

SCIENTIFIC REPORTS



OPEN

NdhV subunit regulates the activity of type-1 NAD(P)H dehydrogenase under high light conditions in cyanobacterium *Synechocystis* sp. PCC 6803

Received: 12 April 2016

Accepted: 01 June 2016

Published: 22 June 2016

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The cyanobacterial NAD(P)H dehydrogenase (NDH-1) complexes play crucial roles in variety of bioenergetic reactions. However, the regulative mechanism of NDH-1 under stressed conditions is still unclear. In this study, we detected that the NDH-1 activity is partially impaired, but the accumulation of NDH-1 complexes was little affected in the *NdhV* deleted mutant ($\Delta ndhV$) at low light in cyanobacterium *Synechocystis* sp. PCC 6803. $\Delta ndhV$ grew normally at low light but slowly at high light under inorganic carbon limitation conditions (low pH or low CO₂), meanwhile the activity of CO₂ uptake was evidently lowered than wild type even at pH 8.0. The accumulation of NdhV in thylakoids strictly relies on the presence of the hydrophilic subcomplex of NDH-1. Furthermore, NdhV was co-located with hydrophilic subunits of NDH-1 loosely associated with the NDH-1L, NDH-1MS' and NDH-1M complexes. The level of the NdhV was significantly increased at high light and deletion of NdhV suppressed the up-regulation of NDH-1 activity, causing the lowered the photosynthetic oxygen evolution at pH 6.5 and high light. These data indicate that NdhV is an intrinsic subunit of hydrophilic subcomplex of NDH-1, required for efficient operation of cyclic electron transport around photosystem I and CO₂ uptake at high lights.

Type-1 NAD(P)H dehydrogenase (NDH-1) complexes function in a variety of bioenergetic reactions, including respiration, cyclic electron transport around photosystem I (PSI)¹ and CO₂ uptake² in cyanobacteria. The subunits of NDH-1 show a high homology with those in chloroplast NDH. Chloroplast NDH consists of more than 28 subunits, among those, NdhA–NdhK are plastid-encoded and others are nuclear-encoded³ and cyanobacterial NDH-1 consists of 17 subunits at least. Structurally, the chloroplast NDH is more complicate compared with the cyanobacterial NDH-1. According to the structural model of chloroplast NDH proposed by Ifuku *et al.*³, in addition to the L-shape structure consisting of subcomplex A, donor binding domain and membrane subcomplex similar with that of cyanobacterial NDH-1, the chloroplast NDH contains a subcomplex B which forms a second hydrophilic arm that extends to the stroma and attaches to the membrane subcomplex with two transmembrane proteins, PnsB4⁴ and PnsB5⁵, and the lumen subcomplex L, which consists of PnsL1–PnsL5 (photosynthetic NDH subunit of luminal location). However, three subunits of Complex I (NuoE, NuoF, and NuoG) involved in accepting electrons from NADH in *Escherichia coli* are missing from cyanobacterial and chloroplast NDH⁶. There are six *NdhD* and three *NdhF* genes in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) (CyanoBase, the genome database for cyanobacteria), which form different NDH-1 complexes involved in diverse physiological functions. Proteomic analysis of cyanobacterial NDH-1 complexes has revealed the presence of three complexes NDH-1L (large size), NDH-1M (medium size) and NDH-1S (small size) in *Synechocystis* 6803⁷. Further research suggested that NDH-1L functions in cyclic electron transport and respiration and NDH-1M and NDH-1S in CO₂

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uptake⁸. In contrast to the crystal structure of Complex I⁹, NDH-1 from cyanobacteria is speculated to possess an oxygenic photosynthesis-specific (OPS) domain¹⁰ comprised of NdhL–NdhO identified in *Synechocystis* 6803^{11,12}. Several NDH subunits function in stabilization of NDH-1. NdhP and NdhQ were found in the purified NDH-1L complex from *T. elongatus*¹³. NdhP is involved in the respiratory and cyclic electron flow¹⁴ and stabilization of the NDH-1L complex¹⁵. NdhQ is also essential for stabilization of the large complex of NDH-1¹⁶. NdhS participates in the activity of cyclic electron flow around PSI in Arabidopsis¹⁷ or in cyanobacteria¹⁸, and serves as the Fd docking site domain, accepting electrons from Fd in chloroplasts¹⁹. Furthermore, He *et al.*²⁰ have found that the NDH-1L complex interacts with Fd via the subunit NdhS in *Thermosynechococcus elongatus*.

Cyanobacteria utilize both CO₂ and HCO₃[−] as carbon species²¹. The dissolved inorganic carbon is pH-dependent. With increasing pH, the ratio of HCO₃[−] to CO₂ continues to rise²². In contrast, the water with acid pH is favorable for providing a sufficient CO₂ supply. Cyanobacteria possess a CO₂-concentrating mechanism (CCM) that enables to raise the concentration of inorganic carbon (Ci, HCO₃[−] and CO₂) at the carboxylation site to a high level for efficient CO₂ fixation despite the low affinity of their Rubisco for CO₂^{23,24}. To date, five inorganic transporters have been found including two Na⁺-dependent HCO₃[−] transporters (BicA and SbtA), one ATPase-dependent HCO₃[−] transporter (BCT1), and two CO₂-uptake NDH-1 complexes in *Synechocystis* 6803 and other cyanobacterial strains^{24–26}. As one of CO₂ uptake systems, NDH-1MS complex, consisting of NdhD3, NdhF3 and CupA (ChyY), is inducible at limiting Ci conditions and has a higher uptake affinity for CO₂^{27–29}. Further research showed that the proteins encoded by *NdhF3/NdhD3/CupA/CupS* formed a small complex NDH-1S, in which CupA and a small protein CupS were identified as subunits of cyanobacterial NDH-1 by proteomic analysis^{30,31}. NDH-1S and NDH-1M form an NDH-1MS complex which has been isolated from a *Thermosynechococcus elongatus* strain in which the C terminus of NdhL has been tagged with 6-His. This complex is easily dissociated into NDH-1M and NDH-1S complexes³¹. As a homologous gene of *cupA*, *cupB* (*chyX*) is involved in constitutive CO₂ uptake system encoded by *NdhD4/NdhF4/CupB* which formed a small complex NDH-1S^{28,29}. Based on the purification of a 450 kDa complex contained both NdhH and CupB protein, it has been suggested that the complex is NDH-1MS' located in the thylakoid membranes³².

Previous studies have shown that the cyclic electron flow around PS I mediated by NDH functions in protection against stressed conditions. Ogawa² has found that deletion of *NdhB* gene resulted in the unable survival phenotype of *Synechocystis* 6803 under low CO₂ (air) condition, suggesting that the NDH-1 functions in inorganic carbon transport. The activity of cyclic electron flow around PSI mediated by NDH-1 has been found to be enhanced under stressed conditions, including low CO₂³³, strong light³⁴. However, how NDH-1 participates in the regulation of photosynthesis is still unclear.

Recently, NdhV is reported to function in stabilization of chloroplast NDH-like complex in Arabidopsis³⁵ and heat tolerance in *Synechocystis* 6803³⁶. However, the mechanism of NdhV in response to stressed conditions is not clear. Here we report that cyanobacterial NdhV localizes in the hydrophilic subcomplex of NDH-1MS', NDH-1L and NDH-1M. Further analysis showed that NdhV functions in regulation of NDH-1 activity for efficient operation of cyclic electron flow around PS I and CO₂ uptake in *Synechocystis* 6803 in response to high lights.

Results

Deletion of *NdhV* partially arrests NDH-1 activity. To reveal the function of *NdhV* in cyanobacteria, we inactivated the cyanobacterial NdhV protein by inserting a kanamycin resistance (*kan^R*) cassette into its coding region (Fig. 1A). The PCR analysis of the *NdhV* locus using the *ndhV*-up-F and *ndhV*-Dn-R primers showed a band of 1.5 Kbp in the WT and 2.7 Kbp in the $\Delta ndhV$ mutant which contained an extra *Kanamycin* resistance cassette of about 1.2 Kbp; it confirmed the complete segregation of the $\Delta ndhV$ allele in this mutant (Fig. 1B).

Post-illumination increase in Chl fluorescence was explained as the reduction of plastoquinone (PQ) by the electrons from photoreductants accumulated in the stroma or cytosol during illumination, which reflects cyclic electron transport around PSI mediated by NDH-1^{37,38} in cyanobacteria and by chloroplast NDH in higher plants³⁹. By comparison with WT, the post-illumination increase in Chl fluorescence was partly impaired in two lines of *ndhV* deleted mutants $\Delta ndhV-1$ and $\Delta ndhV-2$ (Fig. 1C), suggesting that NdhV contributes to the NDH-1 activity.

Growth of $\Delta ndhV$ was suppressed under high light and low pH conditions. In addition to cyclic electron flow around PSI, NDH-1 is also involved in CO₂ uptake². To examine whether NdhV participates in CO₂ uptake, we compared the growth phenotype between WT and $\Delta ndhV$ under various conditions, low (40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or high light (300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), low (below pH 7.5), or high pH (above pH 7.5), high (2%) or low CO₂ (0.04%). With the pH increasing to higher than 7.0, the concentration of HCO₃[−] is predominant but that of CO₂ becomes minor, while at the low pH below 6.5 condition, the concentration of HCO₃[−] becomes minor and that of CO₂ is predominant. The growth phenotype was almost the same under pH 8.0 either at low light or high light (Fig. 2A–D). The growth of $\Delta ndhV$ was slightly slow at low pH, low light, low CO₂ (Fig. 2B) or high light, high CO₂ (Fig. 2C) and more evidently under high light, low CO₂ (Fig. 2D). To confirm the difference, we further compared the growth rate in the liquid culture conditions bubbling with 2% CO₂ at pH 6.5 at low light or high light. The growth rate was identical between $\Delta ndhV$ and wild type under low light condition in liquid (Fig. 2E), consistent with the results on agar (Fig. 2A). However, the growth of $\Delta ndhV$ was slower than WT under the high light condition (Fig. 2F), suggesting that NdhV plays an important role under the stressed conditions. In contrast, the *ndhB* defective mutant (M55) in which both the PSI-cyclic electron flow and CO₂ uptake were inactivated, could hardly grow up at pH 6.5 under either low light or high light conditions (Fig. 2E,F), similar to the observation by Ogawa (1991).

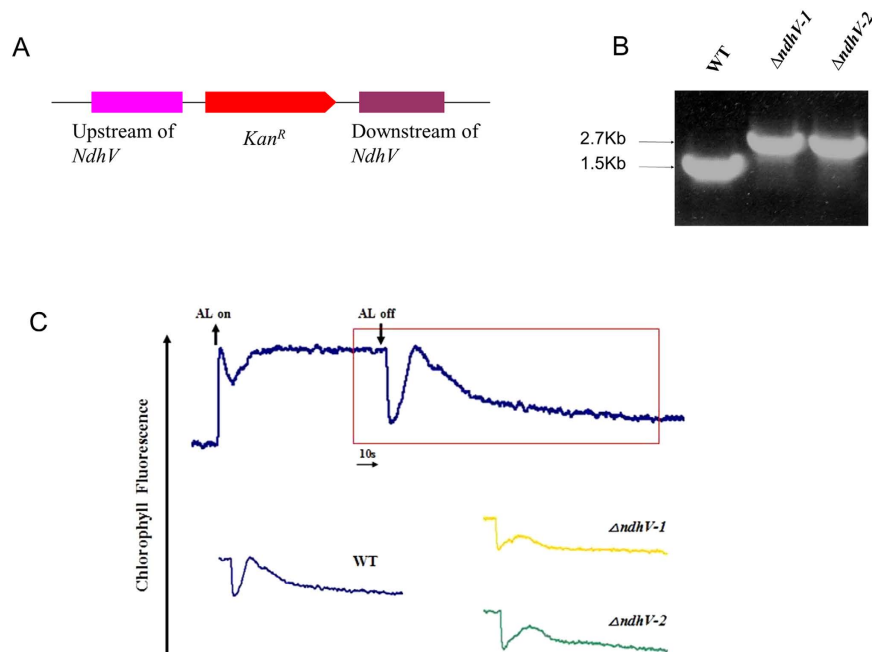


Figure 1. *NdhV* gene deletion and its effect on NDH-1 activity. (A) Construction of plasmid to generate *NdhV* deleted mutant ($\Delta ndhV$). Schematic representation of the $\Delta ndhV$ mutant locus. A kanamycin resistance cassette about 1.2Kb was inserted into the *NdhV* gene. (B) PCR segregation analysis of the $\Delta ndhV$ mutant using the *ndhV*-up-F and *ndhV*-Dn-R primers (Table S1). (C) Monitoring of NDH-1 activity using chlorophyll fluorescence analysis. The top curve shows a typical trace of chlorophyll fluorescence in the WT of *Synechocystis* 6803. The cells (OD_{730} around 0.4) supplemented with 10 mM NaHCO_3 were used for the measurement. After the sample was exposed to the actinic light (AL, $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 90 s, AL was turned off, and the transient increase in chlorophyll fluorescence level was recorded, which was used to ascribe NDH-1 activity. The inset shows magnified traces from the box area.

Suppression of the activity of CO_2 uptake in $\Delta ndhV$. To confirm whether the CO_2 uptake activity was affected by deletion of *NdhV*, we compared the rate of CO_2 uptake in WT, $\Delta ndhV$, and M55 in which only NDH-1S is active in the CO_2 acquisitive systems, using a portable open-flow gas exchange system under high lights and high or low CO_2 conditions. Cell suspensions were placed on the BG-11 agar plate to measure the CO_2 uptake rate. The rate of CO_2 uptake was lowered by about 10% in $\Delta ndhV$ than that in WT at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ either under high CO_2 or low CO_2 conditions (Fig. 3A), and at $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ under low CO_2 , but not under high CO_2 condition (Fig. 3B). In contrast, the rate of CO_2 uptake was almost completely suppressed in M55 (Fig. 3A,B) suggesting the loss of function of both NDH-1MS and NDH-1MS' in the mutant. These results demonstrated that deletion of *NdhV* suppresses the CO_2 uptake activity under high lights and CO_2 limitation conditions.

The proton gradient across thylakoid membranes was lowered in $\Delta ndhV$. It has been suggested that cyanobacterial NDH-1 provides ATP for CO_2 uptake². To investigate whether *NdhV* is involved in this process, the proton gradient across thylakoid membranes, which drives ATPase to synthesis ATP was compared between WT and $\Delta ndhV$. Quinacrine (QA) fluorescence quenching can be used for the determination of ΔpH across the thylakoid membrane for intact cells of *Synechocystis* 6803⁴⁰. The QA fluorescence quenching was decreased by about 20% in $\Delta ndhV$ mutants and 40% in M55 compared with those in WT (Fig. 3C), suggesting that *NdhV* is involved in regulation of proton gradient across thylakoid membrane.

***NdhV* localizes to the thylakoid membrane and its accumulation relies on the electron donor domain of NDH-1M of NDH-1MS', NDH-1L.** The previous study demonstrated that Arabidopsis *NdhV* is mainly localized to the thylakoid membrane^{35,36}. As a homologous protein, the localization of the cyanobacterial *NdhV* was investigated with a polyclonal antibody against the recombinant cyanobacterial *NdhV*. Western blotting analysis detected a band with a molecular mass of about 15 kDa (the theoretical molecular mass of mature *NdhV* is ~18 kDa) in the thylakoid and also in the stromal fractions of WT, but absent in those of $\Delta ndhV$ (Fig. 4A), confirming that the cyanobacterial *NdhV* is mainly localized to the thylakoid membrane.

In cyanobacteria, a total of 4 *NdhD* isoforms are present and they are located in different NDH-1 complexes. Given that NDH-1L, NDH-1MS, NDH-1MS' were respectively defective in $\Delta ndhD1/D2$, $\Delta ndhD3$ and $\Delta ndhD4$ mutant in *Synechocystis* 6803³⁰, suggesting that NDH-1M is stable in those *NdhD* deleted mutants, we checked the amount of *NdhV* in different *NdhD* deleted mutants. As shown in Fig. 4B, there was no much difference in the amount of *NdhV* among WT and the various *NdhD* deleted mutants, suggesting that *NdhV* is present in NDH-1M complex.

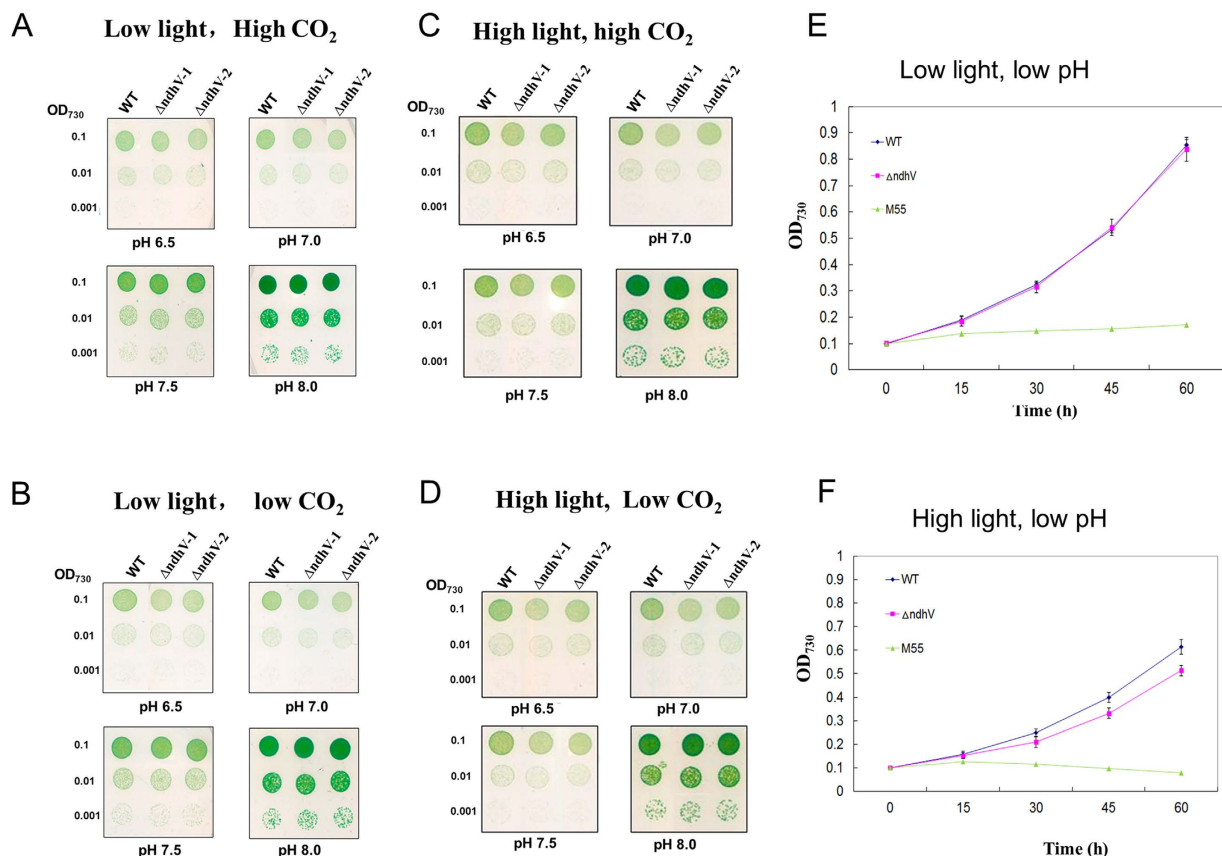


Figure 2. The growth phenotype of WT and $\Delta ndhV$ strains. (A–D) Five microliters of the cell suspensions with the $OD_{730\text{ nm}}$ values of 0.1, 0.01, and 0.001 were spotted on agar plates containing BG11 buffer at different pHs and grown at a low light ($40\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$), high CO_2 (3% v/v) (A); low light, low CO_2 (0.04% v/v) (B); high light ($300\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) and high CO_2 (C); high light and low CO_2 (D) for five days. (E,F) Cell density of WT, $\Delta ndhV$, and M55 strains were measured at different times after grown at low light ($40\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) and low pH (pH 6.5) (E), or high light ($300\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) and low pH (pH 6.5) (F), 2% CO_2 (v/v in air). Values are means \pm SD ($n = 3$). Values are means \pm SD ($n = 3$).

To further confirm the association of NdhV with NDH-1M, the accumulation of NdhV in the mutants defective in different Ndh subunits was investigated by immunoblot. Figure 4C shows that NdhV was detected neither in NdhB defective mutant M55, in which both the NDH-1L and NDH-1M were disassembled³⁰ nor in *NdhM* deleted mutant $\Delta ndhM$ in which all the NDH-1 complexes were disassembled⁴¹. The results indicate that the accumulation of NdhV relies on NDH-1M and the complexes containing NDH-1M, including NDH-1L, NDH-1MS and NDH-1MS'.

We further checked the accumulation of cyanobacterial NdhV in *NdhS* deleted mutant of *Synechocystis* 6803 ($\Delta ndhS$). NdhV was hardly detected in $\Delta ndhS$ (Fig. 4C), consistent with the result obtained in Arabidopsis *ndhS/crr31* mutant (Fan *et al.*)³⁵ or in *Synechocystis* 6803 (Gao *et al.*)³⁶, suggesting that the stability of NdhV is dependent on the electron donor domain of NDH-1 in cyanobacteria. Furthermore, we checked the accumulation of NdhV in the mutant defective in the hydrophilic subunits of NDH-1. The amount of NdhV was reduced significantly in *NdhI* partly deleted mutant ($\Delta ndhI_V$) (Fig. 4C). The result indicates that the accumulation of NdhV is dependent on the presence of the hydrophilic subcomplex of NDH-1 related to its electron donor domain.

NdhV is loosely bound in NDH-1 complexes and its absence hardly affects the accumulation of other Ndh subunits and assembly of NDH-1 complexes under normal growth conditions. To investigate whether the accumulation of the NDH-1 complexes is affected in $\Delta ndhV$, thylakoid protein complexes isolated from $\Delta ndhV$ and WT were separated by a 5–13% gradient blue-native PAGE (BN-PAGE) followed by 2D SDS-PAGE for the immunoblotting analysis. The results show that in the wild type, NdhV presents as a free form and the bands corresponding to NDH-1L and NDH-1M complexes were detected using the antibody of NdhK, and the bands corresponding to NDH-1MS' and NDH-1S' complex could also be detected using the antibody of the key component CupB (Fig. 5A). However, in $\Delta ndhV$, these bands were almost the same as those in wild type, indicating that deletion of NdhV hardly affected the assembly of NDH-1 complexes, including NDH-1L, NDH-1MS' and NDH-1M (Fig. 5A), in accordance with the results in Arabidopsis that the NdhV is a protein easily dissociated from NDH-1 complexes during sample preparation and BN-gel electrophoresis. To overcome this limitation, we performed the chemical crosslinking experiments using the thylakoids isolated from WT. The result shows that NdhV co-migrates with the NdhI and NdhK subunits in the NDH-1M, NDH-1L, NDH-1MS'

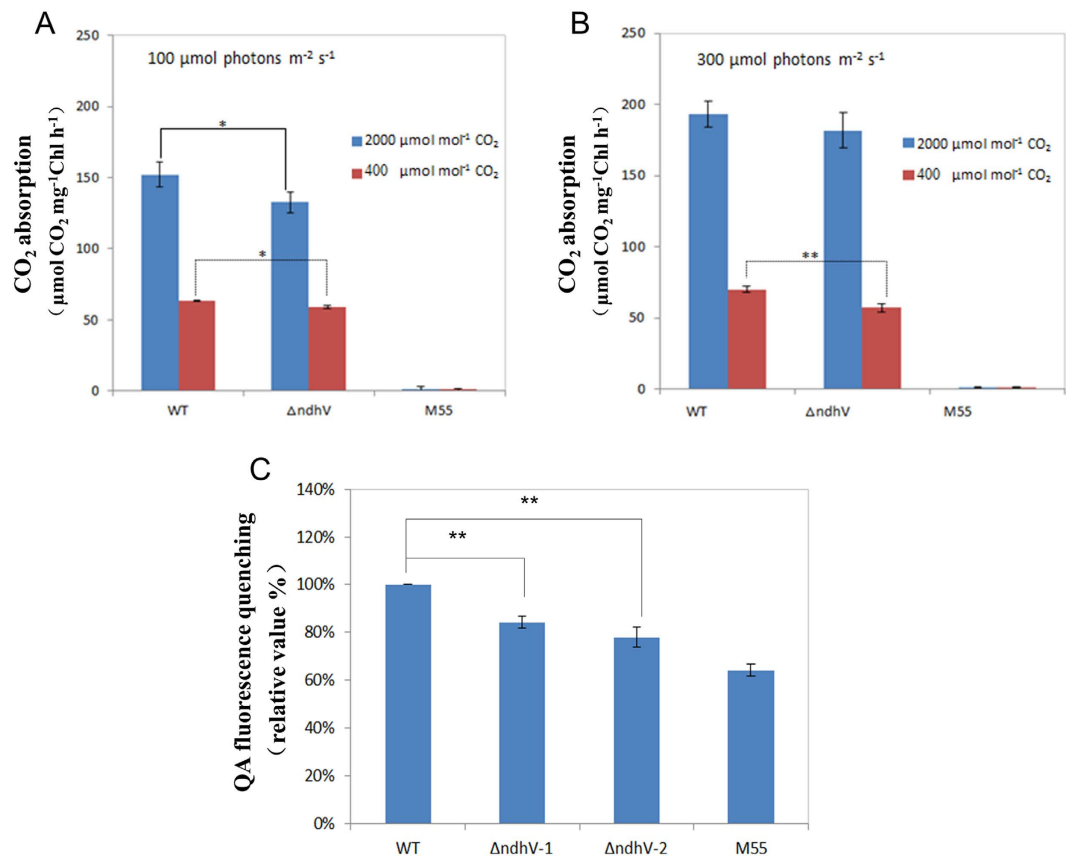


Figure 3. Comparison of the CO₂ uptake and the proton gradient across thylakoid membranes between WT and $\Delta ndhV$ strains. (A,B) The rate of CO₂ uptake in WT, $\Delta ndhV$ and M55 strains at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (A) or at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (B). The cells of WT and mutant strains were harvested at mid-logarithmic phase ($OD_{730} \approx 0.5$) and chlorophyll *a* concentration was adjusted to 400 $\mu\text{g ml}^{-1}$. 30 μl of the cell suspensions were placed on the BG-11 agar plate. The activity of CO₂ uptake was measured at 30 °C. The CO₂ concentration was controlled at 400 or 2000 $\mu\text{mol mol}^{-1}$. Values are means \pm SE of three independent measurements. Asterisk indicates significant differences (t-test, * $P < 0.05$ and ** $P < 0.01$). (C) Analysis of proton gradient across thylakoid membranes using QA (quinacrine) fluorescence quenching in WT, $\Delta ndhV$ and M55 strains. Intact cells of WT and mutant strains were harvested at mid-logarithmic phase ($OD_{730} \approx 0.5$) and then suspended at a final chlorophyll *a* concentration of 150 $\mu\text{g ml}^{-1}$ in a reaction medium contained 5 mM Tris/MES (pH 8.0), 0.3 M mannitol, 2 mM DTT, 5 mM D-Glucose, 1.5 mM ATP, 5 μM quinacridine. The quenching of QA fluorescence was induced by adding the cells sample to 2 ml reaction mixture after the background fluorescence reached steady state about 2 min after started the measurement. The QA fluorescence quenching of WT is 5.02%. Values are means \pm SE of three independent measurements. Asterisk indicates significant differences (t-test, * $P < 0.05$ and ** $P < 0.01$).

and a supercomplex around 1000 kDa, probably due to the aggregation of the NDH-1 (Fig. 5B). These results also confirm that NdhV is a subunit of cyanobacterial NDH-1 but easily dissociated from NDH-1 complexes. To know whether NdhV affects the accumulation of other subunits of NDH-1, we checked the amount of several subunits in different parts of cells from $\Delta ndhV$. The results show that there were no obvious difference of the amount of NdhH, I, K, M either in total proteins, or in the supernatant or in the thylakoid membrane between the WT and $\Delta ndhV$ cultured at pH 6.5 (Fig. 1SA) or at pH 8.0 (Fig. 1SB).

The up-regulation of NDH-1 activity at high lights was suppressed in $\Delta ndhV$. Previous studies have indicated that NDH-1 activity was up-regulated by high light³⁴. To test whether NdhV is required for up-regulation of NDH activity under high light conditions, we compared the NDH-1 activity under different light intensities between WT and $\Delta ndhV$. Figure 6A shows that the NDH-1 activity, reflected by the transient increase in Chl fluorescence after termination of actinic light, increased with the increase of the intensity of actinic light in WT, but the up-regulation of NDH-1 activity in response to high lights was significantly suppressed in $\Delta ndhV$. The results indicate that the up-regulation of NDH-1 activity is almost suppressed when NdhV is absent. Furthermore, we compared the changes of the amount of Ndh subunits after adaptation to the high light between WT and $\Delta ndhV$. As shown in Fig. 6B, after the growth light was shifted to the high light (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 1 h, the amounts of NdhH and NdhK were evidently increased by about 40% and 35%, respectively, both in WT and $\Delta ndhV$. Meanwhile, the amount of NdhV was approximately 2-fold higher at the

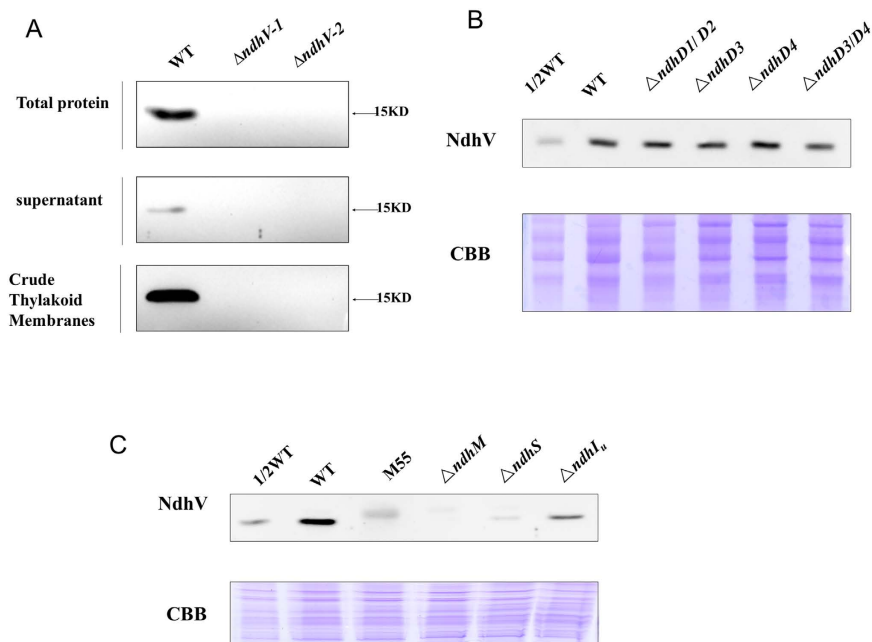


Figure 4. The location of NdhV in WT strain and the effects of mutation of Ndh subunits on NdhV. (A) Immunodetection of NdhV in the total proteins, supernatant and thylakoid membranes of WT and $\Delta ndhV$ strains. Total Proteins: The material obtained after broken by glass beads; supernatant, Crude Thylakoid Membranes: supernatant and precipitation after centrifugation of total proteins at $20,000 \times g$ for 30 min at 4°C , respectively. (B) Immunodetection of NdhV in thylakoid membranes from WT (including indicated serial dilutions), $\Delta ndhD1/D2$, $\Delta ndhD3$, $\Delta ndhD4$ and $\Delta ndhD3/D4$ mutants. Immunoblotting was performed using antibodies against NdhV. Each Lane was loaded with $25\ \mu\text{g}$ total proteins. In the lower panel, a piece of replicated gel stained with Coomassie Brilliant Blue (CBB) was used as a loading control. (C) Immunodetection of NdhV in thylakoid membranes from WT (including indicated serial dilutions), M55, $\Delta ndhM$, $\Delta ndhS$, $\Delta ndhI_v$ (partly deletion of NdhI) mutants. Immunoblotting was performed using antibodies against NdhV. Each Lane was loaded with $25\ \mu\text{g}$ total proteins. In the lower panel, a piece of replicated gel stained with Coomassie Brilliant Blue (CBB) was used as a loading control.

high light in WT. The results suggest that the up-regulation of NdhV is required for regulation of NDH activity in response to high lights in cyanobacteria.

The capacity of photosynthesis was suppressed at the high light and low pH in $\Delta ndhV$. To check whether NdhV functions in protecting the cyanobacterium against the high light stress, we compared the capacity of photosynthesis between WT and $\Delta ndhV$ in response to the high light, as reflected by photosynthetic O_2 evolution at higher pH (8.0) where the HCO_3^- is predominant and lower pH (6.5) where CO_2 is predominant. With the increase in the light intensity, the rate of photosynthetic O_2 evolution increased, but there was not much difference between WT and $\Delta ndhV$ at pH 8.0 (Fig. 7A). However, the rate of photosynthetic O_2 evolution in $\Delta ndhV$ was approximately 10% lower than that in WT at pH 6.5 at higher light intensities (Fig. 7B). The results suggest that NdhV is also required for photosynthetic CO_2 assimilation under the high light and low pH stressed conditions.

Discussion

It has been shown that mutation of *NdhV* caused completely the loss of the NDH activity in Arabidopsis³⁵ but only partly in cyanobacterium *Synechocystis* 6803 (Gao *et al.*)¹⁶, which is confirmed in our work (Fig. 1). The only partial suppression of the NDH-1 activity (Fig. 1C) by deletion of the cyanobacterial *NdhV* is probably attributed to the compensation of the activity from different types of NDH-1 in cyanobacteria⁷. On the other hand, similar with the chloroplast NdhV, the cyanobacterial NdhV is also demonstrated to localize in the fragile part of NDH-1 and may interact with the electron donor domain of NDH-1 by the observation of the severe suppression of the accumulation of NdhV in $\Delta ndhS$ (Fig. 4C). In addition, the accumulation of NdhV was almost lost in the thylakoid membranes in M55 and in $\Delta ndhM$ (Fig. 4C) in which the hydrophilic subcomplex of NDH-1 complexes are degraded (He *et al.*)²⁰, but it was not affected either in *NdhD3/D4* defective mutant (Fig. 4B) in which only NDH-1L and NDH-1M exist, or in *NdhD1/D2* defective mutant (Fig. 4B) in which NDH-1M, NDH-1MS' are still present. Moreover, NdhV co-localized with Ndh subunits of NDH-1MS', NDH-1L and NDH-1M complexes (Fig. 5A). Considering the cells were cultured under high CO_2 culture condition, NDH-1MS would not be visible. Those results let us conclude that NdhV interacts with the electron donor domain of these complexes which contains NDH-1M as a skeleton³⁰. Although defect in NdhS and other hydrophilic subunits affected the accumulation of NdhV (Fig. 4C), but deletion of *NdhV* didn't affect the accumulation of relevant NDH-1 subunits

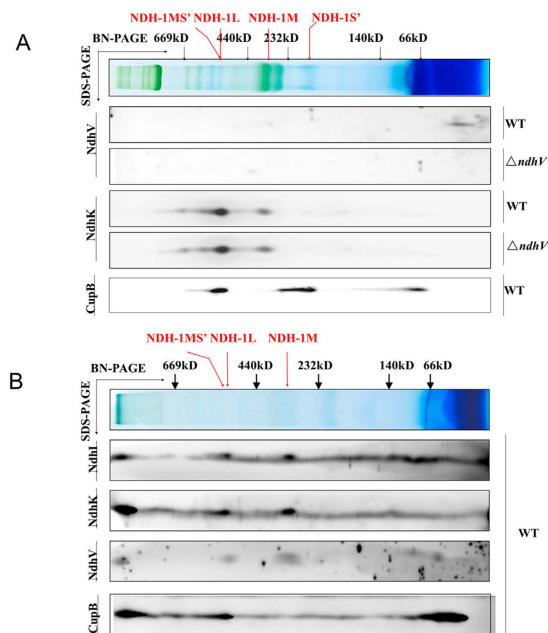


Figure 5. The location of NdhV in NDH-1 complexes in WT. (A) Thylakoid membrane proteins from WT and $\Delta ndhV$ strains were separated by the BN-PAGE and further subjected to a 2D/SDS-PAGE. The proteins were immunodetected with antibodies against NdhK and NdhV. The positions of molecular mass markers in the BN-gel are indicated. (B) Thylakoid membrane proteins from WT strain were crosslinking by DSP, then separated by BN-PAGE and further subjected to 2D/SDS-PAGE. The proteins were immunodetected with antibodies against NdhI, NdhK, NdhV and CupB. The positions of molecular mass markers in the BN-gel are indicated.

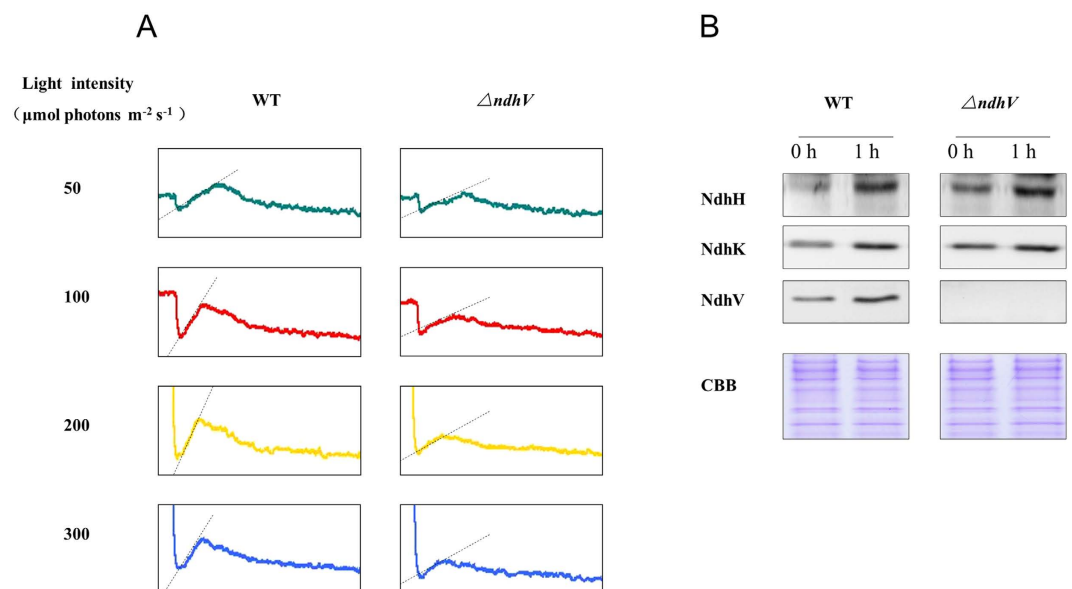


Figure 6. Monitoring of NDH-1 activity of WT and $\Delta ndhV$ strains under different light intensities. (A) Monitoring of NDH-1 activity of WT and $\Delta ndhV$ strains in different light intensities using chlorophyll fluorescence analysis. OD_{730} of the cells was about 0.3. The cells were exposed to the different actinic light (shown in the figure) for 90 s. Then the actinic light was turned off, and the transient increase in chlorophyll fluorescence level was ascribed to NDH activity. (B) Immunodetection of NdhH, K, V in total proteins of WT and $\Delta ndhV$ strains before and after treatment with high light. The cell was cultured to mid-logarithmic phase under normal light (0 h), then the cell cultures were transferred to high light ($\sim 200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 1 hour (1 h). Immunoblotting was performed using antibodies against NdhH, NdhK and NdhV. Each lane was loaded with $25 \mu\text{g}$ proteins. In the lower panel, a piece of replicated gel stained with Coomassie Brilliant Blue (CBB) was used as a loading control.

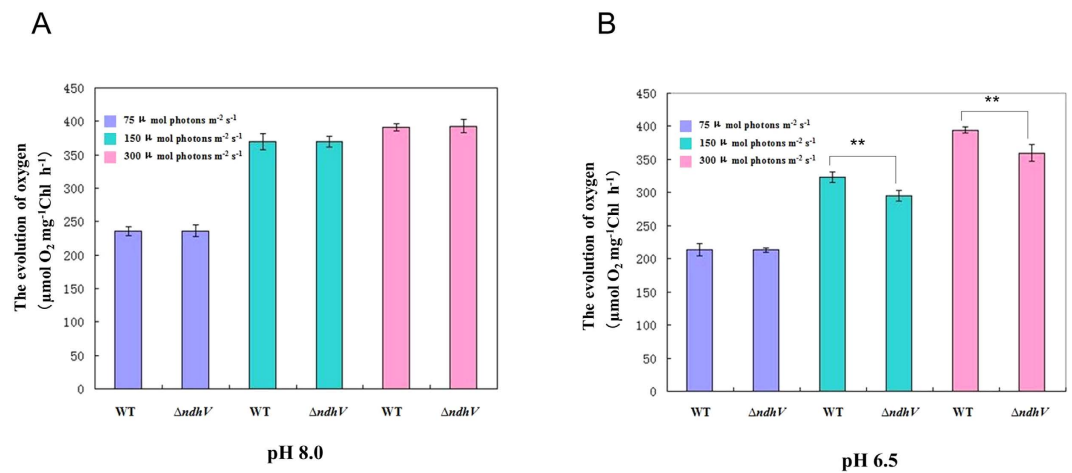


Figure 7. The evolution of oxygen in WT and $\Delta ndhV$ strains at different light intensities and different pHs. (A,B). The evolution of oxygen in WT and $\Delta ndhV$ strains under different light intensities at pH 8.0 (A) and 7.0 (B). WT and $\Delta ndhV$ strains were grown at 30 °C in BG-11 medium buffered with 5 mM Tris-HCl at pH 8.0 and 7.0, respectively, in 2% CO₂ (v/v in air) at 50 μmol photons m⁻² s⁻¹. Then the cells at logarithmic phase (OD₇₃₀ ≈ 0.5) were used to measure the evolution of oxygen in the presence of 200 μM NaHCO₃ at the light intensities of 70, 150 and 300 μmol photons m⁻² s⁻¹, respectively. Values are means ± SE of five independent measurements. Asterisk indicates significant differences (t-test, *P < 0.05 and **P < 0.01).

nor the assembly of NDH-1 complexes (Fig. 1S), implying that NdhV is a peripheral subunit functioning in regulation of NDH-1.

The growth phenotype of both wild type and $\Delta ndhV$ cultured in liquid medium bubbling with 2% CO₂ at pH 6.5 and low light was almost the same (Fig. 2E), which was consistent with the previous observation (Gao *et al.*)¹⁶ who therefore concluded that NdhV didn't play any role in CO₂ uptake. However, when $\Delta ndhV$ was cultured in agar plate in 2% CO₂ at pH 6.5 and low light, the growth was slightly suppressed (Fig. 2B), suggesting that the bubbling may increase the diffusion of HCO₃⁻ into the carboxysome even if it was not predominant, but the ratio of CO₂ to HCO₃⁻ is still about 0.7 at pH6.5. In addition, the growth of $\Delta ndhV$ was evidently suppressed at high light when HCO₃⁻ or CO₂ were limited at low pH or low CO₂ (Fig. 2C,D,F) but not significantly at high light when the inorganic carbon is saturated at high pH or high CO₂ (Fig. 2C,D). The results suggest that NdhV is required for the efficient CO₂ uptake at high light especially under the conditions when the inorganic carbon is limited, which is further supported by the observation that the CO₂ uptake activity was evidently suppressed at the high light of 300 μmol photons m⁻² s⁻¹ under low CO₂ but not under high CO₂ condition (Fig. 2C) and also by the suppression of photosynthetic O₂ evolution at the high light at low pH (Fig. 7B) but not at high pH (Fig. 7A). Defect in the cyanobacterial NdhV caused partially (about 10%) suppression of the rate of CO₂ uptake (Fig. 3A), it might be attributed to the partly contribution of NdhV to the cyclic electron or respiratory electron transports which might function in providing trans-thylakoid membrane proton gradient (Fig. 3C) for ATP synthesis or alkaline pocket (Kaplan and Reinhold, 1999).

The previous study showed that both the cyclic electron flow around PSI and respiratory O₂ uptake mediated by NDH-1 was enhanced at high light (100 μmol photons m⁻² s⁻¹), accordingly the photosynthetic O₂ evolution was also up-regulated³⁴, suggesting that the up-regulation of NDH-1 is required for high activity of photosynthesis. The up-regulation of the NDH-1 activity induced by the high lights was suppressed when *NdhV* is deleted (Fig. 6A), suggesting that the cyanobacterial NdhV is involved in the regulation of NDH-1 activity in response to the high lights. The significant up-regulation of NdhV by the treatment of the high light in the wild type (Fig. 6B) implies its important role in the regulation of photosynthesis, as one of components related with the electron donor domain of NDH-1. The evident suppression of the rate of CO₂ uptake at the high light (100 μmol photons m⁻² s⁻¹) either at high CO₂ or low CO₂ (Fig. 3A) further supports this mention. In addition, the suppression of the photosynthetic oxygen evolution (Fig. 7B) and the growth (Fig. 2D,F) at the high light when CO₂ was limited in the NdhV deleted mutant might be attributed to the loss of the up-regulation for NDH-1 activity under the stressed conditions (Fig. 6A). It has been indicated that cyclic electron flow around PSI plays an important role in providing ATP for carbon assimilation^{42,43}. Actually, the enhancement of cyclic electron flow around PS I by treatment with low concentration of NaHSO₃ enables cyanobacteria to generate sufficient proton gradient across the thylakoid membrane, thereby increased the biomass of *Synechocystis* PCC 6803⁴⁴. Therefore, in NdhV deleted mutant, the partial suppression of NDH-1 activity (Fig. 1C) would lower the building-up of proton gradient across thylakoid membranes (Fig. 3C), resulting in the decrease in the activity of CO₂ uptake (Fig. 3) and suppressed growth, especially when HCO₃⁻ and CO₂ were limited under high light conditions (Fig. 2D).

In conclusion, our work demonstrate that the cyanobacterial NdhV subunit locates at the hydrophilic parts of NDH-1M and plays an essential role in regulation of NDH-1 activity for efficient operation of cyclic electron flow around PS I and CO₂ uptake especially at high light.

Materials and Methods

Cell culture conditions. The *Synechocystis* 6803 wild type and mutant cells were cultured in BG-11 medium⁴⁵ buffered with Tris-HCl (5 mM, pH 8.0) at 30 °C under 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The cells were bubbled with 2% v/v CO₂ in air. The BG-11 solid medium used was BG-11 supplemented with 1.5% agar, Tris-HCl (10 mM, pH 8.0), and 0.3% Na₂S₂O₃. The illumination was provided by fluorescence lamps at 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. According to culturing requirements of wild type and mutant cells, the BG-11 medium was buffered with 10 mM TES-KOH at several other pH conditions.

Construction of $\Delta ndhV$ mutant. The upstream and downstream regions of *sllo272* (*NdhV*) and a DNA fragment encoding a kanamycin resistance (*Kan^R*) cassette were firstly amplified by PCR using specific oligonucleotide primers (Table S1). Then the PCR products of upstream of *sllo272* and *Kanamycin resistance* (*Kan^R*) cassette were used as templates to obtain the second PCR products (SP1), and the PCR products of downstream of *sllo272* and *Kan^R* cassette were used as templates to obtain the second PCR products (SP2). The third round PCR product containing the upstream and downstream regions inserted by a *Kan^R* cassette were amplified by PCR using SP1 and SP2 as templates. Then the final products were ligated to the pMD19T (Fig. 1A), to make the construct for transforming the WT cells of *Synechocystis* 6803 as described by Williams and Szalay⁴⁶. The transformants were spread on agar plates containing BG-11 medium and Kanamycin (10 $\mu\text{g ml}^{-1}$) buffered at pH 8.0, the BG-11 solid medium plates were incubated in 2% v/v CO₂ in air. The mutated *NdhV* in the transformants was segregated to homogeneity as determined by PCR amplification and immunoblotting.

The measurement of chlorophyll fluorescence. The transient increase in chlorophyll fluorescence after actinic light had been turned off was monitored by means of using a PAM Chl fluorometer (Walz, Effeltrich, Germany), emitter-detector-cuvette assembly (ED-101US) and unit 101ED as previously described^{37,38}.

Oxygen exchange. The evolution of oxygen under different light intensities were determined in cultural BG-11 medium that contained the mid-logarithmic cell of *Synechocystis* 6803 at about 2.5 $\mu\text{g chlorophyll ml}^{-1}$ with a Clark-type oxygen electron. The whole monitoring process was performed at 30 °C.

CO₂ uptake measurement. CO₂ uptake was measured with a portable open-flow gas exchange system Li-6400 (LI-COR Biosciences). Air temperature of the leaf chamber was maintained at 30 °C, the photosynthetically active radiation (PAR) was 100 or 300 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and the flow rate of the air in the measuring chamber was 50 $\mu\text{mol s}^{-1}$. CO₂ concentration was controlled at 2% (v/v in air) or 0.04%. The cells of WT and $\Delta ndhV$ strains grown at 30 °C in BG-11 medium at 2% CO₂ were collected at mid-logarithmic stage by centrifugation and re-suspended in fresh BG-11 medium. They were concentrated into 100 of OD₇₃₀, and 30 μl of each cell suspension was placed on the agar plate as a spot incubated in the growth chamber before CO₂ uptake measurement. Each cell spot on agar was cut out into a square of 1 cm \times 1 cm and put on a cover glass to measure the CO₂ uptake rate. Same size of agar was used as control. Measurements were repeated three times and the averages were recorded.

Isolation of crude thylakoid membranes. *Synechocystis* 6803 thylakoid membranes were isolated as described by Gombos *et al.*⁴⁷ with some modifications as follows. The cell cultures (1L) were centrifuged at the logarithmic phase and then resuspended in 20 ml disruption buffer [10 mM HEPES-NaOH, 5 mM sodium phosphate, pH 7.5, 10 mM MgCl₂, 10 mM NaCl, and 20% v/v glycerol], broken by vortexing with glass beads (150–212 μm) for three times at 70 Hz for 30 s with 30 s interval cooling using a TissueLyser-48 system (Shanghai Jingxin). And then the lysis was centrifuged at 5000 g for 5 min at 4 °C to remove glass beads and unbroken cells. The crude thylakoid membranes and supernatant were obtained by centrifugation of the homogenate at 20 000 g for 30 min at 4 °C from the precipitation and supernatant, respectively. The membranes were washed with buffer B [330 mM sorbitol, 50 mM Bis-Tris-HCl, pH 7.0, 0.5 mM PMSF (Sigma, MO)] and re-suspended in buffer C [25 mM BisTris-HCl, pH 7.0, 10 mM MgCl₂, 20% v/v glycerol, 0.1 units RNase-free DNase RQ1 (Promega, Madison, WI), 0.5 mM PMSF].

Cross-linking of the thylakoid membranes. The cross-linking assay was performed as described previously⁴⁸. Thylakoid membranes were resuspended in 1 ml of 20 mM Hepes-KOH, pH 8.0, 5 mM MgCl₂ (0.5 mg chlorophyll ml^{-1}) and incubated with 2.5 mM DSP (Dithiobis[succinimidyl propionate]) for 30 min at room temperature in the dark. After cross-linking, reactions were quenched with addition of 60 mM Tris-HCl, pH 7.5 for 15 min. Thylakoids were pelleted after centrifuged again at 20 000 g for 30 min at 4 °C and solubilized at 0.5 mg of chlorophyll ml^{-1} in a buffer containing 20 mM Hepes-KOH, pH 8.0, 200 mM NaCl, 1.2% Triton X-100, 1 mM PMSF for 30 min on ice. Insoluble thylakoids were removed by centrifugation at 22 000 g for 10 min at 4 °C and the supernatant was transferred to new tubes for further analysis.

Electrophoresis and immunoblotting. BN-PAGE of *Synechocystis* 6803 membranes was performed as described previously⁴⁸ with slight modifications. The chlorophyll a concentration of the membranes solubilized in buffer C was diluted at 0.5 mg ml^{-1} , then the 1/20 volume of 20% *n*-dodecyl- β -D-maltoside (DM) was added. After incubation on ice for 20 min and centrifugation at 20 000 \times g for another 15 min, the supernatants were supplemented with 1/10 volume of BN sample buffer (5% Serva Blue G, 100 mM BisTris-HCl, pH 7.0, 30% w/v sucrose, 500 mM ϵ -amino-*n*-caproic acid and 10 mM EDTA). Solubilized membranes were then applied to a 1.0 mm thick 5–13% acrylamide gradient gel. Samples of 5 $\mu\text{g chlorophyll a}$ were loaded on the gel. Electrophoresis was performed at 4 °C by increasing the voltage gradually from 50–200 V during the 5 h run. For electrophoresis in the second dimension, the lanes of the BN gel were cut out and incubated in 1 \times SDS sample loading buffer containing 2% β -mercaptoethanol for 30 min. SDS-PAGE of the lanes of the BN gel was performed on a 15% polyacrylamide gel as described previously⁴⁹.

For immunoblotting, the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore) and detected using protein-specific antibodies with the ECL assay kit (BioRad) according to the manufacturer's protocol. Antibody against the NdhV protein of *Synechocystis* 6803 was raised in our laboratory. To amplify the *NdhV* gene, primer sequences were listed in Table S1. The PCR product was ligated into vector pET51b (Novagen). The plasmid was used to transform *E. coli* strain BL21 (DE3) pLysS for expression. Polyclonal antibody was raised in a rabbit from purified recombinant protein. The antibodies against CupB, NdhH, NdhI, NdhK, and NdhM were previously raised in our laboratory.

Quinacrine (QA) fluorescence quenching. Fluorescence of Quinacrine (QA) at 503 nm using the PAM chlorophyll fluorometer (Maxi-version, Walz, Effeltrich, Germany) attached with a US-370 emitter with an emission peak at 375 nm and a PM-101/D detector as described previously⁵⁰. Cells were harvested at logarithmic phase and suspended in reaction mixture contained 5 mM Tris/MES (pH 8.0), 0.3 M mannitol, 2 mM DTT, 5 mM D-Glucose, 1.5 μ M ATP, 2.5 μ M QA with a final chlorophyll concentration of 150 μ g/ml. The quenching of QA fluorescence was induced by adding the cells sample to 2 ml reaction mixture after the background fluorescence became stable about 2 min after started the measurement.

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Acknowledgements

We thank retired Prof. Ogawa T in Nagoya University, Japan for providing M55, $\Delta ndhD1/D2$, $\Delta ndhD3/D4$ strains. This work was supported by grants from the State Key Basic Research and Development Plan 973 (No. 2013CB127005, 2015CB150104), the National Natural Scientific Foundation of China (No. 31270286, 31470338) and Shanghai Science Foundation (grant number 13DJ1400102).

Author Contributions

X.C. carried out the main research work and wrote part of the manuscript. Z.H. constructed NdhM, NdhS and NdhI deleted mutants; M.X. revised the manuscript; L.P. and H.M. designed the research and wrote part of the manuscript. All authors reviewed the manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Chen, X. *et al.* NdhV subunit regulates the activity of type-1 NAD(P)H dehydrogenase under high light conditions in cyanobacterium *Synechocystis* sp. PCC 6803. *Sci. Rep.* **6**, 28361; doi: 10.1038/srep28361 (2016).



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