Effects of Phosphorylation of β Subunits of Phycocyanins on State Transition in the Model Cyanobacterium *Synechocystis* sp. PCC 6803

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(Received April 27, 2015; Accepted August 9, 2015)

**Synechocystis** sp. PCC 6803 (hereafter *Synechocystis*) is a model cyanobacterium and has been used extensively for studies concerned with photosynthesis and environmental adaptation. Although dozens of protein kinases and phosphatases with specificity for Ser/Thr/Tyr residues have been predicted, only a few substrate proteins are known in *Synechocystis*. In this study, we report 194 in vivo phosphorylation sites from 149 proteins in *Synechocystis*, which were identified using a combination of peptide pre-fractionation, TiO₂ enrichment and liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. These phosphorylated proteins are implicated in diverse biological processes, such as photosynthesis. Among all identified phosphoproteins involved in photosynthesis, the β subunits of phycocyanins (CpcBs) were found to be phosphorylated on Ser22, Ser49, Thr94 and Ser154. Four non-phosphorylated mutants were constructed by using site-directed mutagenesis. The in vivo characterization of the cpcB mutants showed a slower growth under high light irradiance and displayed fluorescence quenching to a lower level and less efficient energy transfer inside the phycobilisome (PBS). Notably, the non-phosphorylated mutants exhibited a slower state transition than the wild type. The current results demonstrated that the phosphorylation status of CpcBs affects the energy transfer and state transition of photosynthesis in *Synechocystis*. This study provides novel insights into the molecular mechanisms of protein phosphorylation in the regulation of photosynthesis in cyanobacteria and may facilitate the elucidation of the entire regulatory network by linking kinases to their physiological substrates.

**Keywords:** β subunits of phycocyanins (CpcBs) • Phosphoproteomics • Photosynthesis • State transition • *Synechocystis* sp. PCC 6803 • TiO₂ enrichment.

**Abbreviations:** ACN, acetonitrile; APC, allophycocyanin; CPC, C-phycocyanin; CpcB, β subunit of phycocyanin; FA, formic acid; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LHCCI, light-harvesting complex II; OCP, orange carotenoid protein; PBS, phycobilisome; PC, phycocyanin; PE, phycoerythrin; PPI, protein–protein interaction; PQ, plastoquinone; PsbC, CP43 of PSII; ROS, reactive oxygen species; STK, Ser/Thr/Tyr kinase; TFA, trifluoroacetic acid; WT, wild type.

**Introduction**

Cyanobacteria are a diverse group of Gram-negative prokaryotes that show a variation in genome size from 1.6 to 13 Mb and exhibit extraordinary diversity in terms of morphology and cell activity (Abed et al. 2009). They are photoautotrophic prokaryotes that are capable of growing in a wide range of ecological environments and are able to survive fluctuations in temperature, light and chemical concentrations (Crispim and Gaylarde 2005). The diversity of cyanobacteria is also reflected in the complexity of their signal transduction systems. To cope with changing environmental conditions, cyanobacteria have developed a complex signal transduction network to respond to external or internal changes. Two-component regulatory systems, characterized by the transfer of phosphate by a sensor kinase from a histidine residue on the enzyme to an aspartate residue on the response regulator, seem to be the most important mode of signal transduction in cyanobacteria (Zhang et al. 2005). In contrast, Ser/Thr/Tyr kinases (STKs) serve as the backbone of the eukaryote transduction network (Tichy et al. 2011). However, upon the discovery of eukaryotic-like STKs in the bacterium *Myxococcus xanthus* in 1991 (Munoz-Dorado et al. 1991), progress in large-scale genomic sequencing projects has shown ubiquitous distribution of these protein kinases in prokaryotes (Kennelly 2002, Krupa and Sriniivasan 2005, Perez et al. 2008). Approximately two-thirds of all prokaryotes have been estimated to harbor eukaryotic-like STKs (Perez et al. 2008), indicating that signaling involving phosphorylation on Ser/Thr/Tyr residues is also widespread in these organisms.

Protein phosphorylation on Ser/Thr/Tyr residues in cyanobacteria was first revealed via the radioactive labeling of proteins in 1994 (Mann 1994). The unicellular freshwater cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was the first cyanobacterial genome to be sequenced and contains 12 genes for putative STKs (Kaneko et al. 1996, Zhang et al. 1998). Of the 12 putative proteins involved in photosynthesis, the subunit II; PsbC, CP43 of PSII; ROS, reactive oxygen species; STK, Ser/Thr/Tyr kinase; TFA, trifluoroacetic acid; WT, wild type.
STKS in Synechocystis, seven genes encode proteins that belong to the Pkn2 type, named SpkA–SpkG (Kamei et al. 2002), and five encode proteins that belong to the ABC1 type, named SpkH–SpkL (Zorina et al. 2011). The functions of a few Synechocystis STKS have been addressed via the phenotypical characterization of the respective mutants. Thus, the spkA, spkB, spkC, spkD and spkG genes were found to participate in the control of cell motility (Kamei et al. 2001), the oxidative stress response (Mata-Cabana et al. 2012), the regulation of nitrogen metabolism (Galkin et al. 2003), the growth at low concentrations of inorganic carbon (Laurent et al. 2008) and high salt resistance (Li et al. 2011), respectively. Knowledge of the substrates of each of the cyanobacterial STKS is essential to understand their function; however, none of the substrates for any of these enzymes has hitherto been identified, and whether the cyanobacterial STKS are subject to post-translational modification and regulation remains unknown (Zhang et al. 2005).

The first site-specific study of the Ser/Thr/Tyr bacterial phosphoproteome was reported for the bacterium Bacillus subtilis in 2007 by using high accuracy mass spectrometry in combination with the bioinformatic enrichment of phosphopeptides from digested cell lysates (Macek et al. 2007). The same approach has since been employed to analyze the model organisms Escherichia coli (Macek et al. 2008) and Lactococcus lactis (Soffi et al. 2008), as well as a number of bacterial pathogens (Lin et al. 2009, Ravichandran et al. 2009, Prsic et al. 2010, Sun et al. 2010, Misra et al. 2011). As such, this approach has become the standard procedure in bacterial phosphoproteomics (Mijakovic and Macek 2011). These studies have generated large data sets of proteins phosphorylated on serine, threonine and tyrosine in bacteria, with identified phosphorylation sites that represent an excellent starting point for the in-depth physiological characterization of kinases and their substrates. However, so far, phosphorylation sites on serine, threonine or tyrosine have been determined in only a very limited number of Synechocystis proteins according to the prokaryotic Phosphorylation Site Database and a 2D gel electrophoresis-based snapshot of the phosphoproteome in Synechocystis (Wurglar-Murphy et al. 2004, Mijkat et al. 2014).

To obtain a comprehensive understanding of the in vivo Ser/Thr/Tyr phosphorylation events in cyanobacteria, we analyzed the Ser/Thr/Tyr phosphoproteome of Synechocystis using high accuracy mass spectrometry in combination with phosphopeptide enrichment and compared it with the phosphoproteome of other bacteria. These results provided extensive data on Ser/Thr/Tyr phosphorylation and novel insights into the range of functions regulated by Ser/Thr/Tyr phosphorylation in Synechocystis. The bioinformatic analysis of these in vivo phosphoproteins enabled us to identify a phosphorylation site motif shared by several kinases, leading to a model of STK–substrate interaction. Further functional study revealed that the phosphorylation of the β subunits of phycocyanins (CpcBs) is involved in the perception of high light and energy transfer, which influences the state transition of photosynthesis. These results will serve as an important resource for the further investigation of these signal transduction pathways in cyanobacteria and, more broadly, in prokaryotes.

**Results**

**Phosphoproteome of Synechocystis sp. PCC 6803**

We have applied a high accuracy method to study the phosphoproteome of cyanobacterium Synechococcus sp. strain PCC 7002. In the present study, we characterized the in vivo protein phosphorylation events in Synechocystis by using TiO2 affinity chromatography coupled to liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) (Fig. 1A). In addition, we manually checked and removed the suspicious phosphopeptides with unclear MS/MS spectra. Only those hits with a sufficiently high score which passed manual validation were chosen and are listed in Supplementary Table S1. In total, 227 phosphopeptides, from 149 proteins, were identified with a false discovery rate (FDR) <1% for peptides. Among the identified phosphopeptides, 194 non-redundant phosphorylation sites were determined with the highest level of confidence (class I). The ratio of Ser/Thr/Tyr distribution (42.3% : 51.5% : 6.2%) (Fig. 1B) was similar to that of Synechococcus sp. strain PCC 7002 and other bacterial phosphoproteomes, which was a striking departure from findings in euukaryotes, where serine phosphorylation may account for 80–90% of the total phosphorylation sites. Moreover, most identified phosphopeptides possessed only one phosphate group (89.9%) in Synechocystis (Fig. 1B). To validate further the phosphorylation sites identified in our study, we predicted these identified phosphoproteins using NetPhosBac. Approximately 19.6% of the phosphorylation sites identified in Synechocystis showed matches between experimentally detected phosphorylation sites and predicted phosphorylation sites. All raw data and annotated peptide spectra for phosphorylated peptides have been deposited in the publicly accessible database PeptideAtlas (http://www.peptideatlas.org) and can be accessed with the identifier PASS00537 (http://www.peptideatlas.org/PASS/PASS00537).

**Functional classification of the identified phosphorylated proteins**

We used the Blast2GO tool to classify the identified phosphorylated proteins in Synechocystis. Among 149 phosphoproteins identified in this study, 96 (64.4%) were functionally annotated in the biological process (Fig. 1D; Supplementary Table S2). From the biological process perspective, the phosphoproteins were mostly involved in generation of precursor metabolites and energy (16.7%), photosynthesis (15.8%) and other processes. Accordingly, as shown in Supplementary Table S2, 57 (38.3%) phosphoproteins were located in the cytoplasm, 43 were assigned to the cytoplasmic membrane and one to the periplasm. However, the localizations of the majority of the identified phosphoproteins (30.9%) were not predicted. Moreover, of the phosphoproteins with annotated molecular function, the number of phosphorylated proteins in various subcategories was as follows: ion binding, 48;
kinase activity, 13; oxidoreductase activity, 12; RNA binding, 10; and so on. Finally, we have applied a multiple testing correction and performed the statistical test, and the results are shown in Supplementary Fig. S1 and Supplementary Table S3. These findings indicated that phosphorylated proteins were involved in various cellular processes in Synechocystis.

To gain further insight into the functional roles of the Synechocystis phosphoproteome, we conducted Gene Ontology (GO) enrichment analyses for the biological process, molecular function and cellular component categories (Fig. 2A; Supplementary Table S4). In the biological process category, phosphoproteins were markedly enriched in photosynthesis (P-value, 2.07E-04), glucose metabolic process (P-value, 7.55E-03) and hexose metabolic process (P-value, 4.01E-02). For cellular localization, phosphoproteins were over-represented in the phycobilisome (PBS) subcategory (P-value, 3.16E-07), which indicated that the phosphorylation of proteins plays a role in light harvesting in photosynthesis. Supplementary Table S5 lists the 15 motifs found at 14 phosphorylation sites within the top 1% of SCANSITE hit scores in detail. Out of the 15 motifs, the sites phosphorylated by the Acidophilic serine/threonine kinase group (Casein Kinase 1 and Casein Kinase 2), Basophilic serine/threonine kinase (Aurora A and Aurora B) and Proline-dependent serine/threonine kinase group (Pro_ST_kin) were most predicted. Although Motif-X did not find evidence for significantly over-represented consensus sequences, motif analysis was gained from the Phosida algorithm motif matcher by using known motifs. The majority of the analyzed phosphosites were targeted by the CK1 (S-X-X-S/T), CK2 (S/T-X-X-E) and NEK6 (L-X-X-S/T) kinases (see Supplementary Table S6 for details). These results were consistent with those derived from the SCANSITE analysis. Such an analysis may be helpful for the identification of Ser/Thr/Tyr kinases in Synechocystis and identification of motifs within a protein, which may provide a platform for identification of their roles within the cell signaling pathway.

We used the algorithm NetSurfP to investigate the secondary structures of identified phosphoproteins in Synechocystis. Compared with the non-phospho-Ser/Thr/Tyr of the phosphoproteins identified in this study, phospho-Ser/Thr/Tyr showed
Fig. 2 Overview of GO distribution of identified phosphoproteins, bioinformational analysis of phosphorylation sites, and comparative analysis of orthologous phosphoproteins in *Synechocystis*. (A) GO terms enriched in phosphoproteins using BINGO 2.44, a plugin of Cytoscape. A corrected P-value <0.05 was considered as significant. (B) The relative abundance of amino acid residues flanking the phosphorylation sites (S/T/Y) represented by an intensity map. The intensity maps show the enrichment or depletion of amino acids at specific positions of phospho-13-mers (six amino acids upstream and downstream of the phosphorylation site) compared with that of non-phospho-13-mers (six amino acids upstream and downstream of the non-phosphorylation site). The colors in the intensity map represent the log10 of the ratio of frequencies within phospho-13-mers vs. non-phospho-13-mers (red shows enrichment, gray shows depletion). (C) Comparative analysis of orthologous phosphoproteins with other bacterial strains.
similar preferences for the secondary structure, as shown in Supplementary Fig. S2. Our results showed that phospho-
Ser/Thr/Tyr was more frequently found in unstructured coil regions, which is consistent with previous reports (Johnson and Lewis 2001, Iakoucheva et al. 2004). We next performed analyses to attempt to identify the preferred flanking amino acids for the identified phosphorylation site (six amino acids upstream and downstream of the phosphorylation site) with the entire set of the *Synechocystis* proteome as previously described (Beausoleil et al. 2004, Treece et al. 2011, Yang et al. 2013) (Fig. 2B). For all of the pS-containing peptides, the preferred phosphorylation site motifs were identified for the cysteine (C) residues at the −1 and +5 positions. For all of these pT-containing peptides, the major features of the preferred phosphorylation site motif were identified for the methionine (M) and histidine (H) residues at the −2 and −4 positions, respectively. For pY-containing peptides, we observed the most prominent M enrichment at the −1 position. The high abundance of these amino acids suggests the important role of these amino acids in protein phosphorylation in *Synechocystis*.

Comparative analysis with other bacterial phosphoproteomes

To study the conservation of phosphoproteins among bacterial prokaryotes, we compared the *Synechocystis* phosphoproteome with other bacterial phosphoproteomes reported previously (Macek et al. 2007, Voisin et al. 2007, Macek et al. 2008, Soufi et al. 2008, Aivaliotis et al. 2009, Lin et al. 2009, Ravichandran et al. 2009, Parker et al. 2010, Prisic et al. 2010, Schmidl et al. 2010, Sun et al. 2010, Ge et al. 2011, Manteca et al. 2011, Misra et al. 2011, Bai and Ji 2012, Yang et al. 2013). Eleven phosphoproteins have been detected using a 2D gel electrophoresis-based protocol in *Synechocystis* (Mikkat et al. 2014). Among the 93 orthologous phosphoproteins detected in other bacteria, 62 shared common sequences with *Synechococcus* sp. strain PCC 7002 (Fig. 2C; Supplementary Table S7). Additionally, the *Synechocystis* phosphoproteome exhibited greater similarity with Gram-negative bacteria (162 phosphoproteins) than with Gram-positive bacteria (101 proteins). These results point to an evolutionarily conserved and potentially vital role for phosphorylation in their function. The comparative analysis of phosphoproteomes revealed that the phosphorylation of proteins for photosynthesis, several protein kinases and two-component system proteins among these pro-
karyotes was highly conserved between *Synechococcus* and *Synechocystis*; for instance, many phosphoproteins involved in the process of ATP synthase (sll1326), respiratory terminal oxida-
dases (srl1137), CO₂ fixation (sll1031, sll1525), PSI (sll0737) and PSII (sll0928, sll1577, sll1578, sll1579, sll1580, srl0335, srl1986, srl2067), suggesting the conservation of these photosynthesis-
related proteins. Interestingly, in *Synechocystis* and *Synechococcus*, slr1157 and SYNPCC7002_A2210 as well as srl2067 and SYNPCC7002_A1930 in PSBs, and srl0737 and SYNPCC7002_A0682 in PSI exhibit two highly conserved phosphorylated peptides (FPYTT*T*GNFAADQR and IKAFVT* G**R) and one highly conserved phosphorylated site (T15 vs. *Synechococcus* T16), respectively, indicating the crucial role of phosphorylation in these protein complexes.

Phosphorylated proteins involved in photosynthesis

We used the STRING database to assess the direct protein–protein interactions (PPIs) within our data sets, and the network created suggested that a number of phosphoproteins identified in this study were clustered in photosynthesis (Fig. 3A). Seven clusters of putative interacting proteins were retrieved using MCODE (Supplementary Table S8). The first high-ranking complexes are presented in Fig. 3A (cluster 1 in Supplementary Table S8), which consists of the PBS (sll1577, sll1578, sll1579, sll1580, sll0928, srl1986, srl2067, srl3383, srl0335) and orange carotenoid protein (OCP; Slr1963) and sll1734. Most of these phosphorylated proteins are involved in light harvesting and the dissipation of the excess absorbed energy (Fig. 3B) (Wilson et al. 2006). They have also been as-
signed to the photosynthesis pathway via KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis (Supplementary Table S9).

Site-directed mutagenesis of phosphorylation sites of CpcBs

PBSs comprise the primary light-gathering antennae in red algae and in cyanobacteria, which consist exclusively of phy- cobiliproteins and linker polypeptides. Phycocyanins (PCs) and allophycocyanin (APC) are the major phycobiliproteins of cyanobacteria, and phycoerythrins (PEs) are found in some cyanobacteria. They consist of α-type and β-type subunits of 17–22 kDa (MacColl 1998). The β subunits of the phyco-
cyanins (CpcBs) in *Synechocystis* are encoded by the cpcB gene (sll1577). Four sites of the CpcBs, i.e. Ser22, Ser49, Thr94 and Ser154, are targets for phosphorylation. Using the previously described hit-and-run method (Clerico et al. 2007), we successfully converted the amino acid at the four posi-
tions to a non-phosphorylatable alanine or glycine to con-
struct the four mutants S22A, S49A, T94A and S154G. All mutations were verified by both DNA sequencing (Supplementary Fig. S3) and MS analysis (Supplementary Fig. S4). Furthermore, to better understand their potential role, the structure of CpcB was modeled and the location of the identified phosphorylation sites was marked accordingly, as shown in Supplementary Fig. S5.

To make sure that the expression levels of the rod protein CpcB were not affected by the point mutations, we analyzed the abundance of CpcBs in both the wild type (WT) and the mutant strains by Western blotting (Fig. 4). The results showed that CpcBs in the mutants were expressed to the same level as in the WT. In addition, the intrinsic core of PSI and PSII in the mutants and WT were further analyzed using the antibodies of PSI subunit II (PsaD) and PSII CP43 protein (PsbC). Both proteins were expressed in equal amounts between the WT and the mutant strains, indicating that neither PSI nor PSII was affected by the site-directed mutagenesis of the phosphorylation sites of CpcBs.
Fluorescence emission spectra of whole-cell and isolated PBSs

The PBS fluorescence emission spectra at room temperature of the WT and cpcB mutants were examined with an excitation wavelength of 600 nm to excite the PBS. At room temperature, most of the PBS fluorescence is emitted from the APC core (Stadnichuk et al. 2009). Normally, the PBS-excited fluorescence spectrum of the WT shows a major peak at 658 nm emitted from APC and a shoulder at 685 nm emitted from Chl (Fig. 5A). As shown in Fig. 5A, the fluorescence spectra of all of the cpcB point mutants are substantially similar to that of the WT, which indicated that the APC core of the cpcB mutants is not affected under normal growth conditions.

To test the effect of the phosphorylation of CpcBs on the PBS assembly, PBSs were isolated from the WT and mutant of Synechocystis, which had been grown under standard growth conditions. The PBSs were recovered from the 1.0 M sucrose zone of the step gradients, and the sedimentation properties of PBSs from the WT and cpcB mutants were identical. To investigate further whether the energy transfer inside the PBS was affected by the phosphorylation status of CpcBs, the fluorescence emission spectra of isolated PBSs from both the WT and...
Fig. 5 Fluorescence emission spectra of whole cells and isolated phycobilisomes. (A) Room temperature fluorescence emission spectra of wild-type cells (WT) or mutant cells (S22A, S49A, T94A and S154G) were separated by 12% SDS–PAGE and stained with Coomassie Brilliant Blue. (B) Immunoblotting was performed using antibodies specific for CpcB, PsaD and PsbC.

Fig. 4 Western blotting analysis of CpcB protein from the wild type and mutants. (A) Proteins (10 μg) from wild-type cells (WT) or mutant cells (S22A, S49A, T94A and S154G) were separated by 12% SDS–PAGE and stained with Coomassie Brilliant Blue. (B) Immunoblotting was performed using antibodies specific for CpcB, PsaD and PsbC.
all of the point mutants were tested. As shown in Fig. 5B, the fluorescence emission spectra of the intact PBS isolated from both the WT and the mutants showed a single peak at 666 nm, which indicated that the isolated PBSs were intact (Kume and Katoh 1982). However, all four cpcB point mutants had lowered emission quantum yields (F666, Fig. 5B) compared with the WT; taking into account that the expression level of CpcB compared with that of phycocyanin and was amplified to the value in basic fluorescence of samples. The basic fluorescence was very low stant and much more obvious than that of the quenching of CPC fluorescence at 650 nm in the WT was con-

In the PBS, the energy absorbed by C-phycocyanins (CPCs) in the rod is transferred down to the APC core, leading to the fluorescence quenching of CPCs (Yang et al. 2009). The CPC fluorescence decay was determined in order to investigate further the energy transfer efficiency of the protein inside the PBS complex. Light at 625 nm can excite the CPCs to the greatest extent, and the CPCs showed the highest fluorescence emission at 650 nm (Stadnichuk et al. 2009). Fig. 6 shows that the quenching of CPC fluorescence at 650 nm in the WT was constant and much more obvious than that of the cpcB point mutants when excited at 625 nm, which indicated that the energy transfer efficiency down to the APC core in the mutants was reduced, which corresponds well to the fluorescence emission spectra analysis results of the isolated PBSs (Fig. 5B).

**CPC fluorescence decay in vivo**

In the PBS, the energy absorbed by C-phycocyanins (CPCs) in the rod is transferred down to the APC core, leading to the fluorescence quenching of CPCs (Yang et al. 2009). The CPC fluorescence decay was determined in order to investigate further the energy transfer efficiency of the protein inside the PBS complex. Light at 625 nm can excite the CPCs to the greatest extent, and the CPCs showed the highest fluorescence emission at 650 nm (Stadnichuk et al. 2009). Fig. 6 shows that the quenching of CPC fluorescence at 650 nm in the WT was constant and much more obvious than that of the cpcB point mutants when excited at 625 nm, which indicated that the energy transfer efficiency down to the APC core in the mutants was reduced, which corresponds well to the fluorescence emission spectra analysis results of the isolated PBSs (Fig. 5B).

**Light sensitivity of cpcB mutant cells**

As shown above, both the fluorescence emission spectra (Fig. 5B) and quenching analysis (Fig. 6) indicated that the energy transfer rates from the PBS rod down to the APC core were adversely affected in the phosphorylation site-directed point mutants. The decreased PBS energy transfer efficiency normally reduces the light energy use efficiency and improves the high light acclimation. Thus, the cpcB mutants would be expected to be less light sensitive and survive better under high light conditions. Surprisingly, as shown in Fig. 7A, the growth test showed that the WT and the cpcB mutants did not significantly differ when cultured at 7 μmol photons m⁻² s⁻¹ (low light) and 40 μmol photons m⁻² s⁻¹ (normal light); however, when cultured under high light conditions at 250 μmol photons m⁻² s⁻¹, the growth rates of cpcB mutants were significantly lower than that of the WT, especially that of T94A, which could be easily ascertained from the color of the liquid cultures (Fig. 7B).

**State transitions of the cpcB mutant**

In addition to the energy transfer inside the complex itself, PBSs can also affect the light sensitivity of cyanobacteria via state transition. As reported before, the psak2 mutant whose PBS is defective in the PsaK2 subunit cannot adapt to high light because its state transition is affected (Fujimori et al. 2005). The state transition was determined according to Schluchter et al. (1996) and Aspinwall et al. (2004); briefly, dark-adapted cells in state 2 were induced by illumination in the presence of DCMU to block electron flow into the plastoquinone (PQ) pool from PSII, which leads to the oxidation of the intersystem electron carriers and the transition to state 1. The process, which occurs on a time scale of a few seconds to approximately 1 min, could be easily recorded as an increase in the PSII fluorescence at room temperature (Fig. 8). The fluorescence increase was then fitted exponentially (Fig. 8), and the t₁/₂ and amplitude of the state 1 transition were calculated accordingly (Table 1). The state 1 transitions in the S22A, S49A, T94A and S154G strains were slower than that of the WT (Fig. 8, solid lines, and Table 1), which indicated that the time course of the state transition in the mutant strains increased. The amplitudes of the state transition of all mutant strains were significantly lower (10%-40%) than that of the WT (Fig. 8; Table 1). The results suggested that the rapid response and the capacities of state transition were reduced in the cpcB phosphorylation mutant strains.

**Discussion**

Our phosphoproteomic data indicated that protein phosphorylation on serine/threonine/tyrosine residues were involved in diverse cellular processes in the model cyanobacterium *Synechocystis*, including metabolic pathways and signal transduction. In our study, one STK, SpkD (Sll0776), was identified as a phosphorylated protein. Phosphorylation events carried out by kinases have been demonstrated as autophosphorylation or the phosphorylation of the general substrate. An increasing number of STKs were discovered in prokaryotes, and putative STKs homologs were recently identified from several species of sequenced cyanobacteria (Zhang et al. 2007). Some previous surveys have also revealed that several potential eukaryotic protein kinases exist in *Synechocystis* (Kaneoko et al. 1996, Shi et al. 1998, Zhang et al. 1998). Of these putative STKs, seven genes encode proteins that belong to the Pkn2 type, named...
SpkA–SpkG (Kamei et al. 2002), and five encode proteins that belong to the ABC1 type, named SpkH–SpkL (Zorina et al. 2011). Among them, SpkA (Sll1574) (Kamei et al. 2001), SpkB (Slr1697) (Kamei et al. 2003), SpkC (Slr0599), SpkD (Sll0776), SpkF (Slr1225) (Kamei et al. 2002) and SpkE (Slr1443) (Zorina et al. 2014) have been shown to phosphorylate themselves or other substrate proteins. In this work, the kinase motifs recognized by certain protein kinases were also investigated. Some kinases have been identified in prokaryotes (Sun et al. 2010, Yang et al. 2013), including a model cyanobacterium *Synechococcus* sp. PCC 7002. Because of lack of functional experiments associated with these kinases, we were unable to confirm the existence of these kinases in *Synechocystis*. However, the result of the kinase motif search may be helpful for the identification of potential kinases as well as their roles within the cell signaling pathway in *Synechocystis*.

The motif analysis yields motifs with many Xs, where 'X' denotes any amino acid residue. The degenerate amino acid substitutions have often been found in kinase motifs. The reason lies in that protein kinases do not necessarily associate with their substrates and the specificity of the substrate is very relaxed. Several kinases have been well characterized to have multiple substrates in prokaryotes, such as Hanks-type STKs from *Mycobacterium tuberculosis* (Grundner et al. 2005, Molle and Kremer 2010, Baer et al. 2014) and *Bacillus subtilis* (Absalon et al. 2009, Pietack et al. 2010, Ravikumar et al. 2014), PknC from *Anabaena* strain PCC 7120 (Gonzalez et al. 2001) as well as the tyrosine kinase PtkA from *B. subtilis* (Petranovic et al. 2009, Jers et al. 2010, Derouiche et al. 2013). The motif analysis in the present study revealed several motifs identified by some unknown protein kinases. Despite the degenerate amino acid substitutions in this data set, it is interesting that the biological significance of these degenerate motifs has yet to be reported in *Synechocystis*.

It has been widely accepted that reversible protein phosphorylation plays a key role in signal transduction, and many metabolic processes. Consistent with previous reports on Ser/Thr/Tyr phosphorylation, a large proportion of metabolic enzymes involved in central metabolism were found to be phosphorylated, such as enzymes involved in biosynthesis of amino acids, glycolysis/gluconeogenesis and carbon metabolism (Yang et al. 2013). These enzymes in the central metabolism pathway were phosphorylated, indicating the regulatory role of phosphorylation in cellular metabolic processes. Interestingly, we also identified enzymes involved in the microbial metabolism in diverse environments and two-component systems, which are responsible for adaptive responses to a number of environmental stresses. In addition, our study supports the notion that the phosphorylation of hypothetical proteins identified in this study may participate in cellular processes; nevertheless, the potential roles of these phosphorylated proteins have to be further investigated.

PPIs and KEGG pathway analyses showed that a number of phosphoproteins were involved in photosynthesis. These phosphorylation events in PSI, OCP and PBSs were mapped to interpret the molecular mechanism of the photosynthetic process in *Synechocystis* (Fig. 3B). Among these, the PBS...
Fig. 8 The kinetics of the transition to state 1 of the wild type and cpcB mutants and a working model for the phosphorylation-induced PBS-dependent state transition under high irradiance. (A) Cells were resuspended in growth medium at a Chl concentration of 3 μg ml⁻¹. After 10 min of dark adaption to induce state 2, the cells were illuminated in the presence of DCMU to induce state 1. The excitation and emission wavelengths were set to 660 and 695 nm, respectively. To compare the state transition kinetics, the faster phase of the fluorescence rise has been subtracted, and the traces were normalized to the same initial fluorescence. The traces were fitted to exponential curves using Origin 8.0. The inset graph shows an example of exponential fitting, which indicates that all of the original trace and its corresponding fitted curve are nearly coincident. The statistical data obtained from the exponential curve fitting are shown in Table 1. (B) Upon the state 1 (upper) to state 2 (lower) transition, the phycobilisome migrates from PSII to PSI.
represents the primary antenna in cyanobacteria; each native PBS contains a central core and several radial rods. The main functional proteins are phycobiliproteins including APC in the core and PC in the peripheral rod, which consist of two dissimilar polypeptides, designated α and β (Zilinskas and Greenwald 1986). There is well-known evidence of the involvement of protein phosphorylation in the structural and functional process in the PBS (Mann 1994). Piven et al. (2005) concluded that the phosphorylation process of linking proteins is instrumental in the regulation of assembly/disassembly of PBSs and should participate in signaling for their proteolytic cleavage and degradation. Arranging the phycobiliproteins into rods is thought to have an important physiological significance that provides the structural basis for efficient energy transfer to respond to variations of environmental conditions and to adapt to extreme habitats (Westermann et al. 1993). The major component of the PBS rod structure, CpcB, is subject to monooester phosphorylation (Mann 1994). In Synechococcus sp. PCC 6301, labeling experiments performed in vivo with [32P]orthophosphate demonstrated the phosphorylation of an 18.5 kDa protein that was also found in a purified PBS fraction and has been proposed to represent CpcB (Sanders et al. 1989). Consistent with previous findings, the phosphorylation of CpcB has been identified in Synechocystis (Mann 1994, Mukhopadhyay and Kennelly 2011) and Synechococcus sp. strain PCC 7002 (Yang et al. 2013). However, the regulatory role of the phosphorylation process in CpcB remains unknown. Therefore, the role of the phosphorylation status of CpcB in the energy transfer and state transition is tempting to explore.

In this study, we collected cells under normal growth conditions, and four phosphorylation sites, S22, S49, T94 and S154 were found in CpcB under these conditions, and the corresponding single point mutations, S22A, S49A, T94A and S154G were subsequently constructed to evaluate the possible roles of phosphorylation in the subunit. The overlapping fluorescence emission spectra at room temperature from the mutants and WT show that the APC core was not affected (Fig. 5A). The fluorescence emission spectra of isolated PBSs from the mutants exhibited a lower F666 than that of the WT (Fig. 5B), indicating energy losses without fluorescence emission in the PBS. Together with the fluorescence decay analysis results, which indicated that the WT showed constant and much more obvious CPC fluorescence quenching (Fig. 6), our results demonstrated that the phosphorylation status of the PC rod affects the down-transfer of absorbed light energy. The chain fold for PC is mostly α-helical in Synechocystis. Among the four residues, Ser22, Ser49 and Thr94 are in the Y, A and E helices, respectively. The X and E helices of one subunit and the Y and A helices of the other subunit are involved in extensive interactions (Apt et al. 1995). Therefore, phosphorylation may play a role in the efficient maintenance of the energy down-transfer.

However, unexpectedly, we observed that the WT and the mutant strains grow similarly under normal and very low (Fig. 7A) light conditions. These results, together with the observation that an intact PBS with the same sedimentation properties could be obtained, indicates that the phosphorylation of CpcB is not essential for PBS assembly. Furthermore, the losses in the energy transfer of cpcB mutants, while measurable, did not impair growth at low light intensity. Similar results in Synechococcus sp. PCC 7002 and Synechocystis have also been reported by Shen et al. (2008); specifically, they reported that the methylation of CpcB affects the energy transfer without decreasing growth under low light.

Even more surprisingly, we found that the cpcB mutants, which failed to down-transfer light energy efficiently to the APC core, are more sensitive to high light conditions (Fig. 7A). In this study, we speculate that when suffering high light stress, the lower energy down-transfer efficiency might cause a relatively more severe inhibition. Generally, energy absorbed by PC is transferred to the PSII reaction center along the following pathway: PC to APC to the terminal emitters to Chl in PSII (Gao et al. 2012). The energy transferred down is then converted to chemical energy in the form of ATP. Since the down-transfer of the site mutant was slower, the energy captured by the phosphorylation defect in CpcB became relatively more supersaturated than that in the WT, increasing the chances of the formation of long-lived phycobilin triplet states (Muller et al. 2001). The triplet state of phycobiliproteins may generate singlet oxygen (1O2) and superoxide (O2−·) which are important sources of reactive oxygen species (ROS) (Rinalducci et al. 2008). ROS can mediate the damage to the photosynthetic apparatus and further induce the inhibition of photosynthesis (He and Hader 2002, Edreva 2005). Furthermore, since CpcB contains two phycobilin proteins whereas other PBS proteins have only one, and is located at the periphery of the PBS, it can greatly sensitize the formation of ROS (Zolla et al. 2002, Rinalducci et al. 2006). Based on these facts, we speculate that the phosphorylation defect leads to lower down-transfer efficiency and then the generation of more ROS, which finally contribute to the higher light sensitivity of the site mutant. In future studies we will determine whether CpcBs lacking phosphorylation produce more ROS in order to confirm the speculation.

Since the PBS can also affect the light sensitivity of cyanobacteria through the state transition as well as the energy transfer inside the complex itself, we explored the role of phosphorylation states of CpcB in state transitions. State transition monitored by increases in PSII fluorescence (Fig. 8A) indicated that both the t1/2 and the amplitude (Table 1) of

| Table 1 The statistical data obtained by exponential curve fitting |
|---------------------|--------|---------|-------|-------|-------|
|                  | WT     | S22A    | S49A  | T94A  | S154G |
| t1/2 (ms)         | 11.25 ± 0.24 | 13.39 ± 0.40 | 17.15 ± 0.40 | 11.70 ± 0.29 | 13.16 ± 0.32 |
| Amplitude (%)     | 100    | 86.5    | 90.7  | 61.3  | 78.8  |

the state transition were adversely affected in the phosphorylation mutant strains. The state transitions of plants and eukaryotic algae are correlated with the reversible phosphorylation of the polypeptides of the light-harvesting complex II (LHCII) antenna complex (Dépège et al. 2003). Shen et al. (2008) reported that the methylation of Asp71/72 of CpcB in *Synechocystis* (W. Zhao et al. 2014), we speculate that the phosphorylation as soon as OCP-mediated fluorescence quenching occurred in the PBS between the two reaction centers. In cells adapted to state 1, PBSs transfer energy primarily to PSI. In cells adapted to state 2, a significant proportion of energy is transferred to PSI instead (van Thor et al. 1998). Beside that transferred onwards from PSI to PSI and transferred directly from the PBS core to PSI in the same way as to PSI (Mullineaux 1994, Busch et al. 2010, Liu et al. 2013), the PBS rod can transfer energy to PSI by-passing the PBS core (Su et al. 1992). A distinct CpcG2-PBS which retains PC rods but is devoid of a typical central core consisting of APC was reported in 2005; this transfers light energy preferentially to PSI (Kondo et al. 2005, Kondo et al. 2007). Recently, PC has been found to react with the PSI primer only in cells in state 2, but not in state 1 or in the state 2 transition-deficient mutant (J. Zhao et al. 2014). The phosphorylation of CpcB may be specifically involved in the energy transfer from the rod to the PSI reaction center and may further affect the state transition.

In higher plants, the phosphorylation and movement of LHCII are required for state transition (Allen 2002, Mullineaux and Emlyn-Jones 2005). The reversible phosphorylation of LHCII induces the migration of LHCII between the grana (PSII-enriched) and the stroma (PSI-enriched) domains of the thylakoids and therefore regulates the LHCII association with photosystems (Tikkkanen and Aro 2012). The activation of protein kinase for LHCII phosphorylation requires the reduction of the PQ pool (Allen et al. 1981). In cyanobacteria, the main trigger for state transitions is also the reduction or oxidation of PQ or a closely associated electron carrier (Mullineaux and Allen 1986, Mao et al. 2002). Therefore, we speculate that the phosphorylation of the PBS affects the state transition via involvement of the reaction between the PBS and the two photosystems, which is under the control of PQ pool-related redox signaling in a similar way to higher plants. The phosphorylation/dephosphorylation of PBS subunits including CpcB may change the preference for binding to PSI or PSI. When the PBS protein and subunit of PSI are phosphorylated, the PBS will move to PSI and then transfer energy to the PBS (Fig. 8B). Allen et al. (1985) reported that high light can induce the phosphorylation of small photosynthetic proteins and the state transition at the same time, which corresponds to our hypothesis. Furthermore, the phosphorylation of some PBS subunits and the PSI subunit has been shown to be controlled by the same phosphatase SynPPT (open reading frame srl0328) (Mukhopadhyay and Kennelly 2011). The specific signal transduction pathway including the incorporated kinases is still unknown and needs to be studied further. Also, since state transition was reported to stop as soon as OCP-mediated fluorescence quenching occurred (W. Zhao et al. 2014), we speculate that the phosphorylation of OCP may also participate in the regulation of the state transition.

Taken together, the findings of this study imply that phosphorylation at the serine or/and threonine residues of CpcB are important post-translational modifications. The phosphorylation of the protein is directly or indirectly involved in efficient energy transfer and state transitions.

### Materials and Methods

#### Cell culture and protein extraction

The WT strain of *Synechocystis* was acquired from the Pasteur Culture Collection. For phosphoproteomic analysis, cells from cultures in the mid-logarithmic growth phase (A$_{680}$ 0.8–0.9) were inoculated into BG11 medium (Stanier et al. 1971) bubbling with filtered air at 30 °C under continuous illumination from white fluorescent light of 40 μmol m$^{-2}$ s$^{-1}$. For the growth measurement, the WT and the cpcB point mutants were cultivated in 250 ml culture tubes (φ=40 mm × 200 mm) containing 100 ml of BG11 liquid medium with continuous shaking at 60 r.p.m. under different light conditions. For normal growth light conditions (NL), illumination was set at 40 μmol photons m$^{-2}$ s$^{-1}$. For low growth light conditions (LL), the light intensity was lowered to 7 μmol photons m$^{-2}$ s$^{-1}$. To generate high light conditions (HL), 250 μmol photons m$^{-2}$ s$^{-1}$ were used. *Escherichia coli* DH5α competent cells (Novagen) were used to manipulate the plasmids genetically. These strains were grown in Luria–Bertani (LB) medium (Lennox 1955) at 37°C. When required, spectinomycin (50 μg ml$^{-1}$) and/or ampicillin (50 μg ml$^{-1}$) was added to BG11 or LB plates to select the plasmids.

Cells at the exponential growth phase (A$_{680}$ 0.8–0.9) were harvested (6,000 × g at 25°C for 5 min), and the pellet was suspended with lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl]. To inhibit the activities of endogenous protein phosphatases, NaF, Na$_3$VO$_4$ and Na$_4$P$_2$O$_7$ were added at final concentrations of 10, 1 and 10 mM, respectively. The cells were incubated for another 30 min before harvesting by centrifugation (6,000 × g at 4°C for 5 min). The cells were then resuspended in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM β-glycerophosphate, 10 mM NaF, 1 mM Na$_3$VO$_4$, 10 mM Na$_4$P$_2$O$_7$, 1 × protease inhibitor cocktail (Thermo Fisher Scientific). The cells were lysed using sonication (2 s on, 2 s off) for 30 min on ice with a JY93-1N sonicator (Ningbo Scientz Biotechnology Co., Ltd.), which had an output of 135 W. The whole-cell lysate was centrifuged at 3,000 × g for 10 min at 4°C to remove the cell debris. The proteins were precipitated with 5 vols. of ice-cold acetone, dried at room temperature and then redissolved in 50 mM ammonium bicarbonate. The protein concentration was determined with the Bradford assay.

#### In-solution trypsin digestion, reverse-phase chromatography and phosphopeptide enrichment

A 5 mg aliquot of total protein was re-dissolved in 50 mM ammonium bicarbonate and reduced, carboxymethylated and digested with trypsin essentially as described previously (Nielsen et al. 2005). The trypsic digestion was quenched by adding 1% trifluoroacetic acid (TFA), and the mixture was dried in a vacuum centrifuge. Subsequently, AGT Cleanert SPE columns packed with C$_{18}$ material (40 μm, 60 Å pore size, Agilent Technologies Inc.) were prepared for peptide fractionation. The columns were washed with three bed volumes of 100% acetonitrile (ACN), equilibrated with the same volume of 0.1% TFA, and washed five times with 2.0 ml of 0.1% TFA to desalt the sample. The digested peptides were then eluted with a series of elution buffers (2.0 ml each) consisting of 0.1% TFA and different concentrations of ACN (0, 10, 15, 25, 30, 35, 40, 50, 60, 80 and 100%). The fractions were collected, dried with a vacuum centrifuge, and then stored at −20°C until further use.

The phosphopeptides were enriched by using a Phosphopeptide Enrichment TiO$_2$ kit (Calbiochem) according to the manufacturer's instructions with a slight modification. Briefly, each fraction was first re-dissolved in 200 μl of TiO$_2$ Phosphobind buffer with 50 g l$^{-1}$ 2,5-dihydroxybenzoic acid.
The samples were then incubated with 50 μl of TiO₂ Phosphobind resin with mild agitation for 1 h at room temperature. After discarding the supernatant, the resin was washed with wash buffer. The phosphopeptides eluted with 30 μl of elution buffer were then used for LC-MS/MS analysis.

**LC-MS/MS analysis**

The phosphopeptides were analyzed using the UltimateTM 3000 nano-LC system (Dionex) coupled to a HCT ultra electrospray ion-trap mass spectrometer (Bruker Daltonics Inc.) Each sample was loaded on a C₁₈ pre-column (Acclaim PepMap, 300 μm i.d. × 5 mm, Dionex) and then on a C₁₈ reversed-phase analytical column (Acclaim 75 μm × 150 mm; Dionex). The peptides were eluted with a linear gradient of solvents in 0.1% formic acid with an ACN gradient ranging from 5% to 95% over 120 min at a flow rate of 300 nL min⁻¹. The mass spectrometer was set to operate in the positive ion mode at 2 kV, and the heated capillary temperature was set to 180 °C. The MS spectra were acquired in stand-enhanced mode at 8100 m/z s⁻¹ (as termed and specified by the Bruker Esquire Control software). The tandem mass spectra were acquired in the ultra-scan operating mode at 26,000 m/z s⁻¹ (Bruker Esquire Control software). The mass spectrometer was run using the data-dependent neutral-loss method, and the four most abundant ion scans for collision-induced dissociation (CID) MS/MS were selected. The MS/MS spectra were acquired using the following parameters: normalized collision energy, 0.5 V; precursor selection threshold, 100,000 A; and dynamic exclusion of selected ions set to 0.25 min.

The enriched phosphopeptides were also analyzed using the LTQ-Orbitrap XL or LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). In the LTQ-Orbitrap XL mass spectrometer, samples were injected onto a manually packed reversed phase C₁₈ column (3 μm particles, 120 Å pore size, SunChrom) connected to a Finnigan Surveyor HPLC pump (Thermo Fisher Scientific). Peptides were separated using a gradient of increasing mobile phase B (0.1% formic acid, FA) in ACN) from 0 to 80% in 100 min at a 250 nL min⁻¹ flow rate. The peptides were then directly ionized and sprayed into a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific) by a nanospray ion source. The mass spectrometer was operated in a data-dependent mode. Full MS survey with an m/z range of 300–1,800 was acquired with a resolution of 60,000 at m/z = 400 in profile mode. Following every survey scan, up to 10 of the most intense precursor ions were picked for MS/MS fragmentation by CID with a normalized collision energy of 35%. Lockmass at m/z 445,120024 was enabled for full MS analysis. A dynamic exclusion technology was used to detect low abundance proteins which were set as follows: repeat counts, 1; repeat duration, 30 s; exclusion duration, 90 s; exclusion window, ± 10 p.p.m.

In the LTQ-Orbitrap Elite mass spectrometer, samples were pressure-loaded onto an Easy-nano liquid chromatography system (Thermo Fisher Scientific) equipped with an Easy-Spray 50 cm column (C₁₈, 2 μm, 75 μm × 25 cm, Thermo Fisher Scientific). The peptide mixtures were further applied onto a Finnigan Surveyor HPLC pump (Thermo Fisher Scientific) coupled with a LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides were separated using a gradient of increasing mobile phase B (0.1% FA in ACN) from 2% to 90% in 240 min at a 250 nL min⁻¹ flow rate. A full MS survey with an m/z range of 350–1,800 was acquired with a resolution of 42,000 at m/z = 400 in profile mode. Following every survey scan, up to 15 of the most intense precursor ions were picked for MS/MS fragmentation by CID with a normalized collision energy of 35%. Lockmass at m/z 445,120024 was enabled for full MS analysis. A dynamic exclusion technology was used to detect low abundance proteins which were set as follows: repeat counts, 1; repeat duration, 30 s; exclusion duration, 90 s; exclusion window, ± 10 p.p.m.

**Data processing and validation**

The raw files from the electrospray ion-trap mass spectrometer HCT ultra (Bruker Compass software). The resulting files were used for simultaneous searches with a local Mascot server 2.3 (Matrice Science). The raw files from the LTQ-Orbitrap XL and Elite mass spectrometer (Thermo Fisher Scientific) were processed using MaxQuant with an integrated Andromeda search engine (v. 1.3.0.5) (http://www.maxquant.org) (Cox and Mann 2008). The peak files were searched against the Synecochlorella protein database from the Cyanobase database (http://genome.microbed.jp/cyanobase/Synechocystis/; 3,672 sequences) concatenated with a reverse decoy database and protein sequences of common contaminants. The following search parameters were used: tryptic specificity allowing two missed cleavages; carbamidomethyl (Cys) as fixed modification; oxidation (Met), protein N-terminal acetylation, deamination (NQ) and phosphorylation (Ser, Thr and Tyr) as variable modifications. The precursor ion mass tolerance, 0.3 Da, and MS/MS mass tolerance, 0.6 Da, were set for a local Mascot server 2.3. The precursor ion mass tolerance, 6 p.p.m., and MS/MS mass tolerance, 0.5 Da, were set for MaxQuant 1.3.0.5. The minimum peptide length was set at 6. Search results were analyzed and filtered by eliminating all putative hits with a Mascot score < 20 and Andromeda score < 40. The estimated FDR thresholds for modification site, peptide and protein were specified at a maximum of 1%. All phosphopeptide spectra were manually validated as described by Macak et al. (2007, 2008). Generally, a series of at least three successive b- or y-ions were present, and the singly charged peptide fragment ions with Ser (P) and Thr (P) must have lost at least a phosphoric acid residue (–98 Da). Phosphorylation sites with a probability ≥0.75 were reported as class 1 phosphorylation sites (Taus et al. 2011). We have manually inspected all the spectra to improve the reliability of the results, and a spectrum is kept only if it satisfies the criteria as previously reported (Yang et al. 2013, Chen et al. 2014, Tian et al. 2014). All raw data have been deposited in the PeptideAtlas database (http://www.peptideatlas.org) (Desiere et al. 2006, Farrah et al. 2011).

**Bioinformatic analysis**

The identified phosphoproteins were assigned to biological process and molecular function categories according to GO terms by Blast2GO (Conesa et al. 2005). GO enrichment was performed using the Cytoscape plugin BINGO 2.4.4 (Shannon et al. 2003, Maere et al. 2005). In addition, these phosphoproteins were analyzed with PSORTb 3.0 to predict bacterial protein subcellular localization (Yu et al. 2010). SCANSITE 2.0 was used to investigate the consensus sites for kinases and protein-docking motifs (Obenauer et al. 2003). The identified phosphopeptides and phosphoproteins were uploaded to the PHOSIDA database (www.phosida.com) to extract the specific motifs (Olsen et al. 2006).

Analysis of the preferred flanking amino acid for the identified phosphorylation sites was performed as previously described (Beausoleil et al. 2004, Treeck et al. 2011, Yang et al. 2013). The ratio of frequencies was calculated (frequency of residues in the phosphor-13mers/frequency of residues in total the proteome) and plotted as the log, ratio. The position-specific heatmap was generated by plotting the log, ratio of the frequencies. Secondary structures were predicted using the NetSurfP method (Petersen et al. 2009). The serine, threonine and tyrosine phosphorylation sites in phosphoproteins were predicted using the NetPhosBac 1.0 Server (Müller et al. 2009). BLASTP (Altschul et al. 1990), STRING 9.1 (Szklarczyk et al. 2011), the KEGG pathway database (Kanehisa and Goto 2000), and the PPI network Cytoscape (Shannon et al. 2003) and MCODE (Bader and Hogue 2003) were simultaneously used as complementary methods to classify the identified phosphoproteins further. The initial model of the CpcB module was prepared from the previously determined crystal structure (PDB file 4F0T.B) via structural modeling using the CPHmodels-3.0 (Nielsen et al. 2010). The PyMOL Molecular Graphics System (version 1.7.2, http://www.pymol.org) was employed to present the structural results of this study.

**Generation of cpcB point mutants by site-directed mutagenesis**

The serine (S) or threonine (T) residue of CpcB was substituted with alanine (A) or glycine (G) by site-directed mutagenesis according to Clerico et al. (2007). Using genomic DNA as a template, the full-length cpcB gene was amplified with the following primers (5’ – 3’): L1577_f (CCACCCCTGTAGAAGAGTGC) and L1577_r (TGGGGGAGAGGCTCAAGGTT). The PCR amplicon was inserted into pmD18-T (TAKARA). This construct was used as a template with L1577_S22A_f (5’-GCTCACCTATGTCTCTTTGACCC-3’) and L1577_S22A_r (5’-AGCATCTAATGGACACACACAGGTATGCTCCG-3’), L1577_S49A_f (5’-GC GGCGCTATCGTGTTCACCAACTG-3’) and L1577_S49A_r (5’-TGTAAGAACATAGCCGGAGGACATCCATTGCCGACGATGCTCCG-3’), L1577_T94A_f (5’-GCTCGATACTGGCCGGGTCGTGGTCG-3’) and L1577_T94A_r (5’-AGTCGCCCTGCAACACACGACGAGGTCG-3’), L1577_S154G_f (5’-ACACCCATGGCAGAGGATCTCGGGCTTACGC-3’) and L1577_S154G_r (5’-TGCACCCATGGTCAGGCTCTTCATC-3’), L1577_K412A_f (5’-ACACCCATGGCAGAGGATCTCGGGCTTACGC-3’) and L1577_K412A_r (5’-TGCACCCATGGTCAGGCTCTTCATC-3’), L1577_S22A_f (5’-GCTCACCTATGTCTCTTTGACCC-3’) and L1577_S22A_r (5’-AGCATCTAATGGACACACACAGGTATGCTCCG-3’), and as four pairs of primers to generate four cpcB mutants, S22A, S49A, T94A and S154G, using a Quick Change II site-directed mutagenesis kit.
obtain the energy transfer. For the fluorescence transients experiment, 20 fl were recorded over a time scale of approximately 1 min to capture the emission wavelength of 695 nm were used, and the fluorescence was re-
10 min before illuminating with excitation light. Here, a wavelength of 660 nm was added to the resuspended cells, and the cells were then dark-adapted for 10 min before illuminating with excitation light. The fluorescence emission spectra were measured at room temperature and 77K as previously described (Wang et al. 2008) with a PTI Fluorometer (QM-730 0.8–0.9) in order to isolate PBSs as described previously. The cells were resuspended in 750 mM NaKPO₄ (pH 8.0) at a concentration of 0.12 g wet weight ml⁻¹ and lyzed using a French Press at 20,000 p.s.i. Triton X-100 was then added to a final concentration of 1%. After incubation for 45 min with occasional shaking, the cell lysate was clarified by centrifugation (31,000 g for 45 min with 750 mM NaKPO₄ (pH 8.0). The intact PBS-containing fractions of the 1 M sucrose fraction were carefully collected, diluted in 0.75 M phosphate buffer and centrifuged at 250,000 g for 4 h at 4 °C. The PBS pellets were dissolved in 0.75 M phosphate buffer, protected from light and refrigerated until use.

Fluorescence measurement

The fluorescence emission spectra were measured at room temperature and 77K as previously described (Wang et al. 2008) with a PTI Fluorometer (QM-4CW, Photon Technology International Inc.). An excitation wavelength of 375 nm, which could excite PE, PC and APC almost equally, was used for the isolated PBS, and the samples were diluted to the appropriate concentration of approximately 30 μg ml⁻¹ with 750 mM NaKPO₄ (pH 8.0) (Gantt et al. 1979, Wang 2013). Both the excitation and emission slit widths were set to 1 nm. The fluorescence quenching and fluorescence transients at 293K were measured using an alternative wavelength time scanning method using the PTI Fluorometer according to Rahkimberdive et al. (2004) and Aspinwall et al. (2004). During both experiments, the cells were resuspended in growth medium at a Chl concentration of 3.1 μg ml⁻¹. The fluorescence decay of C-phycobiliprotein was examined with an excitation wavelength of 625 nm and emission wavelength of 650 nm. The fluorescence was recorded over a time scale of approximately 10 min to capture the fluorescence quenching due to the energy transfer. For the fluorescence transients experiment, 20 μM DCMU was added to the resuspended cells, and the cells were then dark-adapted for 10 min before illuminating with excitation light. Here, a wavelength of 660 nm and emission wavelength of 695 nm were used, and the fluorescence was recorded over a time scale of approximately 1 min to capture the fluorescence increase due to adaptation to state 1 (Aspinwall et al. 2004). Fluorescence transients were fitted to exponential curves using Origin 8.0 in order to obtain the τ₁, which and relative amplitudes of the state 1 transition.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the National Basic Research Program of China [973 Program, 2012CB518700]; the National Natural Science Foundation of China [grant Nos. 31270094, 31370746]; the Natural Science Foundation of Hubei Province of China [2013CFA109].

Disclosures

The authors have no conflicts of interest to declare.

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