2004; Mikami et al., 2002). Hiks 34, 16, 41 and 33 regulate gene expression under peroxide with Hik33 being the main contributor (Kanesaki et al., 2007). Hik33 is also involved in light-stress and cold-stress response (Hsiao et al., 2004; Suzuki et al., 2001).

1.4.6. MV resistance

MV belongs to family of bipyridylium herbicides. It is effective non selective herbicide acting in plants by generating superoxide anion by reducing oxygen using electrons from Photosystem I (Mehler, 1951) frequently used to induce oxidative stress.

Cells can generally cope with MV by two mechanisms: by increase in activity of ROS-scavenging enzymes in resistant plants; and sequestration of MV away from its site of action in cells. Evidence for the first model relies primarily on measurement of increased enzyme activity and cross-resistance to other oxygen radical-generating stresses in resistant plants. The sequestration model is supported by data showing decreased translocation of MV and absence of MV injury in plant systems that do not have increased levels of scavenging enzymes.

In the *Synechocystis* mutant resistant to MV the second mechanism was observed. Mutation responsible for MV resistance was localized to the regulatory gene *prqR* that belongs to the TetR family of transcriptional regulators (Ramos et al., 2005; Orth et al., 2000). PrqR negatively regulates transcription of a MATE-type exporter (Brown et al., 1999) PrqA

organized in prqRA operon. The mutation resulted in modification of DNA-binding domain of PrqR protein resulting in its inability to bind to DNA. PrqR seems to be also involved in regulation of expression other ROS-scavenging enzymes involved in defense mechanism against oxidative stress in this unicellular cyanobacterium (Nefedova et al., 2003a; Babykin et al., 2003).

2. THE AIMS

ROS are normal by-products of photosynthesis and respiration. Many environmental conditions lead to increased ROS production causing oxidative stress. To overcome oxidative damages the cells employ various anti oxidative processes, primarily detoxification of ROS by scavenging enzymes. These processes can be based on ROS detoxification by scavenging enzymes. The aim of this thesis was to contribute to understanding how *Synechocystis* responds to oxidative stress. We focused on three topics:

1) characterization of several mutants lacking individual scavenging enzymes (superoxide dismutase, catalase-peroxidase, thioredoxin peroxidase, alkyl-hydroperoxide reductase).
2) determination of expression profiles of genes participating in protection against oxidative stress under various stress conditions in several mutants.
3) generation of spontaneous mutants resistant to MV to identify genes responsible for the resistant phenotype.

3. MATERIALS AND METHODS

Growth conditions and stress treatment

The wild type (WT) and mutants of *Synechocystis* sp. strain PCC 6803 (*Synechocystis*) were grown autotrophically or photomixotrophically in liquid BG-11 or in BG-11 medium supplemented with glucose (5 mM) at 30°C and at light intensity of 40 µmol m^{-2} s^{-1} (Ripka et al., 1979). Solid media were supplemented with 1.5% (wt/vol) agar and 0.3% (wt/vol) sodium thiosulfate and for MV-resistant mutants with 20-60 µM MV. The microaerobic growth conditions (N_2) were provided by bubbling liquid cultures or a plastic tank for plates by N_2 with 1%CO_2 (Fig. 3.1). The growth rate was measured as change of optical density at 730 nm (OD_{730}). OD_{750} was used for cultures in microtitre plates with 0.25 mL culture shaken on a rotary shaker (900 RPM). OD_{750} was measured using a microplate reader (Tecan Sunrise, Austria).

Strains were grown to mid-log phase (OD_{730} = 0.5) under standard conditions or under microaerobic conditions (*ΔahpC*, *ΔsodB* or WT) and subjected to various stresses: high light (HL) - 500 µmol m^{-2} s^{-1} of light, low temperature (LT) – 18°C, high salinity (NaCl) – 0.5 M NaCl, methyl viologen (MV) – 5 µM MV, peroxide – 0.5 mM *t*-BuOOH or H_2O_2. Samples for RNA isolation were taken at times 0, 20, 60 and 360 minutes.

Fig. 3.1 The microaerobic cultivation; plastic tank bubbled with N_2:CO_2 (98:2) mixture.

Mutant construction

ΔsodB mutant. The PCR-amplified 1.6-kb DNA fragment on WT template (primers 5´- TATGCTTGAAGCAACATCTC-3´and 5´- AAGAGTACCGCATCACTATC-3´) containing *sodB* (*slr1516*) was cloned into pUC19 vector. The *sodB* gene was inactivated by replacing the fragment between HindII (45) and HindII (498) in the *sodB* coding region with *kan-sacB* cassette (Lagarde et al., 2000) creating pUCΔsodB-kan-sacB construct and used for WT transformation. Transformants were selected on agar plates supplemented with kanamycin up to 25 µg/mL under microaerobic conditions. Latter the kan-sacB cassette was removed by the transformation of segregated pUC*ΔsodB-kan-sacB* mutant with the self

ligated plasmid construct pUC*ΔsodB* and segregated in presence of saccharose under microaerobic conditions yielding markerless *ΔsodB* mutant. Complete segregation was confirmed by PCR.

Δtpx mutant. The PCR-amplified 1.4-kb DNA fragment on WT template (primers 5'-CCAACATGCCTAGAGGCCC–3´ and 5'-CCTCCCGCTTAATTAGGGA–3´) containing *tpx* (*sll0755*) was cloned into the blunt end PvuII site of pUC19 (Fermentas) vector. The *tpx* gene was inactivated by replacing the 360-bp fragment between HindIII (121) and HindII (481) from coding region with a 1.7–kb erythromycin resistance cassette creating pUC*Δtpx* plasmid. This construct was used to transform WT and *ΔkatG* mutant. Transformants were selected on agar plates containing up to 25 µg/mL of erythromycin and segregated routinely under microaerobic conditions although both mutants grew normally under aerobic conditions. Complete segregation was confirmed by PCR.

ΔahpC mutant. The *ΔahpC* strain was prepared by the replacement of the whole ahpC gene with a 1.3-kb kanamycin resistance cassette from pUC4K plasmid using a megaprimer PCR method (Dobáková et al., 2009). Linear deletion constructs were obtained containing upstream and downstream regions of the *ahpC* gene with the kanamycin resistance cassette in the middle. In the first step, upstream and downstream regions of *ahpC* were separately amplified using long fusion primers complementary to the *ahpC* gene in one direction and the kanamycin cassette in the other: (1) 5'-CTTCTTCACGAGGCAGACCTCAGCGTCAATGCTTGCCCTCTTTTC-3' and (2) 5'-CTGTTTCCTGTGTGAAATTGTTATCTAAGTCTGTCAGAGCATTTC-3' (the cyanobacterial part is underlined). These fusion primers were used in pairs with *ahpC* forward and reverse primers: (1) 5'-TGGCATCGTAGCGACAGACC-3' and (2) 5'-GGGAACAGTATGAATTTGAC-3'. In the second step, the kanamycin resistance cassette was amplified using PCR products from the first step as primers. The complete deletion construct was amplified using *ahpC* forward and reverse primers and used for transformation of *Synechocystis* cells. Transformants were selected and segregated on kanamycin-containing agar plates under microaerobic conditions; their full segregation was confirmed by PCR.

Superoxide dismutase activity assay

SOD activity was measured in soluble protein fraction by spectrophotometric method using xanthine/xanthine oxidase/cytochrome *c* system as inhibition of the rate of reduction of cytochrome *c* at 550 nm (McCord and Fridovich, 1969) or by native polyacrylamide gels using negative nitro blue tetrazolium staining (Beauchamp and Fridovich, 1971).

Ezyme activities were determined in soluble protein fractions. 100 mL of cells cultivated to mid-log phase were concentrated to 2 ml by centrifugation. 400µL of cells was mixed with 200 µL of glass beads and disrupted on bead-beater (BioSpec Products, Inc., USA) 3-times for 10 s at maximum speed and cooled on ice. The soluble fraction was obtained by centrifugation at 4°C, 18 000 rpm for 5 min. Protein concentration in the soluble fraction was measured using Protein assay kit (Bio-Rad); according to manual.

Peroxide detoxification.

The dark and light decomposition of 50 µM H_2O_2 or *t*-BuOOH *in vivo* was measured by ferrous oxidation/xylenol orange (FOX) assay (Wolf, 1994). Cells were diluted to OD_{730} = 0.2 and incubated for 60 min in dark or light. Samples were taken at time intervals (0; 10; 20; 30; 40; 60 and 90 min) after addition of peroxide, briefly centrifuged and the supernatant was directly used for the FOX assay. Absorbance at 560 nm was measured on the microplate reader. The concentration was determined using calibration curves with H_2O_2 or *t*-BuOOH. Measurements are average of three parallels.

Lipid peroxidation

A modified ferrous oxidation/xylenol orange assay was used to determine levels of hydroperoxides (LOOH) as H_2O_2 equivalents in cyanobacterial cells (Sakamoto et al., 1998). Exponentially growing cells at OD_{730} of 0.5 and were incubated under standard growth conditions in the presence or absence of 1 µM MV. 800 µL of cells were harvested at times 0; 1; 2; 4 or 6h by centrifugation. To eliminate the presence of H_2O_2 a 30 min pretreatment with 500-1000 U of catalase mL^{-1} (CAT+) was used and followed with the 30 min treatment with 50 µl of 10 mM triphenylphosphine in MetOH (TPP+) or with MetOH (TPP-) (DeLong et al., 2002). The cell pellets were resuspended in 0.75 mL of methanol containing 0.01% butylated hydrotoulene (BHT), 0.1 mL of reagent A (2.5 mM ammonium iron (II) sulfate/0.25 M sulfuric acid) and 0.1 mL of reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol). The mixture was incubated for 60 min at room temperature and then centrifuged to remove cell debris. The absorbance at 560 nm was measured on the microplate reader. As TPP reacts with LOOH, LOOH concentration was determined from the difference TPP treated and untreated samples. H_2O_2 formation was calculated from difference between catalase treated and untreated samples. The concentration determined using calibration curves with H_2O_2. Measurements are average of three parallels.

Oxygen evolution

The tertiary butyl hydroperoxide (*t*-BuOOH)-dependent oxygen evolution was measured using Clark-type electrode (Walz, Germany) at 25°C in according to Gaber et al. (2004). 10 mM glycolaldehyde was added to the cells in the mid-log phase concentrated to OD_{730} = 2 to inhibit of CO_2 fixation via the Calvin cycle. 100 µM or 1 mM *t*-BuOOH was used to measure *t*-BuOOH-dependent oxygen evolution in. Oxygen evolution was measured three times and was normalized to chlorophyll.

Total chlorophyll content and pigment spectra

The total chlorophyll content was determined spectrophotometrically in methanol according to Lichtenthaler (1987). For pigment spectra, cells were extracted with methanol and absorption spectra from 400 nm to 700 nm were recorded (Unicam, USA) and normalized to chlorophyll at 663 nm. Relative carotenoid content was estimated by absorbance at 475 nm.

Diffusion assay

The diffusion assay used to estimate the sensitivity of the WT and mutant strains to various toxic compounds was a modification of standard methods (Bauer et al., 1966; Buchmeier et al., 1997). An 0.1-mL aliquot of a particular exponential culture (adjusted to OD_{750} = 0.5) was spread on a BG11 agar plate grown for 3 days, treated with 5 µL of a solution containing an appropriate concentration of the test compound and incubated for 5 days. Strain sensitivity was estimated from the size of lytic zones on green plaque.

Isolation of chromosomal DNA

Chromosomal DNA was prepared from 1 L culture using NaI/lysozyme for cell lysis, phenol/chloroform for DNA purification and Na-acetate/ethanol for DNA precipitation. Briefly, cells were centrifuged and pellet was resuspended in saturated NaI 2 mL per gram of cells and treated 20 min at 37°C centrifuged and resuspended in 8 mL of TES buffer. After lysozyme (62.5 mg per g of cells) and N-lauryl sarcosine (0.1 g per gram cells) treatments for 20 at min 37°C the lysate was extracted with phenol:TES (1:1) for 60 min, gently mixed and carefully extracted with chloroform. Finally the chromosomal DNA was precipitated using Na-acetate/ethanol overnight at -20°C. During the procedure, cut tips were used to prevent DNA breakage.

DNA digest and isolation of fragments

The complete restriction of 5 µg chromosomal DNA was carried in a large volume (200 µL) overnight with 30-40 units of a particular restriction enzyme. DNA was extracted with chloroform and precipitated with Na-acetate/ethanol. The precipitate was resuspended in 20 µL of Tris/EDTA buffer and separated electrophoretically on 0.5 % low melting agarose (20 V for 8 hours). The gel was briefly stained in ethidium bromide to visualize separated DNA and washed thoroughly with cold water. Each enzyme digest was cut into 15 pieces corresponding to known fragment of Gene Ruler 1Kb DNA Ladder and λ mix Marker 19 (Fermentas) as a reference and gel pieces were frozen at –70°C overnight (Fig. 3.2). For transformation of cyanobacteria, samples were incubated for 30 min at 37°C, than centrifuged (13 000 RPM, 15 min, 4°C) and the liquid phase was used for transformations.

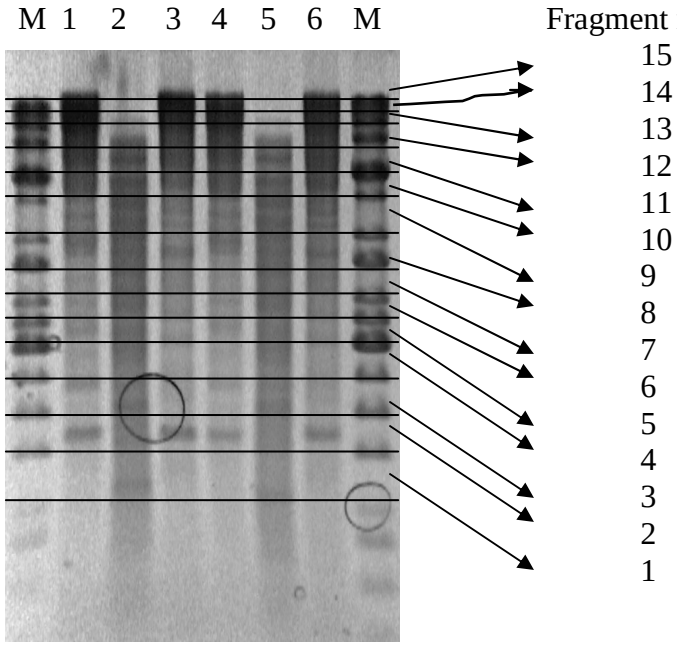


Fig. 3.2 The preparation of DNA fragments. Restriction mixtures were run on LM-agarose gel M: marker; line 1-3: DNA digest of MV-A (BamHI, HindIII, EcoRV); line 4-6: DNA digest of MV-B (BamHI, HindIII, EcoRV) and cut into 15 fragments of known fragment size.

Table 3.1 Sizes of restriction fragments used for transformation.

Fragment number	Fragment size in according to DNA markers (bps)
1	1000-1500
2	1500-2000
3	2000-2500
4	2500-3000
5	3000-3500
6	3500-4000
7	4000-5000
8	5000-6000
9	6000-8000
10	8000-10 000
11	10 000-15 000
12	15 000-20 000
13	20 000- 30 000
14	30 000-40 000
15	more than 40 000

Transformation of WT with restriction fragments

WT cells grown to mid-log phase were concentrated to OD_{730} = 5 and 50 μL were transformed with a particular fragment size (the liquid phase from the gel fragment) for 30 -60 min at room temperature; WT without DNA was used as a negative control. The transformed cells were plated on BG plates with filters (0.85 μm), each plate with the negative control. The filters were moved to plates with gradually increasing concentration of MV (20; 40 and 60 μM; Table 4.8). Transformants growing on BG-11 with 60 μM concentration of MV were counted after 14 days. Size of restriction fragments yielding the highest number of transformants, for particular restriction enzyme, were used for analyses. The region containing the resistance mutation was determined as the intersection of the sets of putative transforming fragments. The point mutation was localized by sequencing of PCR amplification of located genes (Table 3.2).

Table 3.2 Primers used for the localization of mutation in MV-resistant mutants.

Primer name	**Localized gene**	**Genome position start**	**Genome position end**	**Primer sequence 5´- 3´**
MV1	*slr0895*	2762442	2762461	AATGCACTGGTCCAATTCCC
MV2	*slr0895*	2764258	2764239	AGAATGATGGTTGTGTCAGC
MV5	*slr0895*	2763533	2763514	CAGGATTGGTGAAGTTTGCG
MVQ I1	*slr1174*	1943431	1943451	TCAGTGCCAAGGAATCCAATG
MVQ I2	*slr1174*	1947069	1947050	CCTCCTGATCGAAAAGGATG
MVQ I4	*slr1174*	1944157	1944177	ACACCTATCGTCAAACGGTGC
MVQ I5	*slr1174*	1945322	1945303	TTAAGCAGGGCAAATATGCC

Gene expression, RNA isolation, reverse transcription and quantitative PCR

5 mL of *Synechocystis* cells were cooled on ice and harvested by centrifugation at 6,000 rpm for 5 min at 4°C. The pellets were immediately frozen in liquid nitrogen and the samples were stored at -75°C. The total RNA was extracted by the modified hot phenol method (Mohamed and Jansson, 1989). Briefly, the frozen cells were thawed on ice and washed two times with the resuspension buffer, lysed in SDS lysis buffer, two times heated and extracted with hot acid phenol and chloroform. Total RNA was precipitated by LiCl and ethanol. Twenty nanograms of purified RNA were used for cDNA synthesis using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real time-quantitative PCR reactions were performed on the Rotor-Gene 3000 using the iQ SYBR Green Supermix (BioRad) and proper pairs of primers (Table 3.3). Each quantitative PCR experiment was performed on two independent RNA isolations for the steady-state RNA levels and on one for screening. 16S rRNA was used as a reference as its level was found to be proportional to total isolated RNA. The ΔΔCt method was used to calculate gene expression levels. The ΔCt values were reproducible within 0.5 cycle.

Table 3.3 Genes subjected to expression analysis with respective primers.

ORF	Name	Gene product	Probe primers 5´- 3´
Slr1516	*sodB*	Superoxide dismutase	TGCTGGCGATGCTAGCAAAG TTGGTCACTTTCAAAGTGCC
Sll1987	*katG*	Catalase-peroxidase	ACTTATCGCATTGCCGATGG CATCGACTCATAGGCCATGG
Slr1171	*gpx-1*	NADPH-dependent Gpx-like protein	CAAGCCAACAACACAATCTAC ATCGATTGTATAGAGCCTGGA
Slr1992	*gpx-2*	NADPH-dependent Gpx-like protein	CCTATGCCATTACCCACTTC CACACCGATAATTACTAAGCC
Sll0755	*Tpx*	2-Cys Prx, Thioredoxin peroxidase	AGACAGCGAATTTTCACACC TCGAGGACATTATAGGCTTG
Slr1198		1-Cys Prx	AAAGGTGTCCGATCTGTACG ACTGGAGGGAGTCAATTACC
Sll1621	*ahpC*	Type II Prx, hydroperoxide reductase thiol specific antioxidant protein	ACGAATCTGTACCTGGTCCC ATGTCATCTACTCCCAAGGC
Sll0221		Prx-Q	ACCAGAGTTGGATCAACCTGC ATTACCTGGGCATTGAGAGC
Slr0242		Prx-Q	CAGCGCTATTCAACTGTTGC GTTCAACTGCTCAGTGATCC
Sll1159		Probable Prx-Q,	GCATCCTCAGGTTCGTAGCG GTTTTTACCGTTCCCGATGC
Slr1544	*lilA*	Light-harvesting-like protein A	TACCAAAGGACTGCCCTTGG AGTCTTTGCGGAAGACAACG
rrn16Sa	*16S rRNA*	16 S ribosomal RNA	GCTCGCGAGAGTAAGCGAATC GTGTGACGGGCGGTGTGTACAAG
Additional tested genes in MV-resistant mutants			
Slr0895	*prqR*	Transcription repressor of TetR family	GTGGCTAGTGAAGCAGGGGT CTGGATCATTGGCAAGTTCC
Slr0896	*prqA*	Multi-drug efflux transporter	GATCGCCTTTGCTACGCTCCGG CACTGATACTAGCAATGGC
Slr0616	*mvrA*	Sugar and other compounds transporter	TTGGAGGAAGCGGACTACACC CAGTCGCCAGAGCACAATCAC

4. RESULTS

4.1. Characterization of deletion mutants

4.1.1. Superoxide dismutase deletion mutant

The part of *sodB* (*slr1516*) between *Hind* II sites at positions 45 and 498 was replaced by *kan-sacB* cassette (Lagarde et al., 2000); this cassette was later removed by markerless construct completely lacking the *Hind* II fragment, yielding markerless *ΔsodB* mutant. The *ΔsodB* mutant didn't segregate when grown on air but segregated under microaerobic conditions, indicating importance of this enzyme for aerobic metabolism. The complete segregation was confirmed by PCR (Fig. 4.1).

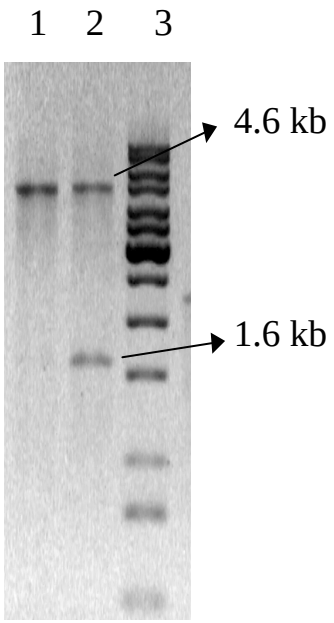


Fig. 4.1 Segregation of the ΔsodB mutant. PCR amplification of sodB gene from the mutant segregating under microaerobic conditions (1) or on air (2). Line 1 - fully segregated ΔsodB (kan-sacB) mutant; line 2 - partially segregated; line 3 - DNA 1 kb ladder.

Phenotype of *ΔsodB* mutant was characterized by growth rates, superoxide dimutase activity and various stress treatments. The growth rate of the *ΔsodB* mutant under microaerobic conditions was comparable to that of wild type. Initially, the mutant didn't grow on air (Table 4.1).

Table 4.1 Growth rates of the ΔsodB mutant and WT under microaerobic and aerobic conditions.

Strain	Doubling time			
	Photoautotrophically		Photomixotrophically	
	Microaerobic	Aerobic	Microaerobic	Aerobic
ΔsodB	24 h	> 60 h	20 h	> 60 h
WT	9 h	12 h	-	9 h

Weak aerobic growth of the mutant was possible only on plates supplemented with catalase (100 U/mL). Also, frozen stock of *ΔsodB* mutant could be prepared only in media supplied with catalase, indicating importance of SodB for cell viability. Prolonged cultivation of *ΔsodB* led to improved growth of the *ΔsodB* mutant on air. The improved growth of *ΔsodB* mutant can be attributed to generation of pseudorevertants, as colonies of *ΔsodB*-pseudorevertants able to grow on air were obtained on plates grown aerobically.

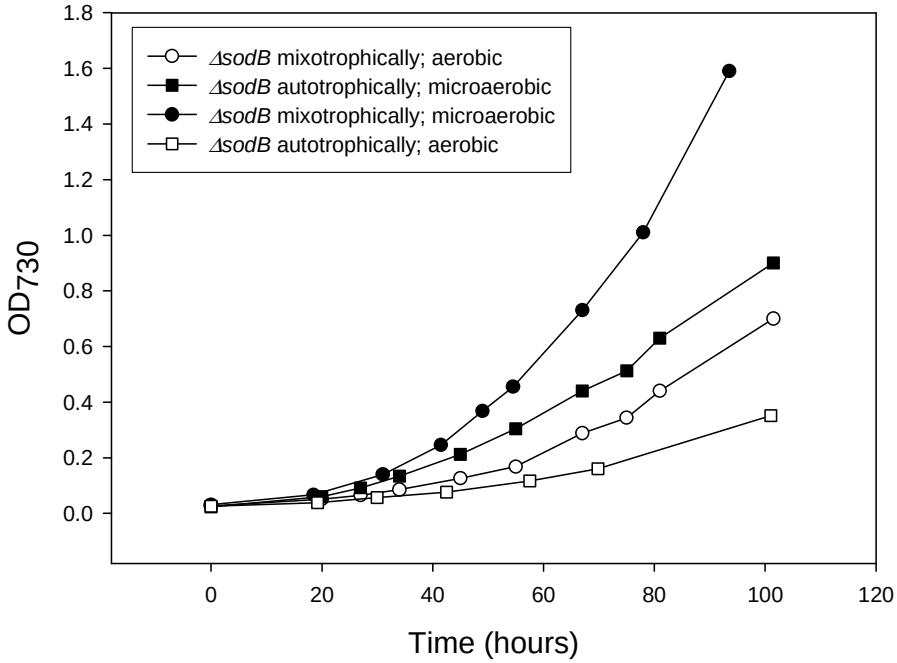


Fig. 4 .2 Growth of ΔsodB mutant under aerobic and microaerobic conditions in BG medium and BG medium supplemented with 5 mM glucose.

SOD activity was measured in soluble protein fraction by spectrophotometric method using xanthine/ xanthine oxidase/ cytochrome *c* system or by native polyacrylamide (PAGE) gels using negative nitro blue tetrazolium staining (Beauchamp and Fridovich, 1971) (Fig. 4.3). SOD activity measured in soluble protein extract was under the detection limit (0.5 U/μg chl) for *ΔsodB* and 29 U/μg Chl for WT. This indicates that no other superoxide dismutase is present in *Synechocystis.*

The *ΔsodB* mutant was also characterized by gene expression pattern of selected ROS-responsive genes in the absence of SOD (Chapter 4.2).

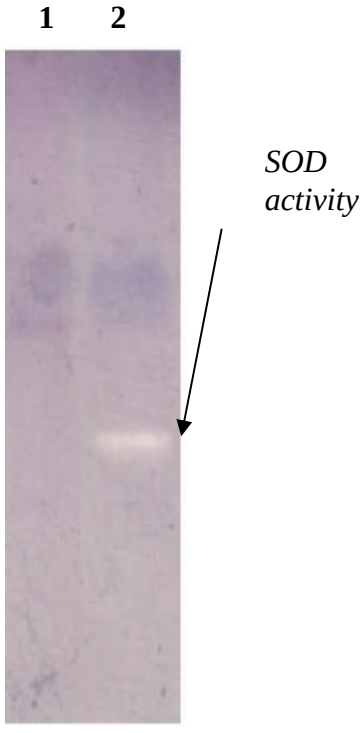


Fig. 4.3 SOD activity on native polyacrylamide gel; line 1: ΔsodB; line 2: WT; 40 μg of soluble proteins loaded.

4.1.2. Thioredoxin peroxidase deletion mutants

The *tpx* (*sll0755*) gene was inactivated by replacing the middle 360-bp part between *Hind*III and *Hind*II sites by an erythromycin resistance cassette. This construct was used to transform WT and the mutant lacking catalase-peroxidase (*ΔkatG*). Both deletion mutants were segregated under microaerobic conditions, although the mutant grew normally under aerobic conditions. The complete segregation was confirmed by PCR (Fig. 4.4).

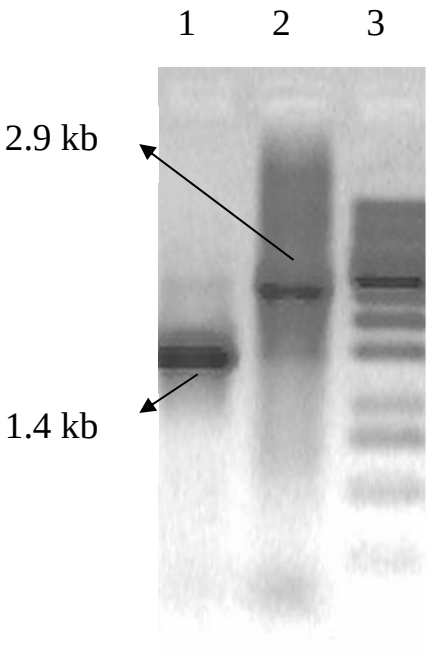


Fig. 4.4 The segregation of the Δtpx mutant. The PCR amplification of the tpx gene in WT and Δtpx mutant yielded 1.4 kb PCR product in WT and 2.9 kb product containing the erythromycin resistance cassette in segregated Δtpx. Line 1 – WT; 2 - Δtpx; line 3 – DNA 1 kb ladder.

The phenotypes of both mutants were characterized by their growth rates, sensitivity to peroxides, peroxide decomposition rates, lipid peroxidation levels and gene expression under various stress treatments.

Δtpx and *ΔtpxΔkatG* mutants grew both photoautotrophically and photomixotrophically. The growth rates of both mutants in liquid media were only slightly decreased in comparison to WT (Table 4.2).

Table 4.2 Growth rates of the Δtpx mutants and WT under microaerobic and aerobic conditions.

Strain	Doubling time			
	Photoautotrophically		Photomixotrophically	
	Microaerobic	Aerobic	Microaerobic	Aerobic
Δtpx	10 h	13 h	-	8.5 h
Δtpx/ΔkatG	10 h	14 h	-	9 h
WT	9 h	12 h	-	9 h

Sensitivity of strains to H_2O_2, *t*-BuOOH and MV was estimated by diffusion assay (Fig. 4.5). All three *Δtpx*, *Δtpx/ΔkatG* and and *ΔkatG* mutants were slightly more sensitive to H_2O_2 than WT (Fig. 4.5A). Strains lacking KatG were more sensitive than *Δtpx*, supporting important role of KatG in peroxide decomposition. All four tested strains exhibited the same sensitivity to *t*-BuOOH (Fig. 4.5B). Interestingly, *Δtpx* exhibited higher resistance to MV than WT and two *ΔkatG* mutants (Fig. 4.5C). Also, the strain *Δtpx/ΔkatG* was 4-times more sensitive to H_2O_2 than *Δtpx* during growth in liquid media (Fig. 4.6A, B). This suggests that no other enzyme can replace the catalase peroxidase in dealing with large amount of H_2O_2.

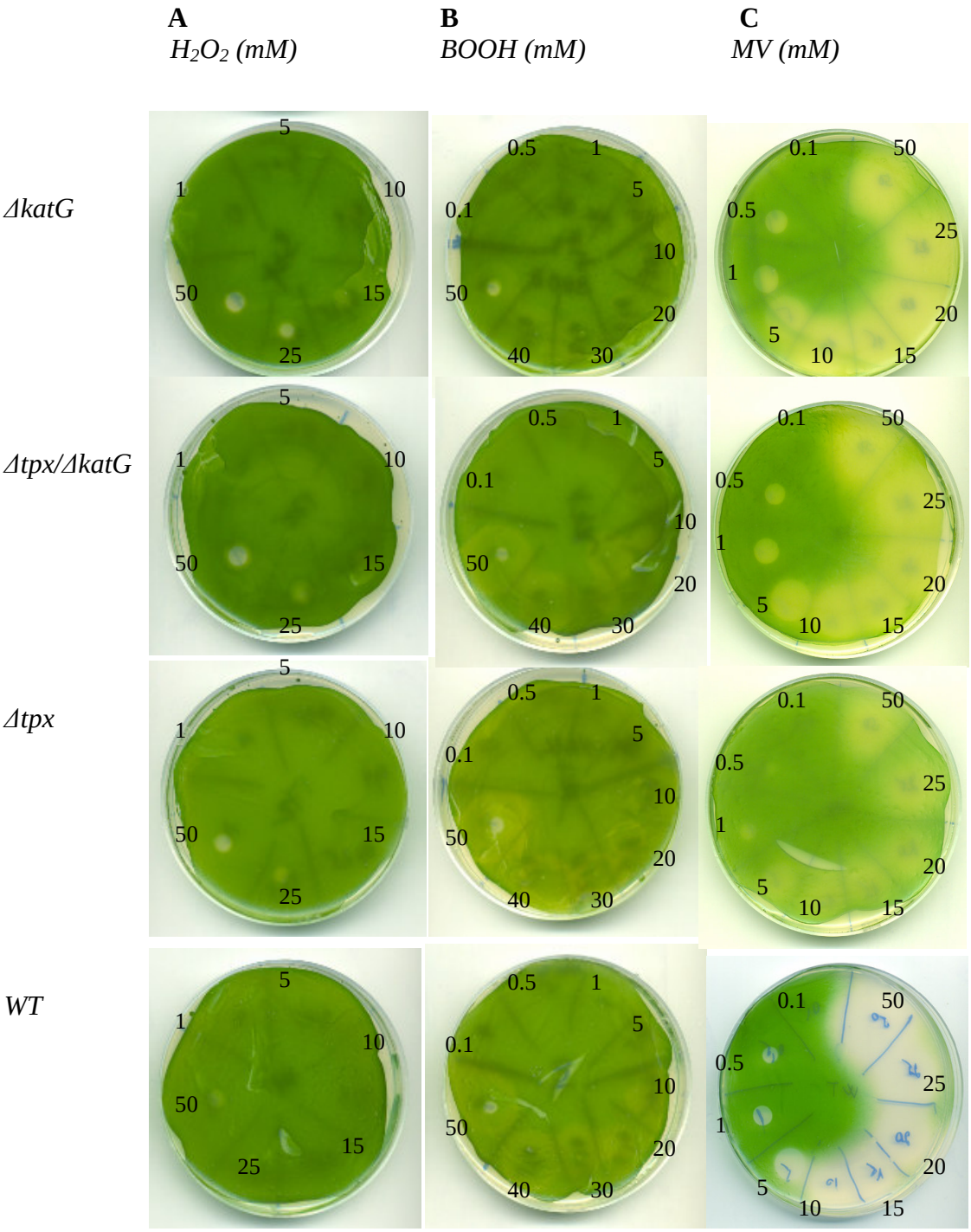
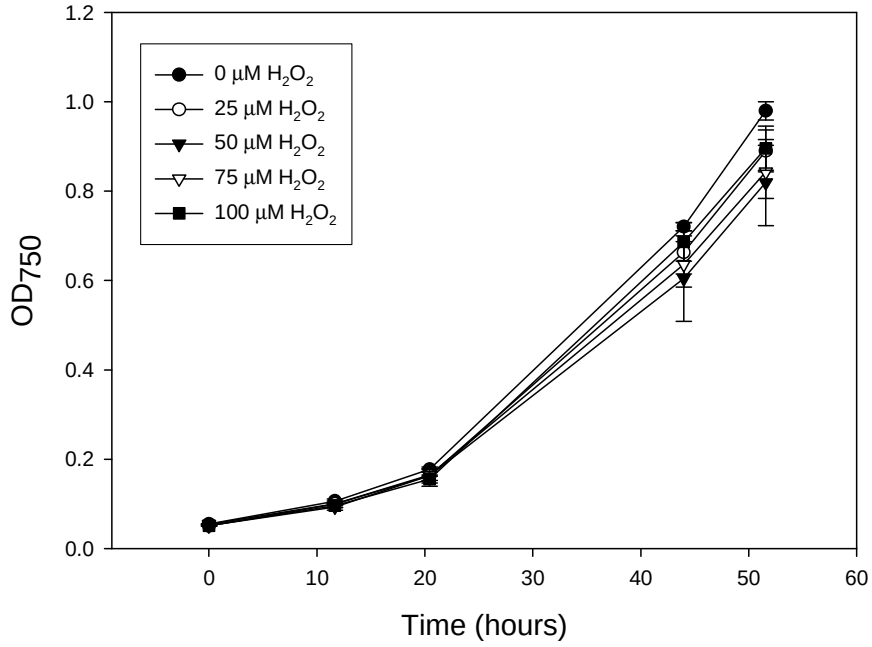


Fig. 4.5 Sensitivity of WT and mutants to peroxides and MV on agar plates. The formation of lytic zones represents lethal effect of applied solution of 1 - 50 mM H_2O_2 (A); 0.1 – 50 mM t-BuOOH (B) or 0.1 – 50 mM MV (C) to the cells.

A



B

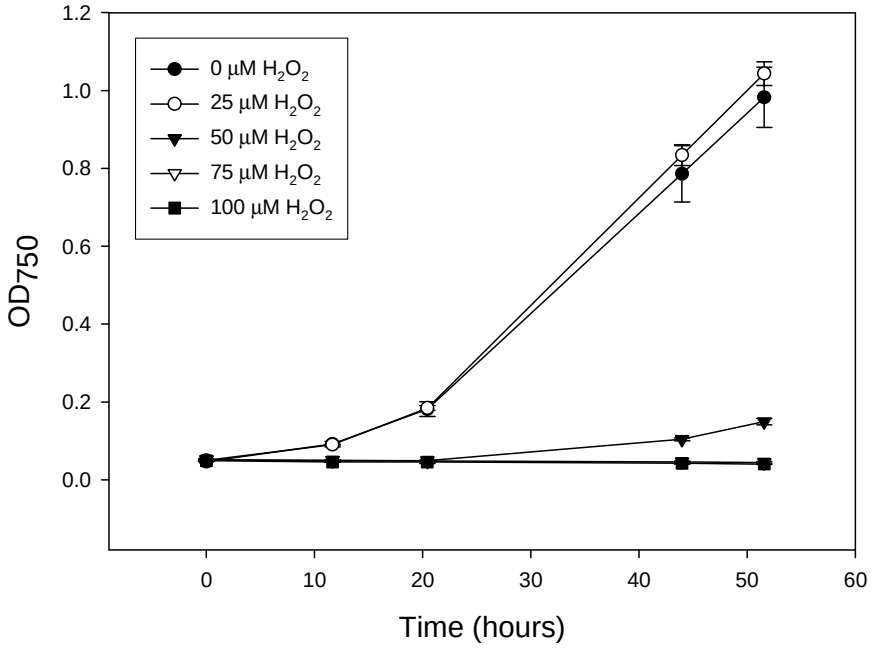


Fig. 4.6 H_2O_2 sensitivity of Δtpx and ΔtpxΔkatG strains. Growth of Δtpx (A) and Δtpx/ΔkatG (B) mutants in presence of 0-100 μM H_2O_2.

Oxidative stress frequently results in increased lipid peroxidation. Therefore, lipid peroxidation was measured in the presence of the superoxide generator MV. The lipid peroxidation was measured by modified FOX assay in WT, *Δtpx*, *Δtpx/ΔkatG* and *ΔkatG* strains during 1 μM MV treatment for 6 hours. Increased lipid peroxidation was not observed in any strain. There was significantly increased amount of H_2O_2 in the strains *Δtpx/ΔkatG* due to *katG* deletion. The same effect was observed for 5μM MV treatment, where the cultures became dead after 6 hours.

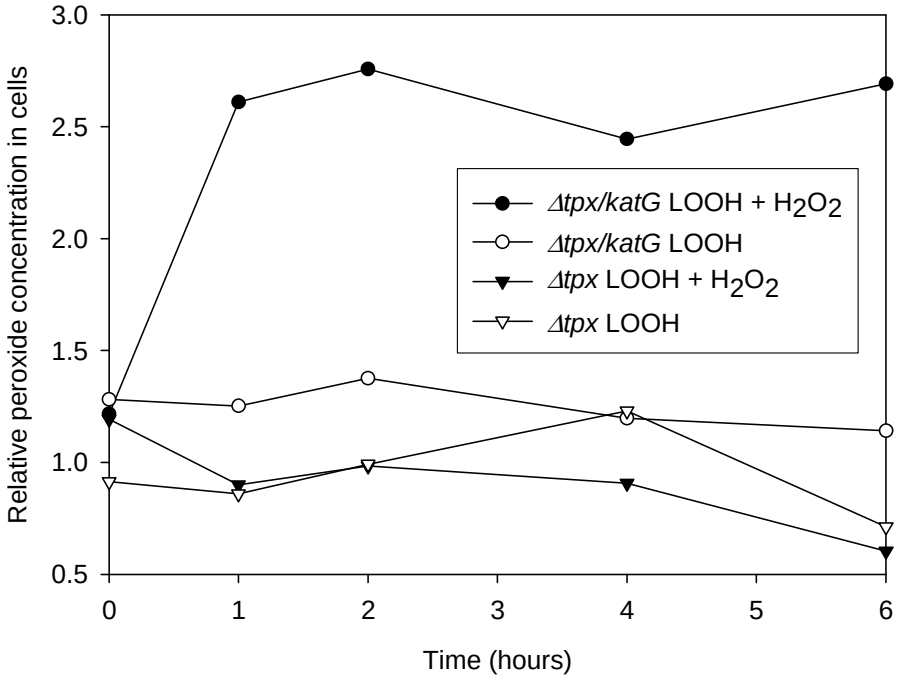
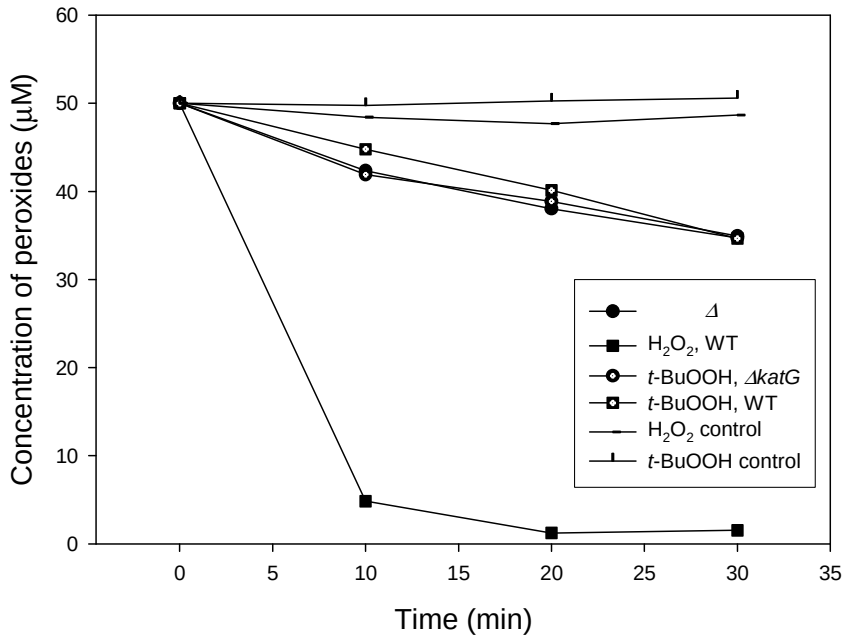


Fig. 4.7 Formation of lipid hydroperoxides and H_2O_2 in Δtpx and Δtpx/ΔkatG strains during 1 µM MV treatment. The formation of lipid hydroperoxides (LOOH) and H_2O_2 was measured by modified FOX assay and related to WT.

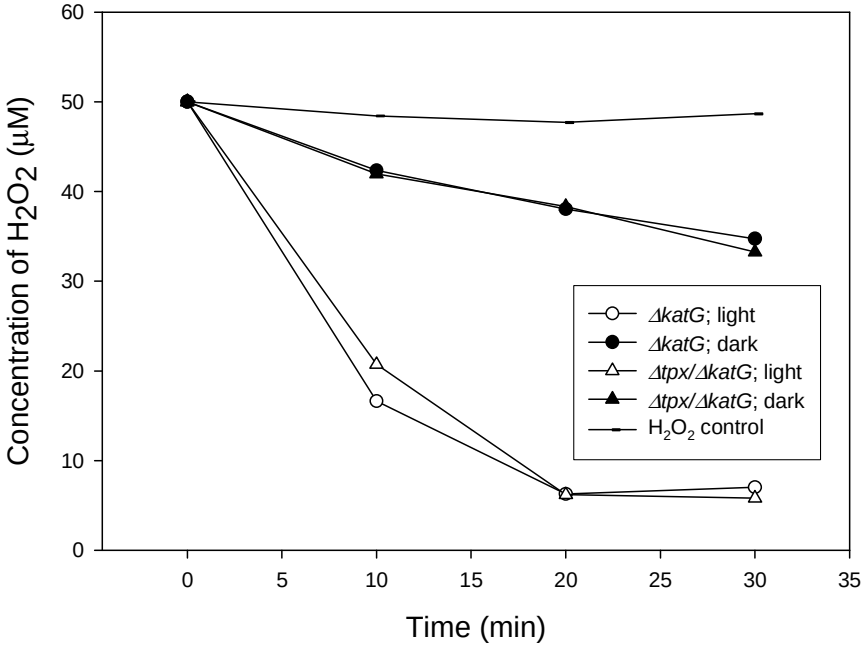
The ability to detoxify H_2O_2 or organic hydroperoxides that may mimic lipid peroxidation, likely contributes to cell survival during oxidative stress. To study the physiological role of Tpx, the ability to detoxify externally added 50 µM H_2O_2 or *t*-BuOOH by FOX assay and peroxide-dependent oxygen evolution by Clark-type electrode were estimated in *Δtpx* deletion mutants.

From previous results it was evident that the H_2O_2 decomposition has to be measured in *ΔkatG* background due to high catalase-peroxidase activity (Tichy and Vermaas, 1999), as WT and *Δtpx* completely decomposed 50 µM externally added H_2O_2 within the first 15 minutes (Fig. 4.8A), in contrast to *t*-BuOOH activity which can be estimated in WT background. The *Δtpx/ΔkatG* mutant exhibited the same H_2O_2 decomposition capacity as the *ΔkatG* mutant (Fig. 4.8B) and the H_2O_2 decomposition in the *Δtpx/ΔkatG* mutant was light-dependent as reported previously for the *ΔkatG* mutant (Tichy and Vermaas, 1999). These indicate that Tpx does not significantly contribute to H_2O_2 decomposition *in vivo* (Fig. 4.8B). Similarly to H_2O_2, no significant difference was observed between strains containing or lacking Tpx in *t*-BuOOH decomposition under both light and dark conditions (Fig. 4.8C). *t*-BuOOH was not completely decomposed by any strain during 30 min. Although the light-dependent decomposition can be clearly seen, there is no evidence that WT and *Δtpx* differ in decomposition of alkyl hydroperoxide (Fig. 4.8C). These indicate that other enzymes are responsible for the light-dependent activities.

A


Concentration of peroxides (μM)
Time (min)
Δ
H2O2, WT
t-BuOOH, ΔkatG
t-BuOOH, WT
H2O2 control
t-BuOOH control

B


Concentration of H2O2 (μM)
Time (min)
ΔkatG; light
ΔkatG; dark
Δtpx/ΔkatG; light
Δtpx/ΔkatG; dark
H2O2 control

C

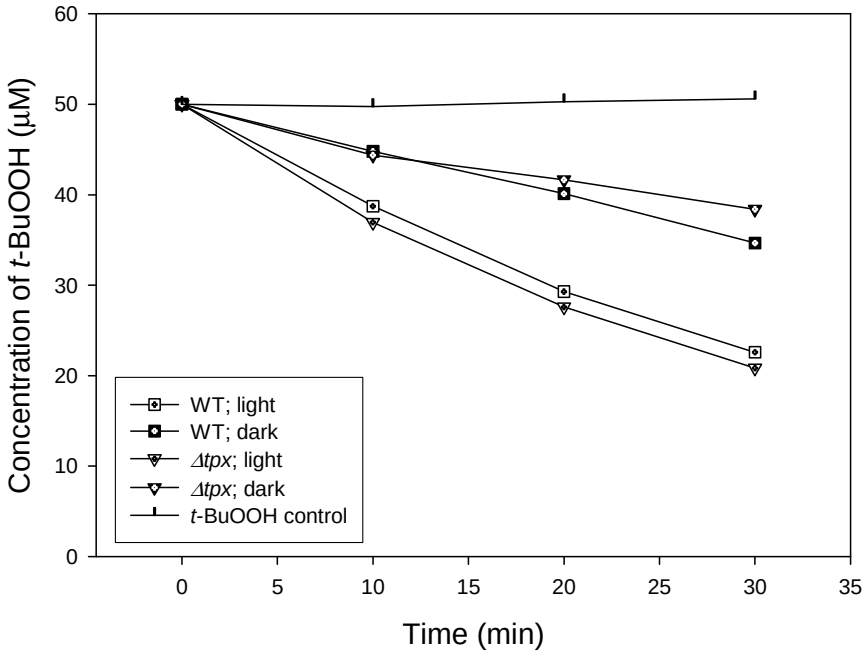


Fig. 4.8 Peroxide decomposition by Synechocystis cells in media. The light-dependent detoxification of externally added peroxides was measured as decrease of peroxide concentration by cells in dark or light using FOX assay; influence of KatG background – decomposition of peroxides by ΔkatG and WT in dark (A); decomposition of H_2O_2 by ΔkatG and Δtpx/ΔkatG mutants (B); decomposition of t-BuOOH by Δtpx mutant and WT (C).

It has been reported that the peroxidases use electrons generated during the photosynthetic electron transport as their activities are not observed in the presence of Photosystem II inhibitors or in the dark (Miyake et al., 1991). In the presence of glycolaldehyde, when the fixation of CO_2 is inhibited (Miller and Canvin, 1989) and the O_2 evolution is stopped in the light, addition of *t*-BuOOH to illuminated cells induce the *t*-BuOOH-dependent O_2 evolution demonstrating the peroxidase activity indicating that electrons used for peroxide reduction are coming from photosynthetic electron flow.

t-BuOOH-dependent O_2 evolution was measured in *Δtpx* mutant and compared to that of WT. In both strains the *t*-BuOOH-dependent O_2 evolution was stopped in the presence of Photosystem II inhibitor DCMU or in the dark. In *Δtpx* the *t*-BuOOH-dependent O_2 evolution was decreased to about half of that in WT (Table 4.3). This indicates that Tpx is using for peroxide reduction electrons from photosynthetic electron flow. It also shows that another peroxidase with the same source of electrons remains in *Δtpx*.

Table 4.3 t-BuOOH-dependent evolution of oxygen in WT and Δtpx was measured in presence of 10 mM glycolaldehyde on light. Data are the mean value ± SD of three independent experiments.

	100 µM *t*-BuOOH	1 mM *t*-BuOOH
Strain	µmol O_2 · mg chlorophyll^{-1} · h^{-1}	
WT	29.8 ± 3.5	63.6 ± 4.2
Δtpx	18.2 ± 1.1	33.6 ± 1.9

Mutant phenotypes were also characterized by gene expression pattern of selected ROS-responsive genes (Chapter 4.2).

4.1.3. AhpC deletion mutant

The *ΔahpC* strain was prepared by the replacement of the whole *ahpC* (*sll1621*) gene with a kanamycin resistance cassette from pUC4K plasmid using a megaprimer PCR method (Dobáková et al., 2009). Linear deletion constructs were obtained containing upstream and downstream regions of the *ahpC* gene with the kanamycin resistance cassette in the middle. Full segregation was reached only under microaerobic conditions with catalase and confirmed by PCR (Fig. 4.9).

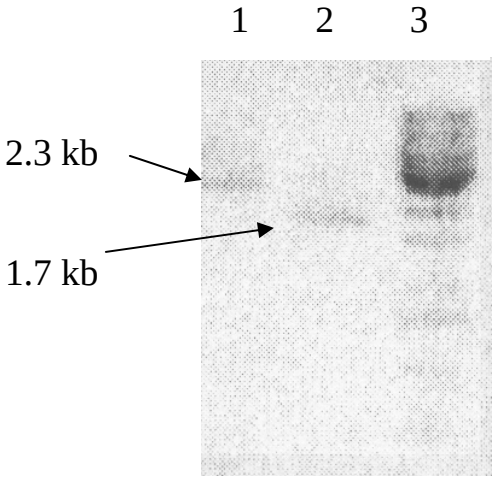


Fig. 4.9 Segregation of the ΔahpC mutant. PCR amplification of ahpC gene from the mutant segregating under microaerobic conditions (1) and WT (2). Line 1 - fully segregated ΔahpC mutant; line 2 - WT; line 3 - DNA 1 kb ladder.

Whereas the *ΔahpC* mutant was segregated under microaerobic conditions it was able to growth on air but its growth severely impaired (Table 4.4) as already reported by Kobayashi et al., (2004) and Hosoya-Matsuda et al., (2005). The cultures on air were turning brown-green color (Fig. 4.10) and could not be cultivated with $OD_{730} < 0.2$ at light intensity of 40 μmol m^{-2} s^{-1}. This is probably due to lower light intensity per each cell in denser cultures. We followed accumulation of carotenoids and also expression levels of selected ROS-responsive genes in WT and in *ΔahpC* during microaearobic and aerobic growth (see Chapter 4.2 for gene expression).

Table 4.4 Growth rates of the ΔahpC mutant and WT under microaerobic and aerobic conditions.

Strain	Doubling time	
	Aerobic	Microaerobic
WT	12 h	9 h
ΔahpC	28 h	12 h

Fig. 4.10 Phenotype of WT and ΔahpC grown under aerobic conditions.

Pigment spectra of WT and *ΔahpC* were measured in methanol cell extract. The WT and *ΔahpC* cultures cultivated under microaerobic and aerobic conditions showed difference in color and growth rates (Fig. 4.10; Table 4.4). The color change was caused primarily by increased carotenoid content in *ΔahpC* (Fig. 4.11). When *ΔahpC* was shifted from 0.5 under microaerobic condition to air it exhibited fast increase in carotenoid content (Fig 4.11). In WT, no such increase in carotenoid content was observed (Fig 4.11). These findings indicate the protection role of carotenoids.

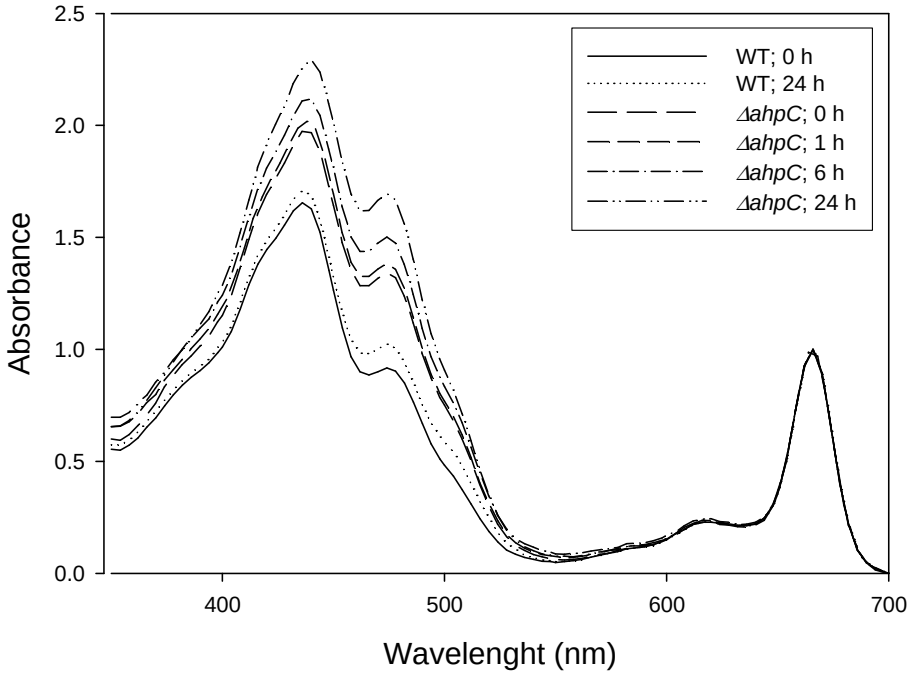


Fig. 4.11 Accumulation of carotenoids in WT and ΔahpC after transition from microaerobic to aerobic conditions. Absorption cell pigment extracts in methanol, normalized to chlorophyll.

WT and *Δtpx* exhibited light-dependent peroxidase activity. In contrast, deletion of *ahpC* dramatically decreased *t*-BuOOH decomposition. Externally added *t*-BuOOH to media was not efficiently scavenged and the decomposition in light and dark did not differ significantly in the *ΔahpC* mutant (Fig. 4.12). This indicates that AhpC is responsible for the observed light-dependent peroxidase activity. This was further supported by measurements of the *t*-BuOOH-dependent oxygen evolution in *ΔahpC*. No oxygen evolution was detected in the *ΔahpC* mutant either in presence of 100 µM or 1 mM *t*-BuOOH (Table 4.5).

Table 4.5 t-BuOOH-dependent evolution of oxygen in WT and ΔahpC mutant were measured in presence of 10 mM glycolaldehyde on light. Data are the mean value ± SD of three independent experiments.

	100 µM *t*-BuOOH	1 mM *t*-BuOOH
Strain	µmol O_2 · mg chlorophyll^{-1} · h^{-1}	
WT	29.8 ± 3.5	63.6 ± 4.2
ΔahpC (N_2)	0	0
ΔahpC (O_2)	0	0

N_2 – microaerobically cultivated
O_2 – aerobically cultivated

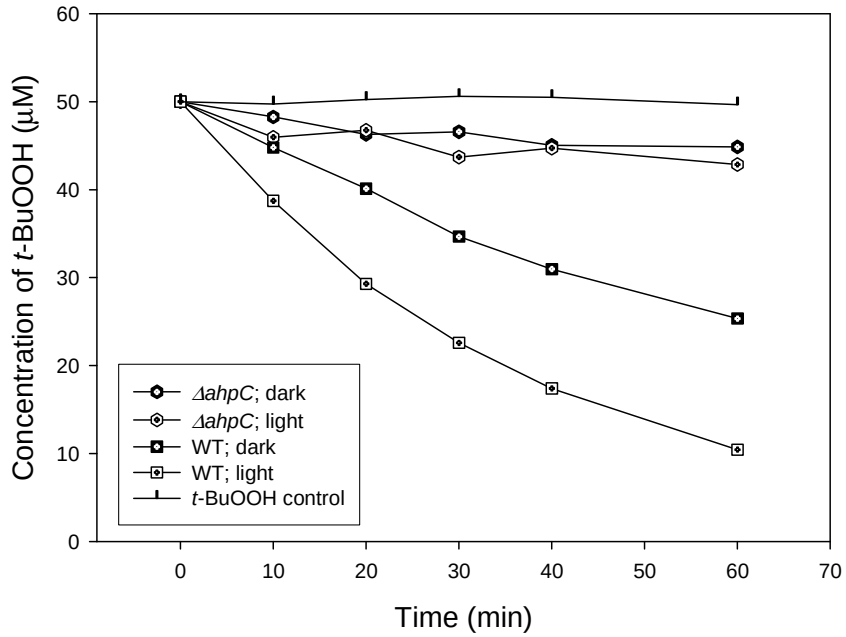


Fig. 4.12 The light-dependent decomposition of 50 µM t-BuOOH in vivo by strains ΔahpC and WT measured with FOX assay.

4.2. Expression pattern of ROS-responsive genes in deletion mutants during normal growth conditions and various stress treatments

4.2.1. Steady-state expression of ROS-responsive genes in WT and mutants

Deletion of individual genes coding for ROS detoxifying enzymes may lead to disturbed cell protection against oxidative stress. This mutation may be complemented by changed expression of other genes involved in response to ROS serving as a backup system. We have followed expression pattern of a set of such genes (Table 3.3) and compared it to WT under standard growth conditions. Expression of *lilA* was used in this and further experiments as a stress indicator (Kufryk et al., 2008). Expression of this gene is transiently highly induced by most stresses as shown in multiple microarray studies (Hihara et al., 2001; Kanesaki et al., 2007; Shoumskaya et al., 2005).

Induced genes with relative expression levels $\geq$ 2 are summarized in Table 4.6 indicating the compensation of deleted ROS-responsive gene by increased expression of others. The permanent expression of *lilA* in *Δtpx* and *ΔkatG* mutants indicates their higher sensitivity to stress. Interestingly *lilA* expression was not induced in microaerobic conditions. The potential backup system for *ΔkatG* or *Δtpx* was demonstrated by the highly induced expression of *gpx-2* gene. The up-regulation of *katG* gene in *ΔahpC* mutant also indicates the compensation of missing peroxidase activity in *ΔahpC* by KatG.

Table 4.6 The steady-state expression in WT and ROS-deletion mutants, up-regulated genes with the relative expression level $\geq$ 2.

Gene	Strains					
	Δtpx	*ΔkatG*	*Δtpx/ΔkatG*	*ΔahpC* (N_2)	*ΔsodB* (N_2)	*WT* (N_2)
sodB	3x	2x	3x	ni	-	ni
katG	ni	-	-	5x	ni	ni
Gpx-2	3x	6x	4x	ni	ni	ni
lilA	7x	14x	4x	ni	ni	ni

N_2 – microaerobically cultivated
- – not determined
ni – not induced

4.2.2. ROS-gene acclimation of ΔahpC, ΔsodB and WT to air

We also followed growth and expression of the same set of genes in WT and *ΔahpC* or *ΔsodB* mutants after transition from microaerobic to aerobic conditions (Table 4.7). The expression of selected genes showed some differences in strain acclimation to air (Fig. 4.13A, B, C). The growth was significantly suppressed in *ΔsodB* mutant and only partially in *ΔahpC* after transition in comparison to WT (Fig. 4.13). The degree of growth suppression indicates the importance of deleted gene product for *Synechocystis* viability on air. The highest stress was also manifested with long-term induction of most ROS-responsive genes and high expression of *lilA* in *ΔsodB* (Fig. 4.14C). Different way of acclimation was characteristic for *ΔahpC* where the gene induction was less pronounced (Fig. 4.14B). This correlates with different carotenoid accumulation in WT and *ΔahpC* (Fig. 4.11) and indicates its differences in acclimation to oxygen.

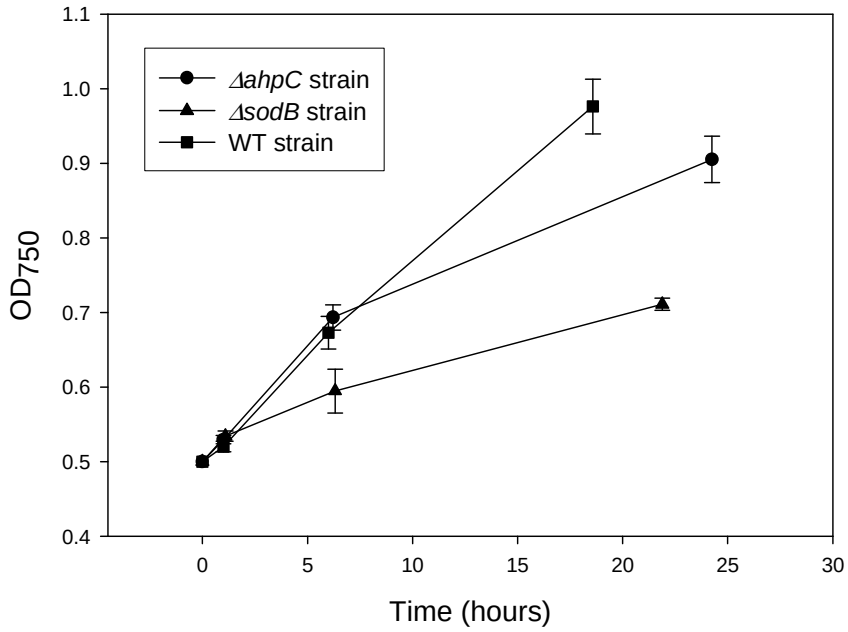


Fig. 4.13 The growth of ΔahpC, ΔsodB and WT strains after transition from microaerobic to aerobic conditions.

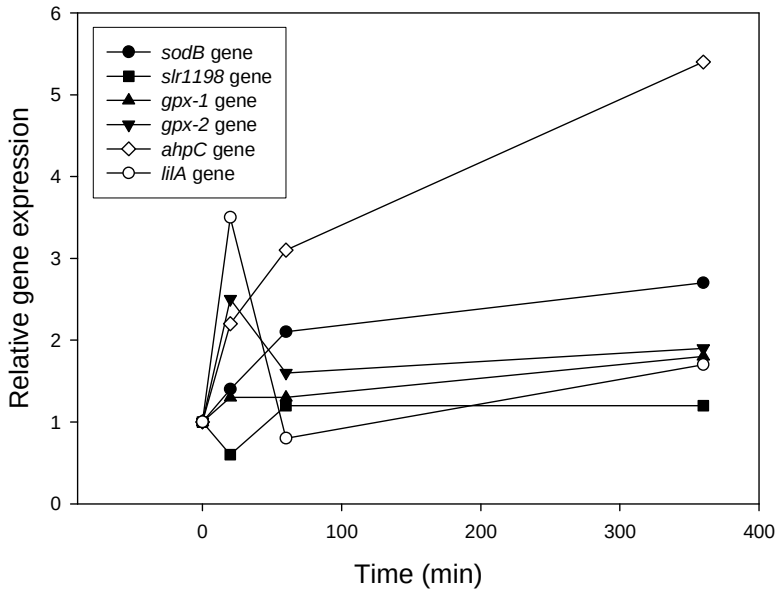
Table 4.7 Genes up-regulated by air treatments in ΔahpC, ΔsodB mutants and WT. The numbers show the relative expression levels of a particular gene after 20, 60 and 360 minutes after transition from microaerobic to aerobic conditions. Only genes with higher relative induction than 2 for particular stress are shown; - inactivated gene.

Fold changes/time period (min)

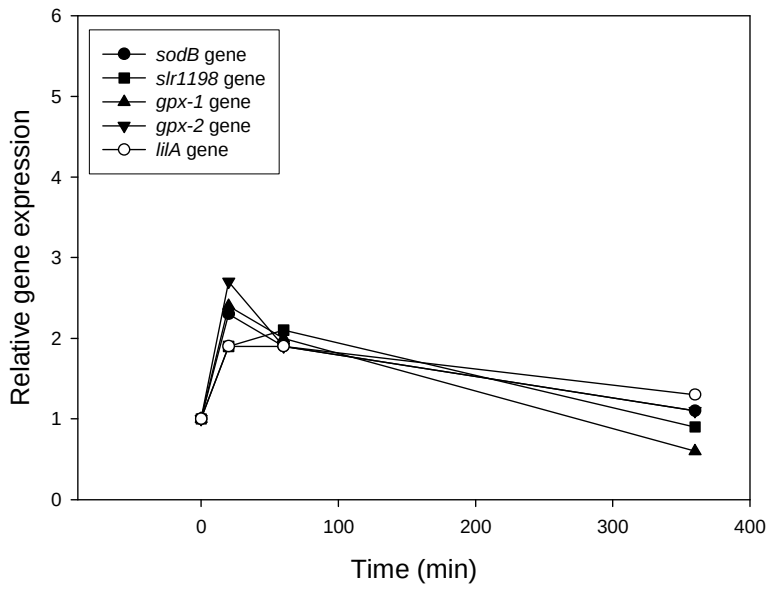
Air stress

Strain	WT			*ΔahpC*			*ΔsodB*		
Gene	20	60	360	20	60	360	20	60	360
sodB	1.4	2.1	2.7	2.3	1.9	1.1	-	-	-
slr1198	0.6	1.2	1.2	1.9	2.1	0.9	0.6	0.7	1.4
gpx-1	1.3	1.3	1.8	2.4	2.0	0.6	2.3	2.4	3.7
gpx-2	2.5	1.6	1.9	2.7	1.9	1.1	5.4	3.5	6.7
ahpC	2.2	3.1	5.4	-	-	-	2.1	2.0	3.4
lilA	3.5	0.8	1.7	1.9	1.9	1.3	15.0	3.5	2.3

A



B



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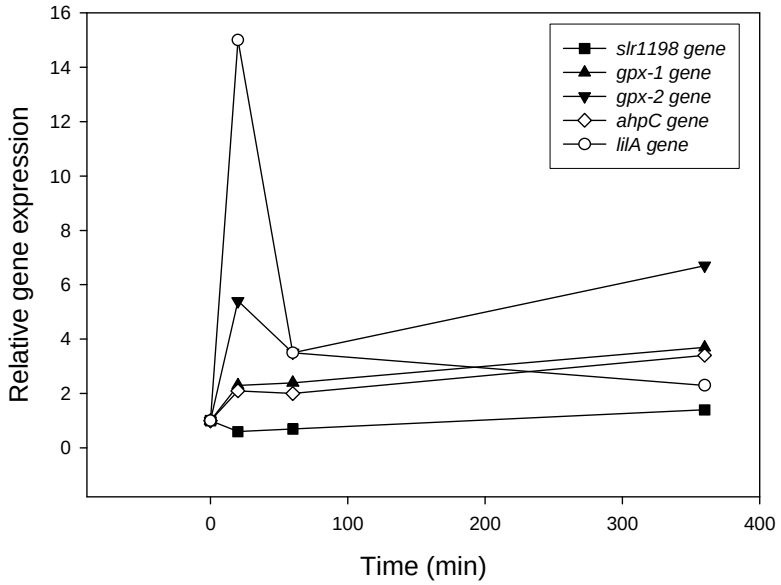

Fig. 4.14 The relative gene expression in WT (A), ΔahpC (B) and ΔsodB (C) strains after transition from microaerobic to aerobic conditions.

4.2.3. Expression pattern of ROS-responsive genes in deletion mutants during various stress treatments

Direct exposure of organisms to changes in their environment leads to stress and results in increased ROS production. Survival depends on the ability of these organisms to acclimate to such changes by efficient scavenging of these ROS. Participation of individual proteins coded by ROS-responsive genes to various stress acclimations is not well understood. Our main attention was focused to screen up-regulated genes after various stress treatments, particularly high light (HL) - 500 μmol m^{-2} s^{-1} of light, low temperature (LT) – 18°C, high salinity (NaCl) – 0.5 M NaCl, MV – 5 μM MV, peroxide – 0.5 mM *t*-BuOOH or H_2O_2. The expression pattern of ROS-responsive genes in mutants was followed at time 0, 20, 60 and 360 minutes, normalized to time 0, and summarized in Table 4.8A-F. Some stress treatments

(MV, *t*-BuOOH, high salinity) led to isolation of degraded RNA indicating their lethal effect. This effect was most pronounced after 360 min treatments. For some of the stresses the changes in growth rates were also measured (Fig. 4.15A-D). The relative gene expression levels in the mutants generally followed that of WT with lower amplitude.

The stress acclimation to ***high light*** (HL; Table 4.8A) conditions, the most common stress inducer in photosynthetic organisms, was represented with a typical short time induction of *lilA* in all strain tested, indicating stress induction. Also *ahpC* was gradually induced in all strains indicating its role in acclimation and elimination of ROS. The *sodB* gene already reported as HL inducible in WT (Hihara et al., 2001; Huang et al., 2002) was significantly induced only in WT and *ΔahpC*. Mutants carrying the *Δtpx and/*or *ΔkatG* background did not reveal any other gene induction whereas WT, *ΔahpC* or *ΔsodB* mutants showed increasing induction of *gpx-2* gene that has been shown to be light inducible by Hihara et al., 2001 and Huang et al., 2002.

Table 4.8 Genes up-regulated by various stress treatments in WT and mutants. The numbers show the relative expression levels of a particular gene after 20, 60 and 360 minutes of stress high light (A); low temperature (B); high salinity (C); MV (D); H_2O_2 (E); organic peroxide (F). Only genes with higher relative induction than 2 for particular stress are shown; nd – not determined; - inactivated gene.

Table 4.8A

Fold changes/time period

High light (HL)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB	2.6	0.9	1.7	0.4	nd	nd	1.1	0.7	0.7
gpx-1	2.1	2.5	2.4	1.0	0.6	nd	1.0	1.1	0.4
gpx-2	6.1	7.3	7.6	1.8	1.4	1.9	0.7	0.6	1.4
ahpC	2.1	2.8	3.7	1.6	1.6	2.1	2	1.7	3.5
lilA	58.0	14.7	7.2	18.0	2.5	0.5	14.3	1.6	0.5

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	0.8	0.4	Nd	2.7	1.7	2.5	-	-	-
gpx-1	1.0	0.7	Nd	0.8	1.1	0.5	2.4	2.6	4.6
gpx-2	1.2	1.0	1.1	2.1	2.9	5.5	3.8	2.8	2.5
ahpC	1.9	2.3	3.0	-	-	-	5.3	3.5	3.4
lilA	11.7	3.0	0.3	18.1	11.6	2.0	54.8	12.1	2.4

Low temperature (LT; Table 4.8B) is known to influence membrane flexibility and limit physiological role of membrane proteins. LT induced expression of *sodB* in all strains. Our results demonstrated its temporal expression, only *ΔahpC* showed maximum induction after 6 hours. In agreement with other studies the *ahpC* gene was not induced by LT (Suzuki et al., 2001). *gpx-2* was significantly induced in all mutants and induction of *gpx-1* was detected in WT and *ΔsodB*, where it gradually increased. The *lilA* gene demonstrated stress induction in all strains.

Table 4.8B

Fold changes/time period

Low temperature (LT)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB	5.3	4.2	1.0	4.0	5.4	2.2	2.4	2.9	1.2
gpx-1	1.3	2.3	1.5	1.0	0.9	1.4	1.2	1.5	1.5
gpx-2	6.1	9.0	7.4	3.4	3.3	3.4	2.0	1.8	0.8
Sll1159	2.3	0.9	0.5	0.5	0.4	0.4	2.7	1.3	0.3
lilA	185.0	198.0	16.0	66.8	45.1	11.1	28.0	20.1	2.6

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	2.1	2.7	1.3	1.1	3.9	4.7	-	-	-
gpx-1	1.1	0.9	1.8	nd	nd	1.6	1.1	2.1	3.3
gpx-2	3.1	2.4	2.7	1.8	2.1	1.7	3.8	4.5	2.6
lilA	33.9	22	7.1	31.7	33.8	25.8	44.8	56.6	25.9

High salinity (HS; Table 4.8C) stress that was intensively studied by many authors in WT due to histidine kinase regulation (Shoumskaya et al., 2005). Our results are in agreement with these authors. We show that this stress had lethal effect to *ΔsodB*, *ΔahpC* and *ΔkatG* manifested as color change and deceased growth (Fig. 4.15A). Our results showed specific induction of stress indicator *lilA*, and also *sodB* which are regulated by histidine kinases (Shoumskaya et al., 2005). The PerR regulated *ahpC* was also strongly induced with the maximum level at 1 hour in all strains. *gpx-2* was specifically induced in WT and *Δtpx* after 20 minutes, indicating that steady-state induction of this gene was not sufficient for stress response to such strong stress in these mutants.

Table 4.8C

Fold changes/time period (min)

High salinity (HS)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB	20.6	21.7	3.4	12.6	19.3	3.0	10.0	11.0	1.4
gpx-1	0.5	0.3	2.0	1.0	0.8	1.4	0.7	0.6	2.1
gpx-2	2.1	1.4	3.5	2.6	0.9	0.8	0.7	nd	nd
ahpC	6.2	14.3	1.8	8.7	17.4	2.2	5.0	11.0	0.6
sll1159	1.4	1.5	1.3	1.6	0.9	0.5	0.6	0.6	0.6
lilA	57.8	43.6	2.0	21.9	4.6	1.9	4.6	0.8	0.5

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	10.8	14.0	0.2	9.8	9.3	2.1	-	-	-
gpx-1	1.0	1.7	1.1	0.4	nd	nd	nd	nd	nd
gpx-2	2.5	1.6	0.8	0.9	0.7	1.0	nd	nd	nd
ahpC	7.1	11.0	0.5	-	-	-	2.8	15.5	2.5
sll1159	2.1	3.4	4.2	0.6	nd	nd	nd	nd	nd
lilA	41.1	7.1	1.2	4.8	0.4	0.5	10.8	22.2	8.4

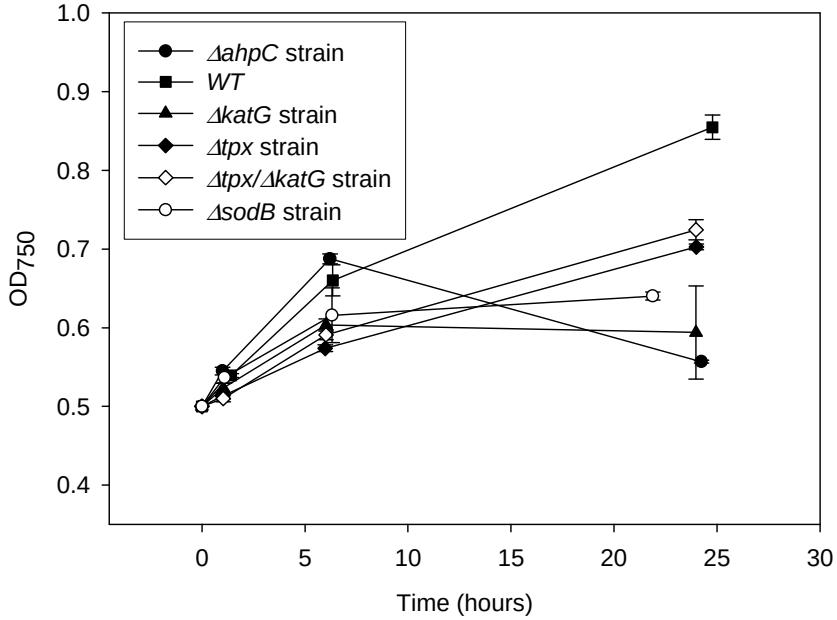

Fig 4.15A Influence of high salinity to growth of WT and mutants.

MV (Table 4.8D), a superoxide generator, is used as strong oxidative stress inducer. Interestingly, no strains expressed *sodB* at significantly higher rates as a response to MV. As expected, *ahpC* expression was induced to high levels in all strains. The increased expression of *sll1159* in WT may indicate its involvement in peroxide detoxification. The specific induction showed also *gpx-2* in WT and *ΔsodB* in comparison to mutants with *ΔkatG* or *Δtpx* backgrounds. This is in accordance to the higher levels expression of *gpx-2* at steady-state *Δtpx* or *ΔkatG* deletion mutants (Table 4.6). Interestingly, slightly in *gpx-1* induction was also detected in some mutants. Surprisingly, the expression of *lilA* was not found in *Δtpx* mutant, possibly due to destabilization of RNA caused by strong oxidative stress.

Table 4.8D

Fold changes/time period (min)

Methyl viologen (MV)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB	1.2	1.2	0.9	1.5	0.8	0.5	0.7	0.8	nd
gpx-1	1.8	1.5	1.1	2.8	2.5	1.9	2.1	0.9	nd
gpx-2	9.7	3.2	2.1	0.8	0.5	nd	0.7	nd	nd
ahpC	47.5	77.9	27.6	13.3	50.1	15.6	34.0	27.5	0.8
slr0242	0.9	0.7	0.7	1.6	0.9	0.5	0.8	0.6	0.6
sll1159	1.9	2.0	2.5	1.4	0.9	1.0	4.8	3.8	1.9
lilA	13.0	5.0	3.0	nd	nd	nd	7.2	8.62	0.4

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	0.9	0.5	0.5	nd	nd	nd	-	-	-
gpx-1	2.1	1.3	0.8	nd	nd	nd	1.6	0.5	1.0
gpx-2	1.8	1.3	1.1	nd	nd	nd	3.9	1.4	0.7
ahpC	14.9	65.0	22.5	-	-	-	5.7	0.8	1.5
slr0242	2.5	0.9	0.8	nd	nd	nd	0.5	0.4	1.3
sll1159	2.3	1.8	1.8	nd	nd	nd	0.5	1.1	4.8
lilA	1.0	1.4	1.3	nd	nd	nd	70.5	59.7	2.7

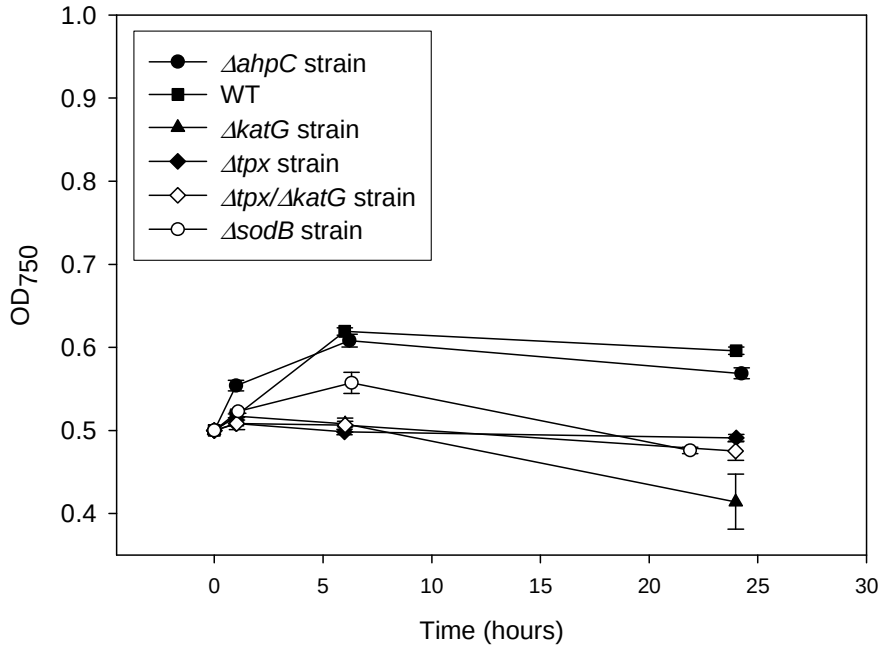

Fig 4.15B Influence of MV to growth of WT and mutants.

Hydrogen peroxide (Table 4.8E), as one of oxidative stress inducer, was not applied to WT due to its high KatG activity (Tichy and Vermaas, 1999). It was used primarily for *ΔkatG* and *Δtpx/ΔkatG* with inactivated KatG but also to *ΔahpC* and *Δtpx* mutants with KatG but impaired in peroxiredoxin genes. Unexpectedly, expression profiles of *Δtpx* and *Δtpx/ΔkatG* were very similar that indicates fast induction by H_2O_2, as gene expression is induced before H_2O_2, is eaten up by KatG. In all tested mutants the presence of H_2O_2 led to stress demonstrated by quick induction of *lilA* gene. Also high induction of *ahpC* gene at 20 minutes and 1 hour was demonstrated, indicating the role of AhpC in hydrogen peroxide detoxification (Li et al., 2004). Interestingly the *gpx-2* was induced only in mutants with *Δtpx* background, although it was up-regulated at steady-state also in *ΔkatG* mutant. Surprisingly up-regulation of *sll1159*, coding for probable peroxiredoxin was detected in all mutants, what has been described for WT by Singh et al. (2004). This indicates the physiological importance and potential peroxidase activity of protein encoded by *sll1159* gene. The lethal effect due to KatG deletion is also seen on growth rates of *ΔkatG* and *Δtpx/katG* mutants in comparison to other strains (Fig. 4.15C), indicating importance of KatG.

Table 4.8E

Fold changes/time period (min)

Hydrogen peroxide (H_2O_2)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB				1.0	0.7	1.1	1.1	1.5	nd
gpx-2				2.7	0.8	0.5	0.5	nd	nd
ahpC				12.9	5.2	2.4	9.7	9.9	nd
sll1159				21.4	28.2	1.0	4.2	2.5	4.0
lilA				52.9	nd	nd	20.0	12.0	nd

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	1.0	1.4	1.7	0.4	1.2	2.4			
gpx-2	2.5	1.1	1.3	0.9	0.9	1.1			
ahpC	9.8	8.6	1.6	-	-	-			
sll1159	5.7	6.0	1.2	2.1	1.4	1.0			
lilA	51.5	26.0	0.7	3.9	0.7	0.4			

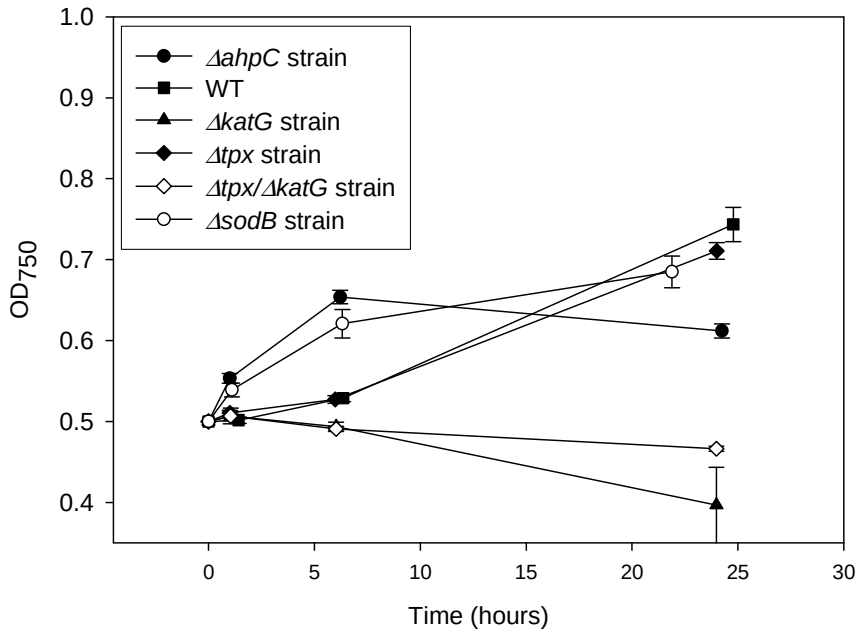

Fig 4.15C Influence of H_2O_2 to growth of WT and mutants.

Organic peroxide stress (Table 4.8F) was induced by *t*-BuOOH that can also cause lipid peroxidation, therefore induction of genes coding for peroxidases with broad substrate specificities should occur. This treatment caused blue-green color changes in *ΔahpC* mutants during first 20 minutes indicating the membrane destruction and death of the cells, also shown by the stopped growth of *ΔahpC* and other strains. Therefore the expression levels were only partially detected. High *lilA* induction was followed by strong *ahpC* up-regulation. Surprising was the high level up-regulation of *sll1159* in all strains tested, indicating possible involvement in lipid peroxide detoxification. Some strains induced also *gpx-2* but surprisingly only *ΔsodB* induced *gpx-1*. Also *sodB* gene was up-regulated only in WT.

Table 4.8F

Fold changes/time period (min)

Organic peroxide (*t*-BuOOH)

Strain	WT			*Δtpx*			*ΔkatG*		
Gene	20	60	360	20	60	360	20	60	360
sodB	3.0	5.7	nd	0.4	0.7	nd	0.7	2.0	0.8
gpx-1	1.3	2.0	nd	1.5	0.8	nd	1.0	0.9	nd
gpx-2	4.1	1.5	nd	3.4	0.6	nd	0.7	0.2	nd
ahpC	21.6	10.0	0.5	16.5	2.9	0.8	28.0	18.7	7.2
sll1159	51.6	9.7	1.3	28.7	5.5	nd	34.0	34.4	0.9
lilA	49.0	8.5	nd	35.0	5.4	nd	3.0	0.4	nd

Strain	*Δtpx/ΔkatG*			*ΔahpC* (N_2)			*ΔsodB* (N_2)		
Gene	20	60	360	20	60	360	20	60	360
sodB	0.3	0.9	nd	0.9	5.1	nd	-	-	-
gpx-1	1.7	0.7	0.8	0.5	nd	nd	2.5	1.5	0.5
gpx-2	2.5	0.2	nd	1.5	0.7	nd	3.9	1.1	nd
ahpC	26.0	13.0	2.8	-	-	-	20.3	9.3	0.8
sll1159	15.3	12.0	nd	5.1	1.1	nd	5.7	1.2	0.4
lilA	35.7	4.1	nd	7.5	9.0	nd	50.0	9.8	0.4

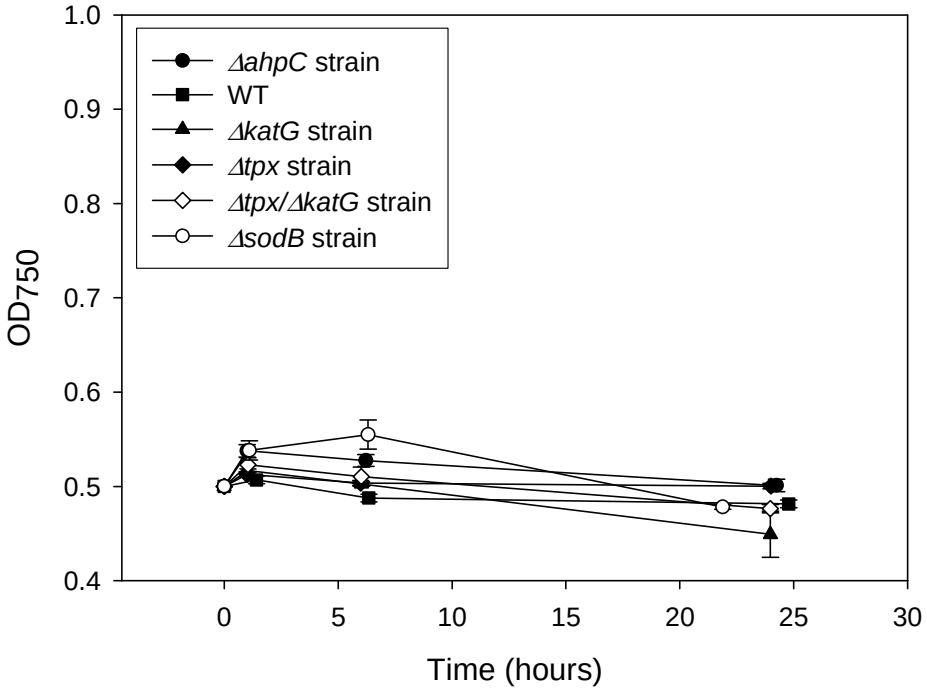

Fig 4.15D Influence of t-BuOOH to growth of WT and mutants.

4.3. Generation of spontaneous MV-resistant mutants and localization of mutations

4.3.1. Generation of spontaneous MV – resistant mutants

To determine how *Synechocystis* copes with MV, an oxidative stress inducer, spontaneous mutants resistant to MV were generated in WT background. Mutants were obtained by cultivation of WT in liquid medium with increasing concentration of MV from 0.5 to 20 μM. From the plates with 20 μM MV single colonies were isolated and cultivated on plates with up to 60 μM MV. Eight colonies from several experiments were isolated and named MV-A, MV-B and MV-1 to MV-6. Mutants were cultivated in liquid BG supplemented with 20 μM MV and subjected to restriction analysis.

4.3.2. Mutation mapping

DNA isolated from both MV-resistant mutants transformed WT with high efficiency, indicating that the mutation is in a single gene (Fig. 4.16). It allowed us to localize the mutation by the restriction enzyme mapping method as previously described by Ermakova-Gerdes and Vermaas (1999), Tichy and Vermaas (2000). This method is based on the restriction of chromosomal DNA with individual restriction endonucleases, separation of fragments according to their size and transformation of WT with pools of fragments with a known size. For each enzyme the size range of complementing fragments was identified and compared. Usually, a single fragment matching all size criteria is found. Unlike in the previous studies where the selection for autotrophic growth was used, the transformation protocol had to be adapted for the selection of MV resistant transformants yielding single colonies with low background of non-transformed cells (Fig 4.17). The optimized two-week schedule is shown in (Table 4.9).

Table 4.9 The optional transformation time schedule. This schedule includes the estimated time-depending increase of MV concentration in BG agar plates for successful transformation with DNA carrying mutation.

Days	Media
1 - 4	BG
5 - 7	20 μM MV
8 - 9	40 μM MV
10 - 14	60 μM MV

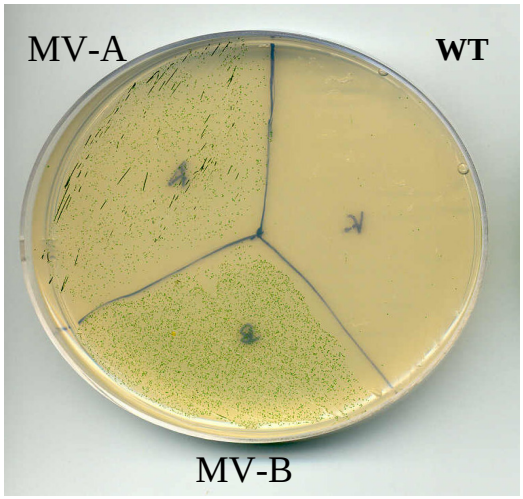

Fig 4.16 Transforming of WT with chromosomal DNA isolated from the MV-A and MV-B mutants. On the plate with 40 μM MV hundreds of transformants are forming single colonies after transformation with DNA from mutants whereas, only several colonies are growing in non transformed WT control.

4.3.2.1. Localization of the MV resistance mutation in MVA, MVB mutants

DNA from both mutants was completely digested by following restriction endonucleases: BamHI, BglII, Bsu15I, EcoRI, EcoRV, HindII, HindIII, PvuII and SmaI. The only chromosome region that satisfied all the restriction data was a 2571 bps long fragment carrying *prqR* gene (Table 4.10; Fig. 4.18). The indication that the MV resistance mutation is in *prqR* gene was confirmed by transformation of WT with *prqR* gene, amplified by PCR with primers MV1, MV2 (Fig 4.18) using DNA from resistant mutants as a template.

Table 4.10 Restriction analysis of MV-A and MV-B mutants.

Restriction endonuclease	Supposed fragment length carrying gene *slr0895* (bps)	The fragment length for MV-A (bps)	The fragment length for MV-B (bps)
BamHI	3 355	3 000-4 000	3 000-4 000
BglII	3 055	3 000-3 500	3 000-3 500
ClaI	7 006	weak 6000-8000	nd
EcoRV	17 887	huge background	longer than 10 000
HindII*	small fragments	nt	nt
HindIII	6 390	5 000-8 000	5 000-10 000
PvuII	longer than 50 000	longer than 40 000	longer than 40 000
SmaI	5 372	nd	4 000-6 000

nd - was not detected
nt – no transformed

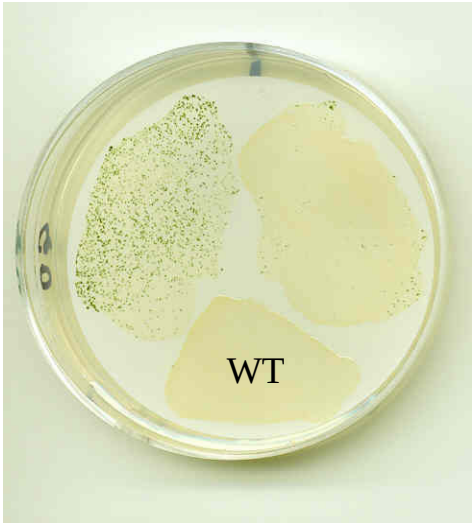

Fig. 4.17 Transformation with restriction fragments. WT transformation with the population of 3 000 – 4 000 bps BamHI fragment of the MV-A mutant. WT – non transformed WT.

Sequencing a of the *prqR* gene from the MV-A, and MV-B mutants´ using the MV5 primer revealed single T to G mutation of the nucleotide 154 leading to the substitution of tyrosine 52 to glutamic acid (Y52E) in the PrqR protein (Fig. 4.18; Table 4.13). This regulator from the TetR family has been previously identified to control expression of the MATE family transporter PrqA (Nefedova et al., 2003a; Babykin et al., 2003). Phenotype of this new Prq mutant was identical to the previously described PrqR20 mutant, suggesting that also Y52E substitution results in impaired binding of mutated PrqR protein to DNA leading to derepression of the PrqA transporter.

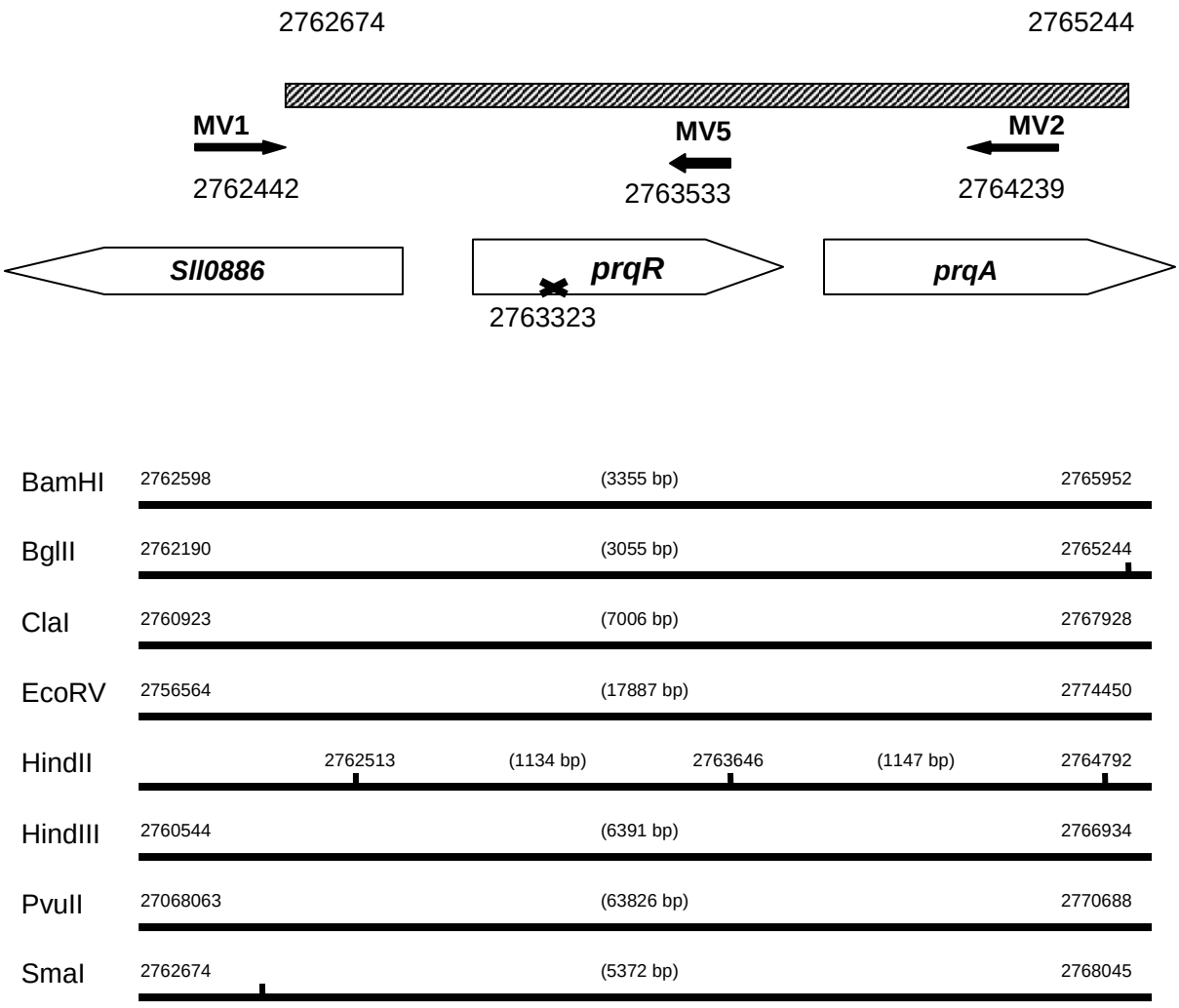

Fig. 4.18 The restriction map of chromosomal region of Synechocystis containing genes prqR, prqA and sll0886. The hatched rectangle with genome positions indicates the resulting fragment (2571 bps) from restriction analyses of MV-A and MV-B strains. The arrows indicate the primers used for the localization of mutation and sequencing. The cross indicates the mutation position. The restriction sites and fragment sizes of restriction enzymes used for localization are shown below.

>*slr0895* {2763170 - 2763733 direct} transcriptional regulator - WT

```
MVSGKRLRSK SIQPSQLLTA ANQVIVSQGV DALTLDAVAS EAGVSKGGLL
HYFPTKEALI AGMVQQALDR FVETLHQELA NDPAPDTPGH WVRAYIRASA
LDDQENYELH FNLLAANFTN PELLAPMRHF WQECHGQIMA SGLDPAVATV
IRLAMDGLWL THLHGFAPLE DPLRSQVIAT ALKLAQL
```

>*slr0895* {2763170 - 2763733 direct} transcriptional regulator – point mutation in MV-A, B strains

```
MVSGKRLRSK SIQPSQLLTA ANQVIVSQGV DALTLDAVAS EAGVSKGGLL
                    α1 – helix          α2 – helix      α3 - helix
HEFPTKEALI AGMVQQALDR FVETLHQELA NDPAPDTPGH WVRAYIRASA
LDDQENYELH FNLLAANFTN PELLAPMRHF WQECHGQIMA SGLDPAVATV
IRLAMDGLWL THLHGFAPLE DPLRSQVIAT ALKLAQL
```

Fig. 4.19 PrqR protein sequences of MV-resistant mutants. Point mutations of prqR gene in MV-A and MV-B mutants leading to substitution of original tyrosine 52 to new glutamic acid (Y52E) in α1-3 – helix (underlined) of conserved DNA binding domain; WT-sequence from CyanoBase (http://genome.kazusa.or.jp/cyanobase/).

4.3.2.2. Localization of the MV resistance mutation in MV-4 and MV-6

We were primarily interested in new mutations not mapping into *prqR*. To avoid generation and characterization of further *prqR* mutants, the *prqR* gene from the MV1 to MV6 resistant strains was amplified by PCR and used directly to transform WT. When MV resistant colonies were formed, indicating that the responsible mutation is in *prqR*, the strain was discarded. Only *prqR* from two mutants MV-4 and MV-6 did not yield MV resistant colonies although their total DNA transformed with high efficiency (Table 4.11). It was probable that the resistance mutations in these two mutants are localized in different gene(s) and the mutants were subjected to new restriction analysis.

Table 4.11 The transformation efficiency of chromosomal DNA and of PCR product of the prqR gene from MV-resistant mutants in WT.

Mutants	Transformation ability	
	DNA	PCR of *prqR* gene
MV-A	Strong +	strong +
MV-B	Strong +	strong +
MV-1	+	+
MV-2	+	weak +
MV-3	+	+
MV-4	Strong +	-
MV-5	Weak +	-
MV-6	Strong +	-

+ yielding MV-resistant transformants
- not yielding MV-resistant transformants

In addition to restriction endonucleases used for *prqR* localization we used also following enzymes: AcyI, BspHI, BstEII KpnI, NheI, NdeI, PstI, ScaI, StuI or XbaI. Localization of this mutation was complicated by the fact that we were not able to find more enzymes yielding reasonable sizes of restriction fragments. The large fragments are more prone to DNA fragmentation complicating data analysis. As a result, not single but ten

possible regions carrying the mutation were identified and screened by transformation with respective PCR product. Finally, the mutations leading to MV resistance and not mapping into the *prqR* gene in both MV-4 and MV-6 were mapped to unknown gene *slr1174* (Table 4.12). These mutations in *slr1174* gene were confirmed by the transformation of WT with specific PCR products amplified with primers MVQI 1-2 from MV-4, MV-6 and from WT, MV-A strains as a control (Fig 4.20). Transformations with the PCR products from MV-4 and MV-6 showed very high efficiency in contrast to WT and MVA. This showed that the mutation is located in *slr1174* in the MV-4 and MV-6 mutants.

Table 4.12 Restriction analysis of the MV-4 strain.

Restriction endonuclease	Supported fragment length carrying *slr1174* (bps)	The fragment length for MV-4 (bps)
AcyI	6332	4000-8000
BamHI	2045	weak transformation 1000-3000
BglII	22190	20000-40000
BspHI	31467	more than 20000
ClaI	15577	15000-40000
EcoRI	5249	5000-8000
EcoRV	17065	(10000-15000) 15000-40000
HindII	1194	nt
HindIII	5613	4000-10000
NheI	8926	6000-15000
PstI	29197	10000-40000
SmaI	2824	nd
BstEII	6100	4000-8000

nd - was not detected
nt – no transformed

Localization was followed by sequencing using MVQI4 and MVQI5 primers. Sequencing revealed surprisingly that point mutations in both MV-4 and MV-6 mapped into the same amino acid R115 and even more surprisingly resulted in different amino acid substitutions, of original arginine with glycine (R115G) in MV-4 or with leucine (R115L) in MV-6 (Fig. 4.20, 4.21; Table 4.13). From the sequence similarity it was probable that Slr1174 may be a part of an ABC transporter (Fig 5.2).

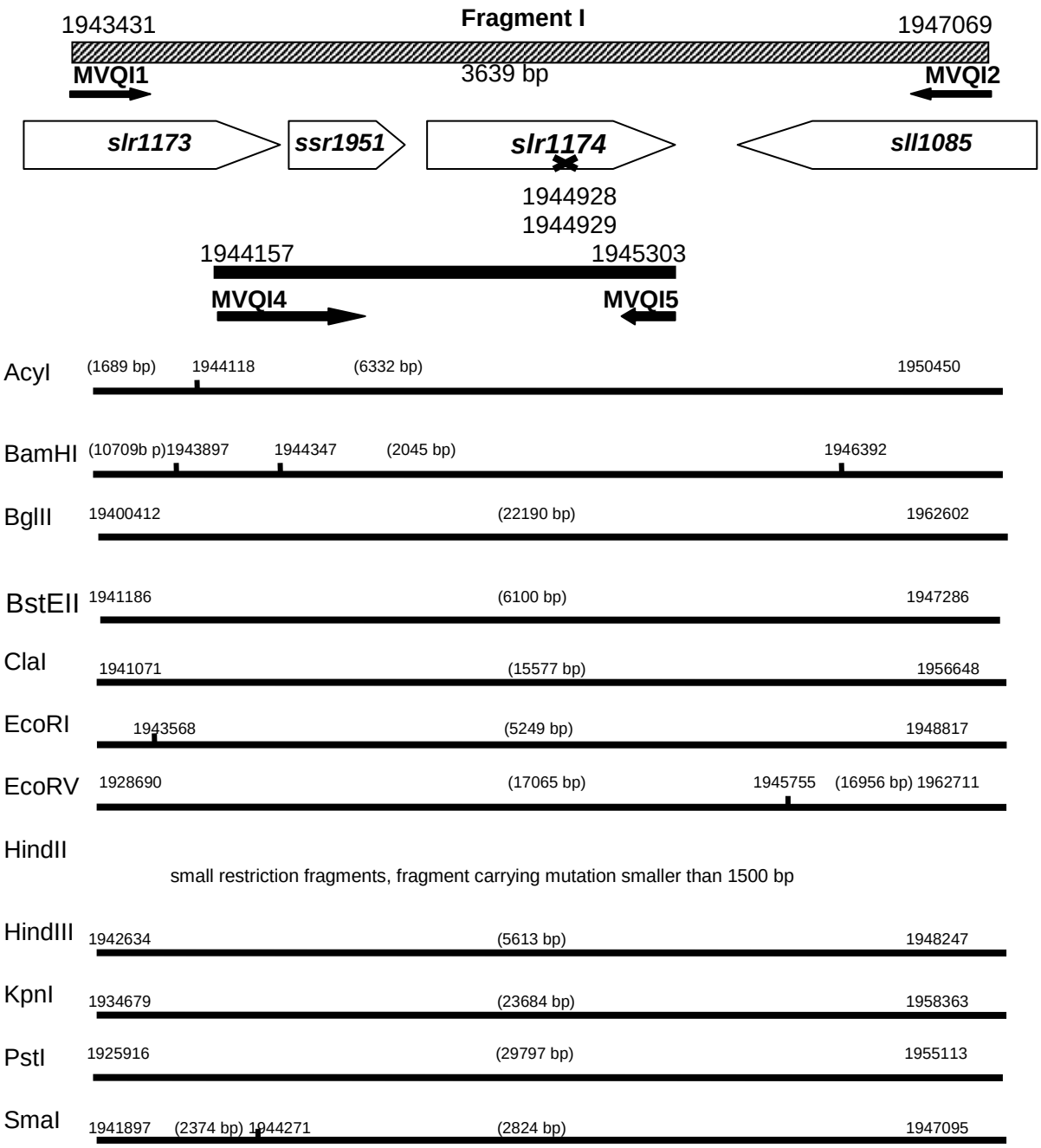

Fig. 4.20 The restriction map of chromosomal region of Synechocystis containing genes slr1174, slr1173, ssr1951 and sll1085. The hatched rectangle with genome positions indicates the resulting PCR fragment I (3639 bps) from restriction analyses in MV-4 strain. The arrows indicate the primers used for the localization of mutation and sequencing. The cross indicates the mutation positions. The restriction sites and fragment sizes of restriction enzymes used for localization are shown below.

>*slr1174* {1944586 - 1945374 direct} hypothetical protein - WT

```
MGHFPHKLRV    LLSVYFAQMV    EYRAEIFFWI    LSGSLPLILM    GVWVKAAESG
DFTLDAIQVA    RYFFAVFVVR    QMTTIWVIWE    FEKEVLEGVL    SFRLLQPLDP
VWHHIARHWA    EKMTRLPILA    ILTVLFFSLY    PEAFWLPDPW    HFIQGLIGIV
CSFTLYFLIQ    YTFALCAFWT    ERASALQDLW    FLFYIFLSGV    IAPLETFPDA
VRQIVLLTPF    PYGVYFPAAA    LVGLPLPFFK    SLLIIGVWIG    IFALLNRWLW
RQGLKQYSGM    GA
```

>*slr1174* {1944586 - 1945374 direct} hypothetical protein - point mutation in MV- 4 strain

```
MGHFPHKLRV    LLSVYFAQMV    EYRAEIFFWI    LSGSLPLILM    GVWVKAAESG
DFTLDAIQVA    RYFFAVFVVR    QMTTIWVIWE    FEKEVLEGVL    SFRLLQPLDP
VWHHIARHWA    EKMTGLPILA    ILTVLFFSLY    PEAFWLPDPW    HFIQGLIGIV
CSFTLYFLIQ    YTFALCAFWT    ERASALQDLW    FLFYIFLSGV    IAPLETFPDA
VRQIVLLTPF    PYGVYFPAAA    LVGLPLPFFK    SLLIIGVWIG    IFALLNRWLW
RQGLKQYSGM0   GA
```

>*slr1174* {1944586 - 1945374 direct} hypothetical protein - point mutation in MV- 6 strain

```
MGHFPHKLRV    LLSVYFAQMV    EYRAEIFFWI    LSGSLPLILM    GVWVKAAESG
DFTLDAIQVA    RYFFAVFVVR    QMTTIWVIWE    FEKEVLEGVL    SFRLLQPLDP
VWHHIARHWA    EKMTLLPILA    ILTVLFFSLY    PEAFWLPDPW    HFIQGLIGIV
CSFTLYFLIQ    YTFALCAFWT    ERASALQDLW    FLFYIFLSGV    IAPLETFPDA
VRQIVLLTPF    PYGVYFPAAA    LVGLPLPFFK    SLLIIGVWIG    IFALLNRWLW
RQGLKQYSGM    GA
```

*Fig. 4.21 Slr1174 protein sequences of MV-resistant mutants. Point mutations of slr1174 gene in MV-4 and MV-6 mutants leading to substitution of original arginine 115 to new glycine and leucine (R115G and R115L); WT-sequence from CyanoBase (*http://genome.kazusa.or.jp/cyanobase/*).*

Table 4.13 The localization of point mutations in MV-resistant mutants.

Mutants	Position of localized point mutation			Genetic background
	Gene	Changed nucleotide	Changed amino acid	
MV-A	*prqR*	**T 154 G**	**Y 52 E**	WT
MV-B	*prqR*	**T 154 G**	**Y 52 E**	WT
MV-4	*slr1174*	**C 343 G**	**R 115 G**	WT
MV-6	*slr1174*	**G 344 T**	**R 115 L**	WT

4.3.3. Phenotype characterization of MV-resistant mutants

For the phenotypic characterization of the MV-resistant mutants, MV-A and MV-4 mutants each representing mutation in different gene were selected. Both mutants were characterized by their growth rates, sensitivity to MV and by the expression of selected genes after MV treatment.

The autotrophic growth rates of both mutants in liquid media were similar to WT, MV-A exhibited significantly slower growth on media with glucose in comparison to WT (Table 4.14).

Table 4.14 Growth rates of the MV-resistant mutants and WT.

Strain	Doubling time	
	Photoautotrophically	Photomixotrophically
MV-A	13 h	12.5 h
MV-4	12 h	10 h
WT	12 h	9 h

Both mutants provided similar level of protection to MV in the diffusion sensitivity assay (Fig. 4.22A), comparable to the previously described MV-A, B. Also both mutants showed similar resistance to MV on plates, up to 60µM. They partially differed in the resistance to MV, when cultivated in liquid media. However, both showed similar growth rate in up to 10 µM MV, growth of MV-4 was partially suppressed in comparison to MV-A in 20 µM MV, both cultures died in 30 µM MV (Fig. 4.22B).

A **B**

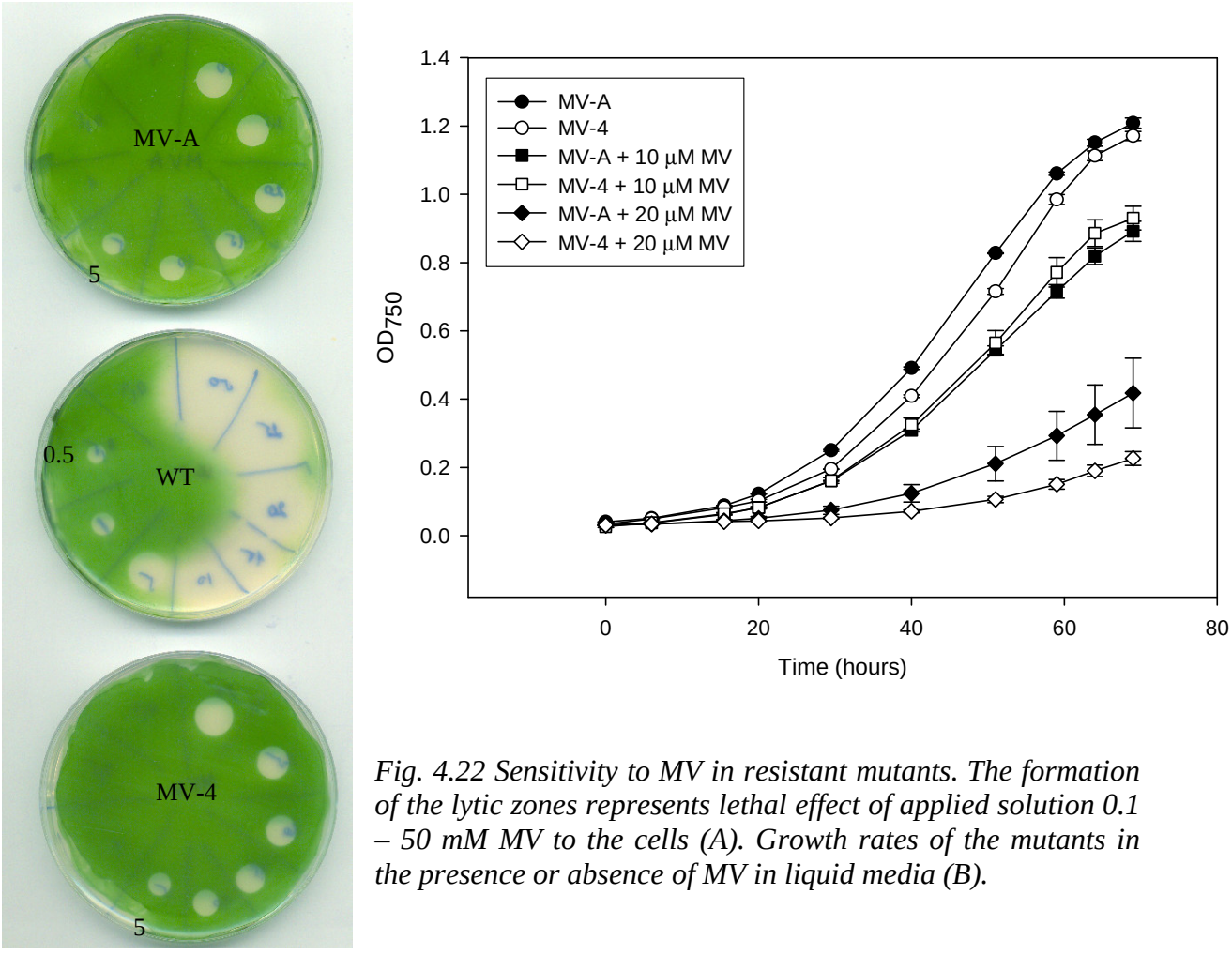

Fig. 4.22 Sensitivity to MV in resistant mutants. The formation of the lytic zones represents lethal effect of applied solution 0.1 – 50 mM MV to the cells (A). Growth rates of the mutants in the presence or absence of MV in liquid media (B).

To elucidate the effect of the mutations we focused on the expression pattern of the *prqR* and *slr1174* genes, and also on genes except to be important for MV resistance as *sodB*, *prqR*, *prqA* or *mvrA* (Babykin et al., 2003; Nefedova et al., 2003a). Also expression of *lilA* that is transiently highly induced by most stresses as shown in multiple microarray studies (Hihara et al., 2001; Kanesaki et al., 2007; Shoumskaya et al., 2005) was used as a stress indicator (Kufryk et al., 2008).

The steady-state expression of selected genes showed the specific up-regulation of *prqR* and *prqA* expression levels in MV-A mutant however, these genes have been only marginally induced in MV-4 mutant (Table 4.15). Mutant MV-A also seems to employ *slr1174* by its expression and shows higher *lilA* levels in contrast to MV-4 in steady state. Surprisingly, *sodB* gene was not induced in either MV-A or MV-4 strain.

To address the possible role of Slr1174 and PrqR in the adaptive response of *Synechocystis* to oxidative stress, the expression pattern of the same genes during 5 µM MV was followed (Table 4.16). The treatment did not influence the relative transcript abundance of *slr1174* neither in mutants nor in WT, indicating the absence of a link between *slr1174* expression and oxidative stress caused by MV. In WT the 10–15 fold increase in the *slr1544* transcript level after 20 minute treatment and also partially increased expression of *prqR*, *prqA* and *mvrA* was demonstrated. No such increases in gene expression have been shown for MV-resistant mutants during 360 minutes of MV treatment. These facts indicate that both mutants can tolerate higher MV concentrations without inducing stress response.

Table 4.15 The steady-state expression in MV-resistant mutants.

Gene	Mutants	
	MV-A	MV-4
prqR	58x	3x
prqA	26x	2.5x
mvrA	19x	3.5x
slr1174	2.5x	ni
lilA	5x	ni

ni – not induced

Table 4.16 The relative expression levels of the selected genes up-regulated by 5µM MV after 20, 60 and 360 minute treatment in WT and MV-resistant mutants.

	Fold changes/time period (min)								
Strain	WT			MV-A			MV-4		
Gene	20	60	360	20	60	360	20	60	360
sodB	1.2	1.3	0.9	0.6	0.9	1.0	1.5	1.0	1.1
prqR	1.9	1.7	2.1	0.7	0.9	1.1	0.9	0.8	1.4
prqA	1.5	2.2	2.7	0.7	0.9	1.1	0.7	0.8	1.3
mvrA	2.4	2.3	2.7	0.5	1.1	0.8	0.5	0.6	1.2
slr1174	1.1	1.1	1.2	0.7	1.1	1.0	0.7	0.7	1.1
lilA	13.0	5.0	3.0	0.5	1.0	0.8	1.0	0.9	0.9

5. DISCUSSION

Although ROS are produced during normal growth of organisms that carry out aerobic respiration and/or oxygenic photosynthesis, their direct exposure to environmental changes increases the ROS production. Survival of aerobic organisms depends on their ability to acclimate to such changes and on efficient ROS scavenging. To understand how *Synechocystis* responds to oxidative stress, we focused on three topics:

To define the physiological role of scavenging enzymes we prepared deletion mutants with inactivated *sodB*, two Prx-s genes *sll0755* (*tpx)* or *sll1621* (*ahpC*) and characterized their phenotypes.

To determine expression profiles of deletion mutants we focused on screening for up-regulated genes reportedly playing important roles in ROS-scavenging: *sodB, katG, gpx-1, gpx-2* and 5 *prx-s* (*slr1198, ahpC, sll0021, slr0242, tpx*), we also followed expression of two, genes *sll1159* and *lilA*, which have been previously shown to be induced by hydrogen peroxide or other stress treatments (Singh et al., 2004; Hihara et al., 2001; Suzuki et al., 2001; Li et al., 2004; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005; Kufryk et al., 2008).

To identify genes responsible for the MV-resistant phenotype we generated spontaneous MV mutants and characterized their phenotypes.

5.1. Physiological role of ROS-scavenging enzymes

5.1.1. Role of superoxide dismutase

Several studies have reported the implication of SODs in protective processes in cyanobacteria as sensitivity to oxygen, light or chilling in *sodB* deletion mutants, where they protect photosynthesis against damage (Herbert et al., 1992; Thomas et al., 1999). SODs also play important role in heterocyst formation where they protect proton-donating system of nitrogen-fixation (Zhao et al., 2007). Although most cyanobacteria contain more SOD isoforms with different metal cofactors (Mn-; Zn-; Cu-; Ni- or Fe-), *Synechocystis* contains only single Fe-SOD. The key role of this cytoplasmic Fe-SOD for *Synechocystis* has been already reported by Nefedova et al., (2003b). Also our *ΔsodB* mutant exhibited similar phenotype with the loss of viability on air. On the other hand, *ΔsodB* mutant prospered under microaerobic conditions and exhibited low expression levels of ROS-scavenging genes (Table 4.6). After shift to air, expression levels of almost all ROS-scavenging genes were increased followed by impaired growth (Fig. 4.13, Table 4.7).

Prolonged cultivation of *ΔsodB* on air led to frequent formation of pseudorevertants. This was observed as formation of colonies on agar plates grown aerobically or by improved performance the strain in liquid media. Localization of such secondary mutations will help us to understand how cells may adapt to higher superoxide levels and what are the main superoxide targets influencing cell viability.

The expression levels of *sodB* in WT and other *Synechocystis* mutants showed strong induction by salt stress and moderate induction by other stresses in agreement with expression data of Hihara et al., (2001). The Hik34 kinase is apparently involved in *sodB* induction under salt and hyperosmotic stress (Shoumskaya et al., 2005; Paithoonrangsarid et al., 2004). Surprisingly, *sodB* expression was not induced by the superoxide generator MV. The fast H_2O_2 accumulation in the presence of MV (Fig. 4.7) indicates that in SOD containing mutants, superoxide is quickly turned into H_2O_2, that does not serve as a *sodB* inducer (table 4.8E).

5.1.2. Physiological differences of Prx-s in peroxide detoxification and ROS response

Recently, peroxiredoxins (Prx-s) became highlighted in many studies due to their important functions in ROS-scavenging as well as in redox homeostasis (Stork et al., 2005). They also function as regulators of redox-mediated signal transduction at least in some eukaryotes (Dietz et al., 2003; Wood et al., 2003) and it is also assumed that the large Prx number in cyanobacteria is linked to various metabolic requirements in context of oxygenic photosynhtesis (Stork et al., 2009).

Thioredoxin peroxidase (Tpx, Sll0755) was the first studied peroxiredoxin in *Synechocystis* (Yamamoto et al., 1999; Baier and Dietz, 1997; Klughammer et al., 1998). It belongs to 2-Cys peroxiredoxin subgroup (Stork et al., 2005). The cyanobacterial 2-Cys Prx-s are highly conserved and similar to plant 2-Cys, originating from former cyanobacterial endosymbiont (Baier and Dietz 1997, Klughammer et al., 1998). Tpx functions in the reduction of both H_2O_2 and alkyl hydroperoxides (Yamamoto et al., 1999), although Tpx predominantly scavenges H_2O_2 rather than alkyl hydroperoxide (Gaber et al., 2004). Regarding the role of Tpx in *Synechocystis*, the *Δtpx* mutants exhibited the same H_2O_2 and *t*-BuOOH decomposition capacities as control strains. This indicates that Tpx does not significantly contribute to peroxide decomposition *in vivo.* However, we have confirmed that Tpx is active in *Synechocystis* as we have observed partially diminished *t*-BuOOH-dependent oxygen evolution in *Δtpx* indicating that electrons for Tpx reduction are derived from PSI *via* ferredoxin:thioredoxin reductase (Baier and Dietz, 1997). The remaining peroxide decomposition activity in *Δtpx* mutants was light-dependent, suggesting existence of other enzyme(s) using reducing equivalents generated in photosynthetic electron flow. We think that Tpx is important but not essential for *Synechocystis* under normal and stress conditions.

Tpx expression in *Synechocystis* did not reveal significant up-regulation under various stress treatment in agreement with microarrays (Hihara et al., 2001; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005). This is in contrast to *Synechococcus elongatus* PCC 7942 or higher plants where 2-cys Prxs are induced and play probably more important role (Pelerman et al., 2003; Stork et al., 2005). It indicates that similar Prxs may differ in their functions among cyanobacteria and higher plants.

Another peroxiredoxin in *Synechocystis* is Prx typeII, called atypical 2-Cys Prx (Li et al., 2004; Kobayashi et al., 2004; Hosoya-Matsuda et al., 2005; Stork et al., 2005) which can use thioredoxins as well as glutaredoxins or glutathione as the reductants and which exists in multiple isoforms in plants while in cyanobacteria is missing or present only in single copy of Prx type II (Stork et al., 2005). *Synechocystis* carries single gene *sll1621* coding for Prx type II protein AhpC localized in cytosol and showing substrate preference for *t*-BuOOH rather than for H_2O_2 *in vitro* (Hosoya-Matsuda et al., 2005). The *in vivo* role of this Prx is not well understood.

Our data demonstrated physiological importance and light-dependence of AhpC in *t*-BuOOH detoxification in *Synechocystis*. Deletion of *ahpC* dramatically decreased *t*-BuOOH decomposition capacity of *Synechocystis* cells (Fig. 4.12) and also their *t*-BuOOH-dependent oxygen evolution was abolished (Table 4.5). There are reports that also Gpx-1 and Gpx-2 reduce *t*-BuOOH using electrons from PSI (Gaber et al., 2004, Yamamoto et al., 1999) as their *t*-BuOOH dependent oxygen evolution was diminished in particular deletion mutants but no complete abolishment of the *t*-BuOOH dependent oxygen evolution like in *ΔahpC* mutant was observed. From *in vitro* experiments it has been suggested, that AhpC is preferably using glutathione as its electron donor (Hosoya-Matsuda et al., 2005). Glutathione is reduced by glutathione reductase using electrons from NADPH and although cyanobacterial cells are known to contain large pool of glutathione this pool would be quickly exhausted in media with 50 μM *t*-BuOOH if not re-reduced. The light-dependency of the peroxidase activity may be explained by the need to recycle the peroxidase electron donor by electrons coming from

the photosynthetic electron transport. We think that AhpC is the key *t*-BuOOH scavenging light-dependent peroxidase in *Synechocystis*.

The physiological importance of AhpC was also demonstrated by the strong phenotype of the *ΔahpC* mutant (Fig 4.10, Table 4.4) in agreement with Kobayashi et al., (2004) and Hosoya-Matsuda et al., (2005). Although the mutant exhibited air and light sensitivity it was able to acclimate to aerobic conditions. The acclimation consisted in accumulation of carotenoids (Fig. 4.11) and in increased constitutive expression of KatG (Table 4.6) but not in air induced expression of ROS-scavenging genes as in WT (Fig. 4.14; Table 4.7). Since, the carotenoids protect the photosynthetic apparatus from extent of light as well as provide an effective elimination of specific ROS, their accumulation is a common response of the cyanobacterial cell to stress (Burton and Ingold 1984; Kelman et al., 2009). We assume that the *ΔahpC* mutant in contrast to WT is unable to compensate for the missing peroxiredoxin by induction of other enzymes under aerobic conditions and reacts by fast increase in the accumulation of carotenoids. It is also seems that carotenoids provide only limited protection in *ΔahpC* as the mutant showed quick bleaching and loss of viability under extreme stress conditions and did not significantly induce ROS responsive genes as WT.

The *ahpC* expression profile in WT and mutants revealed temporal induction of *ahpC* by most of the stresses with the exception of low temperature and with maximum induction by peroxides in complete agreement with microarray data (Hihara et al., 2001; Stork et al., 2005; Li et al., 2004; Shoumskaya et al., 2005; Kanesaki et al., 2007; Suzuki et al., 2001). Additionally, we have demonstrated high *ahpC* induction in response to *t*-BuOOH, a major AhpC substrate (Hosoya-Matsuda et al., 2005). This is interesting as *t*-BuOOH can mimic the action of long-chain lipid hydroperoxides, triggering the peroxidation of cellular membranes via a free-radical mediated process. The major lipid peroxide-induced damage was observed in the *ΔahpC* mutant of *Helicobacter pylori* (Wang et al., 2006). Moreover, as AhpC has affinity for organic and lipid hydroperoxides (Baier and Dietz 1999) we suppose that lipid hydroperoxides are the main target of AhpC in *Synechocystis*. Therefore the AhpC appears to be predominant of all Prx-s presented in *Synechocystis*.

Synechocystis codes for further Prx-s, one 1-Cys Prx (Slr1198) which catalytic cycle is not fully understood, and two PrxQ-s (PrxQ-B2, Sll0221 and PrxQ-B1, Slr0224). One homologous gene coding for 1-Cys Prx is present in *Synechococcus elongatus* PCC 7942, *Anabaena* sp. PCC 7120 or in *Arabidopsis thaliana* (Stork et al., 2009; Latifi et al., 2006). The Slr1198 has been detected in cytosol with notably low peroxidase activity. Also the disruption mutant did not exhibit any strong phenotype (Hosoya-Matsuda et al., 2005). Our experiments did not reveal any significant up-regulation of *slr1198* either in WT or deletion mutants during 6 hours; in accordance to microarrays (Stork et al., 2005; Hihara et al., 2001; Shoumskaya et al., 2005). However, slight up-regulation was observed at 6 hours under NaCl or HL stress in WT and interestingly also after H_2O_2 treatment in *ΔahpC* or *Δtpx/ΔkatG* mutants. *slr1198* up-regulation was described by Stork with maximum 12 h for high light and 48 h for NaCl treatment. Slr1198 has been suggested to protect nucleic acids from oxidative stress (Stork et al., 2005; Stacy et al., 1996). We think that this 1-Cys Prx does not play essential role under normal growth conditions or under short-term stress (under 6 h).

The similarity of the cyanobacterial PrxQ isoforms varies between 53-76% and they do not differ much from higher plant PrxQs. They function in the context to photosynthesis (Dietz et al., 2006). However, the PrxQ-s in *Anabaena sp.* PCC 7120 and *Synechococcus elongatus* PCC 7942 have been shown to play essential roles (Stork et al., 2009; Latifi et al., 2006). In *Synechocystis* the phenotype of deletion in *prxQs* did not reveal such importance (Kobayashi et al., 2004; Stork et al., 2005). We also disrupted *sll0221* but the disruptant did not show any apparent phenotype under normal light conditions (data not shown). In our experiments both *prxQ-s* (*sll0221* and *slr0242*) expressions only slightly responded to MV

and NaCl stress in the double *Δtpx/ΔkatG* mutant. Therefore, in accordance with other authors, we assume that the physiological significance of PrxQ-s in *Synechocystis* and other cyanobacteria is quite different.

5.1.3. Role of Sll1159, the probable Prx

Although *sll1159* gene coding for probable peroxiredoxin protein was not included in bioinformatic study of prx-s genes in *Synechocystis* by Stork et al., (2005) due to its glutaredoxin domain, we included this gene in our study based on the presence of 1 Cys (C_{162}), Cys-X-X-Cys (C_{83} and C_{86}) domain.

This gene exhibited high and specific up-regulation in WT and deletion mutants not only in presence of H_2O_2 but much higher in presence of *t*-BuOOH. Interestingly, *sll1159* was markedly induced in a mutant, highly resistant to H_2O_2 (Singh et al., 2005). Also the increased expression in *ΔkatG* mutant during MV treatment led as to assume that it can compensate for inactivated KatG. It seems that this protein may play an important role in a cell defense against oxidative stress. Moreover, the induction of whole cluster *sll1158-sll1161* (Singh et al., 2004), with sll1161 coding for probable adenylate cyclase, may indicate direct connection to signaling and regulation mechanisms in *Synechocystis*. Despite this, Sll1159 remains the last Prx in *Synechocystis* that was not yet inactivated and characterized.

5.1.4. Role of glutathione peroxidase-like Gpx-1 and Gpx-2 in ROS response

The absence of glutathione peroxidase activity in *Synechocystis* was firstly referred by Tichy and Vermaas (1999). Latter studies showed that two genes coding for glutathione peroxidase-like proteins have high similarity to Gpx but both are NADPH-dependent alkyl hydroperoxidases using electrons from PSI (Gaber et al., 2001, 2004). The importance of these Gpxs in response to different stress condition was also reported (Gaber et al., 2004, Hihara et al., 2001, Suzuki et al., 2001).

gpx-2 is consistently induced by various stresses, changes in *gpx-1* expression levels are less pronounced; again in agreement with microarray data (Hihara et al., 2001; Suzuki et al., 2001; Singh et al., 2008). Interestingly, steady-state expression of *gpx-2* in the *Δtpx* and *ΔkatG* mutants under normal growth conditions was significantly increased (Table 4.6) suggesting that the induced Gpx-2 complements the lack of Tpx, in agreement with Gaber et al., (2004) and/or the lack of KatG. The fact that *gpx-2* is already induced in mutants under normal growth conditions may also explain its lower relative induction under stress in mutants than in WT.

5.1.5. Role of catalase-peroxidase

30% of all known cyanobacterial genomes contain only one *katG* encoding a bifunctional catalase-peroxidase (KatG). It has been suggested that the role of KatG *in vivo* is to scavenge exogenous H_2O_2. It has been also suggested that other peroxidases cope with ROS inside the cells (Tichy and Vermaas, 1999).

We have shown by H_2O_2 formation during MV treatments in strains with inactivated KatG (Fig. 4.7) that the detoxification of endogenously evolved H_2O_2 by other enzymes is not sufficient and KatG may take place in detoxification of higher intracellular concentrations of H_2O_2 under oxidative stress. Increase in gene expression of *ahpC* and *sll1159* in *ΔkatG* mutants in presence of MV may indicate possible substitutes for the missing KatG activity. Also at steady-state the KatG was compensated by Gpx-2 peroxidase, but these peroxidases could not fully compensate for its activity resulting in H_2O_2 sensitive phenotype (Fig. 4.5,6).

Although expression of *katG* have been reported as constitutive in *Synechocystis* (Ushimaru et al., 2002), we demonstrated its inducibility in the *ΔahpC* mutant (Table 4.6).

5.1.6. LilA as a stress indicator in Synechocystis

Gene with the greatest increase in transcript level in most stress conditions was *slr1544*. This gene is coding for protein with molecular mass 12kDa with proposed single transmembrane helix, is related to CAB-like proteins named LilA. This protein was co-purified with PSII (Kufryk et al., 2008). Induction of this gene has been reported in many microarrays and latter demonstrated to be regulated by histidine kinase Hik33 (Suzuki et al., 2001; Mikami et al., 2002; Hihara et al., 2003; Paithoonrangsarid et al., 2004; Shoumskaya et al., 2005). We observed its transient induction, mostly during first 20 minutes. The highest expression was shown for low temperature stress, with almost 200-fold induction in WT. Expression of this gene can be used as a stress indicator in *Synechocystis* (Paper II).

5.2. MV resistance in *Synechocystis*

MV a non-selective herbicide, also known as paraquat (1,1-dimethyl-4,4´-bipyridinium dichloride), is a charged quaternary ammonium compound, that generates O_2^- under aerobic conditions by its cyclical univalent reduction and reoxidation (Hassan and Fridovich, 1978). As such, MV is commonly used to mimic and magnify the oxidative stress that cells normally encounter during photosynthesis and respiration. Cells can generally cope with MV by two mechanisms: by altering MV transport or by increasing the activities of ROS-scavenging enzymes to deal with ROS produced by MV.

Therefore, by selecting spontaneous MV-resistant mutants one can expect two types of mutations. In numerous studies, surprisingly only mutants of the first type were found (Santiviago et al., 2002; Hongo et al., 1994; Cho et al., 2003; Jo et al., 2004; Won et al., 2001; Lomovskaya and Lewis, 1992). A number of bacterial genes have been isolated recently coding for transporters conferring MV resistance. This includes *emrB* of *E. coli*, *smvA* of *Salmonella enterica* (Hongo et al.,1994) *pqrB* of *Streptomyces coelicolor* (Cho et al., 2003) and *pqrA* of *Ochrobactrum anthropi* (Jo et al., 2004; Won et al., 2001), all coding for MFS proteins. Another example is the *E. coli emrE* (or *mvrC*) gene coding for protein belonging to the SMR transporters that are restricted to prokaryotic cells and are the smallest multidrug efflux pumps, with only 4 helices and no significant extra-membrane domain (Lomovskaya and Lewis 1992). Generally, more than half known SMR proteins are capable to export MV (see Borges-Walmsley et al., 2003 for review). Full genome analysis of the cyanobacterium *Synechocystis* sp. PCC 6803 has revealed several putative MDR exporters (http://www.membranetransport.org): 5 TMDs of ABC transporters, 5, 3 and 2 proteins from the MFS, RND and MATE families, respectively. Surprising is the skimpy complement of *Synechocystis* MFS transporters in comparison to 70 in *E. coli*. No putative MV efflux pumps were found based on homology to known MV transporters in *Synechocystis*.

The multi-drug efflux pathways are often controlled by repressors from the TetR family, frequently forming an operon with the transporter (Cho et al., 2003; Ramos et al., 2005). Also in *Synechocystis* the first identified MV transporter was regulated by the PrqR repressor from the TetR family (Babykin et al., 2003; Nefedova et al., 2003a). This regulator controlled expression of the MATE-type exporter PrqA and the point mutation leading to MV-resistance resulted in L17Q substitution in the PrqR regulator (Babykin et al., 2003; Nefedova et al., 2003a). In our study, the Y52E substitution in PrqR was responsible for MV-resistance in MV-A and MV-B mutants. From TetR crystallographic data, Y52 is located in

the α3 helix (Fig 4.19) that is the main element responsible for sequence-specific recognition (Ramos et al., 2005; Orth et al., 2000). The position 52, which was shown to be in contact with the phosphate backbone of DNA, is markedly conserved among TetR family regulators with mostly H or Y appearing at this position (Ramos et al., 2005; Orth et al., 2000). The derepression of the *prqRA* operon has been demonstrated by expression analysis. In MV-A mutant, 60-fold increase in *prqR* transcript and 30-fold increase in *prqA* transcript was observed in comparison to WT (Table 4.15). It is probable, that Y52E similar to L17Q substitution also resulted in the derepression of the *prqRA* operon due to impaired binding of the mutated PrqR to DNA since, both substitutions, L17Q and Y52E, are located in the N-terminal domain of the PrqR regulator from the TetR family, close to DNA-binding helix-turn-helix motif (Ramos et al., 2005). Similar substitution R18Q in the TetR-like regulator of *Streptomyces coelicolor* also led to derepression of the MV efflux pump of the major facilitator superfamily (Cho et al., 2003). It is intriguing that two structurally unrelated MV transporters in two organisms are regulated by the same mechanism (Cho et al., 2003). In most cases including PrqR neither the role of the transporter nor its native inducer are known (Ramos et al., 2005).

Mutations leading to MV resistance but not mapping into the *prqR* gene were localized into *slr1174.* Interestingly, both MV-resistant mutants MV-4 and MV-6 carried different mutations in a single amino acid R115, R115G and R115L. Latter the third point mutation in the same amino acid R115C was described by our colleagues (Paper I). Slr1174 belongs to DUF990 family of proteins with homology to trans-membrane domain (TMD) of ABC transporters. Currently, DUF990 family proteins with unknown function were identified in 135 species across the bacterial kingdom implying their wide distribution (Finn et al., 2008). Advanced membrane topology algorithms, the web application server TRAMPLE (Fariselli et al., 2005), predicted six THMs for *slr1174* (Fig. 5.1). The protein is proposed to be embedded in the cytoplasmic membrane, with the N- and C-terminal being located inside the cell. This structure is common for permease subunits of ABC transporters (Putman et al., 2000; Saier et al., 1989). This topology would place the conserved arginine R115 in Slr1174 in the third TMH, close to the cytoplasmic side of the membrane (Fig 5.1).

Interestingly, the R115 residue replaced by G115, L115 or C115 in Slr1174 of the MV-resistant mutants is highly conserved in the cyanobacterial cluster despite the lower overall transporter homology (36% identity). There was only one conservative replacement for K115 found in *Trichodesmium erythraeum*. All three reported R115 substitutions (R115G, R115L and R115C) replaced positively charged arginine residue and resulted in the same mutant phenotype, though the properties of the three amino acids are quite different: tiny glycine, large and hydrophobic leucine and polar cysteine. The observed variability in R115 substitutions is surprising, taking into account that all other possible single point mutations of the R115 codon CGC could lead only to three more substitutions – R115P (CCC), R115S (AGC) and R115H (CAC). It seems that there is no strict requirement for particular amino acid at the position 115 and that removal of the positively charged arginine from the cytoplasmic side of the Slr1174 is required for increased specificity or processivity for MV. Taking into account that MV has two positive charges, removal of the positively charged arginine from the cytoplasmic side of the ABC transporter could result in increase of its affinity to MV. This is the first example of an involvement of ABC transporters in viologen transport (Paper I).

This hypothesis also corresponds with the expression data. The relative transcript abundance of *slr1174* was not influenced by MV (Table 4.16), indicating the absence of a link between the transporter and oxidative stresses caused by MV.

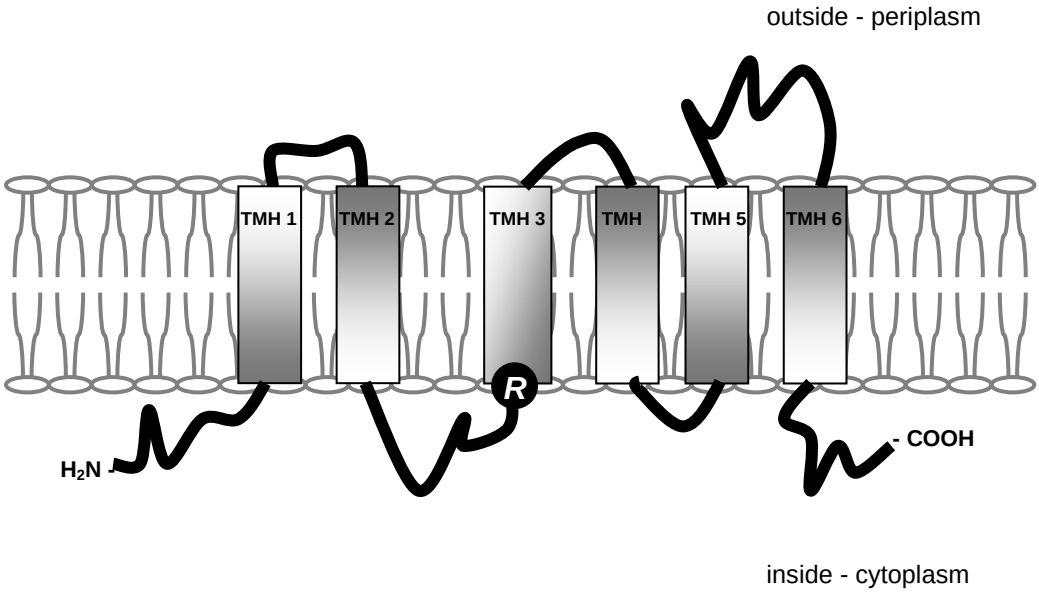

Fig. 5.1 Schematic representation of the Slr1174 (EvrB) polypeptide showing its proposed transmembrane organization and orientation with six THMs. The amino (NH_2) and carboxyl (COOH) termini of the polypeptide are indicated in the cytoplasm. The position of the conserved arginine R115 mutated in MV-resistant mutants is located on the cytoplasmic side of the third TMH.

Bioinformatic search using STRING search tool (von Mering et al., 2007; http://string.embl.de) predicted two possible structural partners of this TMD: another TMD (Slr0610) and nucleotide-binding domain (NBD) (Slr1901) forming a functional ABC transporter (Fig. 5.3; Table 5.1).

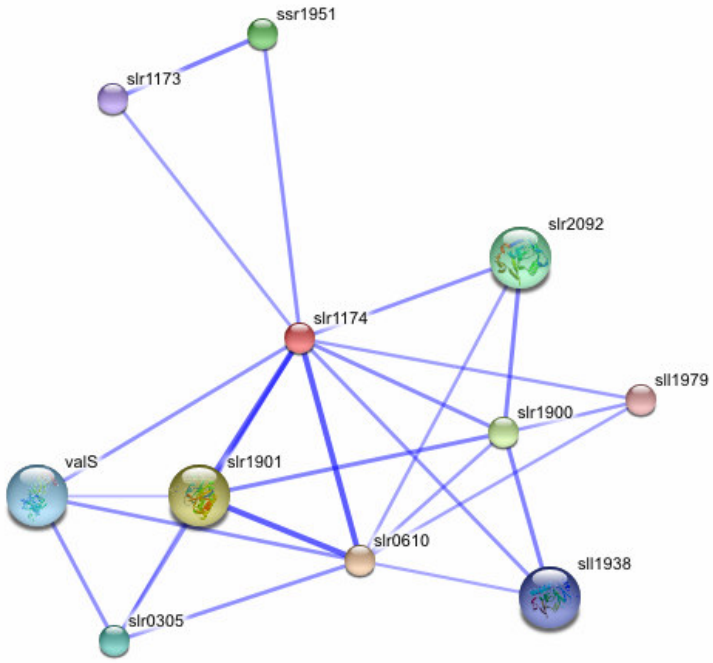

Fig. 5.2 Confidence view of predicted protein interaction by STRING; stronger associations are represented by thicker lines.

Table 5.1 Predicted functional partners of slr1174 with score > 0.6.

ORF	Protein function (length)	Score
slr0610	hypothetical protein (230 aa)	0.944
slr1901	ABC transporter (326 aa)	0.937
slr1900	hypothetical protein (247 aa)	0.678
ssr1951	hypothetical protein (58 aa)	0.661
slr2092	hypothetical protein (133 aa)	0.651
slr0305	TVP38/TMEM64 family membrane protein slr0305 (209 aa)	0.651
valS	Valyl-tRNA synthetase; Catalyzes the attachment of valine to tRNA(Val) (910 aa)	0.640
sll1938	hypothetical protein (203 aa)	0.636

The STRING prediction was primarily based on the fact that in various bacterial genomes the genes encoding orthologous counterparts of the Slr1901 (EvrA), Slr1174 (EvrB) and Slr0610 (EvrC) proteins form a putative operon as in most organisms (Fig. 5.3). Interestingly, these genes are dispersed all over the *Synechocystis* genome. This feature of the *Synechocystis* gene structure has been noted previously for example for the *trp* operon (Fujibuchi et al., 2000). Apparently, in *Synechocystis*, most genes of the ancestral operons are scattered throughout chromosome, possibly because of the abundant repetitive elements that can cause genome rearrangements (Itoh et al., 1999). Existence of this heteromeric ABC transporter in *Synechocystis* was later confirmed by my co-workers using the insertion mutagenesis where inactivation of any single subunit in the MV resistant mutant led to MV sensitive phenotype (Paper I).

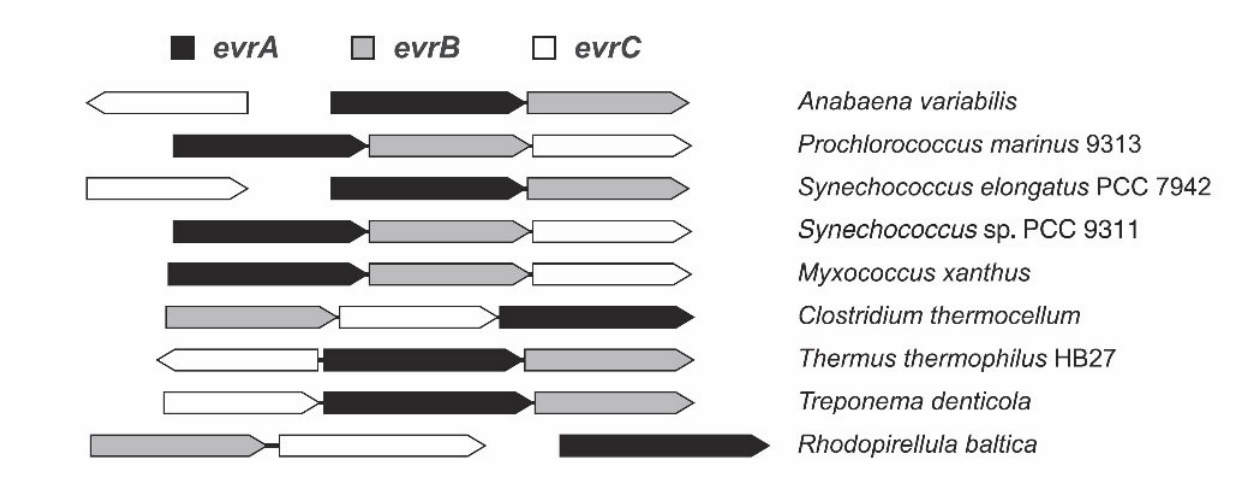

Fig. 5.3 Gene organization of the evr cluster in various organisms. The position and size of the genes encoding proteins that are most homologous to Slr1901 (EvrA), Slr1174 (EvrB) and Slr0610 (EvrC) in different organisms are shown. Note that the genes tend to cluster together despite changes in gene order or even in the gene orientation (in Thermus thermophylus). The disconnected genes are present in different locations of the genome.

Search for spontaneous MV-resistant *Synechocystis* mutants led to identification of two MV transporters PrqA a member of MATE family and Slr1174 a subunit of an ABC transporter. Although both type of mutants (MV-A, MV-4) showed comparable at least 10-fold resistance (Fig. 4.22A) to MV than WT, they basically differ in the mechanism of MV resistance. The resistance to MV in the first type of mutants occurred via the point mutations in PrqR repressor influencing MV transport by PrqA indirectly. The second type of mutants carried mutation directly in the Slr1174 transporter, probably changing its MV transport properties. From the frequency of mutations in both genes it seems that the mutation in PrqR is more frequent than in Slr1174 (Table 4.11) indicating that the number of possible mutations in PrqR is higher then in Slr1174. The fact, that both types of mutants (MV-A, MV-4) can tolerate a much higher MV concentration than WT without inducing the cell stress response, was also demonstrated by expression of *lilA* (Table 4.16) that is transiently induced by various stress (Hihara et al., 2003; Li et al., 2004).

6. CONCLUSION REMARKS

- SodB is indispensable for growth on air but not under microaerobic conditions
- *sodB* is induced under different stress conditions

- KatG scavenges not only exogenous but also endogenous H_2O_2
- KatG is inducible and partially substitutes for AhpC

- AhpC is indispensable for growth on air
- AhpC is the key light-dependent *t*-BuOOH peroxidase in *Synechocystis*
- lipid hydroperoxides are probably the main AhpC substrate *in vivo*
- *ahpC* deletion mutant acclimates to air by the carotenoid accumulation but not by induction of ROS-scavenging genes as WT
- AhpC is the most important of all Prx-s in *Synechocystis*

- Tpx light-dependent peroxidase
- Tpx does not significantly contribute to peroxide decomposition *in vivo*

- Gpx-2 can compensate for missing Tpx or KatG activities

- LilA was transiently induced by all stresses, it can be used as a stress indicator

- Sll1159 was highly induced by both peroxide stress, its role in *Synechocystis* may be significant

- MV-resistance in *Synechocystis* is ensured by at least two different multi-drug transporters
- point mutation in *prqR* repressor of the tetR family resulted in derepression of PrqA MATE-type transporter
- point mutation in Slr1174 apparently resulted in increased affinity of this transmembrane domain of an ABC-transporter for MV

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8. ENCLOSED PAPER

the FEBS Journal

A novel ATP-binding cassette transporter is responsible for resistance to viologen herbicides in the cyanobacterium *Synechocystis* sp. PCC 6803

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Keywords
ABC-type transporter; cyanobacteria; DUF990 family proteins; oxidative stress; viologen herbicide resistance

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(Received 23 January 2009, revised 18 May 2009, accepted 22 May 2009)

doi:10.1111/j.1742-4658.2009.07109.x

The charged quaternary ammonium compounds – methyl, ethyl and benzyl viologens – generate reactive oxygen species in photosynthetic cells. Three independent methyl viologen-resistant spontaneous mutants of *Synechocystis* sp. PCC 6803 were identified, in which the conserved R115 residue of the Slr1174 protein was replaced with G115, L115 and C115. The Slr1174 protein of the DUF990 family is related to the permease subunit of an ABC-2-type transporter and its R115 mutation was found to be solely responsible for the observed methyl viologen resistance. Bioinformatic analysis showed that in various bacterial genomes, two genes encoding another permease subunit and the ATPase component of an ATP-binding cassette transporter form putative operons with *slr1174* orthologs, suggesting that the protein products of these genes may form functional transporters. The corresponding genes in *Synechocystis* sp. PCC 6803, i.e. *slr0610* for the permease and *slr1901* for the ATPase, did not form such an operon. However, insertional inactivation of any *slr1174*, *slr0610* or *slr1901* genes in both the wild-type and the R115-resistant mutant resulted in increased sensitivity to methyl, ethyl and benzyl viologens; moreover, single- and double-insertion mutants did not differ in their viologen sensitivity. Our data suggest that Slr1901, Slr1174 and Slr0610 form a heteromeric ATP-binding cassette-type viologen exporter, in which each component is critical for viologen extrusion. Because the greatest increase in mutant sensitivity was observed in the case of ethyl viologen, the three proteins have been named EvrA (Slr1901), EvrB (Slr1174) and EvrC (Slr0610). This is the first report of a function for a DUF990 family protein.

Introduction

Molecular oxygen is essential for most organisms. However, during aerobic respiration or oxygenic photosynthesis, reactive oxygen species, including the superoxide anion radical (O_2^-), are formed. In the photosynthetic electron transport chain, instead of NADP, O_2 may accept an electron from the reduction

Abbreviations
ABC, ATP-binding cassette; BV, benzyl viologen; DQ, diquat; EV, ethyl viologen; MATE, multidrug and toxic compounds extrusion; MDT, multidrug transporter; MFS, major facilitator superfamily; MV, methyl viologen; NBD, nucleotide binding domain; SMR, small multidrug resistance; TMD, transmembrane domain; TMH, transmembrane helices.

In vivo role of type II peroxiredoxin (AhpC) in the cyanobacterium *Synechocystis* sp. PCC 6803; gene expression during stress acclimation

Running title:
Anti-oxidative stress response in *Synechocystis*

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ABSTRACT

The acclimation of *Synechocystis* sp. PCC 6803 to various stresses depends in part on efficient scavenging of reactive oxygen species (ROS). We constructed mutants with inactivated peroxiredoxins thioredoxin peroxidase (Tpx) and type II peroxiredoxin (AhpC), and characterized their *in vivo* peroxide detoxification capacity. Deletion of *tpx* did not change the rate of peroxide decomposition, while in the Δ*ahpC* mutant decomposition of tertiary butyl hydroperoxide (*t*-BuOOH) was nearly abolished. The Δ*ahpC* mutant accumulated highly increased levels of carotenoids, and showed modified acclimation to various stresses determined as a change in a gene expression profile.

Additionally, we used wild type and several mutants with inactivated genes coding for detoxifying enzymes to screen for differential expression of selected ROS-responsive genes under various stresses (high light, high salinity, low temperature, peroxides and superoxide generator methyl viologen). Most changes in gene expression were transient, specific up-regulation have been shown for genes coding for AhpC, Sll1159, Light harvesting-like protein A (LilA) and NADPH-dependent glutathione peroxidase-like (Gpx-2).