Analysis of the role of potential phytohormone and light-signalling components in photosynthetic organisms

Manti Christiana Schwarzkopf
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<tbody>
<tr>
<td>AHK</td>
<td><em>Arabidopsis</em> histidine kinase</td>
</tr>
<tr>
<td>AHP</td>
<td>histidine phosphotransfer protein</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartate</td>
</tr>
<tr>
<td><em>At</em>, <em>Arabidopsis</em></td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphateadenosine-5'-triphosphate</td>
</tr>
<tr>
<td><em>Bgh</em></td>
<td><em>Blumeria graminis</em> f. sp. <em>hordei</em></td>
</tr>
<tr>
<td>bp</td>
<td>basepairs</td>
</tr>
<tr>
<td>CaMV</td>
<td>cauliflower mosaic virus</td>
</tr>
<tr>
<td>CCM</td>
<td>CO$_2$ concentrating mechanism</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>bis-(3'-5')-cyclic dimeric guanosine monophosphate</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CHASE</td>
<td>cyclase/histidine kinase associated sensory extracellular</td>
</tr>
<tr>
<td>Chl</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>CKX</td>
<td>cytokinin oxidase</td>
</tr>
<tr>
<td>Cyt $b_6f$</td>
<td>cytochrome $b_6f$ complex</td>
</tr>
<tr>
<td>dai</td>
<td>days after inoculation</td>
</tr>
<tr>
<td>DBMIB</td>
<td>dibromothymoquinone</td>
</tr>
<tr>
<td>DCMU</td>
<td>3-(3',4'-dichlorophenyl)-1,1-dimethylurea</td>
</tr>
<tr>
<td>DGC</td>
<td>diguanylate cyclase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>t-DNA</td>
<td>transferred DNA</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2' deoxynucleoside 5' triphosphate</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>f.sp.</td>
<td>forma specialis</td>
</tr>
<tr>
<td>GAF</td>
<td>cGMP phosphodiesterase adenylate cyclase/FhIA</td>
</tr>
<tr>
<td>G6PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescing protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>k</td>
<td>rate constant k</td>
</tr>
<tr>
<td>Hik</td>
<td>histidine kinase</td>
</tr>
<tr>
<td>HKD</td>
<td>histidine kinase domain</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>HKRD</td>
<td>histidine kinase-related domain</td>
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<td>IPT</td>
<td>isopentenyltransferase</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LAHG</td>
<td>light-activated heterotrophic growth</td>
</tr>
<tr>
<td>MASE</td>
<td>membrane associated sensor</td>
</tr>
<tr>
<td>Mb</td>
<td>mega base pairs</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>NDH-1</td>
<td>NADPH type-1 dehydrogenase</td>
</tr>
<tr>
<td>OE</td>
<td>overexpression</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OPPP</td>
<td>oxidative pentosephosphate pathway</td>
</tr>
<tr>
<td>PAS</td>
<td>Per/Arnt/Sim; period circadian protein/aryl hydrocarbon receptor nuclear translocator protein/single-minded protein</td>
</tr>
<tr>
<td>PAM</td>
<td>Pulse Amplitude Modulation</td>
</tr>
<tr>
<td>PAR</td>
<td>photosynthetic active radiation</td>
</tr>
<tr>
<td>PCC</td>
<td>Pasteur Culture Collection</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>Pst</td>
<td><em>Pseudomonas syringae pv. tomato</em> DC3000</td>
</tr>
<tr>
<td>PS I, PS II</td>
<td>photosystem I, II</td>
</tr>
<tr>
<td>PQ</td>
<td>plastoquinone</td>
</tr>
<tr>
<td>pv.</td>
<td>pathovar</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>half-life</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RR</td>
<td>response regulator</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SDH</td>
<td>succinate dehydrogenase</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>WT</td>
<td>wildtype</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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<tr>
<td>v/v</td>
<td>volume per volume</td>
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1 Introduction

For all living organisms it is critically important to adapt quickly and efficiently to changes in their physical and chemical environment. In bacteria, adaptational processes can occur at different levels, at the level of individual genes and proteins, via altered gene expression and allosteric regulation of enzyme activity, the whole-cell level via cellular motility and the multicellular level via cell aggregation and biofilm formation (Galperin, 2010). The ability to mediate these adaptive responses is conferred by sophisticated signal transduction systems, the so-called two-component signalling systems, which are found ubiquitously in prokaryotes and archaea, fungi, yeast and certain plants and have been shown to regulate physiological and molecular processes at each level of adaptation (Galperin, 2010; Perry et al., 2011). Considerable effort has been made in the past years to elucidate these signal transduction processes and the functions of the associated proteins (Stock et al., 2000; Laub & Goulian, 2007; Schaller et al., 2008; Gao & Stock, 2009; Wuichet et al., 2010; Schaller et al., 2011; Jung et al., 2012). However, a thorough understanding of these complex signalling networks remains elusive. Considering the involvement of signal transduction systems in essential processes and the concomitant far-ranging effects, it is of great importance to contribute to the understanding of the underlying molecular mechanisms.

1.1 Synechocystis sp. PCC 6803

Cyanobacteria are considered to be among the evolutionarily oldest organisms on earth since the discovery of 3.5 billion year old putative stromatolites and microfossils, attributed to cyanobacteria (Schopf, 1993). With their ability to perform oxygenic photosynthesis, cyanobacteria played a significant role in the history of earth and the evolution of life as primary producers of atmospheric oxygen (Nakao et al., 2010). Even today, these prokaryotic microorganisms contribute substantially to maintain the biosphere, as they are important primary producers at a global scale with a relevant role in the carbon and nitrogen cycles (Herreiro & Flores, 2008). Considering their long evolutionary history, cyanobacteria are among the most successful groups of microorganisms on earth. One of the main reasons for their evolutionary success can be related to their adaptability to a broad range of environmental conditions, resulting in an almost ubiquitous distribution in marine and terrestrial ecosystems, ranging from fresh and salt water to even more extreme
habitats such as thermal springs, alkaline lakes, glaciers and deserts (van den Hoek et al., 1993; Whitten & Potts, 2000; Koksharova, 2009). According to the endosymbiont theory (Margulis, 1970), chloroplasts in plants and eukaryotic algae have evolved from cyanobacterial ancestors via endosymbiosis (Nakamura et al., 2000). Cyanobacteria and especially the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (order *Chroococcales*) have become important model organisms for research of photosynthesis, respiration, carbon metabolism and its regulation, as well as in signal transduction processes and stress responses. The ability to perform oxygenic photosynthesis with a complete gene set similar to higher plants (Nakamura et al., 2000) and to grow and survive in a wide range of conditions, made *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) one of the most intensely studied cyanobacteria. The original *Synechocystis* strain was isolated from a fresh-water lake and was deposited in the Pasteur Culture Collection (PCC) in 1968 (Rippka et al., 1979). In the early 1980s, the strain was recognised to be spontaneously transformable by integration of exogenous DNA into its genome via double homologous recombination (Grigorieva & Shestakov, 1982). Their natural transformation competence can be used for analysing functions of unknown genes through gene disruption and insertion mutational analysis (Nakamura et al., 2000).

*Synechocystis* cells can either live as single planktonic organisms or in sessile communities, the so-called biofilms or biomats. Biofilms are generally defined as microbial communities adhered to a substratum and embedded in an extracellular polymeric matrix produced by the microbial cells itself (Costerton et al., 1987, 1994). In its natural habitat, *Synechocystis* grows photoautotrophically. Besides photoautotrophic growth, certain strains of *Synechocystis* are able to utilise organic compounds, e.g. glucose as a carbon source concomitant with photosynthetic CO$_2$ fixation (Rippka et al., 1979; Stal & Moezelaar, 1997; Gomez-Baena et al., 2008). Accordingly, the glucose-tolerant wildtype strain of *Synechocystis* is capable of photoautotrophic and photomixotrophic growth under light conditions and of heterotrophic growth in darkness in glucose-supplemented media (Williams, 1988; Anderson & McIntosh, 1991). However, light irradiation is still required for heterotrophic growth, which occurs even with a low light intensity at which photosynthesis can scarcely proceed (Tabei et al., 2009), as *Synechocystis* cells are unable to grow in complete darkness. Occasional light pulses (e.g. for 5 min every 24 h) are sufficient to enable growth and survival of the cells in darkness. This phenomenon is referred to as light-activated heterotrophic growth (LAHG; Anderson & McIntosh, 1991). The ability to carry out heterotrophic growth without loss of viability made
Synechocystis a useful model organism in photosynthesis research by enabling the
disruption of photosynthesis-related genes to investigate functions and contributions
to the photosynthetic pathway (Nakamura et al., 2000). Its role as model organism
became even more important after Synechocystis was the first photosynthetic
organism to be completely sequenced (Kaneko et al., 1996). The availability of the
entire genomic nucleotide sequence provides the possibility to study gene functions
and regulatory mechanisms on a genome-wide level. To summarize, Synechocystis
has become an extremely versatile model organism in research of signal trans-
duction processes and stress responses, as well as in carbon metabolism and
especially in oxygenic photosynthesis.

1.2 The primary energy metabolism in Synechocystis

The primary energy metabolism in cyanobacteria consists of anabolic and catabolic
processes, including photosynthesis, CO₂ fixation, glycogen formation, gluconeo-
genesis and glucose degradation processes, such as glycolysis, the oxidative
pentose phosphate pathway (OPPP) and the incomplete tricarboxylic acid (TCA)
cycle, respectively. Cyanobacteria constitute a unique case where the anabolic and
catabolic carbohydrate mechanisms function in the same compartment of the cell
(Yang et al., 2002; Shastri & Morgan, 2005, 2007; Takahashi et al., 2008; Haimo-
vich-Dayan et al., 2011). Accordingly, this requires strict regulation of the direction of
reactions in response to environmental conditions (Tabei et al., 2009).

Light is one of the most important environmental factors for photosynthetic
organisms. They use light energy to produce chemical energy and reducing power
for the fixation of atmospheric carbon and the assimilation of other nutrients.
Reduced carbon provides energy, as well as building blocks in metabolic reactions
required for growth and development. In their natural habitat, cyanobacteria are
exposed to changing light conditions, such as the diurnal light-dark cycle or daily
changes in light quality and quantity. In order to maintain their metabolic homeo-
stasis, it is of critical importance to adapt to changing light conditions, either by
switching between metabolic modes during the light-dark cycle or by using
mechanisms to adjust the distribution of light energy for maximal energy utilisation
and protection against photodamage. Changing light conditions can cause redox im-
balances and an excessive production of damaging reactive oxygen species (ROS),
when the perceived light is not fully utilised by downstream processes (Apel & Hirt,
2004; Scheibe et al., 2005; Muramatsu & Hihara, 2012). It has been suggested that
photosynthetic organisms integrate nutrient-specific pathways by tightly connecting
photosynthetic processes with other principal metabolic pathways to survive under constantly changing environmental and metabolic cues (Wang et al., 2003; Forchhammer, 2004; Gutierrez et al., 2007; Singh et al., 2010). Furthermore, the tight connection between the activity of photosynthetic processes and metabolic pathways becomes apparent as photosynthesis represents the sole source to generate chemical energy for photosynthetic organisms under photoautotrophic conditions (Singh et al., 2010). When light is available, cyanobacteria assimilate inorganic carbon via the Calvin cycle, using ATP and NADPH generated through photosynthesis. The fixed carbon enters the glycolytic pathway and is subsequently utilised to generate reducing equivalents, cofactors and building blocks for biosynthetic pathways. Besides, excess carbon can be assimilated in the form of glycogen and stored as reserve carbohydrate. Under non-photosynthetic conditions respiration releases the energy stored in those carbon compounds. Glucose residues, derived from glycogen, are catabolized via the OPPP, the lower energy-conserving phase of glycolysis and the TCA cycle, resulting in the production of NAD(P)H and biosynthetic intermediates required for maintenance and growth (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003; Singh & Sherman, 2005; Tabei et al., 2009). The following two sections will give a detailed overview on the primary metabolism and its concomitant processes, beginning with photosynthesis and respiration, followed by the central carbon metabolism.

1.2.1 Photosynthesis and respiration

In cyanobacteria, both metabolic processes, oxygenic photosynthesis and aerobic respiration, are located in the same compartment (Schmetterer, 1994). Unlike in plants, where photosynthesis and respiration are separated in different organelles, cyanobacteria possess, due to the lack of compartmentalization, a highly differentiated membrane system, which harbors the electron transport chains required for photosynthesis and respiration (Vermaas et al., 1994; Mullineaux, 2008b; Nickelsen et al., 2010). This internal membrane system, the thylakoid membrane, which separates the cytoplasm from the lumen, contains a photosynthetic electron transport chain including the two photosystems (PS II and PS I) and a respiratory electron transport chain, including among others, NADPH type-1 dehydrogenase (NDH-1), succinate dehydrogenase (SDH) and a cytochrome aa₃-type terminal oxidase (cytochrome c oxidase) (Tanaka et al., 1997; Cooley et al., 2000). In the thylakoid membrane both electron transport chains intersect and share electron carriers, such as the cytochrome b₆f complex, the plastoquinone pool (PQ) and soluble redox active proteins (Schmetterer, 1994; Vermaas, 2001). In contrast,
the cytoplasmatic membrane, which forms the inner boundary of the periplasmatic space, harbors solely components associated with the respiratory electron transport chain. Therefore, in most cyanobacteria, photosynthetic electron transport occurs solely in the thylakoids, while the respiratory electron flow takes place in both, the thylakoid and the cytoplasmatic membrane systems (reviewed by Vermaas, 2001).

Figure 1: Schematic illustration of the intersecting photosynthetic and respiratory electron transport pathways in the thylakoid membrane of Synechocystis sp. PCC 6803 (modified after Campbell et al., 1998; Vermaas, 2001; Singh et al., 2009)

Bolts designate light energy, starting the redox reactions in both photosystems. Arrows indicate photosynthetic and respiratory electron transfer reactions. Linear photosynthetic electron transport: from water to NADP$^+$ via PS II and PS I (thick solid arrows); respiratory electron transport: from NADPH and succinate to cytochrome $c$ oxidase (dotted arrows) and cyclic electron transport: around PS I via Fd and/or NDH-1 (not indicated). Dashed arrows indicate protons released into the lumen, thereby establishing a proton gradient ($\Delta$H) across the thylakoid membrane, which powers ATP synthase.

Abbreviations: NDH-1, type I NADPH dehydrogenase; SDH, succinate dehydrogenase; Suc, succinate; Fum, fumarate; PS I and II; photosystem I and II; P680, P700, reaction center chlorophyll molecules of PS II and I, respectively; PQ/PQH$_2$, plastoquinone pool; cyt $b$$_{6}$$f$, cytochrome $b$$_{6}$$f$ complex; PC, plastocyanin; cyt $c$ oxidase, cytochrome aa$_{3}$-type cytochrome $c$ oxidase; ATPase, ATP synthase complex; ATP, adenosine-5'-triphosphate; NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced form); PBS, phycobilisome; PC, phycocyanin; AP, allophycocyanin; $hv$, photons; Fd$_{ox}$/Fd$_{red}$, ferredoxin in oxidized and reduced forms, respectively; OPP Pathway, oxidative pentosephosphate pathway.

Electron input in the transport system in cyanobacterial thylakoid membranes is a complex process due to the existence of converging metabolic pathways, feeding electrons into the system. Under light conditions, there is net input of electrons into the transport system from water-splitting activity of PS II (Campbell et al., 1998). Under light or dark conditions, variable electron fluxes from NAD(P)H, which is oxidized by one or more thylakoid-bound dehydrogenases (Scherer et al., 1982; Mi
et al., 1992, 1995; Herbert et al., 1995; Tanaka et al., 1997; Ogawa & Mi, 2007; Battchikova & Aro, 2007; Battchikova et al., 2011) feed into the electron transport system (Campbell et al., 1998). In this electron transport network, three intersecting pathways have been found dominant in Synechocystis thylakoid membranes: linear photosynthetic electron transport from water to NADP⁺ via PS II and PS I, cyclic electron transport around PS I and respiratory transport from NADPH and succinate to cytochrome c oxidase (Cooley & Vermaas, 2001), which will be described in detail in the following section.

Photosynthetic electron transport

Photosynthetic light reactions establish an electron flow in the thylakoid membranes, comprising of linear and cyclic electron transport, leading to the production of NADPH and ATP used for carbon fixation (Iwai et al., 2010). The primary reactions of oxygenic photosynthesis are catalyzed by two large protein complexes, PS II and I, acting in series to drive light-dependent electron transfer reactions (Pakrasi, 1995; Singh et al., 2009). The photosynthetic reaction is initiated by the absorption of light, captured by specialized antennae structures. These antennae transfer the excitation energy to specialized chlorophyll molecules (P680 and P700), the reaction centers in both photosystems, which in turn catalyze light-induced electron transfer across the thylakoid membrane (Pakrasi, 1995). In cyanobacteria, the prevalent antenna pigments for the absorption of light energy are chlorophyll (Chl) and bilin (Glazer, 1977; MacColl, 1998; Mullineaux, 2008a). Chls are mainly associated with PS I and absorb light with maximum absorbance wavelengths (λmax) of 435 and 680 nm, while bilins are covalently attached to light-harvesting proteins, the phycobiliproteins, and are mainly associated with PS II (Singh et al., 2009). The specific combination of apoproteins and bilins in a phycobilisome (PBS) determines its light absorption profile. The two main phycobiliproteins in cyanobacterial PBS are the red light-absorbing allophycocyanin (AP) with λmax ~ 650 nm and phycocyanin (PC) with λmax ~ 620 nm (Singh et al., 2009). Structurally, APs form together with their specific linker polypeptides the “core” of a PBS, whereas PCs (sometimes also phycoerythrins and other phycobiliproteins) represent together with their associated linker polypeptides the “rod” elements, radiating out from the core. PBS are mobile light-harvesting complexes that are located peripheral to the thylakoid membrane (Grossman et al., 1993; MacColl, 1998) and transfer energy directly to the PS (Mullineaux, 2008a). After absorption of excitation energy via the light-harvesting complexes, the photochemical reaction starts with the transfer of the excitation energy to the reaction center molecules of the photosystems. During linear electron transport, PS
II catalyzes the light-driven oxidation of water and the reduction of plastoquinone (PQ). In each charge separation event, one electron is extracted from the Mn-containing oxygen-evolving complex. After accumulation of four positive charges, oxygen is evolved and four H\(^+\) are released into the lumen of the thylakoids. The electron is transferred from P680\(^+\) to plastoquinone (PQ). After two charge separation events, PQ is double reduced, takes up two protons from the cytoplasm and leaves PS II as PQH\(_2\). PQH\(_2\) diffuses through the membrane to the cytochrome b\(_{6f}\) complex, which reduces a soluble electron carrier in the thylakoid. This electron carrier can either be plastocyanin (PC) or cytochrome c\(_{553}\) (cyt c\(_{553}\)), depending on the species and the availability of copper, as plastocyanin is a copper-containing enzyme (Vermaas, 2001). The two protons of PQH\(_2\) are released into the lumen and two additional protons are pumped across the membrane by the cyt b\(_{6f}\) complex. The reduced PC translocates from the cyt b\(_{6f}\) complex to PS I. Light energy, captured by the antenna system of PS I, is transferred to the reaction center of the complex. When the excitation energy reaches P700, P700\(^+\) is formed. An electron is ejected and transferred across the membrane to the final acceptor ferredoxin (Fd) at the cytoplasmatic side of the PS I complex. The reduced ferredoxin is subsequently used for NADPH production, which provides the reducing power for the conversion of carbon dioxide to organic molecules via the Calvin cycle. During linear photosynthetic electron transfer, protons are released into the lumen upon water splitting at PS II and plastoquinol oxidation by the cytochrome b\(_{6f}\) complex, thereby establishing a proton gradient (\(\Delta p\)H) across the thylakoid membrane. This \(\Delta p\)H is used for ATP generation by powering the membrane-bound ATP-synthase. In addition to the linear electron transport, an alternate pathway, the cyclic electron transport, contributes to the \(\Delta p\)H. This electron transport is driven solely by PS I (Fork & Herbert, 1993; Bendall & Manasse, 1995; Munekage & Shikanai, 2005; Iwai et al., 2010) and allows the generation of ATP without the accumulation of reducing equivalents (Singh et al., 2009). Cyclic electron transport describes electron flows around PS I, from PS I via ferredoxin (Fd) and/or NAD(P)H (Herbert et al., 1995; Mi et al., 1995) directly back to the intersystem transport chain (Campbell et al., 1998). Two possible electron transport pathways have been proposed for cyclic electron transport, an Fd-dependent pathway and an NDH-1-dependent pathway (reviewed by Shikanai, 2007). In the NDH-1-dependent pathway, NDH-1 mediates NADPH oxidation and PQ pool reduction similar to complex I in mitochondria (Okhawa et al., 2000), while it remains elusive how electrons on Fd are transferred to the PQ pool in the Fd-dependent cyclic electron transport (Iwai et al., 2010). Hence, linear photosynthetic electron transport leads to NADPH and ATP production and to the
generation of a ΔpH across the thylakoid membranes, whereas cyclic electron flow results only in establishing a ΔpH, which is subsequently used for ATP generation (Tsunoyama et al., 2009).

Depending on environmental or physiological conditions, the ratio between ATP and NADPH can be adjusted by fine-tuning the ratio of cyclic and linear electron transport (Tsunoyama et al., 2009). Cyclic/linear electron transport ratio adjustments support a redox poise of the PQ pool, which is essential for maximal light energy utilisation and protection against photodamage or other types of stress (Fork & Herbert, 1993; Bukhov & Carpentier, 2004; Munekage et al., 2004; Joliot & Joliot, 2006; Shikanai, 2007; Rumeau et al., 2007; Iwai et al., 2010). For example, changes in the spectral composition of light can lead to imbalanced excitation of the photosystems, which in turn leads to reduced photosynthetic efficiency and damages of the photosynthetic apparatus (Anderson et al., 1995; Dietzel et al., 2008; Singh et al., 2009; Muramatsu & Hihara, 2012). In order to cope with such imbalances and to ensure efficient photosynthesis, cyanobacteria, algae and plants have developed adaptation mechanisms, that act on different time-scales, the so-called short-term and long-term mechanisms (Manodori & Melis, 1986; Chow et al., 1990; Anderson et al., 1995; Fujita, 1997; Allen, 2003; Dietzel et al., 2008). Within the range of seconds to minutes, a short-term mechanism, known as state transition, controls the distribution of excitation energy transfer between the two photosystems (Bonaventura & Myers, 1969; Murata, 1969). Accordingly, two states, state I and state II, have been defined, in which the photosynthetic apparatus gives an optimal quantum yield of photosynthesis in light, having a composition favouring its absorption by PS I or PS II, respectively (Wollman, 2001; Allen, 2003). In cells adapted to state I, PBS transfer energy primarily to PS II (Mullineaux, 2008a), resulting in a predominance of the linear electron transport. In cells adapted to state II a significant proportion of PBS transfer energy to PS I instead (van Thor et al., 1998; McConnell et al., 2002; Mullineaux, 2008a), resulting in a predominance of cyclic electron transport. In contrast to higher plants and green algae, cyanobacteria are in state I upon illumination and in state II in the dark or in very low light due to the respiratory electron flow reducing the PQ pool (Mullineaux & Allen, 1986; Mao et al., 2002; Tsunoyama et al., 2009). State transitions are triggered by the redox state of the PQ pool or a closely located electron carrier (Mullineaux, 2008a). Hence, they allow the regulation of light-harvesting in response to the photosynthetic electron transport chain (Mullineaux & Allen, 1990; Mullineaux, 2008a). State transitions require PBS diffusion (Joshua & Mullineaux, 2004) and are assumed to be involved in covalent
modification of PBS, thereby changing their binding properties for PS II or PS I (Allen et al., 1985). Nevertheless, the signal transduction pathway that connects a change in PQ redox state to a change in the binding properties of PBS remains elusive (Mullineaux, 2008a). During prolonged changes in light quality, cyanobacteria utilise long-term mechanisms that occur within hours and days to circumvent reduced photosynthetic efficiency and to protect cells from photodamage. In contrast to the antenna movement during state transitions, the long-term mechanism requires the adjustment of the photosystem stoichiometry to regulate the balance of electron flow between two reaction centers (Chow et al., 1990; Anderson et al., 1995; Dietzel et al., 2008). Hence, cyanobacteria cope with changing light quality by modulating the composition, structure and functions of the photosynthetic apparatus, thereby ensuring an effective balance of electron transfer rates between the two photosystems (Singh et al., 2009).

**Respiratory electron transport**

Electron input into the transport system in the cyanobacterial thylakoid membrane either results from photosynthetic activity or from catabolic pathways providing the respiratory substrates in the absence of light. In the respiratory pathway, electrons from NAD(P)H and succinate enter the electron transport chain via NADPH dehydrogenase 1 (NDH-1) (Mi et al., 1992; Ryu et al., 2003) and succinate dehydrogenase (SDH) (Cooley & Vermaas, 2001). Despite many studies of the respiratory electron transport pathways in cyanobacterial thylakoid membranes, controversy still remains about whether SDH, rather than NDH-1, is the major pathway of electron flow into the PQ pool (Mi et al., 1995; Cooley et al., 2000; Ryu et al., 2003). Besides its function as electron acceptor, the PQ pool serves as electron donor for the respiratory oxidases. The respiratory oxidases function as terminal components of the respiratory electron transport chain, where the electrons are finally transferred to molecular oxygen. So far, three terminal oxidases have been identified in *Synechocystis* (Hart et al., 2005), cytochrome *aa3*-type cytochrome *c* oxidase and cytochrome *bd*-type quinol oxidase (Schmetterer, 1994; Howitt & Vermaas, 1998; Berry et al., 2002) used for respiration and a third terminal oxidase, for which its *in vitro* oxidase activity is still in dispute (Howitt & Vermaas, 1998; Pils & Schmetterer, 2001; Berry et al., 2002; Mogi & Miyoshi, 2009). Electrons are either transferred directly to cytochrome *bd*-type oxidase or via plastocyanin/cyt*553* to a cytochrome *aa3*-type cytochrome *c* oxidase (Schmetterer, 1994; Howitt & Vermaas, 1998; Berry et al., 2002; Mogi & Miyoshi, 2009). The latter functions as the main oxidase for the NADPH-driven electron transport in the thylakoid membrane (Berry et al., 2002).
This electron transport establishes a ΔpH across the thylakoid membrane, which is subsequently utilised for ATP generation through ATP-synthase.

### 1.2.2 Central carbon metabolism

Besides nitrogen, sulphur and phosphorus, reduced carbon is an essential macronutrient required for growth and development of all organisms. Photosynthetic organisms produce reduced carbon through photosynthesis by using light energy to generate chemical energy and reducing power for the fixation of atmospheric carbon and the assimilation of other nutrients (Singh et al., 2008).

![Central Carbon Metabolism Diagram](image)

**Figure 2: Schematic metabolic map of the central carbon metabolism in *Synechocystis* sp. PCC 6803, including the Calvin cycle, glycolysis, the oxidative pentose phosphate pathway and the (incomplete) tricarboxylic acid cycle (modified after Yang et al., 2002).**

**Abbreviations:** G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; Rib5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; X5P, xylulose-5-phosphate; E4P, erythrose-4-phosphate; Rib1,5P₂, ribulose-1,5-bisphosphate; F6P, fructose-6-phosphate; F1,6P₂, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; G1,3P₂, 1,3-bisphosphoglycerate; G3P, 3-phosphoglycerate; G2P, 2-phosphoglycerate; PEP, phosphoenolpyruvate; acetyl-CoA, acetyl coenzyme A; ICI, isocitrate; αKG, α-ketoglutarate; Suc, succinate; FUM, fumarate; MAL, malate, OAA, oxalacetate; Enzymes (italic, grey font): G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; GK, glucokinase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase.
In the absence of light, they must switch to heterotrophic energy generation, which includes the degradation of stored carbon compounds via the OPPP, the lower energy-conserving phase of glycolysis and the TCA cycle (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003; Takahashi et al., 2008). Figure 2 presents a schematic overview of these metabolic pathways and their involvement in the formation and degradation of carbohydrates.

Photosynthetic light reactions provide energy in the form of ATP and NADPH, which is subsequently used for carbon fixation in the Calvin cycle and in the production of hexosephosphates via gluconeogenesis. The first step of the Calvin cycle, the carboxylation of ribulose-1,5-bisphosphate (Rib1,5P₂), is catalyzed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Due to the relatively low affinity of Rubisco for its substrate, Synechocystis utilizes a CO₂-concentrating mechanism (CCM) that is upregulated when the cells are exposed to a limiting CO₂ level, resulting in an increase of the CO₂ concentration in close proximity to Rubisco within the carboxysomes (Kaplan & Reinhold, 1999; Giordano et al., 2005; Badger et al., 2006; Kaplan et al., 2008; Price et al., 2008). The resulting sugar products enter the glycolytic pathway and are subsequently utilized in many biosynthetic pathways or converted into glycogen, the main carbohydrate storage compound in cyanobacteria (Preiss & Sivak, 1998a, 1998b; Knoop et al., 2010), which serves as metabolic energy source under non-photosynthetic conditions/during periods of darkness. The degradation of glycogen provides glucose, which is catabolized via the lower energy-conserving phase of glycolysis, an incomplete TCA cycle and the OPPP (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003; Takahashi et al., 2008).

In the glycolytic pathway glucose is converted into pyruvate by generating small amounts of ATP and reducing power in the form of NADH. Glycolysis is a central pathway that produces important precursor metabolites, including the six-carbon compounds glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and three-carbon compounds of glyceraldehyde-3P (GAP), 1,3-bisphosphoglycerate (G1,3P₂), 3-phosphoglycerate (G3P), phosphoenol-pyruvate (PEP) and pyruvate. The oxidative decarboxylation of pyruvate produces acetyl coenzyme A (acetyl-CoA), the activated form of acetate, which is the starting molecule of the TCA cycle. The two-carbon acetyl group in acetyl-CoA is transferred to the four-carbon compound of oxalacetate (OAA) to form the six-carbon compound of citrate. In a series of reactions, two carbons in citrate are oxidized to CO₂ and the reaction pathway supplies NADH for the use in the oxidative phosphorylation and other metabolic processes. At the end of the cycle, the remaining four-carbon part is transformed...
back to OAA. The TCA cycle is an important pathway for the final steps of carbohydrate oxidation and also supplies important precursor metabolites, such as 2-oxoglutarate. In cyanobacteria, the TCA cycle was assumed to be non-functional due to the absence of the key enzyme 2-oxoglutarate dehydrogenase, which converts 2-oxoglutarate to succinyl-coenzyme A (Pearce et al., 1969; Stanier & Cohen-Bazire, 1977). Consistently, sequence analyses revealed that no fully sequenced cyanobacterial genome encodes the genes for 2-oxoglutarate dehydrogenase (Wood et al., 2004). However, Synechocystis is able to convert 2-oxoglutarate to succinate despite the absence of a 2-oxoglutarate dehydrogenase complex, which indicates the use of an alternate pathway (Cooley et al., 2000; Vermaas, 2001). Only recently, Zhang and Bryant (2011) identified genes encoding a novel 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase in Synechococcus sp. PCC 7002. They demonstrated that these two enzymes convert 2-oxoglutarate to succinate and thus functionally replace 2-oxoglutarate dehydrogenase and succinyl-CoA synthetase in the TCA cycle (Zhang & Bryant, 2011).

In the OPPP, glucose-6-phosphate (G6P) is first converted to 6-phosphogluconolactone (6PGL), catalyzed by the enzyme glucose-6-phosphate dehydrogenase (G6PDH) and further catabolized to glyceraldehyde-3-phosphate (GAP). The catabolic processes of the OPPP provide reducing power in the form of NADPH, as well as metabolic precursors for anabolic processes, such as the biosynthesis of amino acids or nucleic acids (Kruger & von Schaewen, 2003; Osanai et al., 2007). The OPPP is widely accepted to be the main route of glucose catabolism in cyanobacteria, both for the breakdown of glycogen or exogenously supplied glucose (Hagen & Meeks, 2001; Yang et al., 2002). Hence, the enzyme G6PDH (encoded by Zwf, slr1843) is of particular importance, as it catalyzes the initial step of glucose degradation, thereby controlling the carbon flow into the OPPP. The regulation of G6PDH is complex and still not fully understood. Metabolites, including NADPH, ATP, G6P, glutamine and Rib1,5P2 have been implicated in its regulation (Schaeffer & Stanier, 1978; Cossar et al., 1984), as has thioredoxin, which may reductively inactivate and oxidatively reactivate the enzyme in the light and dark, respectively, to prevent futile cycling, which would occur if the Calvin and OPP cycles operated simultaneously (Cossar et al., 1984; Udvardy et al., 1984; Gleason, 1996; Hagen & Meeks, 2001). Biochemical analyses indicated that G6PDH exists in various aggregation states that show different kinetic properties (Schaeffer & Stanier, 1978; Gleason, 1996; Sundaram et al., 1998; Kahlon et al., 2006), however, the biological significance of the aggregation states remains elusive. Besides, a role in regulating
G6PDH activity has been suggested for OPC A (OPP cycle protein A), as this protein is assumed to be involved in the assembly of G6PDH, its oligomerization and activation in various cyanobacteria (Sundaram et al., 1998; Min & Golden, 2000; Hagen & Meeks, 2001; Kahlon et al., 2006).

Variations in the ability of cyanobacteria to consume carbon from their surroundings have been reported. In addition to photoautotrophic growth, where carbon dioxide represents the sole carbon source, some cyanobacteria are also capable of photomixotrophic and heterotrophic growth by utilising exogenous organic carbon (Rippka et al., 1979; Stal & Moezelaar, 1997). It is known that the presence of exogenous glucose affects many physiological and biochemical parameters in Synechocystis, including growth and carbon metabolism (Yang et al., 2002; Knowles & Plaxton, 2003; Lee et al., 2005; Osanai et al., 2005; Singh & Sherman, 2005) and photosynthesis and respiration (Ryu et al., 2004, Lee et al., 2005; Kurian et al., 2006). In contrast to the numerous responses to glucose, the profiles of transcript abundance and protein levels remain almost unaltered in photoautotrophic and photomixotrophic cultures (Yang et al., 2002; Knowles & Plaxton, 2003; Tu et al., 2004; Singh & Sherman, 2005; Kahlon et al., 2006), indicating that the response to glucose is mainly accomplished on alterations at the post-translational level (Kahlon et al., 2006). However, the mechanism by which cyanobacteria sense and respond to the presence of glucose in their environment is still unclear. So far, only a small number of corresponding regulators are known in cyanobacteria. In Synechocystis, several sensors have been identified that are involved in glucose-induced signalling of several glycolytic and OPPP genes under photoautotrophic, photomixotrophic and heterotrophic growth conditions (Lee et al., 2007). Among these sensors are histidine kinases, the sensory components of the so-called two component signalling systems, which are described in detail in Section 1.4. The histidine kinase 8 (HIK8) has been reported to play an important role in glucose metabolism (Singh & Sherman, 2005). HIK8 has significant protein sequence similarity to SASA from Synechococcus sp. strain PCC 7942 (Synechococcus adaptive sensor A), a histidine kinase that interacts with KAIC (Iwasaki et al., 2000), one of the three circadian clock proteins responsible for circadian control in Synechococcus (Golden & Canales, 2003). HIK8 was found to enable cells to grow under heterotrophic growth conditions, probably via transcriptional regulation of Gap1, which encodes one of the two glyceraldehyde-3-phosphate dehydrogenases, catalyzing the catabolic reaction (Singh & Sherman, 2005; Lee et al., 2007). Furthermore, it has been reported that the histidine kinase 31 (HIK31) is involved in the regulation of glucose catabolism in
Synechocystis, either via transcriptional regulation of the IcfG gene, which encodes a protein serine phosphatase (Shi et al., 1999) or by modulation of gluokinase activity (Kahlon et al., 2006). In addition to two-component signalling regulatory proteins, sigma factors represent another group of important regulators determining transcript profiles by directly interacting with the promoter sequence (Paget & Helmann, 2003; Lee et al., 2007). Among the nine sigma factors (SIG) existing in Synechocystis, SIGE has been reported to positively regulate the expression of sugar catabolic genes (Osanai et al., 2005, 2007; Lee et al., 2007).

### 1.3 Phytochrome signalling in plants and cyanobacteria

Light is an important environmental factor for all photosynthetic organisms. Not only does it provide the radiant energy for photosynthesis and hence for growth and metabolism, but also the sensory information for adaptive and developmental processes. Accordingly, photosynthetic organisms possess various photosensors, which enable them to perceive environmental light signals and to respond by regulating the gene expression through signal transduction pathways (Quail, 1994). One such group of photosensors is the red/far-red sensitive phytochrome family. Phytochromes were first discovered in higher plants in the 1950s (Butler et al., 1959), based on the ability to measure the ratio of red light (R) and far-red light (FR) to control plant morphogenesis (Quail, 2002; Smith, 2000). Although originally thought to be restricted to plants, similar genes have been found more recently in several cyanobacteria (Hughes et al., 1997; Yeh et al., 1997; Wu & Lagarias, 2000; Park et al., 2000), in bacteria (Jiang et al., 1999; Bhoo et al., 2001) and even in fungi (Montgomery & Lagarias, 2002; Lamparter et al., 2002; Karniol & Vierstra, 2003; Blumenstein et al., 2005).

Signalling mediated by most phytochromes involves absorption of R light (650-660 nm) and FR light (700-730 nm), which leads to two stable conformations Pr and Pfr, respectively (Figure 3 A) (Smith, 1994; Quail et al., 1995). In most phytochromes, the photoproduct is metastable and can slowly return to the thermally stable dark state in the so-called dark reversion process (Rockwell & Lagarias, 2010). Figure 3 B displays the absorption spectra of Pfr and Pr and their partial overlap. Light establishes a Pr/Pfr photoequilibrium, reflecting environmental light conditions. Changes in the ratio between Pfr and Pr lead to changes in the equilibrium, therefore making phytochromes useful as sensors of critical changes in light quality (Sharrock, 2008). The ability to absorb light is conferred to the phytochrome by a covalently attached tetrapyrrole (bilin) chromophore, which typically absorbs light at 665 nm
This bilin chromophore, produced by the oxidative cleavage of heme, differs slightly among various organisms (Rockwell et al., 2006), ranging from phycobiliprotein utilised by plant phytochromes to phycocyanobilins in cyanobacteria (Yeh et al., 1997; Hughes et al., 1997) and biliverdin, which is used by eubacterial and fungal phytochromes (Rockwell & Lagarias, 2010 and references therein). The chromophore is bound autocatalytically to a conserved cysteine residue within the cGMP phosphodiesterase/adenylcyclase/FhlA (GAF) domain of the photosensory unit of the apoprotein. Upon light absorption, the chromophore undergoes a conformational change comprising of a Z/E photoisomerization (Rockwell et al., 2009).

**Figure 3: Overview of phytochrome properties and domain structures.**

(A) Phytochromes exist in two photointerconvertible forms, Pr and Pfr (modified after Elich & Chory, 1997). (B) Absorption spectra of Pr and Pfr (modified after Nagatani, 2010; Anders et al., 2011; Savakis et al., 2012). The chromophores are covalently linked to the conserved cysteine residues (denoted C) at position 129 and 1022 in the GAF1 and GAF3 domain, respectively. **Abbreviations:** aa, amino acids; NTE, N-terminal extension domain; GAF, domain present in vertebrate cGMP-specific phosphodiesterases, in cyanobacterial adenylate cyclases and in the formate lyase transcription activator FhlA; PHY, phytochrome-associated domain; PAS, domain found in family members including the period clock (PER) protein, the aromatic hydrocarbon receptor nuclear translocator (ARNT) and the single minded (SIM) of Drosophila; HKRD, histidine kinase-related domain; HKD, histidine kinase domain; GGDEF and EAL, domains with conserved sequence motifs, Gly-Gly-Asp-Glu-Phe and Glu-Ala-Leu, respectively, both domains, which are known to be involved in turnover of the second messenger c-di-GMP.

The conformational change of the chromophor triggers a subsequent conformational change in the protein, which initiates a signal transduction cascade (Quail, 1997; Fankhauser, 2001). Thus, by interconverting between the two photochemically distinct forms, phytochromes act as Pr/Pfr-regulated photosensory switches in various controlling processes, ranging from phototaxis, re-setting of the circadian clock, chromatic adaptation and pigmentation in cyanobacteria, to seed germination,
photomorphogenesis, shade avoidance and flowering time in higher plants (reviewed by Ulijasz & Vierstra, 2011; Chen & Chory, 2011). Phytochromes are encoded by multigene families (Elich & Chory, 1997). In the model plant system Arabidopsis thaliana, the phytochrome family consists of five members, designated PHY A to E (Mathews & Sharrock, 1997), which fall into two classes: light-labile (PHY A) and light-stable (PHY B-E) phytochromes (Furuya, 1993). The first prokaryotic genes encoding phytochrome-like proteins have been detected in cyanobacteria (Kehoe & Grossman, 1996; Wilde et al., 1997). The chromosome of Synechocystis sp. PCC 6803 contains a number of open reading frames, exhibiting different degrees of similarity to plant genes encoding putative phytochrome-like proteins (Kaneko et al., 1996). These proteins contain one or several putative chromophore binding domains fused to various signal-transmitting domains, but the red/far red photoconversion of a covalently attached bilin chromophore, typical for plant phytochromes, has only been shown for two Synechocystis proteins: CPH1 (Hughes et al., 1997; Yeh et al., 1997; Hübschmann et al., 2001) and CPH2 (Wu & Lagarias, 2000; Park et al., 2000). The domain architecture of phytochromes (Figure 3 C) implies conserved sequences and domains that are either ubiquitous among phytochromes or conserved in different subfamilies (Rockwell et al., 2009). Plant phytochromes, the cyanobacterial phytochrome CPH1 and most bacterial phytochromes (BphPs) share a common architecture, consisting of an N-terminal photosensory region with three conserved PAS (PER/ARNT/SIM), GAF (cGMP phosphodiesterase adenylate cyclase/FhlA) and PHY (phytochrome-associated) domains and a C-terminal regulatory histidine kinase or histidine kinase-related domain (HKRD) (Quail, 1997). Histidine kinases are involved in signal transduction in prokaryotes and eukaryotes and are best studied as sensors of two-component signal transduction systems that are widely distributed among bacteria (Wilde et al., 2002). Light-regulated protein kinase activity was first reported for the cyanobacterial phytochrome CPH1 from Synechocystis (Yeh et al., 1997). CPH1 was shown to be a true phytochrome by analysing recombinant CPH1 apoprotein from Escherichia coli (Hughes et al., 1997; Yeh et al., 1997; Lamparter et al., 1997), as well as the native holoprotein isolated from Synechocystis (Hübschmann et al., 2001). Once assembled with the chromophore, CPH1 was shown to undergo classic red/far-red reversibility in vitro (Hughes et al., 1997; Yeh et al., 1997). Moreover, CPH1 was found to autophosphorylate at a histidine kinase residue and to transfer the phosphate to the downstream response regulator RCP1 (Yeh et al., 1997). In earlier studies it has been proposed that plant phytochromes also act as light-regulated histidine kinases, based on limited sequence similarity of their C-termini to the histidine kinase domain present in
bacterial two-component histidine kinases (Schneider-Poetsch, 1992; Yeh et al., 1997; Karniol et al., 2005). However, the histidine kinase-related sequence of plant phytochromes lacks several important residues characteristic for two-component histidine kinases, indicating that plant phytochromes work by an alternative mechanism. More recently, plant phytochromes were shown to be light-regulated serine/threonine kinases (Yeh & Lagarias, 1998). In contrast to CPH1, the second cyanobacterial phytochrome CPH2 lacks a histidine kinase domain in the C-terminal module and has a different domain organisation (Figure 3 C). In addition to the two N-terminal GAF domains (GAF1 and GAF2), which form the Pr/Pfr-reversible photosensory module, CPH2 possesses a third C-terminal GAF domain (GAF3). GAF1 and GAF3 contain the bilin-binding sites, two conserved cysteine residues at position 129 and 1022 (cysteine-129 and cysteine-1022) (Park et al., 2000), which have been shown capable of ligating bilin chromophores autocatalytically (Wu & Lagarias, 2000; Anders et al., 2011). Spectroscopic analyses of bilin attachment to the recombinant N-terminal GAF domain showed the typical red/far-red reversibility, while the phycocyanobilin adduct of the recombinant C-terminal part absorbed blue light stronger than red light (Wu & Lagarias, 2000; Moon et al., 2011). Furthermore, GAF3 shows homology to the cyanobacteriochromes (Ikeuchi & Ishizuka, 2008), phytochrome-like proteins that are capable to photointerconvert between green and red (Terauchi et al., 2004; Narikawa et al., 2008; Hirose et al., 2008, 2010), blue and green (Yoshihara et al., 2004; Ishizuka et al., 2006, Rockwell et al., 2008), UV and blue, or violet and orange (Rockwell et al., 2011) absorbing states. This led to the assumption that CPH2 additionally acts as a blue light receptor (Wilde et al., 2002; Fiedler et al., 2005), which was recently confirmed by Savakis and co-workers (2012). They demonstrated that CPH2 undergoes photoconversion between blue and green light absorbing photostates, like other cyanobacteriochromes, e.g. TePixJ (Ishizuka et al., 2006, 2007, 2011; Ulijasz et al., 2009) and Tlr0924 (Rockwell et al., 2009), thereby utilising the same photochemistry including the double tethering of the bilin chromophore by two cysteines, cysteine-994 and cysteine-1022 (Savakis et al., 2012). Thus, based on its molecular architecture, CPH2 can be considered as a hybrid photoreceptor that comprises an N-terminal Pr/Pfr photosensory module and a C-terminal blue/green light-absorbing cyanobacteriochrome module (Savakis et al., 2012).

Following the two N-terminal GAF domains, CPH2 possesses GGDEF and EAL domains. GGDEF and EAL domains are known to be involved in regulating intracellular levels of bis-(3'–5')-cyclic dimeric guanosine monophosphate (c-di-GMP),
a second messenger that is known to regulate motility and biofilm formation in bacteria (Römling et al., 2005; Jenal & Malone, 2006; Cotter & Stibitz, 2007; Yan & Chen, 2010). The cellular c-di-GMP concentration is regulated by GGDEF domains acting as c-di-GMP cyclases to increase the cellular c-di-GMP concentration and by EAL domains, acting as phosphodiesterases to decrease the c-di-GMP content (Yan & Chen, 2010; Anders et al., 2011). Increasing concentrations of c-di-GMP promote the production of extracellular polysaccharides, the matrix that maintains the structural integrity of biofilms, and result in multicellular behaviour and biofilm formation, while low c-di-GMP concentrations promote motility (reviewed by Jenal & Malone, 2006). Furthermore, c-di-GMP also regulates biofilm formation through other multiple factors/signalling pathways, such as quorum sensing, chemotaxis and twitching motility (Tamayo et al., 2007; Yan & Chen, 2010). In Synechocystis, the cells display a flagellar-independent twitching or gliding motility that allows bacteria to move over moist surfaces using type IV pili (Bhya et al., 1999; Fiedler et al., 2005). Interestingly, it has been reported on the photomovement that CPH2 controls phototaxis by inhibiting positive phototaxis towards blue light (Wilde et al., 2002; Fiedler et al., 2005; Moon et al., 2011).

1.4 Histidine kinase signalling

Acclimation to fluctuations in environmental conditions is critically important for the survival of all living organisms. Adaptational processes require the ability to perceive changes in the physical and/or chemical environment in order to adapt by regulating the gene expression and protein synthesis appropriately. In particular unicellular organisms are routinely challenged by changes in their extracellular environment, which makes adaptive responses to altered conditions indispensable for successful competition and survival. The ability to mediate these adaptive responses is conferred by sophisticated signal transduction systems, which represent a fundamental strategy in information processing in both prokaryotes and eukaryotes (reviewed by Schaller et al., 2011). Signal-induced protein phosphorylation is a common regulatory mechanism to transduce intracellular or extracellular signals in both prokaryotes and eukaryotes (Kakimoto, 2003; Schaller et al., 2008). In eukaryotes, regulatory protein phosphorylation predominantly occurs at serine, threonine and tyrosine residues (Hunter, 1995; Hunter & Plowman, 1997; Plowman et al., 1999), while many signal transduction pathways in bacteria use the so-called two-component systems that rely upon phosphorylation of histidine and aspartic residues (Mizuno, 1997; Schaller et al., 2008). However, both phosphorylation
schemes (His→Asp and Ser/Thr/Tyr) can function in prokaryotes and eukaryotes, as His→Asp phosphotransfer systems have been found in several eukaryotic organisms (Wurgler-Murphy & Saito, 1997; Chang & Stewart, 1998; Loomis et al., 1997, 1998) and Ser/Thr and Tyr kinases and phosphatases have been identified in bacteria (Zhang, 1996). Notably, plants also make use of two-component systems, which are involved in important cellular processes, such as the responses to cytokinin, ethylene, red light and osmosensing (Mizuno, 2005, Schaller et al., 2008; Tsai et al., 2012).

The core of two-component signalling pathways is a phosphotransfer reaction between two conserved components, a histidine kinase (Hik) and a cognate response regulator (RR) (Stock et al., 1995; 2000; Gao & Stock, 2009; Bourret & Silversmith, 2010; Jung et al., 2012). The Hik is typically the input component of the pathway, which senses stimuli and correspondingly regulates the signalling pathway, while the RR represents the output component of the system, which is regulated by the Hik and finally affects the cellular response (Gao & Stock, 2009). Structurally, Hiks are generally homodimeric integral membrane proteins with a N-terminal signal-sensing (input) domain, which is located in the extracellular space and a C-terminal cytoplasmatic signal-transducing (transmitter) domain (Stock, 1999; Kakimoto, 2003). Signalling is initiated upon ligand binding to the histidine kinase sensory component. Most frequently, the signal is sensed by an extracellular loop formed between two membrane-spanning regions (Gao & Stock, 2009). However, depending on the corresponding signal, the sensor domains of some Hiks can also be located within the membrane or completely within the cytoplasm (Gao & Stock, 2009). Upon ligand binding, the C-terminal kinase domain is activated, which is followed by autophosphorylation at a specific histidine residue (the H-box) using ATP as phosphate donor (Stock & Da Re, 2000). In prototypical Hiks, the cytoplasmatic kinase core consists of two distinct domains: a well-conserved C-terminal catalytic and ATP-binding domain and a less-conserved dimerization and histidine phosphotransfer domain (Gao & Stock, 2009). Several characteristic sequence motifs are conserved across the kinase core domains in both eukaryotic and prokaryotic Hiks (Stock et al., 1989; Parkinson & Kofoid, 1992; Alex & Simon, 1994). On the basis of specific sequences, they are referred to as H, N, G1, F and G2 boxes, with the H-box containing the phosphorylation site in the dimerization domain and the nucleotide binding pocket in N, G1, F and G2 boxes, located in the catalytic domain (Gao & Stock, 2009). After autophosphorylation of the Hik at its His residue, a high-energy phosphoryl group is created that is transferred from the Hik to an
aspartate residue in the conserved receiver domain of the cognate RR. This phosphorylation event leads to a conformational change that activates an attached output domain of the RR, triggering the cellular response. Response regulatory proteins possess various outputs, which include transcriptional, post-transcriptional, post-translational controls and protein-protein interactions (Perry et al., 2011). Most frequently, output domains are DNA-binding domains so that phosphorylation of the RR is coupled directly to changes in transcription (Laub & Goulian, 2007). However, many other output domains have enzymatic activities, for example the regulation of the c-di-GMP concentration (Hengge, 2009), while other RRs even lack a distinct output domain, concomitant with their regulatory function residing within the receiver domain (Ulrich & Zhulin, 2009). One of those RRs is the chemotaxis protein CheY, which controls the rotational direction of the bacterial flagellar motor (Wadhams & Armitage, 2004).

Besides the typical two-component pathway, involving a single HK and a single RR that participate in an onestep His→Asp phosphotransfer, more complex variants exist (reviewed by Jung et al., 2012). These so-called multistep phosphorelays involve multiple His-containing and Asp-containing domains and a multistep His→Asp→His→Asp phosphotransfer (Appleby et al., 1996; Goulian, 2010; Wuichet et al., 2010). In many cases, Hiks are bifunctional and can catalyze both the phosphorylation and dephosphorylation of their cognate RR (Keener & Kustu, 1988; Aiba et al., 1989; Lois et al., 1993). For bifunctional Hiks, input stimuli can regulate either the kinase or phosphatase activity (Laub & Goulian, 2007). These phosphorelays are usually initiated by a hybrid histidine kinase that autophosphorylates and transfers its phosphoryl group intramolecularly to an RR-like receiver domain. The phosphoryl group is then transferred to a signalling intermediate, a histidine phosphotransfer (HP) protein. HP domains can often be found fused to hybrid histidine kinases or as independent proteins that facilitate the phosphotransfer process (Appleby et al., 1996; Stock et al., 2000). Due to their ability to both receive and transmit phosphoryl groups, HP proteins subsequently transfer them to the receiver domains of a terminal RR, which can induce the corresponding response (Kato et al., 1997; Laub & Goulian, 2007). Phosphotransfer pathways are the most common pathway architecture in prokaryotes, while phosphorelays, which provide a greater number of steps for regulation, are predominant in eukaryotes (Robinson et al., 2000).
1.4.1 Histidine kinase signalling in cyanobacteria

The cyanobacterium *Synechocystis* sp. PCC 6803 contains a total of 47 genes for Hiks and 45 genes for RR on its chromosome and plasmids (Kaneko *et al.*, 1996; Mizuno *et al.*, 1996; Kaneko *et al.*, 2003; Los *et al.*, 2008). There are 44 genes for Hiks on the chromosome, two genes on the plasmid pSYSX and one gene on the plasmid pSYSM (Murata & Suzuki, 2006). The 44 genes for Hiks on the chromosome have been named Hik1 through Hik44. Among them, the three putative Hiks, HIK11, HIK17 and HIK37 are supposed to be inactive as histidine kinases because they lack the conserved histidine residue in the Hik domain (Murata & Suzuki, 2006). The genes for the Hiks on the plasmids have been designated Hik45-Hik47. Likewise, the 42 genes for RRs on the chromosome have been designated Rr1 through Rr42, while Rr43-Rr45 are located on the plasmids.

In *Synechocystis*, all putative Hik genes have been inactivated by insertion of a spectinomycine-resistance gene cassette in order to create a gene-knockout library (Suzuki *et al.*, 2000). The characterisation of these knockout mutants led to the identification of histidine kinases involved in sensing environmental stimuli, including high light, cold and oxidative stress (HIK33), heat (HIK34), salt stress and hyperosmotic stress (HIK33, HIK34, HIK2, HIK41, HIK16 and HIK10), and phosphate limitation (HIK7) (Hirani *et al.*, 2001; Mikami *et al.*, 2002; Hsiao *et al.*, 2004; Paithoonrangsarid *et al.*, 2004; Shoumskaya *et al.*, 2005; Suzuki *et al.*, 2005; Kanesaki *et al.*, 2007), indicating that a single histidine kinase may respond to more than one stimulus and that multiple histidine kinases may respond to any specific environmental cue (Summerfield *et al.*, 2011). One of these histidine kinases, HIK12, encodes a hybrid histidine kinase and a cognate RR (Cyanobase, 2012), which displays structural homologies to the *Arabidopsis* cytokinin receptors AHK2, AHK3 and AHK4, thereby suggesting a putative role for HIK12 as cyanobacterial cytokinin receptor. In addition to the structural homologies, HIK12 possesses a threonine residue within its MASE (membrane associated sensor) domain, which is conserved in all three *Arabidopsis* cytokinin receptors and was found to be one of the five amino acid positions within the ligand binding domain of AHK4, relevant for cytokinin binding (Heyl *et al.*, 2007). First evidence of cyanobacteria responding to cytokinin was observed by Selivankina and co-workers (2006) who demonstrated that the cytokinin trans-zeatin affects the transcriptional activity in *Synechocystis*, leading to the conclusion that cyanobacteria possess the molecular targets for cytokinin signal recognition. During the endosymbiotic event many cyanobacterial genes have been transferred to the plant nucleus (Wuichet *et al.*, 2010; Kieber & Schaller, 2010).
Accordingly, the cytokinin signalling system in plants might also be an inheritance of their cyanobacterial ancestors.

1.4.2 Cytokinin signalling in plants

In *Arabidopsis*, two-component elements are encoded by multigene families (Schaller et al., 2002; Heyl & Schmülling, 2003; Kakimoto, 2003; Ferreira & Kieber, 2005; Maxwell & Kieber, 2005), which fall into three major clades, phytochromes, ethylene receptors and cytokinin receptors (Schaller et al., 2008). The cytokinin receptor family in *Arabidopsis* consists of three histidine kinases, AHK2, AHK3 and AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). AHK4, which is also known as WOL1 or CRE1, was originally identified in two independent screens for *woodenleg* (*wol*) mutants with short roots in *Arabidopsis* (Mähönen et al., 2000) and for *cytokinin response* (*cre*) mutants that were defective in cytokinin induction of shoots in tissue culture (Inoue et al., 2001). Different studies have shown that AHK4 and its paralogs AHK2 and AHK3 act as cytokinin sensors and are capable of mediating cytokinin-dependent histidine kinase activity in heterologous bacterial and yeast systems (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001).

The cytokinin receptors AHK2, AHK3 and AHK4 are hybrid Hiks, displaying a common primary structure consisting of a cyclase/histidine kinase associated sensory extracellular (CHASE) domain, predicted to be extracytosolic, which binds cytokinin (Anantharaman & Aravind, 2001; Heyl et al., 2007) and two flanking N-terminal transmembrane domains. The CHASE domain is followed towards the C-terminus by a Hik and an RR domain in the predicted cytoplasmic part (Heyl & Schmülling, 2003). Functional evidence for cytokinin binding to the CHASE domain was obtained in *in vivo* binding assays (Romanov et al., 2005; Heyl et al., 2007; Wulfetange et al., 2011b). In addition, Heyl and co-workers (2007) identified four amino acid positions within the CHASE domain of AHK4, relevant for cytokinin binding. It was proposed that plants might have acquired the CHASE domain via lateral transfer through their chloroplasts, which have a cyanobacterial ancestry (Anantharaman & Aravind, 2001; Mougel & Zhulin, 2001).

Cytokinin binding to the CHASE domain triggers autophosphorylation of the Hik at a conserved His residue. The phosphoryl group is subsequently transferred from the Hik to a conserved aspartate (Asp) residue in the receptor’s receiver domain. The C-terminal receiver domains of all three receptors interact redundantly with histidine phosphotransfer proteins (AHPs), which transmit the signal to the nucleus (Hwang &
Sheen, 2001; Punwani et al., 2010), where the phosphoryl group is transferred to a conserved Asp residue in the receiver domain of an RR protein (Müller & Sheen, 2007), resulting in the activation of the RR protein. In Arabidopsis, there are two types of RRs: type-A and type-B ARRs. Type-B ARRs directly regulate gene expression, including the transcriptional induction of type-A response regulator genes (Mason et al., 2005; Heyl & Schmülling, 2006; Argyros et al., 2008; Ishida et al., 2008). Type-A ARRs mediate downstream responses to cytokinin and function as negative feedback regulators of the initial cytokinin signalling (Sakai et al., 2001; Hwang et al., 2002; Heyl & Schmülling, 2003; To et al., 2004; Ferreira & Kieber, 2005; Hirose et al., 2007; To & Kieber, 2008; Cheng et al., 2010).

Until recently, models of cytokinin signalling predicted a localization of cytokinin receptors to the plasma membrane (Heyl & Schmülling, 2003; Hwang & Sakakibara, 2006; Müller & Sheen, 2007; To & Kieber, 2008; Pils & Heyl, 2009), based on bioinformatic analysis of the protein sequence and analogy with sensor His kinase localization in bacteria and yeast (Inoue et al., 2001; Ueguchi et al., 2001). Furthermore, the localization to the plasma membrane was experimentally supported by the localization of overexpressed AHK3-GFP fusion proteins to the plasma membrane of Arabidopsis protoplasts (Kim et al., 2006). However, recent findings revealed that cytokinin receptors in monocots and dicots are predominantly located to the endoplasmatic reticulum (ER; Wulfetange et al., 2011a; Caesar et al., 2011; Lomin et al., 2011) and not as previously assumed to the plasma membrane. The novel predominant ER localization makes it necessary to revise the concept of cytokinin perception and signalling. According to these findings, the ligand-binding CHASE domain is not oriented to the apoplast as previously assumed, but exposed to the ER lumen, while the C-terminal his kinase domain is exposed to the cytoplasm. Cytokinin receptors located on the ER membrane could transmit the cytokinin signal via AHP to the nucleus, similar as it was proposed for signal transmission from the plasma membrane (Schaller et al., 1995; Hwang & Sheen, 2001; Heyl & Schmülling, 2003; Kakimoto, 2003; Bishopp et al., 2006). This topology of the receptors is further supported by biochemical studies, revealing an optimal cytokinin binding at pH values of ~6.5 (Romanov et al., 2006), which corresponds to conditions found in the ER lumen (Kim et al., 1998). At pH values of ~5.5, as it has been reported for the apoplast (Li et al., 2005), cytokinin binding activity is almost abolished (Caesar et al., 2011). Cytokinin receptor localization in the ER offers new opportunities for cross talk, as numerous other plant receptor proteins, including the structurally related ethylene receptors (Chen et al., 2002), auxin signalling-related
proteins and specific metabolic enzymes, such as cytokinin oxidases are also located at the ER (Werner et al., 2003; Geldner & Robatzek, 2008; Irani & Russinova, 2009; Friml & Jones, 2010; Wulfetange et al., 2011a).

In *Arabidopsis*, the genes encoding the cytokinin receptors are expressed in almost all tissues, although with different abundance (Riefler et al., 2006; reviewed by Shi & Rashotte, 2012). AHK4 is predominantly expressed in the root, while AHK2 and especially AHK3 transcripts are more abundant in the aerial parts of *Arabidopsis* plants (Mähönen et al., 2000; Ueguchi et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Stolz et al., 2011). Promoter:β-glucuronidase (GUS) fusions revealed that the receptor genes have overlapping, but partly distinct expression domains and are transcribed in almost all cells in the different organs although with different strength (Higuchi et al., 2004; Nishimura et al., 2004; Mähönen et al., 2006). Analyses of T-DNA insertional mutations in AHK2, AHK3 and AHK4 have shown that there is a considerable degree of redundancy of these three receptors (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Stolz et al., 2011; Shi & Rashotte, 2012). Loss-of-function mutation of single receptors displayed no or only minor effects on most of the phenotypes studied, while double *ahk2ahk3* mutants show marked developmental impairments and triple *ahk2,3,4* mutants displayed near complete insensitivity to cytokinins and severe developmental defects, indicating a key regulatory role of cytokinins in plant growth and development (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; To et al., 2007; Kieber & Schaller, 2010; Stolz et al., 2011). However, individual receptors were shown to possess distinct functions and to mediate specific cytokinin activities. These functions include a role for AHK4 in regulating the sensitivity to cytokinin in root elongation and a major role for AHK3 (together with AHK2) in regulating leaf senescence (Inoue et al., 2001; Riefler et al., 2006; Kim et al., 2006; Werner & Schmülling, 2009; Müller et al., 2011). Furthermore, AHK3 appears to be the main regulator of cell differentiation in the transition zone of the root meristem (Dello ioio et al., 2007), while AHK4 regulates embryonic root patterning (Mähönen et al., 2000; Müller & Sheen, 2007), the phosphate starvation response (Franco-Zorrilla et al., 2002, 2005) and sulphate assimilation (Maruyama-Nakashita et al., 2004). For AHK2, no specific function has been assigned so far, although AHK2 alone was shown to be sufficient to maintain normal plant growth under standard growth conditions (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006).
1.5 Cytokinin function in plants

Cytokinins are a class of plant hormones that have been discovered more than 50 years ago, based on their ability to strongly stimulate growth and cell division in tobacco tissue culture (Miller et al., 1955; Amasino, 1955). Cytokinins are involved in numerous developmental and physiological processes, including cell division, shoot meristem initiation, leaf and root differentiation, chloroplast biogenesis, stress tolerance, delay of senescence and also in pathogen resistance (Mok & Mok, 2001; Hwang et al., 2002; Heyl & Schmülling, 2003; Mizuno, 2004; Ferreira & Kieber, 2005; Walters & McRoberts, 2006; Argueso et al., 2009; Choi et al., 2010; Großkinsky et al., 2011). Cytokinins control essential developmental processes in plants by interacting with other plant hormones, including salicylic acid, auxin, gibberellic acid, brassinosteroids, ethylene and abscisic acid (Bishopp et al., 2006; Bari & Jones, 2009; Argueso et al., 2010). These interactions include the reciprocal control of hormone biosynthesis, the expression of components in other signal transduction pathways and cross talk of signal transduction intermediates directly downstream of the receptors (reviewed by Bishopp et al., 2006). Although considerable progress has been made, the understanding of this complex signalling network is still at an early stage.

1.5.1 Cytokinin occurrence and homeostasis

Cytokinins consist of a large array of natural adenine derivatives, which can either be produced by plants and some bacteria through specific enzymatic pathways and degradation of nucleic acids, or can be artificially generated by chemical synthesis (Mok & Mok, 2001; Sakakibara, 2006; Miyawaki et al., 2006). Naturally occurring cytokinins are either isoprenoid or aromatic cytokinins, depending on the configuration of their $N^6$-side chain (Mok & Mok, 2001). Isoprenoid cytokinins occur more often and in greater abundance in plants than aromatic cytokinins (Sakakibara, 2006). Common natural isoprenoid cytokinins are $N^6$-($\Delta^2$-isopentenyl)-adenine (iP), trans-zeatin, cis-zeatin and dihydrozeatin. The major derivatives are usually trans-zeatin, iP and their sugar conjugates, whereas their distinct abundancy varies depending on plant species, tissue, and developmental stage (Sakakibara, 2006). Cytokinin homeostasis in cells is regulated by the rate of de novo synthesis, import, synthesis and breakdown of cytokinin conjugates and finally, the rate of export and breakdown (Mok & Mok, 2001; Schmülling et al., 2003). Key enzymes for cytokinin homeostasis are adenosine phosphate-isopentenyl-transferases (IPT) and cytokinin oxidase/dehydrogenases (CKX), which are involved in cytokinin synthesis and
degradation, respectively (Sakakibara, 2006; Kamada-Nobusada & Sakakibara, 2009; Frébert et al., 2011). In Arabidopsis, both gene families encompass seven members (AtIPT1, AtIPT3-8 and AtCKX1-7) (Takei et al., 2001; Werner et al., 2003; Schmülling et al., 2003; Miyawaki et al., 2006; Kamada-Nobusada & Sakakibara, 2009). Both display differential expression patterns during plant development with highest activities in regions of active growth (Werner et al., 2003).

1.5.2 Cytokinin function in defense reactions

A further function of cytokinin has been suggested that directly links cytokinin effects and plant responses to biotrophic pathogens, due to a striking similarity between several well-established cytokinin functions and changes in host physiology during plant-pathogen interactions (Walters & McRoberts, 2006; Walters et al., 2008). Biotrophic fungal or bacterial pathogens utilise cytokinins to form so-called green islands, sites of green living tissues surrounding the sites of active pathogen growth, which leads to delayed senescence and enhancement of sink activity (Walters & McRoberts, 2006; Walters et al., 2008; Argueso et al., 2009; Choi et al., 2011). It has been assumed that the cytokinin-mediated growth response suppresses plant basal defense mechanisms (Robert-Seilanianzt et al., 2007). However, recent research revealed that plant-originated cytokinins increase plant immunity together with salicylic acid (SA) (Choi et al., 2010). SA is known to play a crucial role in plant defense and is generally involved in the activation of defense responses, such as the induction of pathogenesis-related (PR) genes and in the establishment of systemic acquired resistance (SAR; Grant & Lamb, 2006; Bari & Jones, 2009, Choi et al., 2011). Interplay between SA and cytokinin in plant immunity has been suggested based on phenotypic and genetic data, however, the underlying molecular mechanism remains elusive. In Arabidopsis, the cytokinin receptors and in particular AHK3, are able to recognise both plant- and pathogen-derived cytokinins, but elicit different outputs, either the activation of a defense response or the development of pathogenic symptoms (Pertry et al., 2009; Choi et al., 2011). Together, cytokinins are involved in a variety of plant developmental and physiological processes. In particular, cytokinins have emerged as major factors in plant-pathogen interactions, where they play an essential role in maintaining plant viability by simultaneously enhancing cell division activity and resistance to biotic and abiotic stresses (Choi et al., 2011). Recent findings on the molecular mechanisms underlying the cytokinin-mediated signal transduction network contribute to the understanding of the cytokinin function in plant defense responses. Nevertheless, a thorough understanding of cytokinin receptor function is still at an early stage.
1.6 Objectives

The ability to perceive environmental changes and to respond by adapting appropriately is critically important for the survival and successful competition of all living organisms. Although considerable effort has been made in the past years to elucidate signal transduction processes and the underlying molecular mechanisms, a thorough understanding remains elusive. In the first part of the present study, a cyanobacterial phytochrome, CPH2, and a cyanobacterial histidine kinase of yet unknown function, HIK12, were analyzed with a special focus on their function in the regulation of the central carbon metabolism. For this purpose, knockout and overexpression mutants were used to analyse the function of the respective proteins in the cyanobacterial model organism *Synechocystis* sp. PCC 6803. The mutants were analysed under distinct growth conditions, with particular interest in the transition from exponential growth to a sessile lifestyle in biomats. During the endosymbiotic event, many cyanobacterial genes have been transferred to the plant nucleus. In particular, genes coding for proteins involved in signal transduction are highly conserved among cyanobacteria and plants, e.g. the cyanobacterial histidine kinase HIK12, which shows sequence homologies to the cytokinin receptor family in *Arabidopsis*. Therefore, the goal of this study was to contribute to the understanding of signalling mechanisms in cyanobacteria and to gain new ideas of potentially similar signalling processes in plants.

Besides being involved in essential developmental and physiological processes, cytokinins have recently emerged as important factors in plant-pathogen interactions. The second part of this study focused on the function of cytokinin in regulating plant susceptibility or basal resistance during the interaction with biotrophic/hemibiotrophic pathogens. For this purpose, transiently transformed barley leaves, overexpressing cytokinin-degrading enzymes, were analysed during the interaction with the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Furthermore, a T-DNA insertion line of the *Arabidopsis* cytokinin receptor AHK3 was analysed during the interaction with the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato*. 
2 Materials and methods

2.1 Synechocystis sp. PCC 6803

Synechocystis sp. PCC 6803 strains
All experiments were performed with the unicellular, photoautotrophic, facultative
photoheterotrophic freshwater cyanobacterium Synechocystis sp. PCC 6803 (order
Chroococcales). For our analyses, two independent cph2 knockout lines (cph2-1 and
cph2-2 KO; locus sll0821) and the corresponding glucose-tolerant, parental wildtype
strain were provided by Dr. Young Mok Park (Korea Basic Science Institute,
Daejeon, South Korea). Hik12 (locus sll1672) knockout line (hik12 KO) and the
corresponding wildtype were obtained from Dr. Iwane Suzuki (Graduate School of
Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan). Both
knockout lines (cph2 KO and hik12 KO) were generated by inserting a
spectinomycin resistance cassette into the coding regions of cph2 and hik12,
thereby disrupting the functionality of the corresponding gene (Moon et al., 2011;
Suzuki et al., 2000). In this study, hik12 and cph2 overexpression lines (hik12 OE
and cph2 OE) were generated in the hik12 and cph2-2 background for functional
complementation of the knockout lines with a full-length wildtype coding sequence
(hik12 and cph2, respectively) under the control of the constitutive petJ promoter.
Furthermore, a second independent knockout line of hik12 (hik12 KO_2) was
generated in this study. The parental wildtype strain used for transformation with
hik12 knockout_2 construct was provided by Kay Marin (University of Cologne,
Cologne, Germany).

Cultivation of Synechocystis sp. PCC 6803
Synechocystis cells were cultivated in BG-11 (Blue-Green) medium, prepared as
described in Stanier et al., (1971).

BG-11 medium (10x) | NaNO₃ | 15 g
| K₂HPO₄ | 0.4 g
| MgSO₄·7H₂O | 0.75 g
| CaCl₂·2H₂O | 0.36 g
| Citric acid | 0.06 g
| Ferric ammonium citrate (III+) | 0.06 g
| EDTA Na₂ | 0.01 g
BG-11 medium was prepared in ten-fold concentration, autoclaved at 121°C for 12 min and stored at 4°C. Before use, the ten-fold medium was diluted 1:10 with H₂O.dest. and autoclaved. For solid medium BG-11 was supplemented with 1.5% (w/v) agar, 0.3% sodium thiosulfate (Na₂S₂O₃*5 H₂O) and where appropriate, filter-sterilized antibiotics were added. For cultivation of cph2 and hik12 knockout lines BG-11 medium was supplemented with spectinomycine (Sigma-Aldrich, Hannover, Germany), overexpression lines were cultivated in BG-11 medium containing spectinomycine and chloramphenicol (Merck, Darmstadt, Germany). The second independent knockout line of hik12 was cultivated in BG-11 containing kanamycine (Duchefa, Haarlem, Netherlands). All antibiotics (in liquid and solid medium) were used at a final concentration of 10 µg/ml.

For cultivation and maintenance, the cells were grown under constant light irradiance (NARVA LT 36W/760-010 daylight, Brand-Erbisdorf, Germany) with a light intensity of 20 µmol photons m⁻² s⁻¹ and streaked out at regular 3-week intervals on BG-11 agar plates, supplemented with the appropriate antibiotics. For cultivation in liquid cultures, pre-cultures were inoculated with a sterile inoculation loop directly from the specific cultures, grown on BG-11 agar plates. The cultures were supplemented with 5 mM TES-NaOH (N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid) buffer (pH 8) to maintain a stable pH value. Appropriate antibiotics were added to the pre-cultures of the mutant lines, while the experiments were conducted in BG-11 medium without antibiotics. For acclimatization, cell cultures were grown photoautotrophically on a rotary shaker (Rack-Shaker, A. Kühner GmbH, Birsfelden, Switzerland) at 135 rpm, 28°C and under constant white light (L13W/840 ACTIVE daywhite, Osram, Augsburg, Germany). After 3 to 4 days of acclimatization, the cultures were trans-
ferred to a shaker (Certomat®, Sartorius Stedim Biotech, Aubagne Cedex, France) and grown photoautotrophically at 140 rpm, 28°C and under constant light irradiance (Gro-Lux, F-36W/GRO-T8, Havells Sylvanian Germany GmbH, Erlangen, Germany) of 20 µmol photons m⁻² s⁻¹, enriched with red spectrum.

**Determination of the optical density**

Growth of the cell cultures was monitored by measuring the optical density at a wavelength of 750 nm (OD₇₅₀) in a spectrophotometer (Amersham Pharmacia ULTROSPEC III spectrophotometer, Hertfordshire, GB). H₂O₉ad was used as blank.

**Enduring culture stocks**

5 ml of *Synechocystis* cell culture in mid-exponential growth phase were centrifuged (3 min, RT, 2000 rpm, Rotor A-4-62, Eppendorf 5810 R). After resuspension of the pellet in 10% dimethyl sulfoxide (w/v) (DMSO, in BG-11), the stocks were stored at -20°C.

### 2.1.1 General molecularbiological and cloning methods

**Polymerase Chain Reaction (PCR)**

Standard PCRs and colony PCRs were performed with SupraTherm™ Taq DNA polymerase (Genecraft, Münster, Germany) in a thermocycler (Biometra TPersonal or TProfessional Basic Gradient, Biometra GmbH, Göttingen, Germany). For colony PCRs single colonies of *E. coli* or *Synechocystis* were used as template. For the amplification of coding regions, subsequently used for cloning, PCRs were performed with Phusion® proof-reading Taq-polymerase (Finnzymes, New England Biolabs, Ipswich, UK).

**Table 1: Standard PCR reaction mix and standard PCR program.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Temp.</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10x)</td>
<td>2.5 µl</td>
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<tr>
<td>dNTPs (2 mM)</td>
<td>2.5 µl</td>
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<tr>
<td>Primer 1 (10 pmol)</td>
<td>1.5 µl</td>
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<tr>
<td>Primer 2 (10 pmol)</td>
<td>1.5 µl</td>
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</tr>
<tr>
<td>Template DNA</td>
<td>150-1000 ng</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq-DNA-Polymerase</td>
<td>0.2 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₂O₉ad</td>
<td>ad 25 µl</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Initial denaturation at 95°C for 5 min.

Denaturation at 95°C for 30 sec.

Annealing at x°C for 30 sec.

Elongation at 72°C for x min.

Final Elongation at 72°C for 5 min.

¹ Annealing temperature depends on the melting temperatures of the specific primers.

² Elongation time depends on the estimated fragment size.
**Agarose gel electrophoresis**

To separate DNA fragments, horizontal gel electrophoresis (Sambrook *et al.*, 1989) was used. Due to their intrinsic negative charge, DNA molecules can be separated according to their size by applying an electric field to move the charged molecules through an agarose matrix. 0.8-2% agarose (w/v) in 1x TBE buffer was heated in a microwave until the agarose was melted completely. Before polymerization, ethidium bromide (final concentration 0.5 µg/ml) was added. Ethidium bromide intercalates with double-strand DNA, allowing visualization of the DNA molecules under UV light. The samples, supplemented with DNA loading dye (6x Orange DNA loading dye, Fermentas, St. Leon-Rot, Germany), were loaded on the gel in a gel chamber with 1x TBE. GeneRuler™ 100 bp DNA ladder plus (Fermentas, St. Leon-Rot, Germany) was used as standard. Depending on the size of the DNA fragments and the gel, voltage application was set to 90-120 V for 40-60 min. For documentation DeVision G camera and software (Decon, Hohengandern, Germany) was used.

<table>
<thead>
<tr>
<th>TBE buffer (10x)</th>
<th>Tris</th>
<th>0.9 M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Boric acid</td>
<td>0.9 M</td>
</tr>
<tr>
<td></td>
<td>EDTA pH 6.8</td>
<td>0.025 M</td>
</tr>
<tr>
<td></td>
<td>ad H₂Odest</td>
<td>1 l</td>
</tr>
</tbody>
</table>

TBE buffer was autoclaved at 121°C for 20 min.

**Elution of DNA fragments from agarose gels**

QUIAquick® Gel Extraction Kit (Quiagen, Hilden, Germany) was used according to the manufacturer’s instructions to isolate and purify DNA fragments from excised agarose gel blocks.

**DNA restriction**

For cleaving of double-stranded DNA by restriction endonucleases, restriction enzymes and corresponding buffers from Fermentas (St. Leon-Rot, Germany) were used according to the manufacturer’s instructions. To prevent recircularization during ligation, an alkaline phosphatase (CIAP, *calf intestine alkaline phosphatase*, Fermentas, St. Leon-Rot, Germany) was used to dephosphorylate vector DNA, according to the manufacturer’s instructions.
Ligation

Single DNA fragments can be ligated by linking terminal 3' OH groups with 5' phosphate groups in an ATP-dependent reaction via T4-DNA polymerase. Complement ends as well as blunt ends can be ligated. DNA and linearized vector fragments were mixed in a molar ratio of 3:1. The reaction was carried out in a total volume of 10 µl, containing 1 µl of 10 x ligase buffer, 1-2 U T4-DNA ligase (Fermentas, St. Leon-Rot, Germany). After incubation for 2 h at RT for sticky-end ligation or overnight at 4°C for blunt-end ligation, the ligation mix was used for transformation of competent *E. coli* cells.

*Escherichia coli* - cultivation, transformation and plasmid isolation

*Escherichia coli* (*E. coli*) bacteria cells grown in Luria broth (LB) medium were used for plasmid construction and replication. *E. coli* were either cultivated in overnight cultures in liquid LB medium at 230 rpm and 37°C or on solid LB agar plates at 37°C. When required, filter-sterilized antibiotics, ampicilline (Roth, Karlsruhe, Germany; at a final concentration of 100 µg/ml) or kanamycine (Duchefa, Haarlem, Netherlands; at a final concentration of 50 µg/ml) were added.

**LB medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar (for plates only)</td>
<td>15 g</td>
</tr>
<tr>
<td>ad H₂O₇dest and autoclave</td>
<td>1 l</td>
</tr>
</tbody>
</table>

LB medium was autoclaved at 121°C for 20 min. Antibiotics were added after autoclaving.

For transformation of *E. coli*, the strains K12 Dh5α, provided by Clontech (Heidelberg, Germany) and the more efficient *E. coli* strain XL1-blue, purchased from Stratagene (Amsterdam, Netherlands) were used. CaCl₂ competent *E. coli* cells were prepared as described previously (Sambrook *et al.*, 1989). For transformation, 80-100 µl of competent *E. coli* cells were supplemented with the ligation mix and incubated for 30 min on ice. After a heat shock at 42°C for 90 sec, the cells were supplemented with 0.8 ml LB medium and incubated at 37°C and 230 rpm for 1 h. After brief centrifugation, the supernatant was discarded, cells were resuspended in the reflux and plated out on LB agar plates, containing appropriate antibiotics. For blue/white selection LB agar plates were supplemented with 40 µl X-Gal (20 µg/ml in DMF) and 4 µl IPTG (20 µg/ml in H₂O.bidest). The plates were incubated over night at
37°C. Colonies were picked and cells were grown in LB medium, supplemented with the appropriate antibiotics at 130 rpm, 37°C over night. The isolation of plasmid DNA was achieved by using the NucleoSpin® Plasmid-Kit and the NucleoBond® Xtra Midi Kit from Macherey-Nagel (Düren, Germany) according to the manufacturer’s instructions.

**Isolation of genomic DNA from *Synechocystis* sp. PCC 6803**

For genomic DNA isolation 15 ml of mid-exponential cell cultures (OD$_{750}$ = 0.5-0.8) were harvested by centrifugation (10 min, 4°C, 4000 rpm, Rotor A-4-62, Eppendorf 5810 R). The pellet was resuspended in 200 µl TEN buffer (10 mM Tris/HCL, pH 7.6, 150 mM NaCl, 10 mM EDTA). After addition of 10 µl lysozyme (20 mg/ml; SERVA Electrophoresis GmbH, Heidelberg, Germany), the samples were mixed and incubated for 15 min at 37°C. 5 µl of 10% sodium dodecyl sulfate (SDS) was added and mixed by inverting the tube. After addition of 5 µl protease K (20 mg/ml; Fermentas, St. Leon-Rot, Germany), the samples were incubated for 1 h at 60°C. 200 µl TEN buffer and 400 µl phenol (pH 8; Sigma-Aldrich, Hannover, Germany) were added and mixed by inverting continuously for 5 min. Phase separation was carried out by centrifugation (3 min, RT, 13 000 rpm). The supernatant was transferred to a new tube and mixed with 300 µl of phenol/chloroform/IAA (25:24:1) by inverting continuously for 5 min. After centrifugation, the DNA containing supernatant was transferred to a new tube and mixed with 1/10 of sodium acetate (3 M, pH 5) and 400 µl EtOH (98%, -20°C) and left over night at -20°C for precipitation. After centrifugation the DNA pellet was washed twice with EtOH (70%, -20°C), dried at RT and resuspended in 30 µl H$_2$O$_{bide}$.

**Isolation of genomic DNA from *Arabidopsis thaliana***

Genomic DNA was isolated from 4-week-old *Arabidopsis* leaves. Plant material (1 leaf per plant) was frozen in liquid nitrogen and stored at -80°C. Prior to DNA isolation, plant material was ground under liquid nitrogen and supplied with 0.5 ml of DNA extraction buffer (200 mM Tris, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Samples were thoroughly mixed by vortexing and incubated for 10 min at RT. The samples were supplied with 0.5 ml chloroform and mixed. After centrifugation (10 min, 4°C, 13 000 rpm), the DNA-containing supernatant was transferred to a new 1.5 reaction tube and supplied with 0.5 ml isopropanol and mixed. After centrifugation (10 min, 4°C, 13 000 rpm), the supernatant was discarded. The pellet was washed with ethanol (70%) before air-drying. Finally, the pellets were resuspended in 50 µl H$_2$O$_{bide}$ and stored at 4°C until further analysis.
### 2.1.2 Oligonucleotide primers

All oligonucleotide primers used in this study were purchased from Eurofins MWG Operon (Ebersberg, Germany).

**Table 2: Names, stocknumbers and sequences of all primers used.**

<table>
<thead>
<tr>
<th>Primername</th>
<th>Stock No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotides used for cloning of cph2 OE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cph2 OX-L</td>
<td>857</td>
<td>ACATATGAACCCTAATCGATCC</td>
</tr>
<tr>
<td>Cph2 OX-R</td>
<td>858</td>
<td>TAGATCTTTGGCGGACAACTACAC</td>
</tr>
<tr>
<td><strong>Oligonucleotides used for cloning of hik12 OE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hik12 OX-LB_neu</td>
<td>1008</td>
<td>AATCGATGCAATCAATAACCATGTT</td>
</tr>
<tr>
<td>Hik12 OX-term</td>
<td>991</td>
<td>AAAACGCCCCGCGCGCAACCGAGCTTGATTTTC CATAAT</td>
</tr>
<tr>
<td><strong>Oligonucleotides used for cloning of hik12 KO_2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hik12-L</td>
<td>441</td>
<td>GCTTTATTGCTACAATGT</td>
</tr>
<tr>
<td>L-KM</td>
<td>977</td>
<td>AGATTATTGAACACAACGTTGGCTTTTCCTTAATCAC GGTCTGAG</td>
</tr>
<tr>
<td>Hik12-R</td>
<td>443</td>
<td>CTTTGAATGGTAAACTGAC</td>
</tr>
<tr>
<td>R-KM</td>
<td>978</td>
<td>GATGCTCGTGAGTTTTTTCTAATGTGAGCCGCTATCATAG</td>
</tr>
<tr>
<td>KM-F</td>
<td>850</td>
<td>GGAAAGCCACGTGTGTCT</td>
</tr>
<tr>
<td>KM-R</td>
<td>851</td>
<td>TTAGAAAAACTCATCGAGCATCAAATGT</td>
</tr>
<tr>
<td><strong>Oligonucleotides used for semi-quantitative RT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RnpB-1fw</td>
<td>768</td>
<td>TGTCACAGGGAATCTGAGGA</td>
</tr>
<tr>
<td>RnpB-2rv</td>
<td>769</td>
<td>AAGGGCCGTTATTTTTCTGTG</td>
</tr>
<tr>
<td>G6PDH-1fw</td>
<td>766</td>
<td>GCAAAATCTGATGTTGTGTC</td>
</tr>
<tr>
<td>G6PDH-2rv</td>
<td>767</td>
<td>AATAACCGCTGCTTCTTC</td>
</tr>
<tr>
<td>CtaDI-1fw</td>
<td>1413</td>
<td>CTGGGGGCGACATTAATTTCG</td>
</tr>
<tr>
<td>CtaDI-2rv</td>
<td>1414</td>
<td>GATGCTAAACTGGGCTGGA</td>
</tr>
<tr>
<td>PsaA-1fw</td>
<td>772</td>
<td>GCACCTGCCAAGTTCTGCTG</td>
</tr>
<tr>
<td>PsaA-2rv</td>
<td>773</td>
<td>GTACCCCAAACATCGGATTG</td>
</tr>
<tr>
<td>PsbA3-1fw</td>
<td>770</td>
<td>CTGAGCTTGGAGGCAAATCCTT</td>
</tr>
<tr>
<td>PsbA3-2rv</td>
<td>771</td>
<td>CTGTTCCCAAATGAGCGCT</td>
</tr>
<tr>
<td>Cph2-qPCR-1</td>
<td>1850</td>
<td>TGCGGCTGTATCGAGAAAGGT</td>
</tr>
<tr>
<td>Cph2-qPCR-2</td>
<td>1851</td>
<td>CATTCAATGGGCAATGAGCAA</td>
</tr>
<tr>
<td>Hik12-qPCR-1</td>
<td>1852</td>
<td>AAACCTCTCGCTCCCTTTGG</td>
</tr>
<tr>
<td>Hik12-qPCR-2</td>
<td>1853</td>
<td>GCATTGCTTTGACCTGACC</td>
</tr>
</tbody>
</table>
2.1.3 Generation of mutant lines in *Synechocystis PCC 6803*

Transformation of *Synechocystis* is achieved via the uptake of DNA by type IV pili and incorporation into the host genome by homologous double recombination. *Synechocystis* cells were transformed with psk9 vector-based constructs. The psk9 vector was provided by Annegret Wilde (Justus-Liebig University, Giessen, Germany) and with pGEM-T® vector-based constructs (Promega GmbH, Mannheim, Germany).
### Table 3: Overview of constructs, cloning strategies and genetic background.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Cloning strategy</th>
<th>Transformed in</th>
</tr>
</thead>
<tbody>
<tr>
<td>psK9-cph2 OE</td>
<td>Amplification of full length cph2 (4015 bp) by Cph2 OX-L (857)/Cph2 OX-R (858), NdeI/BglII in psK9</td>
<td>cph2 KO (obtained from Y. Mok Park, Korea Basic Science Institute, Daejeon, South Korea)</td>
</tr>
<tr>
<td>psK9-hik12 OE</td>
<td>Amplification of full length hik12 (2561 bp) by Hik12 OX-LB (1008)/Hik12 Ox-term (991), Clal in psK9</td>
<td>hik12 KO (obtained from I. Suzuki, Univ. of Tsukuba, Tsukuba, Japan)</td>
</tr>
<tr>
<td>pGEM-T-hik12 KO_2</td>
<td>Amplification of flanking regions left border (436 bp, 441/977)/right border (414 bp, 978/443) and Km&lt;sup&gt;R&lt;/sup&gt; (928 bp, 850/851) from pIGA, fusion (441/44) and ligation into pGEM-T®</td>
<td>Wildtype strain (obtained from K. Marin, Univ. of Cologne, Cologne, Germany)</td>
</tr>
</tbody>
</table>

### Construction of overexpression lines

For complementation of the knockout lines, overexpression constructs of hik12 and cph2 were generated. Therefore, full-length coding sequences of *Synechocystis* hik12 and cph2 genes were amplified from genomic DNA of *Synechocystis* by PCR, using a proof-reading Taq-polymerase (Phusion® Taq-polymerase, Finnzymes, New England Biolabs, Ipswich, UK) and the following primers: Cph2 OX-L (857)/Cph2 OX-R (858) and Hik12 OX-LB_neu (1008)/Hik12 Ox-term (991). Primer sequences are listed in Table 2, fragment sizes in Table 3. For subsequent cloning, restriction sites were introduced by oligonucleotides. Clal sites were introduced at the 5' and the 3' of hik12 coding sequence by forward and reverse primers. An NdeI site that overlaps with the ATG start codon of cph2 gene was introduced by the forward primer, while BglII was introduced at the 3' end by the reverse primer. The PCR products were isolated and purified by gel extraction. Using the distinct restriction sites (NdeI/BglII or Clal), the PCR products were ligated in the vector psk9 for chromosomal integration under the control of the petJ promoter. The ligation mixes were used for transformation of competent *E. coli* cells. The correct integration of constructs was checked by restriction analysis and DNA sequencing, prior to *Synechocystis* transformation. DNA sequencing was performed by the Agowa sequencing service (Berlin, Germany). To generate complementation lines, the *Synechocystis* knockout lines of hik12 and cph2 were used as parental strains for transformation with the corresponding overexpression constructs. Positive *Synecho-
cystis transformants were selected on BG-11 agar plates containing 10 µg/ml chloramphenicol and 10 µg/ml spectinomycine as resistance marker.

Construction of knockout lines

For the analysis of the Synechocystis histidine kinase 12 (hik12, locus sll1672) a second independent knockout line, besides the hik12 knockout line obtained from Dr. Iwane Suzuki (Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan) was generated in this study. For this purpose, a deletion mutant was generated by inserting a kanamycine resistance cassette (KM<sup>R</sup>) as resistance gene marker, which disrupts the functionality of the corresponding gene. Chromosomal integration was accomplished by homologous recombination, by replacing the wildtype alleles with the mutated copies via double crossover. For generation of the construct, two fragments with the sizes of 436 and 414 bp, corresponding to the 5' and 3' flanking regions of the hik12 gene, were amplified by PCR using the following primers: Hik12-L (441)/L-KM (977) and R-KM (978)/Hik12-R (443). The kanamycine resistance cassette was amplified by PCR with the primers KM-F (850)/KM-R (851) using the vector pIGA as template. pIGA contains the gene AhpII, coding for kanamycine resistance and was obtained from Martin Hagemann (University of Rostock, Rostock, Germany). In a fusion PCR, using the outer primers Hik12-L (441)/Hik12-R (443), all three fragments (5' flanking region, kanamycine resistance cassette and 3' flanking region) were combined by 3-fragment-PCR, resulting in one assembled construct. After isolation and purification of the assembled PCR product, the construct was ligated into the vector pGEM-T® (Promega GmbH, Mannheim, Germany) according to the manufacturer’s instruction and transformed into E. coli. Positive transformants were identified by blue/white selection and verified by restriction analysis and DNA sequencing, prior to Synechocystis transformation. DNA sequencing was performed by the Agowa sequencing service (Berlin, Germany). For transformation, the Synechocystis wildtype strain, obtained from Kay Marin (University of Cologne, Cologne, Germany) was used. Positive Synechocystis transformants were selected on BG-11 agar plates containing 10 µg/ml kanamycine as resistance marker.

Stable transformation of Synechocystis sp. PCC 6803

Stable transformation of Synechocystis was performed according to Grigorieva and Shestakov (1982) with modifications. Cells were grown photoautotrophically in liquid cultures to an OD<sub>750</sub> of 0.5. 10 ml of the cultures were centrifuged (5 min, 28°C, 3000 rpm, Rotor A-4-62, Eppendorf 5810). The pellet was resuspended in 5 ml BG-11
medium and distributed to 5 sterile reaction tubes. DNA (3 to 5 µg of plasmid DNA) was added and the cells were incubated in darkness (wrapped in aluminium foil) at 28°C over night. 200 µl of the cell cultures were plated on BG-11 agar plates lacking any antibiotics. After 2 days of incubation under constant light with a light intensity of 20 µmol photons m⁻² s⁻¹, the agar layer was lifted with a sterile spatula and the selection marker (1 ml of a solution containing the appropriate antibiotics at a final concentration of 15 µg/ml) was applied beneath the agar layer. Plates were incubated under continuous illumination with a light intensity of 20 µmol photons m⁻² s⁻¹. Colonies appear after 2 to 3 weeks. Single colonies were picked and streaked out on BG-11 agar plates containing the appropriate antibiotics. For segregation of the transformants, restreaks were performed in weekly intervals for 3 to 4 weeks (successive streak purification). To verify the transformants for the presence of the overexpression construct or the absence of the WT genomic sequence in the knockout, colony PCR and PCR on isolated genomic DNA was performed.

2.1.4 Experimental conditions

In addition to photoautotrophic growth, the glucose-tolerant *Synechocystis* sp. PCC 6803 strain is capable of metabolizing exogenous glucose, e.g. in glucose-supplemented medium. Accordingly, *Synechocystis* is capable of photomixotrophic growth under light conditions (Williams, 1988) and heterotrophic growth in darkness, if provided with a daily pulse of several minutes of white light. This minimal amount of light is necessary to enable growth and survival under dark conditions and is referred to as light-activated heterotrophic growth (LAHG) (Anderson & McIntosh, 1991). Furthermore, *Synechocystis* cells can either live as single planktonic organisms or in sessile communities, called biofilms or biomats.

**Photoautotrophic and photomixotrophic growth conditions**

For photoautotrophic growth, *Synechocystis* cells were cultivated in liquid BG-11 medium (TES pH 8.0), in a rotary shaker at 140 rpm, 28°C and under continuous illumination with a light intensity of 20 µmol photons m⁻² s⁻¹. For photomixotrophic growth, BG-11 medium was supplemented with glucose (final concentration 10 mM).

**Biomatforming growth conditions**

For growth conditions, which reflect the lifestyle in sessile communities, photoautotrophically grown mid-exponential cell cultures were supplemented with glucose (final concentration 10 mM) and incubated without agitation, allowing the cells to sediment and form biomats, which is hereafter referred to as biomatforming
conditions. Under biomatforming conditions, the cells were either incubated under continuous illumination (with a light intensity of 20 µmol photons m⁻²s⁻¹) or under LAHG conditions (in total darkness with 15 min light per 24 h, respectively). In this study, hik12 mutant lines (hik12 KO and hik12 OE) and the corresponding wildtype were incubated under continuous illumination, while cph2 mutant lines (cph2 KO and cph2 OE) and the corresponding wildtype were incubated under LAHG conditions.

2.1.5 Growth experiments

Monitoring growth under varying conditions is a fast way to screen mutant lines. Growth of wildtype and mutant strains in liquid cell cultures was monitored by measuring the increase in absorption at 750 nm (OD₇₅₀), during a distinct time course. Growth was analysed under photoautotrophic, photomixotrophic and under biomatforming growth conditions, the latter after resuspension of the cells. For monitoring the cell growth on plates, cells were streaked out on BG-11 agar plates with and without glucose (final concentration 10 mM). Depending on the experimental setup, the plates were grown under constant light, under complete dark conditions and/or under LAHG conditions. Cell growth was monitored by taking pictures at different timepoints.

2.1.6 Determination of cell division activity

Proliferation in *Synechocystis* cells occurs via binary fission, an asexual form of reproduction, which comprises the division process of one cell into two daughter cells. Microscopic analyses allow to distinguish between spherical single cells and dumbbell-shaped cells, which undergo separation. Photoautotrophically grown, mid-exponential cells (OD₇₅₀ = 0.3-0.6) were distributed to sterile 1.5 ml-reaction tubes, supplied with glucose at a final concentration of 10 mM and incubated under biomatforming conditions (without agitation, 28°C, continuous illumination with white light with an intensity of 20 µmol photons m⁻²s⁻¹). The ratio of single cells and dumbbell-shaped cells, which undergo cell division was analysed microscopically before (0 d) and after 14 days of incubation under biomatforming conditions. The ratio was determined by counting 3x50 cells per strain in randomly picked areas, in 15 µl-droplets on a glass slide, thereby distinguishing between single cells and dumbbell-shaped cells. Statistic evaluation was performed using a two-tailed, unpaired Student's *t*-test.
2.1.7 G6PDH enzyme activity measurements

Enzyme activity measurements were performed to determine the specific activity of glucose-6-phosphate dehydrogenase (G6PDH).

Crude protein isolation

For crude protein isolation, cell cultures were harvested by centrifugation (10 min, 4°C, 3500 rpm, Rotor A-4-62, Eppendorf 5810). The pellet was frozen in liquid nitrogen and stored at -80°C till further processing.

**Breakage buffer:**
- HEPES KOH pH 7.5 50 mM
- Glycerol 15% (v/v)
- EDTA pH 8.0 1 mM
- MgCl₂ 3 mM
- DTT (Dithiothreitol) 1 mM
- PMSF (phenylmethyl-sulfonyl fluoride) 1 mM

For isolation of soluble proteins, the method of Knowles and Plaxton (2003) was used with some modifications. Cell pellets were supplied with 0.8 ml breakage buffer and allowed to thaw on ice. The samples were transferred to 2-ml-reaction tubes, containing 0.3 g glass beads (⌀ 0.25-0.50 mm; Roth, Karlsruhe, Germany) and frozen in liquid nitrogen. The samples were allowed to thaw in an ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany) for 1.5 min. The samples were briefly vortexed and frozen again in liquid nitrogen. After repeating the freeze-and-thaw cycle three times, debris was removed by centrifugation (10 min, 4°C, 12 000 g) and the supernatant was transferred to a new reaction tube and kept on ice.

Determination of the protein content

The protein content of the samples was determined using the Bradford-Assay (Bradford, 1976). BSA (bovine serum albumine, Sigma-Aldrich, Hannover, Germany) was used to create a calibration curve. Bradford solution (BioRad Laboratories, Munich, Germany) diluted at the ratio of 1:4 with H₂O<sub>dest</sub>, was mixed with increasing BSA concentrations (5, 10, 20 and 30 µg BSA) in total volume of 1 ml for measurements in a spectrophotometer (Uvikon 931, Kontron Instruments, Rossdorf, Germany) or with BSA concentrations of 1, 2, 4 and 6 µg BSA in a total volume of 200 µl for measurements in a microplate reader (Tecan Infinite, Crailsheim, Stuttgart,
Germany) to create the calibration curve. The absorption was measured at a wavelength of 595 nm. Bradford solution without BSA was used as blank reference. The protein content of the samples was calculated using the BSA calibration curve.

**G6PDH enzyme activity measurements**

In this direct spectrophotometric assay, the specific activity of G6PDH was determined. As key metabolic enzyme of the oxidative pentose phosphate pathway, G6PDH converts glucose-6-phosphate into 6-phosphogluconolactone by reducing NADP⁺ to NADPH. As the coenzyme strongly absorbs UV light at 340 nm only in its reduced form, the course of the reaction can be measured by following the change in absorbance at 340 nm.

The reaction mix contained 50 mM HEPES KOH (pH 8.0), 10 mM MgCl₂·6 H₂O, 0.4 mM NADP, 5 mM G6P and 100 µg of crude protein in a final volume of 1 ml for measurements in a spectrophotometer (Uvikon 931, Kontron Instruments, Rossdorf, Germany) or 20 µg of total protein in a final volume of 200 µl per well (96-well microplates, Greiner Bio-one, Frickenhausen, Germany) for measurements in a microplate reader (Tecan Infinite, Crailsheim, Germany). The reaction was started by addition of glucose-6-phosphate (G6P). H₂O bidest replaced G6P as substrate control. The samples were measured by reading the absorbance at 340 nm for 10 min in 20 sec intervals. The specific enzyme activity Aₜₚₑₑₑₑ using the following equation after Lambert-Beer-Law:

\[
A_{spec} \text{ unit mg}^{-1} = \frac{\Delta \text{slope min}^{-1} \ast V_{cuvette} \text{ l}^{-1}}{\varepsilon \text{ mM}^{-1} \text{ cm}^{-1} \ast \text{ d cm}^{-1} \ast \text{ protein mg}^{-1}}
\]

\[\varepsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}, \text{ molar extinction coefficient}\]

\[\Delta \text{slope} = \text{ initial linear increase of } A_{340}\]

Statistic evaluation was performed using a two-tailed, unpaired Student’s t-test.

**2.1.8 Glycogen determination**

The glycogen content in *Synechocystis* cells was quantified using anthrone reagent according to Schneegurt and co-workers (1994) with modifications. 2 ml cell culture were harvested by centrifugation (10 min, RT, 15 000 g), washed twice with H₂O bidest and resuspended in 0.2 ml H₂O bidest. After addition of 0.4 ml of 40% KOH, the samples were boiled in a thermo-block (Biometra, Göttingen, Germany) at 90°C for 1 h. After cooling down, the samples were supplemented with 1.2 ml of EtOH (98%) and stored over night at -20°C. After centrifugation (30 min, RT, 15 000 g) and
removing the supernatant, pellets were dissolved in 0.1 ml of H$_2$SO$_4$ (conc.) and incubated for 10 min at RT. After filling up to 1 ml with H$_2$O$_{bidest}$, 0.5 ml of each sample was mixed with 1 ml anthrone reagent (0.2% anthrone [9,10-dihydro-9-oxoanthracene] Sigma-Aldrich, Hannover, Germany, in sulfuric acid) and incubated for 10 min at 90°C. Muscle glycogen (Sigma-Aldrich, Hannover, Germany) was used as reference. For this purpose, muscle glycogen (5, 10, 30 and 60 µg glycogen, dissolved in H$_2$O$_{bidest}$) was supplemented with 0.1 ml H$_2$SO$_4$ (conc.), filled up with H$_2$O$_{bidest}$ to 0.5 ml and treated similar to the samples. After samples have been cooled down to RT, the absorbance at 625 nm was measured in a spectrophotometer (Amersham Pharmacia ULTRO-SPEC III spectral-photometer, Hertfordshire, GB) in a quartz cuvette against H$_2$O$_{dest}$. Specific glycogen contents were given as µg glycogen per OD$_{750}$. Statistic evaluation was performed using a two-tailed, unpaired Student’s t-test.

### 2.1.9 Measurement of P700 reduction kinetics

Chlorophyll a fluorescence measurements were conducted using Pulse-Amplitude-Modulation (PAM) fluorometry. In order to analyse electron flows at PS I, reduction kinetics of P700, were recorded in presence and absence of specific electron transport inhibitors. Photoautotrophically grown, mid-exponential cell cultures were set to an OD$_{750}$ of 0.6 and adapted to biomatforming conditions for 24 h, either under continuous illumination (hik12 KO, hik12 OE and corresponding WT) or in total darkness (cph2 KO, cph2 OE and corresponding WT). Measurements were taken before the addition of glucose and the transition to biomatforming conditions and after 24 h of incubation under biomatforming conditions (24 h), in presence of glucose at a final concentration of 10 mM, without agitation and under continuous illumination with a light intensity of 20 µmol photons m$^{-2}$ s$^{-1}$or in complete darkness). By applying specific inhibitors (Table 4) different components of the electron transport chain can be blocked. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) inhibits PS II by blocking the plastoquinone-binding site, thereby inhibiting the linear electron flow. 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) inhibits the electron transport chain by preventing the re-oxidation of plastoquinone by the cytochrome $b_{6f}$ complex. Inhibitors used were provided by Sigma-Aldrich (Hannover, Germany).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration</th>
<th>Dissolved in</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCMU</td>
<td>10 µM</td>
<td>EtOH (98%)</td>
</tr>
<tr>
<td>DBMIB</td>
<td>60 µM</td>
<td>EtOH (98%)</td>
</tr>
</tbody>
</table>
Reduction kinetics of oxidized P700 (P700+) in adapted wildtype and mutant cells were recorded \textit{in vivo} with a dual PAM-100 measuring system (Heinz Walz GmbH, Effeltrich, Germany) equipped with a standard emitter-detector unit DUAL-E and detector unit Dual-DB and the data acquisition software DualPAM. Blue actinic illumination at 460 nm was provided by a LED lamp with max. 700 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PAR. \textit{Synechocystis} cell cultures (set to an OD\textsubscript{750} of 0.6 at timepoint 0 h) were placed in an emitter-detector cuvette assembly unit (ED-101US/MD) at the growth temperature of 28°C. An automated script file for sequential measurements was programmed. A schematic illustration of a measurement sequence is displayed in Figure 4.

![Figure 4: Schematic illustration of a P700\(^+\) reduction kinetic measurement sequence.](image)

Black rectangles indicate the 60-s light pulses of blue actinic illumination. Dashed lines indicate completely oxidized (upper line) and reduced (lower line) P700. Grey arrows indicate the addition of inhibitors before and during the measurement.

Complete P700\(^+\) oxidation was achieved by 60 s blue light pulses with an intensity of 700 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \). The light pulses of actinic illumination (indicated by black bars) were followed by the reduction of P700\(^+\) in the dark. Dashed lines indicate completely oxidized (upper line) and reduced (lower line) P700. The measurement implies 2x3 individual single traces. In the first half of the measurement, the P700\(^+\) reduction kinetics were recorded either in absence of an inhibitor (A1 control) or in presence of DCMU (B1 DCMU). After a 120 s break, in the second half of the measurement, the reduction kinetics were recorded after addition of a second inhibitor (B2 DBMIB). Resulting data were analysed as described by Tsunoyama et al., (2009) and Bernát et al., (2009). Rate constants \((k)\) in presence and absence of specific inhibitors, respectively, were determined by fitting the decays with single exponential functions according to the following equation:

\[
y = A_1 e^{-kt} + y_0 \quad \text{with} \quad k = \frac{1}{t_1}
\]
The half-life \( t_{1/2} \) of the re-reduction of P700\(^+\) gives an indication of the rate of electron donation to PS I (Howitt et al., 2001). Given the rate constant \( k \) and \( \ln (2) \), \( t_{1/2} \) for each decay was determined:

\[
t_{1/2} = \frac{\ln(2)}{k}
\]

For statistical analysis an ANOVA test was performed, combined with a post-hoc Tukey’s highly significant difference (HSD) test at \( P = 0.05 \). The evaluation was performed using the program IBM SPSS Statistics 19.

### 2.1.10 ROS detection by luminol-based assay

Luminol-based assays were used to detect ROS generation in cyanobacterial cell cultures in presence and absence of 10 mM glucose. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich, Hannover, Germany) is stepwise oxidized to aminophthalate. When the excited aminophthalate returns into its ground state, blue light of 428 nm is emitted. This chemoluminescence reaction can be detected by a highly sensitive microtiter plate luminometer (Dynatech Laboratories, Houston, Texas, USA). 100 \( \mu \)l of cyanobacterial cell culture were added to the wells of a 96-well multi-titer plate (Greiner Bio-one, Frickenhausen, Germany) containing 150 \( \mu \)l of the reaction mixture (0.02 mM luminol, horseradish peroxidase at 0.02 U/ml and 5 mM sodium phosphate buffer at pH 8). Chemoluminescence was followed for 60 min at 150 s intervals. Recordings of relative luminescence kinetics were plotted over time.

### 2.1.11 Genexpression analysis

**Isolation RNA from Synechocystis**

Total RNA of *Synechocystis* was isolated according to McGinn and co-workers (2003) with modifications. 10-25 ml of photoautotrophically grown, mid-exponential cell culture was mixed with the same volume of a pre-cooled stop solution (5% [v/v] acidic phenol, 95% [v/v] ethanol). After centrifugation (15 min, 4°C, 3500 rpm, Rotor A-4-62, Eppendorf 5810), the supernatant was discarded and the pellet was washed twice with 0.5 ml STE buffer (50 mM Tris/HCl, pH 8.0, 5 mM EDTA, 0.5% SDS) and stored at -70°C. For RNA isolation the pellets were allowed to thaw on ice and resuspended in pre-cooled STE buffer. After addition of 0.5 ml phenol (prewarmed at 65°C), the samples were mixed by vortexing and incubated at 65°C for 15 min. Phase separation was carried out by centrifugation (15 min, 4°C, 13 000 rpm). The supernatant was transferred to a new reaction tube and mixed with 0.4 ml phenol by
vortexing, followed by centrifugation. The phenol treatment was repeated twice. After transferring the aqueous phase to a new reaction tube, 0.4 ml phenol/chloroform (1:1, [v/v]) was added. After centrifugation, 0.3 ml chloroform was added to the supernatant, followed by mixing and centrifugation. The RNA containing supernatant was transferred to a new reaction tube and mixed with 20 µl sodium acetate (3 M, pH 4.0) and 0.4 ml EtOH (98%, -20°C) and left over night at -20°C for precipitation. After centrifugation and washing the pellet twice with EtOH (80%), the pellets were dried at RT and subsequently resuspended in 30 µl DEPC-treated H2O. At this stage, the isolated RNA was stored at -70°C. The quantity and purity of the RNA was determined photometrically by absorption at 260 and 280 nm using NanoDrop® (Peqlab Biotechnology, Erlangen, Germany) and by denaturing gel electrophoresis.

Denaturating agarose gel electrophoresis

Denaturating gel electrophoresis was used to analyse the quality and quantity of the isolated RNA. 1.2% agarose (w/v) was heated in 1x MOPS buffer until the agarose was completely melted. Before polymerization, 5% formaldehyde (w/v) (Roth GmbH, Karlsruhe, Germany) was added. 1 µg of RNA, supplemented with RNA loading buffer (Fermentas, St. Leon-Rot, Germany) was denaturated at 95°C for 5 min and loaded on the gel. Voltage application was set to 90 V for 35 min. For documentation DeVision G camera and software (Decon, Hohengandern, Germany) was used.

MOPS buffer (10x) 3- (N-morpholino)propan sulfonic acid 200 mM
Sodium acetate 50 mM
EDTA 10 mM
ad H2O<sub>DEPC</sub> 1 l

DEPC-treated water (H2O<sub>DEPC</sub>): autoclaved H2O<sub>bidest</sub> was supplied with 0.1% (w/v) DEPC (diethylenepyrocarbonate) and incubated at RT for 2 h under agitation. The solution was incubated at 37°C over night and autoclaved thereafter.

cDNA synthesis and semi-quantitative RT-PCR

To analyse gene expression semi-quantitative two-step reverse-transcription polymerase chain reactions (RT-PCR) was performed. cDNA was generated using the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) according the manufacturer’s instructions. 1 µg of the isolated RNA was reversely transcribed to first strand cDNA. 500 ng of the first strand cDNA was subsequently used as template for PCR amplification with gene-specific primers. As control for constitutive gene expression, a house-keeping gene RnpB (slr0249), encoding for the RNase P
subunit B was used. Optimal PCR cycle numbers varied between 31 to 32 cycles (31 cycles for \textit{PsaA}, 32 cycles for \textit{RnpB}, \textit{Zwf}, \textit{CtaDI}, \textit{PsbA3}, 33 cycles for \textit{Cph2} and 36 cycles for \textit{Hik12}).

**Table 5: Target genes, functions, forward (fw) and reverse (rv) primer sequences, product sizes and optimal annealing temperatures.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>T (°C)</th>
<th>size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{slr0249} (\textit{RnpB})</td>
<td>RNaseP subunit B</td>
<td>RnpB-1fw</td>
<td>TGTCACAGGGAATCTGAGGA</td>
<td>54.5</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RnpB-2rv</td>
<td>AAGGGCGGTATTCTTGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{slr1843} (\textit{Zwf})</td>
<td>G6PDH</td>
<td>G6PDH-1fw</td>
<td>GCAAAATCTGATGGGTTCC</td>
<td>54.5</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G6PDH-2rv</td>
<td>AATAACGGGTCGTTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{slr1137} (\textit{CtaDI})</td>
<td>Cyt c-ox subunit I</td>
<td>ctaDI-1fw</td>
<td>CTGGGGGGCATTAATTTTGT</td>
<td>54.5</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ctaDI-2rv</td>
<td>GATGCTAACACTGGGTTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{slr1834} (\textit{PsaA})</td>
<td>P700 subunit Ia</td>
<td>PsaA-1fw</td>
<td>GCACCTGCAAAGATCTGCTT</td>
<td>54.0</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PsaA-2rv</td>
<td>GTACCCAAACATCGGATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{sll1867} (\textit{PsbA3})</td>
<td>PSII/D1</td>
<td>PsbA3-1fw</td>
<td>CTGAGCTTGAGGCCAAATCCTT</td>
<td>54.0</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PsbA3-2rv</td>
<td>GTGAGCTGAGGCCAAATCCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{sll0821} (\textit{Cph2})</td>
<td>Cph2</td>
<td>Cph2-1fw</td>
<td>TCGGCGTGTATCGAGAAGGT</td>
<td>55.5</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cph2-2rv</td>
<td>CATTCATGAGGAAGCGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{sll1672} (\textit{Hik12})</td>
<td>Hik12</td>
<td>Hik12-1fw</td>
<td>AAACCTCGCTCCTTTTGG</td>
<td>56.0</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hik12-2rv</td>
<td>GCATTGCCTTGTACCTGACC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 6: Reaction mix and PCR program used for semi-quantitative RT-PCR.**

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10x)</td>
<td>2.5 µl</td>
<td>Initial denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>2.5 µl</td>
<td>Denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Primer 1 (10 pmol)</td>
<td>1.5 µl</td>
<td>Annealing</td>
<td>54.0-54.5°C</td>
</tr>
<tr>
<td>Primer 2 (10 pmol)</td>
<td>1.5 µl</td>
<td>Elongation</td>
<td>72°C</td>
</tr>
<tr>
<td>template cDNA (500 ng/µl)</td>
<td>1.0 µl</td>
<td>Final Elongation</td>
<td>72°C</td>
</tr>
<tr>
<td>Taq-DNA-Polymerase</td>
<td>0.15 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{H}<em>2\text{O}</em>{\text{bidest.}}</td>
<td>ad 25 µl</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( ^1 \) All annealing temperatures used for semi-quantitative RT-PCRs performed here, varied between 54.0 and 54.5°C, depending on the melting temperatures of the specific primers.
2.2 Plants and pathogens

2.2.1 Plant material

*Hordeum vulgare*

The barley (*Hordeum vulgare* L.) cultivar Pallas, provided by Lisa Munk (Royal Veterinary and Agricultural University, Copenhagen, Denmark) was used for gene function analysis in transient transformation assays. Barley plants were grown for 7 days in a growth chamber (Conviron, Winnipeg, Canada or Sanyo, Munich, Germany) at 18°C, with relative humidity of 65% and a photoperiod of 16 h light irradiance at 90 µmol photons m⁻²s⁻¹. For cultivation of barley plants soil Typ ED 73, (Einheitserde- und Humuswerke, Gebr. Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) was used.

*Arabidopsis thaliana*

An *Arabidopsis* T-DNA insertion line, deficient in the cytokinin receptor AHK3 (locus *At1g27320*) was analysed in pathogen assays. The AHK3-deficient (loss-of-function) T-DNA-insertion line was acquired from the Nottingham *Arabidopsis* Stock Centre (NASC, Loughborough, UK; accession no. NASC N 6562). The *ahk3-1* allele contains a T-DNA insertion in the 4th intron, which is adjacent to exons encoding the third transmembrane segment. *Arabidopsis Wasilewska* (*Ws*), provided by Erwin Grill (Chair of Botany, Technische Universität München, Munich, Germany) was used as wildtype in all experiments performed. *Arabidopsis* seeds were incubated for 24 h in H₂O dest at 4°C for stratification prior sewing into soil. For cultivation, soil (Fruhstofer Erde, Typ P, Vechta, Germany) was mixed with sand (Quarzsand, granulation: 0.1-0.5 mm Sakret® Trockenbaustoffe Europa GmbH & Co. KG, Wiesbaden, Germany) in a ratio of 2:1. *Arabidopsis* plants were cultivated in a growth chamber (Conviron, Winnipeg, Canada) at 22°C with a relative humidity of 64% and a photoperiod of 10 h light irradiance at 120 µmol photons m⁻²s⁻¹.

2.2.2 Pathogens and inoculation procedures

*Blumeria graminis* f. sp. *hordei* (*Bgh*)

The powdery mildew fungus *Blumeria graminis* forma specialis *hordei* (*Bgh*) race A6, provided by Jörn Pons-Kühnemann (Justus-Liebig Universität, Giessen, Germany) was used to inoculate transiently transformed barley leaves. *Bgh* was maintained on *Hordeum vulgare* cultivar Golden Promise in climate chambers (Conviron, Winnipeg, Canada or Sanyo, München, Germany) at 18°C, with relative humidity of 65% and a
photoperiod of 16 h light irradiance at 90 µmol photons m⁻²s⁻¹. Transiently transformed barley leaf segments were fixed with the abaxial side up on 0.5% (w/v) water agar. The inoculation was accomplished under a tent-like frame by evenly spreading the fungal spores, giving a density of 150 conidia/mm⁻².

*Pseudomonas syringae pv. tomato (Pst)* DC3000

*Pseudomonas syringae pv. tomato (Pst)* strain DC3000 was provided by Jörg Durner (Helmholtz Zentrum, Munich, Germany). For cultivation and maintainance, bacteria cells were grown on solid King's B medium, containing 50 µg/ml rifampicine and 25 µg/ml tetracycline, and incubated at 28°C for 3 days. Afterwards, the plates were stored at 4°C and streaked out at 4-week intervals. For cultivation in liquid cultures cells were grown in King's B medium, supplemented with the appropriate antibiotics, at 150 rpm, at 28°C for 2 days.

King' s B medium
- Difco protease peptone 10 g
- K₂HPO₄ 1.5 g
- Glycerol 1.5 g
- Agar (for plates only) 15 g
- ad H₂O dest 1 l

use HCl to adjust to pH 7.0

After autoclaving (121°C, 20 min), the medium was supplemented with filter-sterilized MgSO₄•7 H₂O (final concentration 5 mM) and the antibiotics rifampicine (dissolved in DMSO; final concentration 50 µg/ml,) and tetracycline (dissolved in EtOH [70%], final concentration 25 µg/ml).

For pathogen assays, the optical density of the Pst cell culture was determined photometrically (Amersham Pharmacia ULTROSPEC III spectrophotometer, Hertfordshire, GB) at a wavelength of 600 nm. An OD₆₀₀ of 0.2 is equivalent to 10⁸ cfu ml⁻¹. The bacterial suspension was diluted to 10⁴ cfu ml⁻¹ in 10 mM MgCl₂ and used for inoculation. Mature leaves of 6-week old *Arabidopsis* plants were infiltrated at the abaxial sites with 20 µl of the bacterial suspension using a needleless syringe. Control leaves were mock infiltrated with 10 mM MgCl₂. The infiltrated plants were incubated at 20°C, covered with a transparent plastic dome to maintain high humidity. To determine bacterial proliferation, one infiltrated leaf per plant was harvested at 0, 2, 4 and 8 dai (days after infiltration). The marked infiltrated areas were excised and scaled. After washing with EtOH (70%), the infiltrated area of each
leaf was ground with a small pistil in 200 µl 10 mM MgCl₂. Dilution rows (1:10, 1:100, 1:1000 and 1:10 000) were prepared for each sample and dropped in 7x10 µl droplets on King’s B medium agar plates. The dilution of 1:10 000 was used for the evaluation. After 2 days of incubation at 28°C, bacterial colonies were counted and the quantity of bacteria per gram of leaf material was evaluated. Statistic evaluation was performed using a two-tailed, unpaired Student’s t-test. Infiltration assays as described above were also performed on detached Arabidopsis leaves. The infiltrated leaves were placed on water agar (0.5% [w/v]) and kept under light (10 h light irradiance at 120 µmol photons m⁻²s⁻¹) or dark conditions. The development of the disease symptoms was monitored and pictures were taken at different timepoints.

2.2.3 Transient transformation of barley epidermal cells

Construction of pGY1-AtCKX1 and pGY1-AtCKX2

The cDNAs of AtCKX1 (At2g41510) and AtCKX2 (At2g19500), coding for two cytokinin oxidases in Arabidopsis, cloned into the pCR-BluntII-TOPO vector (Invitrogen, Darmstadt, Germany), were obtained from Thomas Schmülling (FU Berlin, Berlin, Germany) (Schmülling et al., 2003). The coding sequences of AtCKX1 and AtCKX2 were released with SalI (AtCKX1) and EcoRI (AtCKX2) from the pCR-BluntII-TOPO vector and subcloned via SalI (AtCKX1) and blunt-end via Smal (AtCKXX2) into the plant expression vector pGY1. pGY1 was provided by Patrick Schweizer (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany). Prior to ballistic delivery into barley epidermal cells, pGY1-AtCKX1 and pGY1-AtCKX2 were sequenced to verify the correct insertion of the coding sequences into the vector. Sequencing was performed by the Agowa sequencing service (Berlin, Germany). Primer sequences are listed in Table 2.

Preparation of tungsten particles

Tungsten particles were used as microcarriers in transient transformation assays. 50 mg tungsten particles were mixed with 1 ml H₂O bidest and incubated for 20 sec in an ultrasonic bath. After centrifugation (1 min, RT, 11 000 g), the supernatant was removed and this process was repeated once with water and then twice with EtOH (98%). Particles were allowed to dry for 10 min by incubation at 80°C in a thermoblock. To set a final concentration of 25 mg/ml, particles were resuspended in 2 ml of 50% (v/v) glycerol and 0.1% (v/v) DEPC. After 1 h of incubation on a shaker at 37°C, the particles were incubated for 30 min at 100°C in a thermoblock to inactivate DEPC. Before coating the tungsten particles with plasmid DNA, the particles were
incubated for 10 min in an ultrasonic bath for separation. The plasmids containing the reporter gene (GFP under control of CaMV 35S promotor), together with pGY1-AtCKX1, pGY1-AtCKX2 or empty pGY1 vector control, were added to 12.5 µl of tungsten particles (25 mg/ml). To attach the plasmids to the particles, 12.5 µl calcium chloride (20 mM CaCl₂, pH 10) was added drop by drop during continuous vortexing. After 10 min of incubation at RT, the coated particles were spun down and the supernatant was removed. 6 µl of coated tungsten particles per shot remained in the reaction tube.

Table 7: Components per volume/shot.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/shot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tungsten particles (25 mg/ml)</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Plasmid with reporter gene</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>Plasmid with gene of interest/empty vector control</td>
<td>1 µg</td>
</tr>
<tr>
<td>20 mM CaCl₂ (pH 10)</td>
<td>12.5 µl</td>
</tr>
</tbody>
</table>

Ballistic transformation

For transient transformation of barley epidermal cells, leaf segments of 7-day-old barley plants were placed on petri dishes containing 0.5% (w/v) water agar. The segments were fixed with the abaxial site up. Petri dishes with the fixed leaf segments were placed in the Particle Inflow Gun (Finer et al., 1992), where the bombardment took place under the negative pressure of -0.9 bar vacuum and 8.5 bar helium gas pressure. Right after particle bombardment, the leaf segments were incubated in a growth chamber at 18°C, with relative humidity of 65% and a photoperiod of 16 h light irradiance at 90 µmol photons m⁻²s⁻¹. To allow progression of overexpression, the inoculation with Bgh spores was conducted 4 h after bombardment as described in Section 2.2.2.

Microscopic analysis and evaluation of penetration efficiency

48 h after inoculation the penetration efficiency of Bgh spores on transformed epidermal cells, was analysed using fluorescence and brightfield microscopy using a Leica DM 1000 microscope (Leica microsystems, Wetzlar, Germany). Transformed cells, expressing the green fluorescent protein (GFP) were evaluated for penetration efficiency, distinguishing between attacked and successfully penetrated cells (haustorium-containing cells) and attacked and non-penetrated cells (papilla forming cells). For each individual variant, a minimum of 80 interactions was evaluated. The
quotient of all penetrated cells and all attacked (penetrated and non-penetrated) cells multiplied by 100 describes the penetration efficiency. Statistic evaluation was performed using a two-tailed, paired Student’s t-test.

2.2.4 ROS detection by luminol-based assay

Luminol-based assays were used to detect ROS production in plants. *Arabidopsis* leaves of 6 week old plants were harvested and cut in small square pieces of 4 mm$^2$ in size and incubated floating on water over night in a climate chamber at 22°C with a relative humidity of 64% and a photoperiod of 10 h light irradiance at 120 µmol photons m$^{-2}$s$^{-1}$. Prior to the measurement, 2 leaf pieces each were transferred to the wells of a 96-well multi-titer plate (Greiner Bio-one, Frickenhausen, Germany) containing 200 µl of the luminol reaction mixture (0.02 mM luminol, horseradish peroxidase at 0.02 U/ml and 5 mM sodium phosphate buffer at pH 8). Measurements were taken in presence and absence of 10 mM glucose. Chemoluminescence was followed for 60 min at 150 s intervals. Recordings of relative luminescence kinetics were plotted over time.
3 Results

3.1 Functional characterisation of CPH2, a phytochrome photoreceptor in *Synechocystis* sp. PCC 6803

In order to characterise the physiological function of the *Synechocystis* phytochrome photoreceptor CPH2 (sll0821), a knockout mutant and the corresponding complementation line were analysed under different growth conditions. *Synechocystis* sp. PCC 6803 cells (hereafter referred to as *Synechocystis*) are able to live as single, planktonic organisms or in sessile communities, the so-called biofilms or biomats. This study was aimed at elucidating the function of CPH2 with a special focus on its effect on the central carbon metabolism during the transition from fast exponential growth to slowed down growth and high cellular density within a biomat.

3.1.1 Generation of *cph2* complementation lines

Two independent *cph2* knockout lines (*cph2*-1 and *cph2*-2) and the corresponding glucose-tolerant, parental wildtype strain were provided by Dr. Young Mok Park (Korea Basic Science Institute, Daejeon, South Korea). The knockout lines were generated by inserting a spectinomycin resistance cassette into the coding region of *Cph2* gene, thereby disrupting the functionality of the gene (Moon et al., 2011). In this study, the *cph2*-overexpression line was generated in the *cph2*-2 background for functional complementation of the knockout line with a full-length wildtype *cph2* coding sequence under control of the constitutive petJ promotor (Figure 5 A). The *cph2* full length coding sequence was amplified by PCR using Cph2 OX-L (P1)/Cph2 OX-R (P2) primers, thereby introducing NdeI (5' end) and BglII (3' end) restriction sites for subsequent cloning into the vector psk9 (Figure 5 A). Stable transformation of *Synechocystis* via chromosomal integration was performed essentially according to Grigorieva and Shustakov (1982), as described in detail in Section 2.1.3. For complementation, the *cph2* knockout line 2 (*cph2*-2) was used as parental strain. Transformation resulted in five chloramphenicol-resistant colonies. After restreaking on selective media for segregation, the analysis of the transformants by PCR, using the primers psk9-fw-seq (P3) and Cph2-OX-L1 (P4) (Figure 5 A), led to the identification of four mutants, *cph2* OE1, 2, 7 and 8, carrying the OE construct (Figure 5 B). Primer sequences are given in Table 2. Gene expression analysis using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed the overexpression of *cph2* (Figure 5 C). The parental strain, *cph2*-1 and
cph2-2 knockouts and the cph2 complementation/overexpression mutants are in the following referred to as WT, cph2 KO 1, cph2 KO 2 and cph2 OE, respectively.

Figure 5: Generation of cph2 overexpression construct (A), verification of successful genomic integration (B) and transcript overexpression of cph2 (C).

(A) Cph2 overexpression construct used for Synechocystis transformation. Primer P1/P2 used for amplification of full length cph2 (4015 bp) and Primer P3/P4 used for verification of the correct insertion in the transformants are indicated. Restriction sites used for cloning: Ndel and BglII.

(B) Verification of positive genomic integration of the construct in four transformants (designated as cph2 OE1, OE2, OE7 and OE8) by PCR on genomic DNA (estimated fragment size: 1986 bp, black arrow). For complementation, the cph2 knockout line 2 (cph2-2) was used as parental strain. MW, molecular mass standard.

(C) Verification of cph2 overexpression by semi-quantitative RT-PCR. Total RNA of WT and cph2 OE clone 1, 2, 7 and 8 was isolated. After reverse transcription, 500 ng of first strand cDNA was used as template for PCR (33 cycles for RnpB and Cph2). PCR products were analysed on a 1.5% agarose gel and stained with ethidium bromide. The constitutively expressed RNase P gene (RnpB) was included as control.

3.1.2 Growth characteristics: cph2 KO is impaired in growth under biomat forming conditions

Besides photoautotrophic growth, certain strains of Synechocystis are capable of mixotrophic growth in presence of metabolizable sugars, e.g. glucose (Williams, 1988). These glucose-tolerant Synechocystis strains are capable to grow photomixotrophically under light conditions, as well as heterotrophically in darkness, if supplied with glucose and a daily pulse of white light of about 5 min at least. The latter type of growth is referred to as light-activated heterotrophic growth (LAHG) (Anderson & McIntosh, 1991). To provide insights into growth characteristics and to screen for physiological effects of the non-functionality of Cph2, growth of WT and cph2 KO and OE strains was analysed under different growth conditions (photoautotrophic, photomixotrophic and LAHG/biomat forming conditions).
To analyse growth under photoautotrophic or photomixotrophic growth conditions, photoautotrophically grown precultures of WT, cph2 KO and OE strains were diluted to an initial OD$_{750}$ of 0.3 and incubated at 140 rpm, 28°C, under continuous illumination with a light intensity of 20 µmol photons m$^{-2}$ s$^{-1}$ and in presence (photomixotrophic) or absence (photoautotrophic) of 10 mM glucose. In photoautotrophic cultures, growth was monitored by daily measurements of OD$_{750}$ in a time course from 0 to 4 days. Due to the acceleration of growth after addition of glucose in mixotrophic cultures, OD$_{750}$ was recorded in a time course from 0 to 9 hours in measuring intervals of 3 hours. For growth under biomatforming conditions, cultures of WT, cph2 KO and cph2 OE were grown photoautotrophically to an OD$_{750}$ of 0.6-0.8. After addition of glucose (final concentration 10 mM), the cell cultures were incubated without agitation, allowing the cells to sediment and form biomats for 96 h (hereafter referred to as biomatforming conditions). Under biomatforming conditions, WT, cph2 KO and OE strains were incubated in darkness with 15 min light per 24 h, respectively, which is considered as LAHG conditions (Anderson & McIntosh, 1991; Tabei et al., 2009). To determine the growth of the biomats, the cells were resuspended at 0, 48 and 96 h and the OD$_{750}$ was determined.

The growth kinetics of WT, cph2 KO and OE strains are presented in Figure 6. Under photoautotrophic growth conditions (A), WT, cph2 KO and cph2 OE grew at similar rates. The accelerated growth under photomixotrophic growth conditions (B) compared to photoautotrophic conditions, indicated that WT, cph2 KO and cph2 OE are capable to utilise exogenous glucose and therefore are capable of photomixotrophic growth. However, no significant differences in growth of WT, cph2 KO and cph2 OE were detectable under both conditions. In contrast, incubation without agitation under LAHG conditions (C) resulted in growth cessation of cph2 KO, which becomes further obvious in the comparison of the slopes (m) between 0-48 h ($m_{WT}$, 0-48 h = 0.010 ± 0.001, $m_{cph2 KO}$, 0-48 h = 0.007 ± 0.0004, $m_{cph2 OE}$, 0-48 h = 0.012 ± 0.001) and between 48-96 h ($m_{WT}$, 48-96 h = 0.004 ± 0.001, $m_{cph2 KO}$, 48-96 h = 0.001 ± 0.0004, $m_{cph2 OE}$, 48-96 h = 0.004 ± 0.002). The complementation of cph2 KO 2 with the cph2 overexpression construct (cph2 OE clone 8) resulted in growth kinetics similar to those observed for the WT under LAHG/biomatforming conditions, thereby indicating that the growth phenotype of cph2 KO was due to the deletion of Cph2.
Figure 6: Growth kinetics of *Synechocystis* WT, cph2 KO and cph2 OE under distinct growth conditions.

Exponentially growing cultures of WT, cph2 KO and cph2 OE were diluted to an OD<sub>750</sub> of 0.3 (for photoautotrophic and photomixotrophic growth) or 0.6 (for growth under LAHG/biomat-forming conditions) and incubated under photoautotrophic (A), photomixotrophic (in presence of 10 mM glucose) (B) and LAHG/biomat-forming conditions (C). WT (filled rectangle), cph2 KO 2 (open circle) and cph2 OE clone 8 (open triangle). OD<sub>750</sub> was determined at indicated timepoints. Error bars represent standard error, based on mean values of 6 (A, B) and 3 (C) independent experiments.

### 3.1.3 Incubation under LAHG/biomatforming conditions results in a reduced G6PDH activity in cph2 KO

In their natural environment, *Synechocystis* cells are subjected to a diurnal light-dark cycle, during which the organism has to adapt its metabolism by switching between a photoautotrophic and a dark heterotrophic metabolic mode (Sundaram *et al.*, 1998). When light is available, *Synechocystis* grows photoautotrophically by assimilating inorganic carbon via the Calvin cycle, using energy provided by photosynthetic light reactions. The resulting sugar products are subsequently converted into the storage carbohydrate glycogen, which serves as metabolic energy source under non-photosynthetic conditions (Osanai *et al.*, 2007). The degradation of glycogen provides G6P, which is catabolized via the OPPP, the lower energy-conserving phase of glycolysis and the incomplete TCA cycle (Stal & Moezelaar, 1997). In *Synechocystis*, the OPPP is the major pathway of glucose catabolism under heterotrophic growth conditions (Pelroy *et al.*, 1976; Schaeffer & Stanier, 1978; Yang *et al.*, 2002; Singh *et al.*, 2004). The enzyme glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the
rate-determining step of the OPPP, the conversion of glucose-6-phosphate to 6-
phosphoglucono-1,5-lactone, thereby controlling the carbon flow into the pathway (Singh
et al., 2004). Due to its key function in glucose catabolism, enzyme activities of
G6PDH were analysed in cph2 KO, cph2 OE and WT cells, grown under photoauto-
trophic, photomixotrophic and LAHG/biomat forming conditions.

Photoautotrophically grown, mid-exponential cultures of WT, cph2 KO and OE
strains were incubated in presence (photomixotrophic conditions) and absence
(photoautotrophic conditions) of 10 mM glucose for 24 h (140 rpm, 28°C, continuous
illumination with a light intensity of 20 µmol photons m⁻² s⁻¹). Specific G6PDH
enzyme activities of WT, cph2 KO and cph2 OE strains were determined and are
presented in Figure 7 A. Data are normalized relative to the WT under photoauto-
trophic growth conditions. Under photoautotrophic and photomixotrophic conditions
no significant differences in G6PDH activity of WT, cph2 KO and cph2 OE were
observable. Under photomixotrophic conditions there is a tendency of increas
G6PDH activity in all lines, which indicates that WT, cph2 KO and OE mutants are
capable to utilise exogenous glucose and therefore are capable of mixotrophic
glucose metabolism. In order to analyse specific G6PDH activities under LAHG/bio-
mat forming conditions, photoautotrophically grown, mid-exponential cultures of WT,
cph2 KO and OE strains were supplied with glucose and transferred to LAHG
conditions without agitation. After 96 h of incubation under LAHG/biomat forming
conditions, the specific G6PDH enzyme activities of WT, cph2 KO and OE strains
were determined and are presented in Figure 7 B. Data are normalized relative to
the WT. Enzyme activity measurements after 96 h of incubation under LAHG/bio-
mat forming conditions revealed that G6PDH activity in extracts of both independent
cph2 knockout clones was significantly reduced compared to the WT (P < 0.01, Student’s t test). Overexpression of CPH2 resulted in complementation of G6PDH
activity in cph2 OE clone 8, while cph2 OE clone 2 and 7 partially complement
G6PDH activity, indicating that the decreased enzyme activity in cph2 KO under
LAHG/biomat forming conditions results from the deletion of cph2. The G6PDH
activity in cph2 overexpression clones 7 and 8 was proved significantly different to
cph2 KO1 and KO2 (P < 0.05, Student’s t test) and in cph2 OE clone 2 significantly
different to cph2 KO1 (P < 0.05, Student’s t test).
Figure 7: Specific G6PDH enzyme activity of WT, cph2 KO and cph2 OE strains under photoautotrophic and photomixotrophic (A) and under LAHG/biomatforming (B) growth conditions.

(A) Photoautotrophically grown, mid-exponential (OD750 = 0.6-0.8) cell cultures of Synechocystis WT and cph2 mutant strains (cph2 KO clone 2 and cph2 OE clone 8) were assayed for the activity of G6PDH in presence and absence of glucose. Where indicated, the cells were supplied with glucose at a final concentration of 10 mM (Glc) for 24 h, prior to the extraction of proteins. 100 µg of crude protein were used in a total volume of 1 ml assay solution to determine G6PDH activity. Data are normalized to the WT under photoautotrophic growth conditions (0.073 ± 0.010 U/µg protein, set as 100% for each individual experiment). Columns represent average values of minimum 7 independent experiments. Bars represent standard errors. Asterisks indicate significant difference at $P < 0.05$, according to Student’s t test.

(B) Photoautotrophically grown, mid-exponential cell cultures (OD750 = 0.6-0.8) of WT and cph2 mutant strains (cph2 KO clone 1 and 2, cph2 OE clone 2, 7 and 8) were supplied with glucose at a final concentration of 10 mM and incubated without agitation under LAHG conditions (darkness with 15 min light per 24 h) for 96 h. 100 µg of crude protein were used in a total volume of 1 ml assay solution to determine G6PDH activity. Data are normalized to the WT (0.036 ± 0.013 U/µg protein, set as 100% for each individual experiment). Columns represent average values of minimum 3 independent experiments. Bars represent standard errors. Asterisks indicate significant difference at $P < 0.01$, according to Student’s t test.
3.1.4 Incubation under LAHG/biomatforming conditions induces downregulation of CtaDI expression in cph2 KO

To analyse the expression of key metabolic and photosynthetic genes in WT, cph2 KO and OE under different growth conditions (photoautotrophic, photomixotrophic and LAHG/biomatforming conditions), semi-quantitative reverse-transcription polymerase chain reactions (RT-PCR) were conducted. Total RNA of mid-exponential WT, cph2 KO and cph2 OE cell cultures was isolated after 24 h under photoautotrophic/photomixotrophic conditions and after 96 h under LAHG/biomatforming conditions and subjected to semi-quantitative two-step RT-PCR.

![Figure 8: Gene expression analysis of key metabolic and photosynthetic genes in WT, cph2 KO and cph2 OE by semi-quantitative RT-PCR.](image)

Effects of different growth conditions on the expression of key metabolic and photosynthetic genes were examined by semi-quantitative RT-PCR. Total RNA of WT and cph2 mutants (cph2 KO clone 2 and cph2 OE clone 8; OD\textsubscript{750} = 0.6-0.8) was isolated after 24 h under photoautotrophic (A) and photomixotrophic (in presence of 10 mM glucose) (B) conditions and after 96 h under LAHG/biomatforming conditions (C). After reverse transcription, 500 ng of first strand cDNA was used as template for semi-quantitative RT-PCR (32 cycles, except for PsaA [31 cycles]). PCR products were analysed on a 1.5% agarose gel and stained with ethidium bromide. Data presented are representative for three independent experiments. Ribosomal RNA was tested before first strand cDNA synthesis to ensure non-degradation of total RNA (data not shown).

Analysed genes: RnpB (slr0249), housekeeping; Zwf (slr1843), G6PDH; CtaDI (slr1137), cytochrome c oxidase; PsaA (slr1834), photosystem I; PsbA3 (sll1867), photosystem II.

Besides the housekeeping gene RnpB (slr0249), encoding for subunit B of ribonuclease P (Vioque, 1992), the transcript levels of four genes involved in glucose metabolism and photosynthesis were analysed in WT, cph2 KO and cph2 OE under different growth conditions (Figure 8). Zwf (slr1843) and CtaDI (slr1137) were among the analysed genes involved in glucose metabolism, encoding for G6PDH and for subunit I of the cytochrome c oxidase complex, the terminal component of the respiratory chain, respectively. Among the photosynthesis-related genes, the transcript levels of PsaA (slr1834) and PsbA3 (sll1867) were analysed, encoding for subunit Ia of the P700 apoprotein and for PS II D1 protein, respectively. Gene expression analysis after 24 h of incubation under photoautotrophic (Figure 8 A) and
photomixotrophic (Figure 8 B) growth conditions revealed similar expression patterns for Zwf and CtaDI in WT, cph2 KO and cph2 OE with exception of PsaA and PsbA3, which display a slightly induced transcript accumulations in cph2 OE under both conditions. In contrast, incubation under LAHG/biomatorming conditions for 96 h led to distinct changes in the gene expression pattern (Figure 8 C). The transcript level of CtaDI in cph2 KO was significantly downregulated in comparison to WT and cph2 OE, whereas WT and cph2 OE showed induced transcript accumulation of CtaDI under LAHG/biomatorming conditions compared to photoautotrophic and photomixotrophic conditions. Complementation of cph2 KO resulted in a CtaDI expression pattern similar to the WT, thereby suggesting that the downregulation of CtaDI in cph2 KO under LAHG/biomatorming conditions resulted from the deletion of Cph2.

3.1.5 Analysis of electron transport activities

Electron transport in the cyanobacterial thylakoid membrane is a complex process due to the existence of converging metabolic pathways. Three intersecting electron flows have been found dominant in *Synechocystis* thylakoid membranes: linear photosynthetic electron transport from water to NADP$^+$ via PS II and PS I, respiratory electron transport from NADPH and succinate to cytochrome c oxidase and cyclic electron transport around PS I. Electron input converges at the PQ-pool, from where the electrons are subsequently passed on to PS I via cytochrome $b_6f$ complex and/or finally transferred to molecular oxygen by the respiratory oxidases.

**Figure 9: Overview of the electron transport pathways in the thylakoid membrane of *Synechocystis* and sites of action of inhibitors used (modified after Berry et al., 2002).**

**Abbreviations:** NDH-1, NADPH dehydrogenase 1; SDH, succinate dehydrogenase; PS I and II, photosystem I and II; PQ, plastoquinone pool; cyt $b_6f$, cytochrome $b_6f$ complex; PC, plastocyanin; cyt $c_6$, cytochrome $c_6$; cyd $bd$, cytochrome $bd$-type quinol oxidase; cyt aa3, cytochrome aa3-type cytochrome c oxidase; NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced form); Inhibitors (indicated as thick black bars): DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone;

Chlorophyll a fluorescence measurements represent a non-invasive method, providing real-time information of key aspects of photosynthetic light capture and electron transport. Using Pulse-Amplitude-Modulation (PAM) measurements, reduction kinetics of oxidized P700 (P700$^+$) were recorded in WT, cph2 KO and cph2 OE, in presence and absence of specific electron transport inhibitors to analyse...
electron transport activities in the thylakoid membrane. Figure 9 gives a schematic overview of the described electron flows and the sites of action of the inhibitors used. P700$^+$ reduction kinetics were recorded in photoautotrophically grown, mid-exponential WT, cph2 KO and cph2 OE before the addition of glucose and the transition to biomatfoming conditions (0 h), which refers to photoautotrophic growth conditions and after incubation under biomatfoming/dark conditions for 24 h. Following, the half-life ($t_{1/2}$) of the re-reduction of P700$^+$ was determined, giving an indication of the rate of electron donation to PS I (Howitt et al., 2001). In order to illustrate the principles of this quantification, P700$^+$ reduction kinetic decays of untreated and DCMU-treated WT, cph2 KO and cph2 OE after 24 h of incubation under biomatfoming/dark conditions are displayed in Figure 10 A and B. Cells were irradiated with blue light to keep P700 in its oxidized form (indicated by black bars). When the blue light is switched off, P700$^+$ returns into its reduced state. Corresponding rate constants ($k$) for the calculation of $t_{1/2}$ were obtained by fitting the decays with single exponential functions, as described in Section 2.1.9. $t_{1/2}$ of P700$^+$ reduction in untreated, DCMU- and DCMU/DBMIB-treated samples of WT, cph2 KO and cph2 OE are summarized Figure 10 C and D, before and after 24 h of incubation under biomatfoming conditions, respectively. In untreated (control) samples, $t_{1/2}$ of P700$^+$ reduction of WT, cph2 KO and cph2 OE displayed no significant differences before and after 24 h of incubation under biomatfoming/dark conditions (Figure 10 C, D). DCMU is a PS II inhibitor, which inhibits the linear electron transport by blocking the plastoquinone-binding site. Treatment with DCMU resulted in increased $t_{1/2}$ of P700$^+$ reduction in samples of WT, cph2 KO and cph2 OE adapted to photoautotrophic conditions (Figure 10 C), indicating a reduced electron flow to P700$^+$. Since the linear electron transport is blocked, electron input into the PQ pool originates from electron flows via SDH and NDH-1, from NADPH generated by the heterotrophic metabolism or PS I under blue light illumination. Due to the missing electron input from PS II, the PQ pool is more oxidized in DCMU-treated samples compared to untreated samples. This results in increased $t_{1/2}$ of P700$^+$ reduction in DCMU-treated cells, as it takes longer to re-reduce P700$^+$ after switching off blue light illumination. In samples adapted to biomatfoming/complete dark conditions, treatment with DCMU resulted in a significantly increased $t_{1/2}$ of P700$^+$ reduction in cph2 KO compared to the WT (ANOVA, followed by Tukey’s test $P = 0.05$) (Figure 10 D), which is also reflected in the P700$^+$ reduction kinetic decays displayed in Figure 10 B. The higher increase in $t_{1/2}$ of P700$^+$ reduction indicates that cph2 KO adapted to biomatfoming/dark conditions, has less electron input into the PQ-pool compared to the WT.
Figure 10: P700⁺ reduction kinetics and corresponding half-life in WT, cph2 KO and cph2 OE before and after 24 h of incubation under biomforming, dark conditions in presence and absence of specific electron transport inhibitors.

Cell cultures of WT, cph2 KO and cph2 OE were photoautotrophically grown to an OD₇₅₀ of 0.6. P700⁺ reduction kinetics were recorded before the addition of glucose and the transition to biomforming conditions (0 h) and after 24 h of incubation under biomforming conditions (24 h), in presence of glucose at a final concentration of 10 mM, without agitation and in darkness. Measurements were conducted in untreated (control), DCMU and DCMU/DBMIB-treated cells (final concentrations, 10 µM [DCMU], 60 µM [DBMIB]). P700⁺ reduction kinetic decays of untreated (A) and DCMU-treated (B) WT, cph2 KO and OE cells after 24 h of incubation under biomforming/dark conditions. Curves correspond to three averaged traces of the same sample. Black bars symbolize time intervals (60 s) when blue light is on to keep P700 in its oxidized form. When the blue light is switched off, P700⁺ is reduced. Corresponding t₁/₂ of the P700⁺ reduction was determined in untreated (control) cells and DCMU- and DCMU/DBMIB-treated cells, before (C) and after 24 h (D) of incubation under the described conditions. Data represent the average value of three independent experiments ± standard error. For statistical analysis an ANOVA test was performed, combined with a post-hoc Tukey’s test for each group (control, DCMU, DCMU/DBMIB). Letters A, B and AB show the grouping of data sets according to Tukey’s highly significant difference (HSD) test; columns without a common letter are significantly different at $\ P = 0.05$.

DBMIB is a Q₀ site inhibitor of cytochrome b₅₅₃, which prevents the reoxidation of plastoquinone, thereby inhibiting the electron input from the PQ pool into the cytochrome b₅₆₃ complex. However, the inhibition with DBMIB does not result in a complete electron blockage. Therefore, it takes longer to re-reduce P700⁺ after switching off the blue light, which is reflected in increased t₁/₂ of P700⁺ reduction. Since most of the electron input into the PQ pool originates from PSII in the presence of light, the additional blockage of linear electron transport by DCMU upon simultaneous DBMIB/DCMU-treatment should result in a more effective inhibition by DBMIB. In samples adapted to photoautotrophic conditions, simultaneous treatment
with DCMU and DBMIB resulted in increased $t_{1/2}$ of P700* reduction in WT, cph2 KO and cph2 OE (Figure 10 C), indicating a strongly restricted electron flow to P700*. In samples adapted to biomat forming/dark conditions, this effect is no longer detectable as WT, cph2 KO and cph2 OE showed similarly increased $t_{1/2}$ of P700* reduction (Figure 10 D). Compared to samples adapted to photoautotrophic conditions, the increase in $t_{1/2}$ of P700* reduction upon DCMU/DBMIB-treatment was lower under biomat forming/dark conditions, which might indicate a generally reduced electron flux under biomat forming conditions. Together, the increase in $t_{1/2}$ of P700* reduction upon DCMU-treatment in cph2 KO adapted to biomat forming/dark conditions and the similar $t_{1/2}$ of P700* reduction upon DCMU/DBMIB-treatment in WT, cph2 KO and cph2 OE adapted to biomat forming/dark conditions, indicates that the electron input into the PQ pool might be affected in cph2 KO. This further supports the assumption of a reduced ability to metabolize glucose in cph2 KO.

3.2 Functional characterisation of HIK12, a histidine kinase in Synechocystis sp. PCC 6803

*Synechocystis* includes 44 putative genes for histidine kinases (Hiks) on its chromosome (Kaneko *et al*., 1996; Mizuno *et al*., 1996) and three putative genes for Hiks on its plasmids (Kaneko *et al*., 2003), all of which are potential sensory histidine kinases. Among them, the histidine kinase 12 (HIK12) shows structural homology to the histidine kinases of the cytokinin receptor family in *Arabidopsis*. A sequence alignment of the ligand-binding domains of the cytokinin receptors in *Arabidopsis* and the structurally homologous domain in *Synechocystis* HIK12 is given in Figure 23 and is discussed in detail in Section 4.2. To provide insights into the function of the *Synechocystis* cytokinin receptor-like protein HIK12 (sll1672), a hik12 knockout mutant and the corresponding complementation were characterised with a special focus on its effects on the central carbon metabolism during the shift from planktonic lifestyle to a sessile lifestyle within a biomat.

3.2.1 Generation of hik12 complementation lines

The hik12 (sll1672) knockout mutant (hereafter referred to as hik12 KO) and the corresponding glucose-tolerant wildtype were obtained from Dr. Iwane Suzuki (Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan). The knockout line was generated by inserting a spectinomycin resistance cassette into the coding region of hik12 gene, thereby disrupting the functionality of the gene (Suzuki *et al*., 2000). In this study, a hik12 overexpression line was generated in the background of hik12 KO for functional complementation of
the knockout line with a full-length hik12 coding sequence under the control of the constitutive active petJ promotor (Figure 11 A). The hik12 full length coding sequence was amplified by PCR, using Hik12 OX-LB_neu (P1)/Hik12 OX-term (P2) primers, thereby introducing ClaI restriction sites at the 5' and 3' end for subsequent cloning into the vector psk9 (Figure 11 A). Besides the ClaI restriction sites, an oop terminator was introduced at the 3' end by the primer P2 (Figure 11 A).

Figure 11: Generation of hik12 overexpression construct (A), verification of successful genomic integration (B) and transcript overexpression of hik12 (C).

(A) Hik12 overexpression construct used for Synechocystis transformation. Primer P1/P2, used for amplification of full-length hik12 (2561 bp) and primer P3/P4, used for verification of the correct insertion in the transformants, are indicated. The oop terminator was introduced at the 3' end by primer P2. Restriction sites used for cloning: ClaI.

(B) Verification of positive genomic integration of the construct in five transformants (designated as hik12 OE3, OE4, OE7, OE9 and OE17) by PCR on genomic DNA (estimated fragment size: 1654 bp, black arrow). MW, molecular mass standard.

(C) Verification of hik12 overexpression by semi-quantitative RT-PCR. Total RNA of WT and hik12 OE clone 4 and 7 was isolated. After reverse transcription, 500 ng of first strand cDNA was used as template for PCR (32 cycles for RnpB and 36 cycles for Hik12). PCR products were analysed on a 1.5% agarose gel and stained with ethidium bromide. The constitutively expressed RNase P gene (RnpB) was included as control.

Stable transformation of Synechocystis via chromosomal integration was performed essentially according to Grigorieva and Shestakov (1982), as described in Section 2.1.3. For complementation, the hik12 knockout strain was used as parental strain. Transformation resulted in 30 chloramphenicol-resistant colonies. After restreaking on selective media for segregation, the analysis of 12 transformants by PCR on genomic DNA, using psk9-fw-seq (P3) and Hik12 SDM2 (P4) primers (Figure 11 A), led to the identification of five mutants (hik12 OE clone 3, 4, 7, 9 and 17) carrying the OE construct (Figure 11 B). Gene expression analysis using semi-quantitative RT-PCR confirmed the overexpression of hik12 (Figure 11 C). The parental strain, hik12 knockout and hik12 overexpression mutants are in the following referred to as WT, hik12 KO and hik12 OE, respectively.
3.2.2 Growth characteristics: incubation under biomat forming conditions leads to growth cessation in hik12 KO

To screen for physiological effects of the loss of Hik12, growth of WT, hik12 KO and hik12 OE strains was analysed under different (photoautotrophic, photomixotrophic and photomixotrophic/biomat forming) growth conditions. To analyse growth under photoautotrophic or photomixotrophic conditions, photoautotrophically grown precultures of WT, hik12 KO and hik12 OE strains were diluted to an initial OD<sub>750</sub> of 0.3 and incubated at 140 rpm, 28°C, under continuous illumination with a light intensity of 20 µmol photons m<sup>-2</sup> s<sup>-1</sup>, in presence (photomixotrophic) or absence (photoautotrophic) of 10 mM glucose. In photoautotrophic cultures, growth was monitored by measurements of the OD<sub>750</sub> in a time course from 0 to 4 days. Due to the accelerated growth in presence of glucose, the OD<sub>750</sub> in photomixotrophic cultures was recorded in a time course from 0 to 9 hours, in measuring intervals of 3 hours.

For growth under biomat forming conditions, cell cultures of WT, hik12 KO, hik12 OE clones 4 and 7 were grown photoautotrophically to an OD<sub>750</sub> of 0.6-0.8. After addition of glucose (final concentration 10 mM), the cell cultures were incubated without agitation, allowing the cells to sediment and form biomats, under continuous illumination with a light intensity of 20 µmol photons m<sup>-2</sup> s<sup>-1</sup> for 96 h (hereafter referred to as biomat forming conditions). To determine the growth of the biomats, the cells were resuspended at 0, 48 and 96 h and the OD<sub>750</sub> was determined.

The growth kinetics of WT, hik12 KO, hik12 OE clones 4 and 7 are presented in Figure 12. No differences in growth were observed for WT, hik12 KO and both hik12 OEs, neither under photoautotrophic growth conditions (A), nor under photomixotrophic growth conditions (B). The accelerated growth under photomixotrophic growth conditions (B) indicated that WT, hik12 KO and both hik12 OEs are capable of mixotrophic growth. The analysis of the growth kinetics under biomat forming conditions (C) revealed a growth cessation in hik12 KO, while WT, hik12 OE 4 and 7 continued to grow, hik12 KO ceased in growth after 48 h, which is further reflected in the slope (m) between 48 and 96 h (m<sub>WT</sub>, 48-96 h = 0.009 ± 0.0033, m<sub>hik12 KO</sub>, 48-96 h = 0.002 ± 0.0018, m<sub>hik12 OE 4</sub>, 48-96 h = 0.008 ± 0.0041, m<sub>hik12 OE 7</sub>, 48-96 h = 0.011 ± 0.0008). The complementation of hik12 KO with the hik12 overexpression construct resulted in growth kinetics similar to those observed for the WT.
Exponentially growing cultures of WT, *hik*12 KO and *hik*12 OE clone 4 and 7 were diluted to an OD<sub>750</sub> of 0.3 (for photoautotrophic and photomixotrophic growth) or 0.6 (for growth under biomatatforming conditions) and incubated under photoautotrophic (A), photomixotrophic (in presence of 10 mM glucose) (B) and biomatatforming conditions (C). WT (filled rectangle), *hik*12 KO 2 (open circle), *hik*12 OE clone 4 (open triangle) and *hik*12 OE clone 7 (open rectangle). OD<sub>750</sub> was determined at indicated timepoints. Error bars represent standard error, based on mean values of 4 (A), 3 (B) and minimum 3 (C) independent experiments.

### 3.2.3 Incubation under biomatatforming conditions results in decreased cell division activity in *hik*12 KO

Growth in *Synechocystis* implies proliferation via binary fission, an asexual form of reproduction, which includes the cell division process of one cell into two daughter cells. Microscopic analyses enable to distinguish between these cells, either being spherical, single cells or dumbbell-shaped cells, which undergo separation. Besides the growth kinetics in liquid cultures, microscopic analyses of the cell division activity were conducted. For this purpose, photoautotrophically grown, mid-exponential cells (OD<sub>750</sub> = 0.3-0.6) were distributed to sterile 1.5 ml-reaction tubes, supplied with glucose at a final concentration of 10 mM and incubated under biomatatforming conditions (without agitation, 28°C, continuous illumination with an intensity of 20 µmol photons m<sup>-2</sup>s<sup>-1</sup>). Cell division activity in WT and *hik*12 KO was analysed before the addition of glucose and the transition to biomatatforming conditions (0 d) and after incubation under biomatatforming conditions for 14 days by determining the relative amount of dumbbell-shaped cells (Figure 13). The analysis revealed that in photo-
autotrophically grown WT and hik12 KO no significant differences in the relative amount of dumbbell-shaped cells and thus in cell division activity were observable before incubation under biomatforming conditions (0 d). In contrast, continuous incubation under biomatforming conditions for 14 days led to a significant decrease in the cell division activity in hik12 KO ($P < 0.001$, Student’s $t$ test), thereby confirming the results of the growth kinetics under biomatforming conditions (Figure 12 C).

**Figure 13:** Cell division activity in WT and hik12 KO before (0 d) and after 14 d of incubation under biomatforming conditions.

Cell division activity in WT and hik12 KO was analysed microscopically by determining the ratio between single, spherical cells and dumbbell-shaped cells undergoing separation, before incubation under biomatforming conditions and the addition of glucose (0 d) and after incubation under biomatforming conditions for 14 d. Data are presented as the relative amount of dumbbell-shaped cells. Columns represent average values of three independant experiments. Bars represent standard errors. Asterisks indicate significant difference at $P < 0.001$ according to Student’s $t$ test.

### 3.2.4 Incubation under biomatforming conditions results in increased glycogen content in hik12 KO

In cyanobacteria, photosynthetic carbon assimilation results in the accumulation of polysaccharides, mostly glycogen (Nakamura *et al.*, 2005). Glycogen serves as reserve carbohydrate to meet the metabolic needs under non-photosynthetic conditions (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003; Singh & Sherman, 2005; Takahashi *et al.*, 2008) or for adaptatational processes in response to environmental stress stimuli (Preiss, 1984; Suzuki *et al.*, 2010). To analyse the glycogen content within the cells, the glycogen content was determined in hik12 KO and WT after incubation under biomatforming conditions. For this purpose, photo-autotrophically grown, mid-exponential cultures were set to an $\text{OD}_{750}$ of 0.4, supplied with glucose at a final concentration of 10 mM and incubated for 14 d under biomatforming conditions (without agitation, 28°C, continuous illumination with a light intensity of 20 µmol photons m$^{-2}$ s$^{-1}$).
The glycogen content in \textit{hik12} KO and WT cells was determined after 14 days of incubation under biomatformation conditions. The resulting glycogen contents are presented as \(\mu g\) glycogen/OD\textsubscript{750} and are displayed in Figure 14. The analysis revealed a significant increase in the glycogen content in \textit{hik12} KO cells (\(P < 0.01\), Student’s \(t\) test), which amounts to more than twice as much as in WT cells.

![Glycogen content in WT and \textit{hik12} KO after 14 days of incubation under biomatformation conditions.](image)

**Figure 14:** Glycogen content in WT and \textit{hik12} KO after 14 days of incubation under biomatformation conditions.

Specific glycogen contents are given as \(\mu g\) glycogen per OD\textsubscript{750}. Columns represent average values of 3 (WT) and 5 (\textit{hik12} KO) independent experiments. Bars represent standard errors. Asterisks indicate significant difference at \(P < 0.01\) according to Student’s \(t\) test.

### 3.2.5 Incubation under biomatformation conditions results in a reduced G6PDH activity in \textit{hik12} KO

Due to its key function in the OPPP, the central glucose degrading pathway in cyanobacteria (Schaeffer & Stanier, 1978; Yang \textit{et al.}, 2002; Singh \textit{et al.}, 2004), the enzyme activity of G6PDH was analysed in WT, \textit{hik12} KO and \textit{hik12} OE, grown under photoautotrophic, photomixotrophic and biomatformation growth conditions. Photoautotrophically grown, mid-exponential cultures of WT, \textit{hik12} KO and \textit{hik12} OE were incubated in presence (photomixotrophic conditions) and absence (photoautotrophic conditions) of 10 mM glucose for 24 h (140 rpm, 28°C, continuous illumination with a light intensity of 20 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)). Specific G6PDH enzyme activities were determined and are presented in Figure 15 A. Data are normalized to WT under photoautotrophic growth conditions. Under photoautotrophic conditions, \textit{hik12} KO and \textit{hik12} OE 4 displayed slightly, but not significantly reduced G6PDH activities compared to the WT. The exposure to glucose for 24 h under photomixotrophic conditions resulted in slight, but not significantly reduced G6PDH activity in WT, \textit{hik12} KO and \textit{hik12} OE. The decrease of G6PDH enzyme activity in WT, \textit{hik12} KO and OE might be explained by a less developed glucose tolerance of this particular WT strain and its deriving KO and OE. For the analysis of specific G6PDH activities under biomatformation conditions, photoautotrophically grown, mid-
exponential cultures of WT, hik12 KO and hik12 OE were supplied with glucose and incubated without agitation, at 28°C, under continuous illumination with a light intensity of 20 µmol photons m⁻² s⁻¹. After 96 h of incubation under biomatatforming conditions, the cells were assayed for the activities of G6PDH. Data are presented in Figure 15 B and are normalized to the WT.

![Figure 15: Specific G6PDH enzyme activity of WT, hik12 KO and hik12 OE under photoautotrophic and photomixotrophic (A) and under photomixotrophic/biomatatforming (B) growth conditions.](image)

(A) Photoautotrophically grown, mid-exponential (OD₇₅₀ = 0.6-0.8) cell cultures of WT and hik12 mutant strains (hik12 KO and hik12 OE clone 4) were assayed for the activities of G6PDH in presence and absence of glucose. Where indicated, the cells were supplied with glucose at a final concentration of 10 mM (Glc) for 24 h, before the extraction of proteins. 100 µg of crude protein were used in a total volume of 1 ml assay solution to determine G6PDH activity. Data are normalized to the WT under photoautotrophic growth conditions (0.116 ± 0.007 U/µg, set as 100% for each individual experiment). Columns represent average values of 4 independent experiments. Bars represent standard errors.

(B) Photoautotrophically grown, mid-exponential cell cultures (OD₇₅₀ = 0.6-0.8) were supplied with glucose at a final concentration of 10 mM and incubated without agitation for 96 h. 100 µg of crude protein were used in a total volume of 1 ml assay solution to determine G6PDH activity. Data are normalized to the WT (0.064 ± 0.013 U/µg, set as 100% for each individual experiment). Columns represent average values of minimum 3 independent experiments. Bars represent standard errors. Asterisks indicate significant difference at $P < 0.001$ according to Student’s t test.

Incubation under biomatatforming conditions for 96 h resulted in a significantly decreased G6PDH activity in hik12 KO compared to the WT ($P < 0.001$, Student’s t test), which indicates a deficiency in the ability of hik12 KO to utilise exogenous glucose as carbon source. Overexpression of HIK12 resulted in a partial complementation of G6PDH activity, reaching more than double the value than in hik12 KO. A reduced G6PDH activity under biomatatforming conditions was verified in the background of another WT strain (provided by K. Marin, University of Cologne, Cologne, Germany). This KO line was generated by full replacement of the coding sequence of Hik12 with a kanamycin resistance cassette as detailed in Section
2.1.3 and in Supplementary figure 1. As shown in Supplementary figure 2, the KO of Hik12 results in reduced G6PDH activities compared to the WT.

3.2.6 hik12 KO is impaired in the upregulation of CtaDI gene expression under biomat forming conditions

The expression patterns of genes involved in glucose metabolism were analysed in WT, hik12 KO and hik12 OE under different growth conditions (photoautotrophic, photomixotrophic and biomat forming conditions). Total RNA of mid-exponential (OD<sub>750</sub> = 0.6-0.8) WT, hik12 KO and hik12 OE cell cultures was isolated after 24 h under photoautotrophic/photomixotrophic conditions and after 96 h under biomat forming conditions and subjected to semi-quantitative two-step RT-PCR.

![Gene expression analysis of key metabolic genes in WT, hik12 KO and hik12 OE by semi-quantitative RT-PCR.](image)

Figure 16: Gene expression analysis of key metabolic genes in WT, hik12 KO and hik12 OE by semi-quantitative RT-PCR.

Effects of different growth conditions on the expression of key metabolic genes were examined by semi-quantitative RT-PCR. Total RNA of WT and hik12 mutants (hik12 KO and hik12 OE clone 4) was isolated after 24 h under photoautotrophic (A) and photomixotrophic (in presence of 10 mM glucose) (B) conditions and after 96 h under biomat forming conditions (C). After reverse transcription, 500 ng of first strand cDNA was used as template for semi-quantitative RT-PCR (32 cycles). PCR products were analysed on a 1.5% agarose gel and stained with ethidium bromide. Data presented are representative for three independent experiments. Ribosomal RNA was tested before first strand cDNA synthesis to ensure non-degradation of total RNA (data not shown). The constitutively expressed RNase P gene (RnpB) was included as control.

Analysed genes: RnpB (slr0249), housekeeping; Zwf (slr1843), G6PDH; CtaDI (slr1137), cytochrome c oxidase.

The expression patterns of two genes, encoding for key enzymes in glucose metabolism (Zwf and CtaDI) and one housekeeping gene (RnpB) were analysed in WT, hik12 KO and hik12 OE under different growth conditions (Figure 16). The analysis of the transcript levels after 24 h of incubation under photoautotrophic conditions (Figure 16 A) and photomixotrophic conditions (Figure 16 B) revealed relatively low, but similar expression levels of Zwf and CtaDI in WT, hik12 KO and hik12 OE. After incubation under biomat forming conditions for 96 h, distinct changes in the gene expression pattern were observable. The transcript levels of CtaDI in WT and hik12 OE were significantly upregulated compared to photoautotrophic and photomixotrophic conditions, whereas hik12 KO showed no induced transcript
accumulation of CtaDI under biomat forming conditions (Figure 16 C). The complementation of hik12 KO with hik12 OE construct resulted in a CtaDI expression pattern similar to the WT, indicating that the deficiency in the KO mutant to up-regulate CtaDI under biomat forming conditions resulted from the deletion of Hik12.

3.2.7 Analysis of electron transport activities

To analyse electron transport activities in the thylakoid membrane, reduction kinetics of oxidized P700 (P700\(^+\)) were recorded in samples of WT, hik12 KO and hik12 OE in presence and absence of specific electron transport inhibitors. P700\(^+\) reduction kinetics were recorded in photoautotrophically grown, mid-exponential WT, hik12 KO and hik12 OE before the addition of glucose and the transition to biomat forming conditions (0 h), which refers to photoautotrophic growth conditions and after incubation under biomat forming conditions (in presence of glucose, without agitation, under continuous illumination) for 24 h. In terms of evaluation, the half-life (t\(_{1/2}\)) of the re-reduction of P700\(^+\) was determined as described in Section 2.1.9. Detailed information about electron flows and the sites of action of the inhibitors used are given in Figure 9. T\(_{1/2}\) of P700\(^+\) reduction in untreated, DCMU- and DCMU/DBMIB-treated samples of WT, hik12 KO and hik12 OE are presented in Figure 17 A and B, before and after 24 h of incubation under biomat forming conditions, respectively.

![Graph A](image)

**Figure 17:** Half-life (t\(_{1/2}\)) of P700\(^+\) reduction in WT, hik12 KO and hik12 OE before (0 h) and after 24 h of incubation under biomat forming conditions in presence and absence of specific electron transport inhibitors.

Cell cultures of WT, hik12 KO and hik12 OE were photoautotrophically grown to an OD\(_{750}\) of 0.6. P700\(^+\) reduction kinetics were recorded before the addition of glucose and the transition to biomat forming conditions (0 h) and after 24 h of incubation under biomat forming conditions (24 h), in presence of glucose at a final concentration of 10 mM, without agitation and under continuous illumination with a light intensity of 20 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\). Measurements were conducted in untreated (control), DCMU and DCMU/DBMIB-treated cells (final concentrations, 10 \(\mu\)M [DCMU], 60 \(\mu\)M [DBMIB]). Data represent the average value of 3 independent experiments. For statistical analysis an ANOVA test was performed with a post-hoc Tukey’s highly significant difference (HSD) test at \(P = 0.05\) for each group (control, DCMU, DCMU/DBMIB).
of P700$^+$ reduction of WT, hik12 KO and hik12 OE displayed no significant differences before and after 24 h of incubation under biomat-forming conditions, in presence and absence of both inhibitors tested. In samples of WT, hik12 KO and hik12 OE adapted to photoautotrophic conditions, treatment with DCMU resulted in increased $t_{1/2}$ of P700$^+$ reduction, indicating a reduced electron flow to P700$^+$. Since the linear electron transport is blocked by DCMU, the reduced electron flow to P700$^+$ indicates a reduced electron input into the PQ pool via SDH and/or via NDH-1 (originating from NADPH, which was generated by glucose degradation or PS I during blue light illumination) in samples adapted to photoautotrophic conditions. Simultaneous treatment with DCMU and DBMIB, the latter inhibits the electron input from the PQ pool into the cytochrome $b_{6}f$ complex, resulted in highly increased $t_{1/2}$ of P700$^+$ reduction in WT, hik12 KO and hik12 OE compared to DCMU-treated samples. The inhibition with DBMIB does not result in a complete electron blockage, causing a strongly reduced electron flow to P700$^+$, which is reflected in the observed increase in $t_{1/2}$ of P700$^+$ reduction in WT, hik12 KO and hik12 OE before and after incubation under biomat-forming conditions. In comparison to samples adapted to photoautotrophic conditions, treatment with DCMU after incubation under biomat-forming conditions resulted in only slightly increased $t_{1/2}$ of P700$^+$ reduction in WT, hik12 KO and hik12 OE, indicating an increased electron input under biomat-forming conditions. As a result, $t_{1/2}$ of P700$^+$ reduction in DCMU-treated samples after biomat induction amounts to approximately half the value compared to samples adapted to photoautotrophic conditions. This could be explained by a higher electron input into the electron transport chain from heterotrophic sources after biomat induction in presence of glucose. In conclusion, electron transport activities do not differ between WT, hik12 KO and hik12 OE after 24 h of biomat induction in the light.

3.2.8 hik12 KO shows constitutive ROS production

Reactive oxygen species (ROS) are highly reactive compounds, which are produced as toxic byproducts of respiratory and photosynthetic electron transport and during stress situations (Tichy & Vermaas, 1999; Apel & Hirt, 2004; Pérez-Pérez et al., 2009; Bernroitner et al., 2009). The most important ROS include the superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and the hydroxyl radical (OH$^-$) and are produced during the stepwise reduction of molecular oxygen (O$_2$) to water (H$_2$O). In plants, ROS are known to play an important role in defense reactions against pathogen attacks (Lamb & Dixon, 1997). As the phytohormone cytokinin recently emerged to play a major role in plant-pathogen interactions (Walters et al., 2008; Pertry et al., 2009; Choi et al., 2011) and due to sequence homology of HIK12 to the
Arabidopsis cytokinin receptor family, the ROS production in the cyanobacterial cell cultures was determined, using a luminol-based assay. ROS-mediated oxidation of luminol was recorded as relative chemoluminescence units for 60 min in photoautotrophically grown, mid-exponential WT and hik12 KO cells, in presence and absence of glucose. Luminol assay solution-buffer without cells was included as control. Measurements were carried out in 96-well plates in a microtiter plate luminometer. The total volume of 250 µl per well consisted of 100 µl of cell culture and 150 µl of luminol assay solution. As shown in Figure 18, no distinct chemoluminescence reactions and thus no ROS production was detectable in WT cells, except from background emissions. A striking increase in chemoluminescence emissions, indicative of ROS production, was detectable in hik12 KO cells during the initial 20 min of the measurement. Thereafter, a phase of relatively constant chemoluminescence emission signals was reached, which slowly declined in the last 20 min of the measurement. Furthermore, the addition of glucose, prior to the measurement, did not affect chemoluminescence emissions, neither in WT nor in hik12 KO cells.

Figure 18: ROS production in WT and hik12 KO cells in presence and absence of exogenous glucose.

ROS-mediated oxidation of luminol was recorded as relative chemoluminescence units for 60 min in photoautotrophically grown, mid-exponential WT (circles) and hik12 KO (rectangles) cells, in presence (open symbols) and absence (filled symbols) of 10 mM glucose. Luminol assay solution-buffer without cells was included as control (filled triangle). Error bars represent standard deviation, based on mean values of five samples per strain and condition (+/− glucose). Data presented are representative for three independent experiments.
3.3 Analysis of cytokinin function in plant-pathogen interactions

Cytokinins are a class of aminopurine-based phytohormones that regulate various physiological and developmental processes in higher plants, which include cell division and differentiation, chloroplast development, seed germination, delay of leaf senescence, nutrient mobilization and stress responses (Kakimoto, 2003; Balibrea Lara et al., 2004; Sakakibara, 2006; Müller & Sheen, 2007, Bari & Jones, 2009; Hwang et al., 2012). Recently, evidence has been found that cytokinins are involved in processes related to defense mechanisms during plant-pathogen interactions (Argueso et al., 2009; Choi et al., 2010). To provide further insight into cytokinin function in plants and their involvement in defense mechanisms, different approaches, implying transient overexpression of cytokinin-degrading enzymes in the biotrophic host-pathogen system barley-Blumeria graminis f.sp. hordei and the analysis of an Arabidopsis cytokinin receptor-deficient mutant in interaction with Pseudomonas syringae pv. tomato DC3000 (Pst) were conducted.

3.3.1 Simultaneous overexpression of two cytokinin oxidases in barley cells increased resistance to powdery mildew

To examine the function of cytokinin in defense mechanisms, the effect of altered cytokinin levels was analysed during plant-pathogen interaction. Therefore, two cytokinin degrading enzymes of Arabidopsis, the cytokinin oxidases AtCKX1 and AtCKX2, were transiently overexpressed in barley cv. Pallas leaves and analysed in interaction with the barley-powdery mildew fungus Blumeria graminis f.sp. hordei (Bgh). In this compatible interaction, the fungus penetrates the plant cell and develops its characteristic digitate-shaped feeding organ, the haustorium. Ballistic transformation of single cells is a reliable method for assessing gene functions in plant-pathogen interactions, in particular in interactions of members of the tribe Triticeae and powdery mildew fungi (Panstruga, 2004). Barley epidermal cells were transiently transformed via particle bombardment, inoculated with Bgh spores and analysed microscopically. Tungsten particles served as microcarriers to deliver the expression vectors into single epidermal cells. The particles were coated with plasmid DNA of the expression construct of AtCKX1, AtCKX2, AtCKX1 and AtCK2 together or the empty vector as control, together with plasmid DNA of the green fluorescent protein (GFP) as reporter gene. All genes were subcloned into the plant expression vector pGY1 to be controlled by the constitutive cauliflower mosaic virus (CaMV) 35S promoter (Hückelhoven et al., 2003; Schweizer et al., 1999). Due to the
CaMV 35S promoter, pGY1 can be used for overexpression of genes in plant cells. Four hours after transformation, the barley leaves were inoculated with spores of *Bgh* giving a density of 150 conidia mm$^{-2}$. 48 hours after inoculation (hai), transformed cells were identified by fluorescence microscopy for the presence of GFP fluorescence. Using fluorescence microscopy, the outcome of interactions was evaluated as penetration efficiencies (PE), thereby distinguishing between attacked and successfully penetrated cells (haustorium-containing cells) and attacked and non-penetrated cells (papilla forming cells). Microscopic analysis revealed, that single overexpression of AtCKX1 (Figure 19 A) and AtCKX2 (Figure 19 B), resulted in no significant differences in PE of *Bgh* in transformed barley epidermal cells. To analyse potential synergistic effects, both cytokinin oxidases were simultaneously overexpressed. As shown in Figure 19 C, simultaneous overexpression of AtCKX1 and AtCKX2 resulted in a significantly reduced PE of *Bgh* ($P < 0.05$, Student’s $t$ test).

![Figure 19: Penetration efficiencies of *Bgh* spores on transiently transformed barley epidermal cells, overexpressing *Arabidopsis* cytokinin oxidases AtCKX1 (A), AtCKX2 (B) and AtCKX1/AtCKX2 simultaneously (C).](image)

Penetration efficiencies (PE) of *Blumeria graminis* f. sp. *hordei* (*Bgh*) spores on barley epidermal cells (cv. Pallas), transiently overexpressing *Arabidopsis* cytokinin oxidases AtCKX1 (A), AtCKX2 (B) and AtCKX1/AtCKX2 simultaneously (C). As control, cells were transformed with the empty vector pGY1. Co-transformed GFP served as reporter gene, allowing the detection of transformed cells by means of fluorescence microscopy. Columns in A, B and C represent average values of three independent experiments (in each experiment a minimum of 80 interactions was analysed). Bars represent standard errors. The asterisk indicates significant difference at $P < 0.05$ according to Student’s $t$ test.

### 3.3.2 The *Arabidopsis* AHK3 cytokinin receptor mutant is impaired in the defense response to *P. syringae*

Besides the involvement in various developmental processes, cytokinins are known to have a profound effect on leaf senescence (Mok, 1994; Kim *et al.*, 2006). A direct link between cytokinin effects and plant responses to biotrophic pathogens has been suggested (Walters *et al.*, 2008), as defense responses are associated with altered senescence phenotypes, well known as the phenomenon of “green island” formation.
In Arabidopsis, the cytokinin signal is perceived by three sensor histidine kinases AHK2, AHK3 and AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Among them, AHK3 has been identified as the cytokinin receptor controlling senescence in leaves (Kim et al., 2006). To investigate the function of cytokinins in plant-pathogen interactions, the AHK3 loss-of-function T-DNA insertion line (NASC Nr. N6562; Higuchi et al., 2004; Nishimura et al., 2004) was analysed in pathogen assays during the interaction with the hemibiotrophic pathogen Pseudomonas syringae pv. tomato DC3000 (Pst). The insertion of T-DNA in AHK3-T-DNA insertion plants used was confirmed by PCR on genomic DNA (Supplementary figure 3). To monitor the development of disease symptoms, detached leaves of 6-week old Arabidopsis WT and AHK3 cytokinin receptor-deficient (ahk3 KO) plants were infiltrated with Pst at a density of $10^4$ cfu ml$^{-1}$ in 10 mM MgCl$_2$ and incubated on water agar in darkness. Pictures of infiltrated leaves were taken at 5 and 8 days after inoculation (dai) and are representative for three independent experiments (Figure 20 A).

![Figure 20: Responses of Arabidopsis AHK3 receptor-deficient mutant and WT plants to Pseudomonas syringae pv. tomato DC3000 (Pst).](image)

(A) Disease symptom development in detached leaves of 6-week old Arabidopsis WT (Wasilewska) and AHK3 receptor-deficient (ahk3 KO) plants, infiltrated with a bacterial suspension of virulent Pseudomonas syringae pv. tomato DC3000 at a density of $10^4$ cfu ml$^{-1}$ in 10 ml MgCl$_2$. Mock inoculation with 10 mM MgCl$_2$ was used as control. The infiltrated leaves were incubated on water agar (0.5% w/v) in darkness. Pictures of infiltrated leaves were taken at 5 and 8 dai and are representative for 3 independent experiments.

(B) Bacterial proliferation in WT and ahk3 KO. Infiltrated, non-detached leaves were sampled at 2 and 4 dai. The infiltrated areas were cut out, scaled, ground in 10 mM MgCl$_2$ and dropped in 7x10 µl droplets in dilution rows on King’s B medium agar plates. After 2 days of incubation, bacterial colonies were counted and quantified by evaluating the amount of bacteria per gram leaf material. Data are expressed in colony forming units (cfu) and are normalized to the WT at 2 dai ($1.48\times10^7$ cfu/g fresh weight). Columns represent average values of 3 independent experiments. Bars represent standard errors.

Pst is infectious for Arabidopsis and becomes visible as chlorotic leaf spots turning into necrosis. As shown in Figure 20 A, ahk3 KO leaves displayed a clear phenotype in disease symptom development, comprising of necrotic areas at the site of Pst.
infiltration, while mock infiltration with 10 mM MgCl₂ did not lead to the development of necrotic lesions. The disease symptoms in ahk3 KO leaves developed markedly faster compared to the WT. The necrotic lesions in WT leaves were not formed until 8 dai, while the leaves of ahk3 KO were rendered almost entirely chlorotic at 8 dai.

To determine bacterial proliferation in WT and ahk3 KO, non-detached leaves were infiltrated as described above. Samples were harvested at 2 and 4 dai. The infiltrated areas were cut out, scaled, ground in 10 mM MgCl₂ and dropped in dilution rows on King’s B medium agar plates. After 2 days of incubation, bacterial colonies were counted and quantified by evaluating the amount of bacteria per gram leaf material. Data are expressed in colony forming units (cfu) and are normalized to the WT at 2 dai. The analysis of bacterial growth sampled from infiltrated WT and ahk3 KO revealed that Pst proliferates faster in ahk3 KO compared to the WT (Figure 20 B). The amount of bacteria sampled from ahk3 KO leaves at 2 dai was more than three times as high as in WT leaves. From 2 dai to 4 dai, the amount of Pst sampled from WT leaves almost doubled, while Pst from ahk3 KO leaves increased almost three-fold. These results further support the observed phenotype in disease symptom development, thereby indicating a function of AHK3 in the defense response of Arabidopsis to Pseudomonas syringae pv. tomato.

### 3.3.3 Arabidopsis ahk3 KO displays constitutive ROS production

To analyse the ROS production in Arabidopsis WT and cytokinin receptor-deficient mutant (ahk3 KO) leaves, luminol-based assays were conducted. ROS-mediated oxidation of luminol was recorded as relative chemoluminescence units for 60 min in samples of Arabidopsis WT and ahk3 KO leaves, in presence and absence of glucose. Luminol assay solution/buffer without leaf samples was included as control. Prior to the measurement, the excised leaf samples were incubated floating on a water surface. Measurements were carried out in 96-well plates in a microtiter plate luminometer. Two leaf pieces per well were added to 200 µl assay solution/buffer, immediately before the measurement. As shown in Figure 21, no distinct chemiluminescence reactions and thus no ROS production was detectable in the WT, except from background emissions. In contrast, increasing chemoluminescence emission signals, indicative of ROS production, were detectable in ahk3 KO, until a phase of relatively constant chemoluminescence emission signals was reached after 30 min. The addition of glucose prior to the measurement, did not affect chemoluminescence emission in WT and ahk3 KO.
Figure 21: ROS production in *Arabidopsis* WT and ahk3 KO leaf explants in presence and absence of glucose.

ROS-mediated oxidation of luminol was recorded as relative chemoluminescence units for 60 min in leaf samples (cut in square pieces of 4 mm$^2$) of 6-week old *Arabidopsis* WT (circles) and ahk3 KO (rectangles) samples. Measurements were carried out in presence (open symbols) and absence (filled symbols) of 10 mM glucose. Luminol assay solution/ buffer without leaf samples was included as control (filled triangle). Prior to the measurement, excised leaf samples were incubated floating on a water surface over night in a climate chamber at 22°C with a relative humidity of 64% and a photoperiod of 10 h light irradiance at 120 μmol photons m$^{-2}$s$^{-1}$. Data presented are representative for three independent experiments. Error bars represent standard deviation, based on mean values of five samples per strain and condition (+/- glucose).
4 Discussion

Analysing the metabolic and physiological responses to individual gene deletions can provide deeper insights into an organism’s control and regulation of the metabolism. During the endosymbiotic event, many cyanobacterial genes have been transferred to the plant nucleus. In particular, genes coding for proteins involved in signal transduction are highly conserved among cyanobacteria and plants, which renders cyanobacteria into suitable model organisms for the analysis of signal transduction mechanisms.

This thesis contributes to the understanding of signalling processes and their effects on the metabolism by analysing the functions of the signalling components CPH2 and HIK12 in the cyanobacterium *Synechocystis* sp. PCC 6803. For CPH2, an involvement of this cyanobacterial phytochrome photoreceptor in various light-dependent processes has been demonstrated (Park et al., 2000; Wilde et al., 2002; Fiedler et al., 2004; Moon et al., 2011; Savakis et al., 2012), while HIK12 is one of more than 40 histidine kinases in *Synechocystis* of yet indefinite function, but with similarity to plant cytokinin receptors.

In the present study, knockout and overexpression mutants were physiologically analyzed under distinct growth conditions, with particular interest in the central carbon metabolism and the transition from fast exponential to slowed down growth in biomats. The obtained results show a clear regulatory function of both proteins on the central carbon metabolism, which is substantiated in a strongly retarded growth phenotype in the knockout mutants, correlating with a reduced G6PDH activity and altered transcript levels of cytochrome c oxidase encoding *CtaDI* gene, which manifests after the transition to biomatformating conditions, in *cph2* KO under heterotrophic, in *hik12* KO under photomixotrophic conditions.

Besides the well-known involvement in essential developmental and physiological processes throughout the plant life, the phytohormone cytokinin has recently emerged as main factor in plant-pathogen interactions. The last part of this thesis focuses on the function of cytokinin in regulating susceptibility or basal resistance in the interaction of pathogens with barley and *Arabidopsis*. The results obtained further support an essential function of cytokinins in plant-pathogen interactions by confirming that cytokinins affect plant immunity.
4.1 Physiological characterisation of the cyanobacterial phytochrome CPH2 and its regulatory function on the central carbon metabolism

Light is a basic need for all photosynthetic organisms. With the diurnal changes in light quality and quantity, photosynthetic organisms are in the constant need to adapt to changing light conditions, which requires appropriate adjustments of the metabolism in order to maintain energy homeostasis. In cyanobacteria, the response to changes in light intensity and spectral qualities is accomplished by various different photoreceptors coupled to signal transduction networks (Montgomery, 2007). Among them, the best-studied photoreceptors in cyanobacteria are the bilin-containing photoreceptors of the phytochrome family (Rockwell & Lagarias, 2010). These photoreceptors photointerconvert between red (Pr) and far-red (Pfr) absorbing forms by utilising a tetrapyrrrole chromophore that is covalently bound to a cysteine residue. Like in plants, the cyanobacterial phytochrome CPH1 (slr0473) contains an N-terminal photosensory module, which confers Pr/Pfr-like photochromicity (Yeh et al., 1997; Hughes et al., 1997). The related phytochrome-like photoreceptor CPH2 (sll0821) differs in its domain organisation. CPH2 is a hybrid photoreceptor, comprising of an N-terminal Pr/Pfr photosensory module (Park et al., 2000; Wu & Lagarias, 2000) and a C-terminal cyanobacteriochrome module that photointerconverts between blue and green light absorbing forms (Savakis et al., 2012). Furthermore, CPH2 possesses GGDEF and EAL domains, whose diguanylate cyclase (DGC) and phosphodiesterase (PDE) activities are known to be involved in the synthesis and breakdown of the second messenger c-di-GMP, respectively (Ryan et al., 2006; Cotter & Stibitz, 2007; Pesavento & Hengge, 2009; Yan & Chen, 2010). Most GGDEF and EAL domains found in bacteria are coupled to a signal input or sensory domain, which include blue light sensing (Barends et al., 2009), red/far red light sensing (GAF-PHY domain), domains involved in oxygen sensing (PAS domains) and phytochrome binding (GAF-PHY domains) (Hengge, 2009; Galperin, 2006; Schirmer & Jenal, 2009). Upon perception of environmental changes, e.g. changes in light abundance or oxygen, the primary signals are transmitted to DGC or PDE that activate the biosynthesis or degradation of c-di-GMP, which functions as allosteric regulator of downstream target proteins (Yan & Chen, 2010). Although much about the downstream regulation cascades remains unknown, c-di-GMP has been shown to play an important role in adhesion, cell-to-cell communication, exopolysaccharide formation and motility (Tischler & Camilli, 2004) and in particular, in many bacterial species in regulating the transition between
motile and sessile lifestyles (Römling et al., 2005; Jenal & Malone, 2006; Cotter & Stibitz, 2007; Pesavento & Hengge, 2009; Yan & Chen, 2010).

Based on the structural organisation of CPH2, including the ability to perceive red/far-red and blue light and the existence of GGDEF and EAL domains, it is conceivable that light perceived by CPH2 modulates the activity of GGDEF and EAL domains. This was recently confirmed by Savakis and co-workers (2012), who demonstrated that c-di-GMP is a functional second messenger in *Synechocystis* sp. PCC 6803 and that CPH2 functions as its primary, light-regulated modulator. Aside from its involvement in adaptionial processes during light-dark transitions (Park et al., 2000) and the regulation of growth under changing light conditions (Fiedler et al., 2004), CPH2 was reported to be involved in phototactic motility, in particular in the inhibition of phototaxis towards blue light (Wilde et al., 2002) and UV-A light (Moon et al., 2011). Savakis and co-workers (2012) provided the first evidence that c-di-GMP is directly involved in the light-dependent regulation of cyanobacterial phototaxis.

In this study, the physiological effects of the loss of CPH2 in *Synechocystis* sp. PCC 6803 were analysed with a special focus on the central carbon metabolism. For this purpose, growth kinetics, specific enzyme activities and gene expression patterns for WT, *cph2* knockout (loss-of-function) and *cph2* overexpression (complementation) mutants were analysed under different growth conditions.

### 4.1.1 Deletion of CPH2 negatively affects growth under LAHG/biomatforming conditions

To screen for physiological effects, growth kinetics were analysed under different growth conditions in WT, *cph2* KO and *cph2* OE. The analysis of the growth kinetics revealed no differences in growth between WT, *cph2* KO and *cph2* OE under photoautotrophic and photomixotrophic growth conditions (Figure 6 A, B). The accelerated growth upon addition of glucose under photomixotrophic conditions (Figure 6 B) compared to photoautotrophic conditions, indicates that WT, *cph2* KO and *cph2* OE are capable of utilising exogenous glucose and therefore are capable of photomixotrophic growth (Williams, 1988; Anderson & McIntosh, 1991). This is in accordance with findings of Knowles and co-workers (2003) and Anderson and McIntosh (1991), who reported similar growth profiles for *Synechocystis* sp. PCC 6803 under photoautotrophic and photomixotrophic growth conditions.

For CPH2, Fiedler and co-workers (2004) demonstrated an involvement in photoautotrophic growth under different light qualities and quantities, which was reflected in decreased growth rates of *cph2* knockout mutants under red light (660 nm).
illumination. The present study focuses on the function of CPH2 under LAHG/biomatatforming conditions. To this end, growth of WT, cph2 KO and cph2 OE was analysed after the transition from light to darkness with a daily amount of 15 min white light, in presence of glucose as exogenous carbon source. These conditions are referred to as light-activated heterotrophic growth (LAHG) (Anderson & McIntosh, 1991; Tabei et al., 2009). For Synechocystis to grow heterotrophically on glucose, a minimal amount of light, e.g. for 5 min every 24 h, is necessary to keep the cells metabolically active (Tabei et al., 2009). LAHG was chosen as it allows to exclude almost entirely the effect of photosynthesis on the metabolism of the cells. Furthermore, due to the existence of GGDEF and EAL domains in CPH2, we speculated about a function of CPH2 in the switch between motile and sessile lifestyles. The analysis of growth kinetics revealed that incubation under LAHG/biomatatforming conditions led to a considerably lower growth rate compared to that obtained under photoautotrophic or photomixotrophic conditions. Most of all, the cph2 KO mutant was characterized by growth cessation under LAHG conditions (Figure 6 C). Under non-photosynthetic conditions, the cells have to meet their energy demands for maintenance and growth by switching their metabolic mode to light-activated heterotrophic energy generation (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003). The primary energy source for heterotrophic metabolism is glucose, deriving from glycogen catabolism or exogenous glucose. Hence, the growth cessation of cph2 KO might reflect a deficiency in utilising either exogenous glucose or glucose residues derived from carbon stores, thereby indicating a regulatory function of CPH2 on growth under LAHG/biomatatforming conditions. To elucidate the cause of the observed growth phenotype of cph2 KO further research of the sugar catabolism and its corresponding pathways was performed. For this purpose, the specific activity of G6PDH, an enzyme indispensable for glucose catabolism and the transcript levels of key metabolic genes were analysed.

4.1.2 Deletion of cph2 affects G6PDH enzyme activity and the transcript level of CtaDi, encoding cytochrome c oxidase, under LAHG/biomatatforming conditions

In cyanobacteria, the OPPP is widely accepted to be the main route of glucose catabolism, for both the breakdown of glycogen and exogenously supplied glucose (Hagen & Meeks, 2001; Yang et al., 2002). The initial step of glucose degradation is the conversion of glucose-6-phosphate to 6-phosphogluconolactone, which is catalyzed by the enzyme glucose-6-phosphate dehydrogenase (G6PDH). G6PDH represents the rate-determining step of glucose degradation by controlling the
carbon flow into the OPPP (Yang et al., 2002; Singh et al., 2004; Singh & Sherman, 2005). The analysis of the specific G6PDH enzyme activity provides further information about the assumed deficiency of cph2 KO in metabolising glucose, which is substantiated by a significantly reduced G6PDH enzyme activity in both independent cph2 loss-of-function mutants after incubation under LAHG/biomatforming conditions (Figure 7 B). After the transition from photoautotrophic to dark heterotrophic conditions in presence of glucose, CO₂ assimilation via the Calvin cycle is subsequently inactivated and the major metabolic pathway switches from the Calvin cycle to the OPPP (Pelroy et al., 1976). This is in accordance with the analysis of metabolic flux distributions by Yang and co-workers (2002), which revealed that over 90% of glucose is catabolized through the OPPP during heterotrophic growth. Thus, the strongly reduced G6PDH activity in cph2 KO should result in significantly lower glucose degradation by the OPPP. This in turn could explain the inability of cph2 KO to grow under LAHG/biomatforming conditions. In addition to the shift from photoautotrophy to dark heterotrophy, the cells were subjected to biomatforming conditions, which includes the transition from a motile, fast growing lifestyle to a sessile, slow-growing lifestyle with high cellular density within a biomat. In this context, the reduced enzyme activity in cph2 loss-of-function mutant might also indicate that CPH2 has a special function in the induction of G6PDH enzyme activity or in protein expression under biomatforming conditions. Notably, overexpression of CPH2 in the cph2 KO background resulted in complementation of enzyme activity and growth kinetics, confirming that the observed phenotype is caused by the deletion of Cph2. Furthermore, the fact that no significant differences in enzyme activities of WT, cph2 KO and cph2 OE were observable under photoautotrophic and photomixotrophic conditions (Figure 7 A) indicates a specific function of CPH2 during the shift from light to dark heterotrophy under biomatforming conditions.

The supply of glucose to photoautotrophically grown cells leads to various changes in growth and carbon metabolism (Yang et al., 2002; Knowles & Plaxton, 2003; Lee et al., 2005; Osanai et al., 2005; Singh & Sherman, 2005), as well as in photosynthesis and respiration (Ryu et al., 2004, Lee et al., 2005; Kurian et al., 2006). Despite the numerous responses to glucose, profiles of transcript abundance and protein levels did not show significant differences in photoautotrophic and photomixotrophic cultures (Yang et al., 2002; Knowles & Plaxton, 2003; Tu et al., 2004; Singh & Sherman, 2005; Kahlon et al., 2006), indicating that the response to glucose is mainly accomplished on alterations at the level of post-translational modifications or modulation of enzyme activities (Kahlon et al., 2006). To examine the effects of
cph2 deletion on the transcriptional level, gene expression profiles of two key metabolic and photosynthetic genes, respectively, were analysed using semi-quantitative RT-PCR. In addition to Zwf (slr1843), encoding for G6PDH, a second metabolism-related gene, CtaDI (slr1137), encoding for subunit I of the cytochrome c oxidase complex, the terminal component of the respiratory chain, and two photosynthesis-related genes, PsaA (slr1834) and PsbA3 (sll1867), encoding for subunit Ia of the P700 apoprotein and for PS II D1 protein, respectively, were analysed under different growth conditions. Consistent with the results obtained in growth and enzyme activities, the presence or absence of exogenous glucose did not significantly alter the gene expression patterns of both metabolic genes in WT, cph2 KO and cph2 OE, which is reflected in similar expression patterns for Zwf and CtaDI under photoautotrophic and photomixotrophic conditions (Figure 8 A, B). This is in accordance with Yang and co-workers (2002), who demonstrated that levels of mRNA transcripts encoding various key enzymes of the central carbon metabolism were not significantly altered upon the presence or absence of exogenous glucose. Solely, under both photoautotrophic and photomixotrophic growth conditions, the photosynthesis-related genes, PsaA and PsbA3 displayed a slightly induced transcript accumulation in the cph2 overexpression mutant (Figure 8 A, B). Using DNA microarrays, Singh and co-workers (2009) demonstrated that illumination of *Synechocystis* with light that preferentially excites PS II or PS I, has a significant impact on transcript levels of genes involved in various processes. They found that preferential excitation of *Synechocystis* cells with PS I light (\(\lambda_{\text{max}} \sim 455\) nm) or PS II light (\(\lambda_{\text{max}} \sim 627\) nm) led to the complementary regulation of photosystem genes. Possibly, the observed slightly induced transcript accumulation in cph2 complementation mutant (Figure 8 A, B) might be due to the spectral composition of light in the incubator and/or a side effect of the expression of cph2 under the control of the constitutive petJ promotor. Beyond that, changes in the redox poise of the PQ pool, possibly due to the overexpression, might also be considered to cause the observed changes in the expression levels in cph2 complementation mutant, since the expression levels of certain photosynthetic genes are regulated by the redox status of the PQ pool (Bendall & Manasse, 1995; Alfonso *et al*., 1999; Li & Singh, 2000; Ma & Mi, 2008).

The transition from photoautotrophic to LAHG and biomatiforming conditions revealed an interesting transcriptional pattern for cph2 KO. In contrast to the strong effect on the enzyme activity level (Figure 7 B), the transcript level of Zwf, encoding for G6PDH, was not significantly altered in WT, cph2 KO and cph2 OE under
LAHG/biomatforming conditions (Figure 8 C). This inconsistency of the Zwf transcript level and the enzymatic activity of G6PDH support the previously assumed regulation of G6PDH at the post-translational level. This corresponds to the findings of Yang and co-workers (2002), who demonstrated, using carbon isotope labelling, that the metabolic flux through G6PDH was enhanced under heterotrophic conditions, even though the transcript levels of Zwf remained unaltered. This might further indicate that the regulation of the metabolic flux through G6PDH was accomplished post-translational. This is also in accordance with the finding that the activities of enzymes involved in the glycolytic and OPP pathway, such as G6PDH, Fructose-1,6-bisphosphatase, phosphofructokinase, Fructose-1,6-bisphosphatase-aldolase and pyruvate kinase, are regulated by changes in metabolite concentrations (Stal & Moezelaar, 1997; Sundaram et al., 1998; Yang et al., 2002; Knowles & Plaxton, 2003; Lee et al., 2007). In the case of G6PDH, this includes NADPH, ATP, G6P, glutamine and Rib1,5P2 (Schaeffer & Stanier, 1978; Cossar et al., 1984; Plaxton & Podesta, 2006; Lee et al., 2007).

Under heterotrophic conditions, in the absence of light or at very low light intensities, the respiratory electron transport chain has a much higher capacity of electron flow than the photosynthetic electron transport chain (Vermaas, 2001). Under these conditions, catabolic pathways mainly provide electron input into the electron transport chain. Electrons from NADPH, generated through glucose oxidation, mainly by G6PDH, enter the electron transport chain via NDH-1, are transported via the PQ pool/cytochrome b$_{5}$f complex and are finally transferred to molecular oxygen. Among the three terminal oxidases identified so far in Synechocystis (Hart et al., 2005), cytochrome c oxidase has been confirmed to function as main terminal oxidase (Schmetterer, 1994) for electrons generated from NADPH (Howitt & Vermaas, 1998; Ryu et al., 2003). According to its function as terminal component of the respiratory electron transport chain, cytochrome c oxidase encoding gene CtaDI was analysed as second metabolism-related gene, thereby displaying an interesting transcriptional pattern in cph2 KO under LAHG/biomatforming conditions. In contrast to the unchanged transcript levels of Zwf and both photosynthetic genes, the transcript level of CtaDI, is clearly downregulated in cph2 KO mutant under LAHG/biomat- forming conditions (Figure 8 C). Furthermore, the complementation of cph2 KO displays a gene expression pattern for CtaDI similar to the WT (Figure 8 C). The phenotype in CtaDI expression has been observed specifically during acclimation to LAHG/biomatforming conditions. Thus, one could assume that CPH2 has a function in the regulation of cytochrome c oxidase expression under LAHG/biomatforming
conditions. The strong underexpression of CtaDI in cph2 KO under LAHG/biomat-forming conditions should result in a significantly reduced cytochrome c oxidase activity. Recent data (Rheinhard Pröls, Technische Universität München, Munich, Germany, personal communication) indeed show strongly reduced cytochrome c oxidase activity under these conditions for cph2 KO. This in turn could result in an overreduced electron transport chain, as the electrons cannot be removed. As G6PDH activity is inactivated under reducing conditions (Plaxton & Podesta, 2006; Lee et al., 2007), the underexpression of CtaDI might cause the lower G6PDH activity in cph2 KO. Therefore, cytochrome c oxidase might be a target of CPH2 in regulating the heterotrophic metabolism under LAHG/biomat-forming conditions. This hypothesis has been supported since recent measurements of fluorescence induction, which allow to monitor the redox state of the PQ pool, evidence the assumed overreduction of the PQ pool in cph2 KO (Rheinhard Pröls, Technische Universität München, Munich, Germany, personal communication).

4.1.3 P700⁺ reduction kinetics: Analysis of electron transport activities

To further sustain the regulatory function of CPH2 on the glucose metabolism under heterotrophic/biomat-forming conditions, P700⁺ reduction kinetics were used to analyse electron transport activities in the cyanobacterial thylakoid membrane. In Synechocystis, the thylakoid membrane contains both photosynthetic and respiratory electron transport chains, which intersect and share electron carriers (Figure 1) (Schmetterer, 1994; Vermaas, 2001). Among them, the PQ pool represents the central switching point of both respiratory and photosynthetic electron transport, where the electron inputs from photosynthetic linear, cyclic and respiratory electron transport pathways, converge. The PQ pool is oxidized by PS I and by cytochrome c oxidase via cytochrome b₅f complex or directly by cytochrome bd-type quinol oxidase (Endo, 1997). In addition to its central role in the electron transport chain, the redox state of the PQ pool regulates the transcription of various genes, among them, genes encoding for photosynthetic proteins (Pfannschmidt et al., 1999; Alfonso et al., 2000; Li & Sherman, 2000; Hihara et al., 2003; Kufryk & Vermaas, 2006). Furthermore, the redox poise of the PQ pool can directly mediate the regulation of metabolic processes (Alfonso et al., 1999, 2000; El Bissati & Kirillovsky, 2001; Vermaas, 2001; Ma & Mi, 2008).

In the light, the photosynthetic electron transport chain has a higher capacity of the electron flow than the respiratory chain (Vermaas, 2001). Accordingly, linear electron
transport from water to NADP⁺ via PS II and PS I, is the prevalent electron transport pathway, which implies that the PQ pool is mainly reduced by electrons deriving from water-splitting activity of PS II and not by electrons deriving from the heterotrophic metabolism. In samples adapted to photoautotrophic conditions, treatment with the PS II inhibitor DCMU resulted in increased t₁/₂ of P700⁺ reduction in WT, cph2 KO and cph2 OE compared to untreated samples (Figure 10 C). Since the linear electron transport is blocked by DCMU, electron input into the PQ pool originates from electron flows via SDH and NDH-1, from NADPH generated mainly by glucose oxidation in the heterotrophic metabolism or by PS I during blue light illumination. Due to the reduced amount of electrons, the PQ pool is more oxidized in DCMU-treated samples compared to untreated samples, resulting in increased t₁/₂ of P700⁺ reduction, as it takes longer to re-reduce P700⁺ after switching off blue light illumination.

In darkness or at very low light intensities, the respiratory electron transport chain has a higher capacity of electron input than PS II (Vermaas, 2001). In samples adapted to dark/biomatforming conditions, treatment with DCMU resulted in a significantly increased t₁/₂ of P700⁺ reduction in cph2 KO compared to the WT (Figure 10 D). The higher increase in t₁/₂ of P700⁺ reduction in cph2 KO indicates a reduced electron input into the PQ pool. This might either be caused by a reduced electron input from NADPH, generated by PS I during blue light illumination or by a reduced electron input from the heterotrophic metabolism, which would further sustain the previously assumed reduced ability of cph2 KO in metabolising glucose. A possible explanation for the reduced electron input from the heterotrophic metabolism is that G6PDH activity is significantly lower under LAHG/biomatforming conditions (Figure 7 B). An alternative explanation could be an overreduced PQ pool, resulting from the non-functionality of cytochrome c oxidase, which could inactivate G6PDH. DBMIB prevents the reoxidation of plastoquinone, thereby inhibiting the electron input from the PQ pool into the cytochrome b₆f complex. The inhibition with DBMIB does not result in a complete electron blockage, which explains why P700⁺ reduction is detectable. In samples adapted to photoautotrophic conditions, simultaneous treatment with DCMU and DBMIB resulted in increased t₁/₂ of P700⁺ reduction in WT, cph2 KO and cph2 OE (Figure 10 C), indicating a strongly restricted electron flow to P700⁺. After the transition to dark/biomatforming conditions, this effect is no longer observable, as WT, cph2 KO and cph2 OE displayed increased, but not significantly different t₁/₂ of P700⁺ reduction (Figure 10 D). Notably, the increase in t₁/₂ of P700⁺ reduction upon DCMU/DBMIB-treatment was higher in
samples adapted to photoautotrophic conditions than to dark/biomatforming conditions. This might indicate a reduced electron flux in samples adapted to dark/biomatforming conditions, resulting from a generally slowed down metabolism under dark/biomatforming conditions.

Together, the increase in $t_{1/2}$ of P700$^+$ reduction upon DCMU-treatment in cph2 KO adapted to dark/biomatforming conditions and the similar $t_{1/2}$ of P700$^+$ reduction upon DCMU/DBMIB-treatment in WT, cph2 KO and cph2 OE adapted to dark/biomatforming conditions, indicate that the electron input into the PQ pool, from heterotrophic sources via SDH or NDH-1 or from NADPH, generated by PS I during blue light illumination, might be affected in cph2 KO. This corroborates a regulatory function of CPH2 on glucose metabolism during the reorganisation of the metabolism, concomitant with the transition from photoautotrophic to heterotrophic/biomatforming growth conditions.

### 4.1.4 CPH2 has an essential regulatory function on glucose metabolism under LAHG/biomatforming conditions

This study provides evidence for the cyanobacterial phytochrome CPH2 playing a significant role in the regulation of the central carbon metabolism of *Synechocystis* sp. PCC 6803 under heterotrophic (LAHG)/biomatforming conditions. The transition from photoautotrophic to LAHG/biomatforming conditions and concomitant the reorganisation of the metabolism, revealed a physiological phenotype in the cph2 loss-of-function mutant, including reduced growth, reduced G6PDH enzyme activity and a strongly downregulated transcript level of cytochrome c oxidase encoding CtaDI gene. Based on these data, it is most likely that the primary effect of cph2 deletion on the regulation of the metabolism is the downregulation of cytochrome c oxidase. Cytochrome c oxidase might further regulate the activity of G6PDH, possibly at the post-translational level via the redox state of the PQ pool or via a so far unknown (feedback) mechanism, leading to a reduced ability in metabolising glucose, which causes the reduced growth phenotype under LAHG/biomatforming conditions. The mechanism by which CPH2 regulates cytochrome c oxidase expression remains elusive and is a topic for further studies.

In photosynthetic organisms, glucose metabolism encompasses indispensable metabolic pathways. However, only few of the corresponding regulatory proteins have been identified so far. In *Synechocystis*, for example, some sensors have been identified that affect the glucose metabolism under different trophic conditions. Kahlon and co-workers (2006) demonstrated that the histidine kinase 31 (HIK31) in
Synechocystis is involved in the regulation between photoautotrophic and photomixotrophic modes of growth. Furthermore, histidine kinase 8 (HIK8) and the RNA polymerase sigma factor SIGE have been identified as regulatory proteins on glucose catabolism (Singh & Sherman, 2005, 2007). With the results obtained in this study, the cyanobacterial phytochrome CPH2 has been identified as a new regulatory protein of glucose metabolism. This includes the identification of a new function of CPH2, in addition to its already known function in phototaxis and motility (Wilde et al., 2002; Fiedler et al., 2005; Moon et al., 2011; Savakis et al., 2012).

The hypothetical model in Figure 22 connects the physiological functions with the structural organisation of CPH2, suggesting that CPH2 may act as a light-responsive, non-kinase enzyme that possibly regulates various physiological processes via c-di-GMP signalling. C-di-GMP-mediated signalling encompasses enzymes that synthesize and degrade c-di-GMP and downstream c-di-GMP-binding effector proteins. Upon binding of the first messenger (e.g. light in certain wavelength regions, oxygen and phosphorylation) to the sensory domain (e.g. PAS, GAF-PHY and GAF domains) (Galperin, 2006; Hengge, 2009; Schirmer & Jenal, 2009; Yan & Chen, 2010), the cellular concentration of the second messenger c-di-GMP is altered through diguanylate cyclase (DGC) or phosphodiesterase (PDE) activities (Galperin et al., 2001). The GGDEF domain-containing DGC utilises guanosine triphosphate (GTP) as substrate and catalyzes the formation of c-di-GMP, while EAL domain-containing PDE degrades c-di-GMP into guanosine monophosphate (GMP) through the linear intermediate pGpG (Ryan et al., 2006; Jenal & Malone, 2006; Tamayo et al., 2007; Yan & Chen, 2010). C-di-GMP can function as an allosteric regulator of downstream proteins (effectors or receptors). Upon binding of c-di-GMP, these proteins undergo conformational changes, leading to the either positive or negative regulation of important cellular functions or phenotypic transitions, including exopolysaccharide synthesis, cell-to-cell communication, virulence, motility and biofilm formation (Tischler & Camilli, 2004; Pesavento & Hengge, 2009; Yan & Chen, 2010).
Figure 22: Hypothetical model of the phytochrome photoreceptor CPH2 and its regulatory function on glucose metabolism under heterotrophic (LAHG)/biomat-forming conditions.

According to the working hypothesis, the perception of light leads to conformational changes within CPH2, which affect the activity of GGDEF and EAL domains. This in turn activates the biosynthesis or degradation of c-di-GMP. The altered c-di-GMP level might regulate the transcription of cytochrome c oxidase encoding CtaDI gene. The activity of cytochrome c oxidase could further regulate the activity of G6PDH at the post-translational level, via the redox poise of the PQ pool or another unknown posttranslational regulatory mechanism. The reduced G6PDH activity could cause the reduced ability in metabolising glucose, leading to the observed strongly retarded growth phenotype under LAHG/biomat-forming conditions.

Abbreviations: GAF, domain present in vertebrate cGMP-specific phosphodiesterases, in cyanobacterial adenylate cyclases and in the formate lyase transcription activator FhlA; C, cysteine residues in the GAF-domains, which covalently bind the chromophore; GGDEF and EAL, domains with conserved sequence motifs, Gly-Gly-Asp-Glu-Phe and Glu-Ala-Leu, respectively, known to be involved in turnover of c-di-GMP; GTP, guanosine triphosphate; c-di-GMP, bis-(3’-5’) -cyclic dimeric guanosine monophosphate; GMP, guanosine monophosphate; CtaDI, gene encoding for cytochrome c oxidase subunit I; G6P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; G6PDH, glucose-6-phosphate dehydrogenase; NDH-1, type I NADPH dehydrogenase; SDH, succinate dehydrogenase; Suc, succinate; Fum, fumarate; PS I and II; photosystem I and II; PQ pool, plastoquinone pool; cyt b6f, cytochrome b6f complex; PC, plastocyanin; cyt c oxidase, cytochrome c oxidase; ATPase, ATP synthase complex; NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced form); OPP Pathway, oxidative pentosephosphat pathway; TCA cycle, tricarboxylic acid cycle.
CPH2 possesses a unique domain organisation, including two N-terminal (GAF1 and GAF2) and a C-terminal GAF domain (GAF3). GAF1 and GAF3 contain bilin-binding sites, conserved cysteine residues, which confer Pr/Pfr-like photochromicity (Wu & Lagarias, 2000) and the ability of blue light perception (Wilde et al., 2002; Fiedler et al., 2005; Ulijasz et al., 2009). Similar to the known mechanism in plant phytochromes (reviewed by Nagatani, 2010; Rockwell & Lagarias, 2010), the perception of light (Pr, Pfr or blue light or possibly cross talk of red and blue light) might lead to a conformational change of the bilin-chromophore in CPH2, which triggers a subsequent conformational change in the protein. This primary signal might cause changes in the activity of GGDEF and EAL domains in CPH2, which activate the biosynthesis or degradation of c-di-GMP. Recently, Savakis and co-workers (2012) confirmed that the EAL domain and the C-terminal GGDEF2 domain are catalytically active in CPH2. Moreover, they demonstrated that the light-regulated activity of c-di-GMP-synthesizing or degrading enzymes is modulated by CPH2. CPH2 is known to be involved in phototactic motility, in particular in the inhibition of phototaxis towards blue light (Wilde et al., 2002) and UV-A light (Moon et al., 2011). The recent work by Savakis and co-workers (2012) links the involvement of CPH2 in phototaxis and motility to c-di-GMP and its light-dependent regulation.

To conclude, CPH2 may regulate the glucose metabolism primarily via transcriptional control of cytochrome c oxidase encoding CtaDI gene. Thereby, light-induced conformational changes in CPH2 might affect the activity of GGDEF and EAL domains and consequently the intracellular concentration of c-di-GMP, a second messenger known for its involvement in phenotypic transitions, phototaxis and motility (Park et al., 2000; Wilde et al., 2002; Fiedler et al., 2004; Moon et al., 2011). Recent data by Savakis and co-workers (2012) made this hypothesis even more intelligible by confirming that GGDEF and EAL domain are functional in CPH2, that c-di-GMP is a functional second messenger in Synechocystis and that CPH2 functions as its primary, light-regulated modulator. The next steps would be to confirm that c-di-GMP is responsible for the regulation of the glucose metabolism and the existence of cross talk between red light and blue light signalling. The latter could be analysed by point mutations of the cysteine residues within the chromophor binding domains. Additionally, a detailed understanding of light-mediated glucose metabolism in cyanobacteria would further help to understand the regulation of photosynthesis and heterotrophy in other photosynthetic organisms.
4.2 Physiological characterisation of the cyanobacterial histidine kinase HIK12 and its regulatory function on the central carbon metabolism

Histidine kinases are sensory components of sophisticated signal transduction systems, which confer the ability to monitor environmental and intracellular cues and to respond appropriately by regulating adaptational processes. In *Synechocystis*, over 40 histidine kinases have been identified, all of which hold potential sensor domains. Although considerable progress has been made to identify histidine kinases and their involvement in sensing (stress) stimuli, e.g. high light, cold and oxidative stress, heat, salt stress, hyperosmotic stress and phosphate limitation (Hirani *et al.*, 2001; Mikami *et al.*, 2002; Hsiao *et al.*, 2004; Paithoonrangsarid *et al.*, 2004; Shoumskaya *et al.*, 2005; Suzuki *et al.*, 2005; Kanesaki *et al.*, 2007), the physiological functions of many histidine kinases remain still elusive. Among them, the histidine kinase 12 (HIK12) is of particular interest due to its structural homology to the sensor histidine kinases AHK2, AHK3 and AHK4 of the cytokinin receptor family in *Arabidopsis* (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Ueguchi *et al.*, 2001; Yamada *et al.*, 2001). The structural homology is based on the existence of an approximately 250 amino acid long domain in the N-terminal parts of all three AHK receptors and in HIK12. In *Arabidopsis*, this so-called CHASE (cyclases, histidine kinase associated sensory protein) domain was identified to be responsible for cytokinin binding (Heyl *et al.*, 2007), whereas the structurally homologous domain in *Synechocystis*, the so-called MASE (membrane associated sensor) domain is of unknown function so far.

<table>
<thead>
<tr>
<th></th>
<th>Protein Sequence</th>
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<tbody>
<tr>
<td>HIK12 (505)</td>
<td>TTEGEVVL[SNFRNAEQ</td>
</tr>
<tr>
<td>AHK2 (418)</td>
<td>RASGKGVLSPPFKLKS</td>
</tr>
<tr>
<td>AHK3 (281)</td>
<td>RSSGKGVLLAPFPLIK</td>
</tr>
<tr>
<td>AHK4 (301)</td>
<td>RETGKAVLSPFRLLLET</td>
</tr>
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Figure 23: Sequence alignment of the ligand-binding CHASE domain in *Arabidopsis* cytokinin receptors AHK2, AHK3 and AHK4 and the putative ligand-binding MASE domain in HIK12 of *Synechocystis* sp. PCC 6803.

Protein sequences of *Arabidopsis* AHK2 (AT5G35750.1), AHK3 (AT1G27320.1) and AHK4 (AT2G01830.2) were retrieved from the TAIR database (www.arabidopsis.org). The protein sequence of *Synechocystis* sp. PCC 6803 HIK12 (sll1672) was retrieved from the cyanobase database (www.genome.kazusa.or.jp/cyanobase/Synechocystis). The alignment was conducted with the Multiple Sequence Alignment (MSA) tool of ClustalW (www.ebi.ac.uk/Tools/msa/). Numbers in brackets indicate the amino acid positions of the conserved threonine residue (indicated by a black frame) of the respective proteins. Grey boxes indicate fully or partly conserved amino acids within the protein sequences. Asterisks indicate conserved amino acids in all protein sequences tested.
Sequence analysis by aligning the (putative) ligand-binding domains revealed that the cyanobacterial HIK12 (sll1672) possesses a threonine residue, which is part of a Valine/Leucine/Threonine motif in its MASE domain, which is conserved in all three Arabidopsis cytokinin receptors (Figure 23). In Arabidopsis, this conserved threonine at position 301 (T301) within the CHASE domain of AHK4 was identified to be among the amino acids essential for cytokinin binding, including tryptophane at position 244 (W244), lysine at position 297 (K297), phenylalanine at position 304 (F304), arginine at position 305 (R305) and threonine at position 317 (T317) (Heyl et al., 2007). Mutagenesis of T301 led to a complete loss of cytokinin binding activity (Yamada et al., 2001) and was originally discovered as woodenleg (wol) mutation in a screen for altered root morphology (Mähönen et al., 2000). Heyl and co-workers (2007) could further confirm the importance of these amino acids in cytokinin binding by substitution of W244, F304, R305 and T317 to alanine, resulting in dramatic alterations of cytokinin binding, ranging from strong reductions to total loss of cytokinin binding. Although the conservation at the amino acid level of the ligand-binding CHASE domains in AHK2, AHK3 and AHK4 is relatively low with only 65% (Spíchal et al., 2004; Heyl et al., 2007), the secondary structure is highly homologous with a characteristic pattern of α-helices and β-sheets (Heyl & Schmülling, 2003). Moreover, the mutated amino acids, which led to the strongest effect (W244, F304, R305 and T301), are located in close vicinity to two predicted β-sheets within the center part of the CHASE domain. This led to the hypothesis that these β-sheets are part of a binding pocket for cytokinins (Heyl et al., 2007). In addition to the conserved threonine residue at position 301 in AHK4 and position 505 in HIK12, several but not all of the amino acids (Figure 23), which have been identified to be important for cytokinin binding in plants, are also conserved in Synechocystis. Considering the evolutionary relationship between plant chloroplasts and their cyanobacterial ancestors, it is conceivable that the cytokinin signalling system in plants is an inheritance from their cyanobacterial ancestors (Anantharaman & Aravind, 2001; Mougel & Zhulin, 2001). Following the endosymbiotic event, corresponding genes could have been transferred to the plant nucleus and developed into cytokinin receptors. Vice-versa, one could suggest a putative function of HIK12 in cytokinin sensing in Synechocystis. In this context, Selivankina and co-workers (2006) found first evidence of cyanobacteria responding to cytokinin by demonstrating that the cytokinin trans-zeatin affects the transcriptional activity in Synechocystis. This led to the conclusion that cyanobacteria hold at least the molecular targets for cytokinin signal recognition. However, not much is known about cytokinin signalling in Synechocystis. In this study the function of HIK12, a
cyanobacterial histidine kinase with homology to the cytokinin receptor family in *Arabidopsis* was analysed. For this purpose, *hik12* loss-of-function and overexpression mutants, the latter for functional complementation of the knockout, were physiologically analysed. Results show a regulatory function of HIK12 on glucose metabolism after the transition from a planktonic lifestyle to a sessile lifestyle within a biomat.

### 4.2.1 Deletion of HIK12 negatively affects growth under biomat forming conditions

The analysis of the growth kinetics revealed no differences in growth between WT, *hik12* knockout (loss-of-function) and both *hik12* overexpression (complementation) mutants under photoautotrophic and photomixotrophic conditions (Figure 12 A, B). The accelerated growth upon addition of glucose under photomixotrophic conditions (Figure 12 B) compared to photoautotrophic conditions indicates that WT, *hik12* KO and both *hik12* OE mutants are capable of photomixotrophic growth (Williams, 1988; Anderson & McIntosh, 1991). Similar growth patterns under photoautotrophic and photomixotrophic conditions were reported by Kahlon and co-workers (2003) and Anderson and McIntosh (1991). In contrast, after 48 h of incubation under biomat forming conditions led to growth cessation in *hik12* KO, while the overexpression of HIK12 in the *hik12* KO background complemented this phenotype (Figure 12 C).

This indicates that HIK12 has a critical role in the regulation of growth under biomat forming conditions. *Synechocystis* cells proliferate via binary fission, an asexual form of reproduction, which comprises the cell division process of one cell into two daughter cells. Microscopic analysis enables to distinguish between single spherical cells and dumbbell-shaped cells, undergoing cell division. Accordingly, in an actively growing culture, which implies high cell division activity, more dumbbell-shaped cells than single cells are expected. The finding that HIK12 is involved in the regulation of growth under biomat forming conditions is further corroborated by the results obtained in microscopic analyses. In *hik12* KO, the relative amount of dumbbell-shaped cells was significantly decreased after 14 days of incubation under biomat forming conditions in presence of glucose, while that in the WT remained almost unchanged (Figure 13). The reduced cell division activity in *hik12* KO might suggest either a direct regulatory function of HIK12 on cell division processes or an indirect function, e.g. via regulation of the metabolism.
4.2.2 Deletion of HIK12 results in reduced G6PDH enzyme activity and a lower transcript level of CtaDI, encoding cytochrome c oxidase, under biomatfoming conditions

Synechocystis performs oxygenic photosynthesis to convert light energy to chemical energy. Using NADPH and ATP, generated through photosynthesis, inorganic carbon is assimilated via the Calvin cycle. The fixed carbon enters either the glycolytic pathway or is stored as reserve carbohydrate in the form of glycogen. Glycogen can be degraded to provide glucose residues to meet the metabolic needs under non-photosynthetic conditions (Stal & Moezelaar, 1997; Knowles & Plaxton, 2003; Singh & Sherman, 2005; Takahashi et al., 2008) or for adaptational processes in response to environmental stress stimuli (Preiss, 1984; Suzuki et al., 2010). In order to determine the cause for the observed growth phenotype in hik12 KO both directions of glucose processing, the degradation via the OPPP and the accumulation of glycogen were analysed. The analysis of the specific G6PDH enzyme activity, the rate-limiting enzyme in the OPPP (Yang et al., 2002; Singh et al., 2004; Singh & Sherman, 2005) revealed a reduced ability of hik12 KO in degrading glucose. This is substantiated by a significant decrease in G6PDH activity after incubation under biomatfoming conditions (Figure 15 B). Overexpression of HIK12 in the hik12 KO background complemented this phenotype. Under photosynthetic and photomixotrophic conditions, however, no significant differences in G6PDH activity between WT, hik12 KO and hik12 OE were observed (Figure 15 A). In Synechocystis, excess carbon is stored in the form of glycogen, the main carbon storage compound in cyanobacteria (Nakamura et al., 2005). Under biomatfoming conditions, hik12 KO had a higher amount of glycogen compared to the WT (Figure 14). Thus, the reduced rate of G6PDH activity together with the increased accumulation of glycogen within hik12 KO, indicate a regulatory function of HIK12 on the glucose metabolism. The increased glycogen content in hik12 KO could result from the redirection of G6P into glycogen, caused by the reduced G6PDH activity. This would also be an explanation for the observed growth retardation of hik12 KO under biomatfoming conditions.

To further analyse the effects of hik12 deletion, gene expression profiles of two key metabolic genes, Zwf (slr1843), encoding for G6PDH and CtaDI (slr1137), encoding for subunit I of the cytochrome c oxidase complex, the terminal component of the respiratory chain, were examined under different growth conditions using semi-quantitative RT-PCR. The analysis of the gene expression patterns revealed no significant differences in the transcript levels of Zwf and CtaDI in WT, hik12 KO and
hik12 OE under photoautotrophic or photomixotrophic conditions (Figure 16 A, B). This is in accordance with previous reports, demonstrating that transcript levels of various genes encoding key enzymes of the central carbon metabolism are not significantly affected upon the presence or absence of exogenous glucose (Yang et al., 2002; Tu et al., 2004; Singh & Sherman, 2005; Kahlon et al., 2006). Although the expression patterns for Zwf and CtaDI are not significantly altered under photoautotrophic and photomixotrophic conditions, incubation under biomatforming conditions resulted in an interesting transcriptional pattern. Under biomatforming conditions, the transcript level of cytochrome c oxidase encoding CtaDI, was significantly upregulated in WT and hik12 OE, while the hik12 KO displayed no induced transcript accumulation (Figure 16 C). The incapability of hik12 KO to upregulate CtaDI expression under biomatforming conditions was reversed in the complementation mutant, indicating that the observed transcriptional pattern is caused by the deletion of hik12. Notably, the lower CtaDI expression in hik12 KO was only observed after the transition to biomatforming conditions. Other than the strong effect on the enzyme activity level (Figure 15 B), the transcript level of Zwf, encoding for G6PDH, was not significantly altered in WT, hik12 KO and hik12 OE under biomatforming conditions (Figure 16 C). The fact that the Zwf transcript level is similar in WT, hik12 KO and hik12 OE, although the G6PDH activity differs, further substantiates the previously mentioned regulation of G6PDH at the post-translational level. This is in accordance with reports, demonstrating that the activity of enzymes involved in glycolytic and OPP pathways is regulated at the post-translational level by changes in metabolite concentrations (Stal & Moezelaar, 1997; Sundaram et al., 1998; Yang et al., 2002; Knowles & Plaxton, 2003; Lee et al., 2007).

Together, the results obtained in this study clearly indicate a regulatory function of HIK12 on the glucose metabolism under biomatforming conditions. The incapability of hik12 KO to upregulate CtaDI expression under biomatforming conditions should result in a reduced cytochrome c oxidase activity and accordingly in a limited respiration. As previously discussed for cph2 KO, the reduced cytochrome c oxidase activity could result in an overreduced electron transport chain, as the electrons cannot be removed. As G6PDH is inactivated under reducing conditions (Plaxton & Podesta, 2006; Lee et al., 2007), the incapability to upregulate CtaDI expression might be the cause for the reduced G6PDH activity in hik12 KO. Thus, cytochrome c oxidase might be the direct target of HIK12 in regulating the glucose metabolism under biomatforming conditions. For future work it is important to determine the activity of cytochrome c oxidase in hik12 KO and the redox state of the PQ pool.
4.2.3 P700\(^+\) reduction kinetics: Analysis of electron transport activities

The analysis of P700\(^+\) reduction kinetics revealed no significant differences in determined t\(_{1/2}\) of P700\(^+\) reduction between WT, hik12 KO and hik12 OE, neither before nor after incubation under biomat forming conditions (Figure 17). However, there is an induction in glucose metabolism observable after biomat induction. The less increased t\(_{1/2}\) of P700\(^+\) reduction in DCMU-treated samples after biomat induction compared to that under photoautotrophic conditions indicates a higher electron input from heterotrophic sources, indicating that the cells have adapted to the presence of exogenous glucose. A possible explanation for the absence of any significant differences in t\(_{1/2}\) of P700\(^+\) reduction between WT, hik12 KO and hik12 OE could be that the biomat induction takes place under light conditions, which implies that the general energy metabolism is still highly active in the cells, other than under dark conditions as seen in Section 3.1.5. Furthermore, as HIK12 is not a light sensor, other than the phytochrome photoreceptor CPH2 (Section 4.1.3), which showed a clear effect on P700\(^+\) reduction kinetics after the switch from light to dark conditions, it could take longer to see an effect of hik12 KO on electron transport activities after biomat formation. Considering that HIK12 might be involved in sensing signals associated with the cell cycle activity, the time span of 24 h might be too short to see HIK12-mediated effects on the metabolism. As the strong effects of hik12 deletion on G6PDH enzyme activity (Figure 15 B) and on the transcript level of cytochrome c oxidase encoding CtaDI (Figure 16 C) were observed after 96 h of incubation under biomat forming conditions, it will be inevitable for future work to repeat the measurements after 96 h of incubation under biomat forming conditions.

4.2.4 Deletion of hik12 leads to constitutive ROS production

Inherently, oxygenic photosynthesis and aerobic respiration give rise to the generation of reactive oxygen species (ROS), which are continuously formed as byproducts of these aerobic processes (Tichy & Vermaas, 1999; Apel & Hirt, 2004; Pérez-Pérez et al., 2009). The different ROS types, including hydrogen peroxide (H\(_2\)O\(_2\)), superoxide anion radical (O\(_2^•\)) and the hydroxyl radical (OH\(^•\)) cause oxidative damage to nucleic acids, proteins and lipids (Apel & Hirt, 2004; Latifi et al., 2009). ROS are inevitably generated during photosynthetic electron transport, at PS II, where singlet oxygen is produced by energy input to oxygen from photosensitized chlorophyll and at PS I, where superoxide anion radicals are generated by the Mehler reaction (reviewed by Latifi et al., 2009). H\(_2\)O\(_2\) and OH\(^•\) are produced during
the stepwise reduction of molecular oxygen (O\textsubscript{2}) to water (H\textsubscript{2}O). To cope with the cytotoxic properties of ROS, the cells possess detoxification systems, ROS-scavenging enzymes, such as superoxide dismutases, catalase-peroxidases and peroxiredoxins (Jeanjean \textit{et al.}, 2007; Pérez-Pérez \textit{et al.}, 2009, Bernroitner \textit{et al.}, 2009). Accordingly, the cells are in the constant need to strictly control ROS production and detoxification, in order to maintain their cellular redox balance. However, the equilibrium between ROS production and scavenging can be perturbed by environmental stress factors, such as high light intensities, leading to redox imbalances and subsequently to increased ROS production (Apel & Hirt, 2004; Scheibe \textit{et al.}, 2005). The luminol-based assay, conducted to analyse the ROS production within \textit{hik12 KO} and WT cells, revealed a constitutive ROS production in \textit{hik12 KO} (Figure 18). Environmental stress factors, such as high light, which might cause the ROS production precedent redox imbalances, can be excluded, as the measurement took place in darkness. Moreover, the WT displayed no further chemoluminescence emissions, indicative for ROS production, than the background emissions. Glucose was added prior to the measurement to check for electron imbalances in the respiratory chain. The presence or absence of exogenous glucose did not influence the observed ROS production, neither in \textit{hik12 KO} nor in the WT (Figure 18), indicating that the redox status of the PQ pool or imbalances in the electron transport chain are rather unlikely to cause the constitutive ROS production in \textit{hik12 KO}. Furthermore, the measurements were conducted in exponentially growing, photoautotrophic cultures. Therefore, no differences in cytochrome c oxidase activity and consequently no overreduction of the electron transport chain are expected. Thus, these results indicate rather defects in ROS-scavenging or reduced activities of the scavenging enzymes to cause the constitutive ROS production in \textit{hik12 KO}. However, further research is inevitable to elucidate the cause of the constitutive ROS production and to clarify the correlation between the deletion of \textit{hik12} and the striking ROS production.

4.2.5 HIK12 has a regulatory function on glucose metabolism under biomatfoming conditions

Histidine kinases are the essential sensory components of sophisticated signal transduction systems that confer the ability to perceive a wide range of signals and to respond appropriately by regulating various adaptational processes. Although considerable progress has been made in the past years, the physiological functions of many Hiks in \textit{Synechocystis} have not yet been characterized. In \textit{Synechocystis}, the histidine kinases HIK8 and HIK31 are involved in the regulation of glucose
catabolism (Singh & Sherman, 2005) and in the regulation between different trophic modes of growth (Kahlon et al., 2006), respectively. The results obtained in this study provide evidence that with HIK12 another histidine kinase with a regulatory function on the central carbon metabolism of *Synechocystis* sp. PCC 6803 has been identified. After the transition from photoautotrophic to photomixotrophic/biomat-forming conditions, *hik12* KO shows a clear phenotype, comprising of growth cessation, reduced G6PDH enzyme activity, an increased amount of the carbon storage compound glycogen and the incapability to upregulate the transcript level of cytochrome *c* oxidase encoding *CtaDI* gene. The results obtained in this study confirm a function of HIK12 in the regulation of glucose metabolism. Based on these results, two hypotheses have been developed, which are summarized in a hypothetical model presented in Figure 24.

With regard to the observed growth phenotype and the significantly reduced cell division activity in *hik12* KO, it is assumable that HIK12 has a regulatory function on cell division processes under biomat-forming conditions. In this context, the reduced cell division activity and concomitantly the reduced growth might be the consequence of the restricted availability of biosynthetic intermediates required for growth, resulting from the reduced glucose degradation by G6PDH, the rate-limiting enzyme of glucose catabolism. This would also explain the increased amount of glycogen, probably resulting from the redirection of G6P to glycogen, caused by the reduced G6PDH activity. According to the endosymbiont theory (Margulis, 1970), cyanobacteria are the evolutionary ancestors of plant chloroplasts (Anantharaman & Aravind, 2001; Mougel & Zhulin, 2001). In the course of the endosymbiotic event, many cyanobacterial genes were transferred into the plant nucleus. Especially, genes coding for proteins involved in photosynthesis and signal transduction are highly conserved among cyanobacteria and plants. Considering the homology of HIK12 to the cytokinin receptor family in *Arabidopsis*, in particular the conserved threonine residue in the cytokinin binding domain of *Arabidopsis* and in the putative ligand-binding domain of HIK12, the cytokinin signalling system in plants might be an inheritance of their cyanobacterial ancestors during the evolution of the chloroplast. A putative function for HIK12 in cytokinin signalling in *Synechocystis* is assumable. HIK12 could either be a cytokinin receptor or could recognize similar molecules. Furthermore, the assumed regulatory function of HIK12 on cell division activity under biomat-forming conditions corresponds to the growth-stimulating effect of cytokinins in plants (Mok & Mok, 2001; Dello Ioio et al., 2008; Perilli et al., 2010).
Figure 24: Hypothetical model of the histidine kinase 12 and its regulatory function on glucose metabolism under photomixotrophic/biomatrating conditions.

According to the working hypothesis, signal perception by HIK12 leads to the phosphorylation (P) of its response regulator (RR). This in turn could modulate the transcription of cytochrome c oxidase encoding CtaDI gene or another so far unknown target gene, which could be involved in the regulation of cell division processes. The activity of cytochrome c oxidase might further regulate the activity of G6PDH at the posttranslational level, via the redox poise of the PQ pool or another unknown posttranslational regulatory mechanism. The reduced G6PDH activity might cause the reduced ability in metabolising glucose, leading to the observed strongly retarded growth phenotype under biomatrating conditions. The down-regulation of G6PDH could also be a consequence of a feedback inhibition, caused by the reduced cell division events and concomitantly the reduced cellular demand for biosynthetic intermediates. Alternatively, the reduced cell division events could affect the glucose metabolism.

Abbreviations: CPH2, cyanobacterial phytochrome 2; c-di-GMP, bis-(3’-5’)-cyclic dimeric guanosine monophosphate; CtaDI, gene encoding for cytochrome c oxidase subunit I; G6P, glucose-6-phosphate; GPGL, 6-phosphogluconolactone; G6PDH, glucose-6-phosphate dehydrogenase; NDH-1, type I NADPH dehydrogenase; SDH, succinate dehydrogenase; Suc, succinate; Fum, fumarate; PS I and II; photosystem I and II; PQ pool, plastoquinone pool; cyt b6f, cytochrome b6f complex; PC, plastocyanin; cyt c oxidase, cytochrome aa3-type cytochrome c oxidase; Fdox/Fred, ferredoxin in oxidized and reduced forms, respectively; ATPase, ATP synthase complex; NADP(H), nicotinamide-adenine dinucleotide phosphate (reduced form); OPP Pathway, oxidative pentosephosphate pathway; TCA cycle, tricarboxylic acid cycle.

Based on the results obtained by Selivankina and co-workers (2006), Synechocystis possesses the molecular targets for cytokinin signal recognition. Nevertheless, it is inevitable for future work to provide evidence that the putative ligand-binding domain in HIK12 binds cytokinin. In Arabidopsis, definitive evidence for AHK2, AHK3 and AHK4 functioning as cytokinin receptors was provided by complementation ex-
experiments in heterologous bacterial and yeast systems (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). When expressed in E. coli cells, AHKs confer the ability to complement the ΔrcsC histidine kinase mutant, which is impaired in the regulation of extracellular polysaccharide synthesis, in a cytokinin-dependent manner (Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Similarly, the budding yeast histidine kinase Δsln1 mutant, which is lethally defective in osmosensing, was complemented by expressing AHKs in a cytokinin-dependent manner (Inoue et al., 2001; Ueguchi et al., 2001). Accordingly, due to the homology of HIK12 to AHKs, expression of HIK12 in these heterologous bacterial or yeast systems could be used to give evidence for HIK12 functioning as cytokinin receptor. Interestingly, Reiser and co-workers (2003) demonstrated that the yeast osmosensor SLN1, which is one of the two sensory components of the high osmolarity glycerol (HOG) pathway (Wurgler-Murphy et al., 1997), monitors changes in turgor pressures caused by hyperosmotic stress. They showed that the plant cytokinin receptor AHK4 can substitute the SLN1 osmosensing function and that its kinase activity is similarly regulated by turgor pressure, leading to the suggestion that AHK4 could have dual sensor functions as cytokinin receptor and osmosensor in plants (Reiser et al., 2003). Due to the homology of AHKs and HIK12, it is further conceivable that HIK12 is involved in osmosensing in Synechocystis.

The reduced G6PDH activity/CtaDI expression could also be the consequence of a feedback inhibition, caused by the reduced cell division events and concomitantly the reduced cellular demand for biosynthetic intermediates. The strong effect of hik12 deletion on the expression of cytochrome c oxidase encoding CtaDI indicates that cytochrome c oxidase is the primary target of HIK12, with HIK12 possibly acting as positive regulator of cytochrome c oxidase transcription under biomatforming conditions. In this context, cytochrome c oxidase might further regulate the activity of G6PDH, possibly at the post-translational level via the redox state of the PQ pool or via a so far unknown (feedback) mechanism. The reduced G6PDH activity could result in the redirection of G6P to glycogen, which would be an explanation for the increased amount of glycogen in hik12 KO cells. To further clarify the regulatory function of HIK12, it is inevitable for future work to determine the activity of cytochrome c oxidase. Another important aspect is to provide evidence for a correlation between the observed effects of hik12 deletion and the hormone cytokinin. A possible approach would be to analyse the enzyme activities of cytochrome c oxidase and G6PDH and the transcript levels of the corresponding genes in presence and absence of cytokinin.
4.3 Cytokinin function in plant-pathogen interactions

Cytokinins are a class of phytohormones that regulate various physiological and developmental processes throughout the plant life (Kakimoto, 2003; Balibrea Lara et al., 2004; Sakakibara, 2006; Müller & Sheen, 2007; Bari & Jones, 2009; Hwang et al., 2012). Besides processes associated with active growth, metabolism and plant development, cytokinins have further been suggested to be involved in plant-pathogen interactions (Walters & McRoberts, 2006; Robert-Seilaniantz et al., 2007; Walters et al., 2008). This suggestion was based on the striking similarity of several well-established cytokinin functions and changes in host physiology during plant-pathogen interactions (Walters & McRoberts, 2006; Walters et al., 2008). The role of cytokinins in plant-pathogen interactions, the underlying mechanisms and eventual cross talk with other defense-related plant hormones remain elusive. The results obtained in this study further support an essential function of cytokinin in plant-pathogen interactions. Altered cytokinin levels within transiently transformed barley epidermal cells influenced the susceptibility against powdery mildew spores in this biotrophic host-pathogen system. Furthermore, an Arabidopsis cytokinin receptor-deficient mutant was impaired in the defense response against the hemibiotrophic pathogen Pseudomonas syringae pv. tomato (Pst), which is in accordance with the recent finding by Choi and co-workers (2010), who demonstrated that modulated cytokinin levels or signalling activity correlates with altered resistance. Interestingly, the Arabidopsis AHK3 cytokinin receptor-deficient mutant displayed a constitutive ROS production. Modulation of ROS production is often associated with plant defense responses to pathogen challenge (Hückelhoven, 2007).

4.3.1 Overexpression of cytokinin oxidases positively affects resistance in the barley-powdery mildew interaction

The powdery mildew fungus Blumeria graminis f. sp. hordei (Bgh) is an obligate biotrophic phytopathogen. In a compatible interaction, the fungus successfully colonizes its host and develops its feeding organ, the haustorium, within the host cell. The fungus continues its growth by developing secondary hyphae and haustoria and finally accomplishes its infection cycle by spreading new spores from arising conidiophores. During the interaction with biotrophic fungal pathogens, the host undergoes characteristic alterations in physiology and metabolism, which include reduced photosynthetic rates, the accumulation of nutrients at infection sites and in later stages of the infection, the formation of the so-called green islands (Walters & McRoberts, 2006). Green islands describe the phenomenon of infection sites, which
remain green while the rest of the leaf senesces (Walters et al., 2008). Notably, the changes in host physiology strikingly resemble well-established functions of the phytohormone cytokinin during leaf senescence (Balibrea Lara et al., 2004), thereby linking cytokinin effects and plant responses to biotrophic pathogens. Beyond that, there has been some debate about the origin of cytokinin in infected leaves. It is not clear yet, if it is the pathogen producing cytokinin or the host or even both (Werner & Schmülling, 2009). Biotrophic fungal pathogens, including Bgh, might produce cytokinins to modulate the hormonal balance of their host, leading to the suppression of defense responses (Walters & McRoberts 2006; Robert-Seilaniantz et al., 2007). In any case, green islands, which are formed by biotrophic pathogens, function as a nutritional source for pathogen growth and propagation (Walters & McRoberts, 2006). Furthermore, in green islands that are associated with pustule formation by fungal pathogens, increased levels of cytokinin have been detected (Lopez-Carbonell et al., 1998). A possible role of cytokinins in plant-pathogen interactions has been proposed by Walters and McRoberts (2006), who suggested that in the early stage of plant-pathogen interaction, the accumulation of cytokinins, probably released by the fungus, leads to increased cell-wall invertase activity. This in turn causes nutrient mobilisation towards the infection site, reductions in photosynthetic metabolism and the development of green islands.

To investigate the impact of an altered cytokinin level during the early stage of host-pathogen interaction, cytokinin-degrading enzymes were transiently overexpressed in barley epidermal cells. Transformed cells were analysed during the early stage of interaction with the biotrophic powdery mildew fungus Bgh, in terms of resistance to penetration attempts of the fungus. The analysis revealed that simultaneous overexpression of the Arabidopsis cytokinin oxidases 1 and 2 (AtCKX1 and 2) mediated a significant decrease in the penetration success of Bgh, while single overexpression of AtCKX1 or AtCKX2 had no significant influence on the penetration efficiency of Bgh (Figure 19). In Arabidopsis, cytokinin synthesizing and degrading enzymes are located in different subcellular compartments (Werner et al., 2003; Sakakibara, 2006). In particular, cytokinin degradation is compartmentalized to the endoplasmatic reticulum (ER), the vacuole, the extracellular space and the cytoplasm (Motyka et al., 1996; Werner et al., 2003; Köllmer, 2009), suggesting that cytokinin homeostasis needs to be controlled at different locations of the cell (Wulfetange et al., 2011a). In this context, the different subcellular localization of both cytokinin oxidases, with AtCKX1 being targeted to the vacuole and AtCKX2 being located in the ER lumen (Werner et al., 2003; Galuszka et al., 2004;
Gajdosová et al., 2010) points to distinct functions for both isoenzymes. Supposedly, the simultaneous overexpression of AtCKX1 and 2 is necessary to alter the endogenous cytokinin level at different cellular compartments to a certain extent, which affects host-pathogen interactions.

A possible explanation for the decreased penetration efficiency of Bgh in AtCKX1/2 overexpressing barley cells might be attributed to the function of cytokinin in suppressing programmed cell death (Gan & Amasino, 1995; Walters & McRoberts, 2006). Cytokinins, secreted by the pathogen in the early host-pathogen interaction, might suppress host cell death, thereby enabling the pathogen to feed on living host tissue (Murphy et al., 1997; Ashby, 2000). Accordingly, a decreased cytokinin level, resulting from the overexpression of AtCKX1/AtCKX2 could result in a regression of cell death suppression. This in turn leads to a decreased susceptibility of the host, as Bgh as a biotrophic fungus is dependent on nutrient acquisition from living host tissue. This leads to another possible explanation, based on the function of cytokinins in nutrient mobilisation and in source-sink regulation (Roitsch et al., 2003; Balibrea Lara et al., 2004; Walters & McRoberts, 2006). During plant-pathogen interaction, biotrophic fungi establish strong metabolic sinks for nutrients and carbohydrates (Walters & McRoberts, 2006; Walters et al., 2008). A central modulator of sink activity is the extracellular invertase, which is a key sucrose-cleaving enzyme, having an essential function in source-sink regulation and in supplying carbohydrates to sink tissues (Tang et al., 1999; Goetz et al., 2001; Roitsch et al., 2003). Extracellular invertases are upregulated by several stimuli affecting carbohydrate requirements, which include growth-stimulating phytohormones such as cytokinins (Roitsch, 1999; Roitsch et al., 2003). Walters and McRoberts (2006) suggested that accumulated cytokinins, probably released by the fungus, lead to an increase in invertase activity and concomitantly to the establishment of a strong nutrient sink. The reduced penetration efficiency of Bgh in cells overexpressing AtCKX1/AtCKX2 could be explained by a reduced cytokinin level, which in turn leads to the downregulation of invertase expression and hence in a less efficient sink induction. Accordingly, the nutrient acquisition of the pathogen is less efficient, which negatively affects the fungal growth and development.
4.3.2 Loss-of-function of AHK3 results in enhanced susceptibility of Arabidopsis to Pseudomonas syringae pv. tomato

Interaction between signalling pathways is an important mechanism for regulating plant defense responses against various pathogens. In addition to salicylic, ethylene and jasmonates, which are considered as the main hormones mediating defense signalling (Bari & Jones, 2009), only recently the involvement of other hormones, such as cytokinins, abscisic acid, auxin, gibberellic acid and brassinosteroids in defense signalling pathways has been recognized. In this context, the very recent finding that Arabidopsis cytokinin receptors are located in the endoplasmatic reticulum (ER; Wulfetange et al., 2011a; Caesar et al., 2011) and not as previously assumed at the plasma membrane, offers new opportunities for hormonal cross talk, as numerous other plant receptor proteins, including the structurally related ethylene receptors (Chen et al., 2002), auxin signalling-related proteins and specific metabolic enzymes, such as cytokinin oxidases are also located at the ER (Werner et al., 2003; Geldner & Robatzek, 2008; Irani & Russinova, 2009; Friml & Jones, 2010; Wulfetange et al., 2011a). In Arabidopsis, cytokinins are perceived by the three membrane-bound sensor histidine kinases AHK2, AHK3 and AHK4 (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). Although genetic studies have shown a considerable degree of functional redundancy among the three receptors, individual receptors were reported to possess distinct functions and to mediate specific cytokinin activities (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). AHK3 has been identified as the cytokinin receptor that plays a major role in mediating senescence (Kim et al., 2006). As cytokinin-mediated defense responses are associated with altered senescence phenotypes (Walters & McRoberts, 2006), an AHK3 receptor-deficient Arabidopsis T-DNA insertion line was chosen as a candidate to analyse cytokinin-related effects in pathogen interaction. To this end, leaves of ahk3 KO mutant were infiltrated with the hemibiotrophic pathogen Pseudomonas syringae pv. tomato DC3000 (Pst), a non-cytokinin-producing pathogen (Choi et al., 2010) and the outcome of interaction was analysed. The analyses revealed that the AHK3 receptor-deficient mutant exhibited enhanced susceptibility to Pst, which was reflected in faster development of disease symptoms (Figure 20 A) and faster proliferation of bacteria (Figure 20 B). This is in accordance with Choi and co-workers (2010), who reported that plant-derived cytokinins positively affect plant immunity to Pst by showing that elevated levels of endogenous cytokinin in Isopentenyltransferase (IPT) overexpressing plants enhance plant resistance against Pst, while ahk2ahk3 cytokinin receptor-double knockout plants
displayed enhanced susceptibility. Based on these findings, Choi and co-workers (2010) proposed a model in which cytokinin perception through the receptors AHK2 and AHK3 in Arabidopsis elicits a defense response against non-cytokinin producing pathogens by modulating defense signalling. They suggested that mainly AHK3 is responsible for eliciting the defense response. There is no clear evidence for AHK3 being the main receptor mediating plant defense responses, as the results by Choi and co-workers (2010) were obtained from ahk2ahk3 double knockout plants. The results obtained in this study clearly show an enhanced susceptibility phenotype in the single ahk3 loss-of-function mutant. This phenotype is similar to the ahk2ahk3 phenotype and supports a predominant role of AHK3 in defense signalling.

4.3.3 Loss-of-function of AHK3 cytokinin receptor leads to constitutive ROS production in Arabidopsis

As described in detail in Section 4.2.4, reactive oxygen species (ROS) are continuously generated as toxic byproducts of photosynthetic and respiratory electron transport (Tichy & Vermaas, 1999; Apel & Hirt, 2004; Pérez-Pérez et al., 2009). In plants, also mitochondria and the plasmamembrane NADPH oxidase can be a source of ROS (Torres et al., 2010). To cope with the cytotoxic properties of ROS, the cells hold efficient mechanisms for scavenging and detoxification of ROS. The equilibrium between generation and removal of ROS can be perturbed by environmental stress stimuli, leading to disturbances in cellular integrity and the metabolic balance (Apel & Hirt, 2004 and references therein). These external stress factors leading to redox imbalances within the plant cells can be of abiotic or biotic origin. In plants, ROS are known to play an important role in defense reactions against pathogen attacks. In addition of being toxic to microorganisms, ROS are involved in various defense-related processes, such as cross-linking of cell wall components during the formation of papillae, in the hypersensitive reaction, in defense gene expression and also as signalling molecules in defense-related signal transduction (Lamb & Dixon, 1997).

A luminol-based assay was conducted in this study to analyse the ROS production in the Arabidopsis ahk3-loss-of-function mutant. Ahk3 KO showed a constitutive ROS production, while the WT displayed no ROS production under the conditions tested (Figure 21). Environmental stress factors, such as high light, which might cause redox imbalances leading to the production of ROS, can be excluded as the measurement took place in darkness. The addition of exogenous glucose prior to the measurement did not affect the observed ROS production, neither in ahk3 KO nor in
the WT (Figure 21). Glucose was added to check for electron imbalances in the respiratory chain. Due to the non-affected ROS production in presence or absence of glucose, redox imbalances in the electron transport chain and/or the PQ pool are rather unlikely to cause the constitutive ROS production in ahk3 KO. These results rather point to defects in scavenging or possibly reduced activities of ROS-scavenging enzymes, such as superoxide dismutases, catalase-peroxidases and peroxiredoxins (Jeanjean et al., 2007; Pérez-Pérez et al., 2009, Bernroitner et al., 2009), causing the constitutive ROS production in ahk3 KO. However, as ROS production in plant cells can have various causes, further research is required to elucidate the cause for the constitutive ROS production in ahk3 KO. Moreover, especially with regard to the fact that the cyanobacterial hik12 loss-of-function mutant shows a similar phenotype of constitutive ROS production (Figure 18), it will be interesting for future work to clarify the underlying mechanisms.
5 Summary

The ability to perceive environmental changes and to respond by adapting appropriately is indispensable for the survival and successful competition of all living organisms. Sophisticated signal transduction systems confer the ability to mediate these adaptive responses. In the present work, two signalling components, the phytochrome photoreceptor CPH2 and the histidine kinase 12 (HIK12) of the cyanobacterium Synechocystis sp. PCC 6803 were investigated with a special focus on the central carbon metabolism.

The phytochrome photoreceptor CPH2 of Synechocystis is known to be involved in phototaxis and motility. In the present work, a new function of CPH2 as a regulator of the glucose metabolism was shown. The knockout of cph2 results in a strongly retarded growth phenotype under biomat forming conditions in darkness and presence of glucose as sole energy source for heterotrophic growth. This phenotype correlates with a reduced glucose-6-phosphate dehydrogenase (G6PDH) enzyme activity in the knockout and a strongly down-regulated transcript level of cytochrome c oxidase encoding CtaDI gene. These phenotypes can be complemented by overexpression of CPH2 in the knockout background.

The second signalling component HIK12, a histidine kinase of Synechocystis, displays homology to the cytokinin receptor family in Arabidopsis thaliana. The knockout of hik12 results in a strongly retarded growth phenotype under biomat forming conditions in the light and in the presence of glucose. This phenotype correlates with a reduced G6PDH enzyme activity, increased glycogen content and the incapability to upregulate the transcript level of cytochrome c oxidase encoding CtaDI gene. These phenotypes can be complemented by overexpression of HIK12 in the knockout background, confirming a new regulatory function of HIK12 on the central carbon metabolism. Interestingly, hik12 KO displayed constitutive production of reactive oxygen species (ROS).

The obtained results show a clear regulatory function of the phytochrome photoreceptor CPH2 and the histidine kinase HIK12 on the central carbon metabolism of Synechocystis sp. PCC 6803. These phenotypes manifest under biomat forming conditions, which implies the transition from fast exponential to slowed down growth.

Cytokinins are a class of phytohormones that regulate various physiological and developmental processes in higher plants, including cell division and differentiation,
chloroplast development, seed germination, delay of leaf senescence, nutrient mobilization and stress responses. Recently, cytokinins have emerged to be involved in processes related to defense mechanisms during plant-pathogen interaction. In the present work, the role of cytokinins during the interaction with biotrophic/hemibiotrophic pathogens was investigated. Transiently transformed barley cells, over-expressing cytokinin-degrading enzymes, were analysed during the interaction with the biotrophic powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*). The simultaneous overexpression of cytokinin oxidases 1 and 2 resulted in reduced penetration success of *Bgh*, indicating that a lowered cytokinin level in the cells positively affects resistance against *Bgh* spores. An AHK3-cytokinin-receptor deficient *Arabidopsis* T-DNA-insertion line was analysed during the interaction with the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*). The AHK3 cytokinin receptor-deficient T-DNA insertion line exhibited enhanced susceptibility to *Pst*, which is reflected in faster development of disease symptoms, indicating that cytokinins promote resistance of *Arabidopsis* to *Pst*. Interestingly, a constitutive ROS production in the *ahk3* KO mutant in the absence of any elicitors was shown. Together, the results obtained in the present work further support an essential function of cytokinins in plant-pathogen interactions by confirming that cytokinins affect plant immunity.
6 Zusammenfassung


Die Ergebnisse dieser Arbeit zeigen eine klare regulatorische Funktion des Phytochrom-Photorezeptors CPH2 und der Histidin-Kinase HIK12 auf den zentralen Kohlenstoff-Metabolismus von *Synechocystis* sp. PCC 6803. Diese Phänotypen
manifestieren sich unter Biofilm-bildenden Bedingungen, die einen Wechsel von schnellem, exponentiellem zu verlangsamtem Wachstum einschliessen.

7 References


Mullineaux, C.W. & Allen, F. (1990) State 1-State 2 transitions in the cyanobacterium Synechococcus 6301 are controlled by the redox state of electron carriers between photosystem I and II. Photosynth Res. 23:297-311.


Supplementary figure 1: Verification of genomic integration of the kanamycine (KM<sup>R</sup>) resistance cassette in 3 transformants (designated 1, 2, 3) by PCR on genomic DNA.

A) Schematic illustration of hik12 KO construct. Primer P1 (Hik12 KO Check fw_1241)/P2 (Hik12 KO Check rev_1242) bind within the flanking regions of hik12 (sll1672), amplifying parts of the flanking regions and the inserted KM<sup>R</sup> (estimated fragment size: 1396 bp, black arrow).

B) Schematic illustration of wildtype hik12 (sll1672). Primer P1 (Hik12 KO Check fw_1241, also used in A), primer P3 (Hik12 SDM-2) binds within hik12 ORF (estimated fragment size in WT: 1744 bp, black arrow). MW, molecular mass standard.
Supplementary figure 2: Specific G6PDH activity in WT and *hik12 _2 KO* mutants under photomixotrophic/biomatforming growth conditions.

Photoautotrophically grown, mid-exponential cell cultures (OD\textsubscript{750} = 0.6-0.8) of WT and *hik12 _2 KO* mutants (clone 2-2 and clone 2-3) were supplied with glucose at a final concentration of 10 mM and incubated without agitation for 96 h, 28°C, under continuous illumination with a light intensity of 20 µmol photons m\textsuperscript{-2} s\textsuperscript{-1}. 100 µg of crude protein were used in a total volume of 1 ml assay solution to determine G6PDH activity. Data are normalized to the WT (0.051 ± 0.001 U/µg protein, set as 100% for each individual experiment). Columns represent average values of 3 independent experiments. Bars represent standard errors.
Supplementary figure 3: PCR-based verification of T-DNA insertion in AHK3-T-DNA insertion plants.

To verify the T-DNA insertion in AHK3-T-DNA insertion plants, genomic DNA was isolated as described in 2.1.1. To detect the presence of the T-DNA insertion in 10 transgenic AHK3-T-DNA insertion line plants (designated 1-10), a T-DNA-insert-specific primer (T-DNA, stocknumber: 356) and a genomic reverse primer (AHK3-7 rev, stocknumber: 907) were used for PCR. Primer sequences are given in Table 2. Estimated fragment size: 550 bp. MW, molecular mass standard.