

# The role of chloroplastic NAD(P)H dehydrogenase in photoprotection

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**Abstract** After a brief exposure to supra-saturating light, leaves of a tobacco transformant, in which chloroplastic NAD(P)H dehydrogenase (NDH) was defective, showed more severe photoinhibition than the wild-type, when judged by the parameter of chlorophyll fluorescence  $F_v/F_m$ . Repeated application of supra-saturating light eventually resulted in chlorosis in the NDH-defective mutant, while the wild-type sustained less photodamage and was able to recover from it. The mechanism of the phenomena is discussed with respect to the potential role of NDH in photosynthesis.

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**Key words:** Chloroplast transformation; Chlororespiration; Ferredoxin-quinone reductase; Ferredoxin-NADP<sup>+</sup> reductase; NAD(P)H dehydrogenase

## 1. Introduction

NADH dehydrogenase (NDH) complex is the primary electron mediator in the respiratory electron transport in mitochondria, transferring electrons from NADH to ubiquinone. In the cyanobacterium *Synechocystis* PCC6803, the NDH complex is bound to the thylakoid membranes and mediates a cyclic electron transport around photosystem I as well as the respiratory electron transport [1–3]. The cyanobacterial NDH is essential to provide energy for the uptake of inorganic carbon, and NDH-defective mutants can proliferate only in CO<sub>2</sub>-enriched conditions [4].

Genes encoding homologs of mitochondrial NDH subunits (*ndhA-K*) have been found in chloroplast genomes [5,6]. Considering close genetic relationship between cyanobacteria and chloroplasts, it was not surprising to find that NDH-defective mutants of tobacco lack a cyclic electron transport [7–9]. Importance of NDH-mediated cyclic photophosphorylation was suggested in a C<sub>4</sub> plant *Sorghum bicolor* in which high levels of NDH proteins are expressed in bundle-sheath chloroplasts where a high level of ATP is required to sustain C<sub>4</sub> type CO<sub>2</sub> fixation [10]. However, growth of the NDH-defective transformant of tobacco *AndhB* is normal in growth chambers [7], as are transformants reported by Burrows et al. [8]. Thus, the physiological role of NDH has not yet been assigned. Another independent cyclic pathway mediated by ferredoxin quinone reductase (FQR) [11–13] may compensate for the function of NDH under optimal growth conditions. Kofer et al. [9] re-

ported abnormal growth of NDH-disruptants due to a defect in starch degradation, a different phenotype from the transformant lines produced by other groups [7,9]. The reason for the difference in the phenotype has not yet been clarified [14,15].

In this report, we show that after a brief exposure to strong light, photoinhibition of photosystem II (PS II), as judged by the parameter of chlorophyll fluorescence maximum yield of fluorescence at closed PS II centers minus minimum fluorescence yield at open PS II centers ( $F_v$ )/maximum yield of fluorescence at closed PS II centers ( $F_m$ ), is more severe in an NDH-defective tobacco than in the wild-type. The mechanism and physiological significance of this phenomenon are discussed.

## 2. Materials and methods

Transformant plants of *Nicotiana tabacum* c.v. Xanthi in which the chloroplastic *ndhB* gene was insertionally inactivated (*AndhB*) [7] were cultivated along with wild-type plants in a growth chamber at 25°C under fluorescent lamps (about 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , 16 h light; 8 h dark). Young leaves (55–100 mm in length) from 2–3 month old tobacco plants were used for the experiments. Chlorophyll fluorescence was measured with a PAM-2000 portable fluorometer (Walz, Effeltrich, Germany). Oxidation and reduction of P700 was measured at 820 nm with a PAM fluorometer with an emitter-detector unit ED800T (Walz, Effeltrich, Germany).

Leaf discs (10 mm diameter) were excised from young leaves (90–100 mm in length) of the wild-type and *AndhB* plants. The lower epidermis of the leaf discs was peeled off, then the leaf discs were floated on buffer A containing 50 mM sorbitol, 10 mM KCl, 1 mM MgCl<sub>2</sub> and 10 mM MES (pH 6.3) for 24 h under fluorescent lamps (30  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ).

## 3. Results

### 3.1. Effects of a brief exposure to supra-saturating light

Repeated illumination by strong light induced significant lowering of maximum yield of chlorophyll fluorescence ( $F_m$ ), especially in *AndhB*. A lower  $F_m$  in *AndhB* resulted in a lower  $F_v/F_m$ , a parameter indicating photoinhibition of PS II, the photodegradation of the reaction center protein of PS II (Fig. 1). Recovery of  $F_v/F_m$  to the original  $F_v/F_m$  level requires 1–2 days in darkness. This slow recovery supports that the lowering of  $F_v/F_m$  was caused by photoinhibition [16,17]. Increases in minimum fluorescence yield at open PS II centers ( $F_o$ ) due to non-photochemical reduction of plastoquinone as judged by the significant decrease in  $F_o$  by far-red light (FR) have been reported [18]. However, no such change in apparent  $F_o$  by FR was found in the presented experiments.

Repeated illumination of strong light caused not only photoinhibition but also severe irreversible photodamage in *AndhB*, while the wild-type sustained less photodamage and

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**Abbreviations:**  $F_m$ , maximum yield of fluorescence at closed PS II centers;  $F_o$ , minimum fluorescence yield at open PS II centers;  $F_v$ ,  $F_m - F_o$ ; PS II, photosystem II

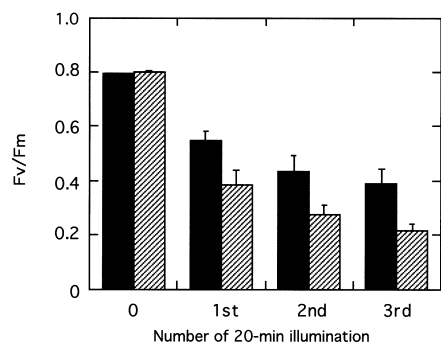


Fig. 1. Changes in  $F_v/F_m$  after repeated illumination with white light (20 min,  $3000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ).  $F_o$  and  $F_m$  were measured 15 min after the actinic light was extinguished. The dark interval between the light periods was 20 min. Values are the averages of five independent measurements. The vertical bars represent the S.E.M.

was able to recover from it (Fig. 2). During incubation for 48 h under a moderate light ( $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) after the light stress, the  $\Delta\text{ndhB}$  leaves lost photosynthetic activity as judged from negligible  $F_v/F_m$  and showed complete chlorosis (loss of chlorophyll). Eight out of 10 leaves from independent  $\Delta\text{ndhB}$  plants showed complete chlorosis after the light stress as shown in Fig. 2, and the other two leaves showed partial chlorosis. In contrast, eight out of ten wild-type leaves recovered from photodamage ( $F_v/F_m$ , 0.7–0.8), and only two out of 10 showed partial chlorosis. The threshold  $F_v/F_m$  value measured after the light stress for the induction of chlorosis was about 0.2 under the conditions employed. If the  $F_v/F_m$  after the photostress was above the threshold value, the  $F_v/F_m$  recovered gradually during incubation under moderate light, and there was no difference in the recovery kinetics of  $F_v/F_m$

between the wild-type and  $\Delta\text{ndhB}$ . Thus, a small difference in  $F_v/F_m$  lowering after light stress between the wild-type and  $\Delta\text{ndhB}$  could be critical for survival under certain stress conditions. Considering the function of complex I in mitochondria, it seems reasonable to assume that the chloroplastic NDH functions in dark respiration in chloroplasts, so called chlororespiration [24]. However, the rates of recovery of  $F_v/F_m$  during 48 h, a process of synthesis of D1 protein and its assembly into PS II, are identical in wild-type and  $\Delta\text{ndhB}$  leaves, suggesting that the NDH has no contribution in this ATP-consuming process in the moderate light. At present, we have no experimental support for the above assumption that the NDH functions in chlororespiration.

Photoinhibition by strong light is normally induced by accumulation of reducing equivalents in stroma, which produce active oxygen species. Therefore, from the observations above it is proposed that in the NDH-defective mutant, exposure to strong light induced a reduction of the stromal redox pool, resulting in a more severe photoinhibition of PS II than that occurring in the wild-type possibly due to increased formation of active oxygen species. The extreme reducing condition, observed after exposure to strong light, has been often regarded as over-reduction [19,20]. Extra ATP formation by the NDH-mediated cyclic flow might be required for the operation of the Calvin cycle and to consume all NADPH photoproduced, because the linear electron transport cannot produce enough ATP to drive the Calvin cycle. Alternatively, downregulation of PS II by the thylakoidal proton gradient produced by the NDH-dependent cyclic flow might be essential to dissipate light energy to prevent stromal components from over-reduction [20]. Recently, Joët et al. [21] used chlorophyll fluorescence to show a stronger reducing level in an NDH-defective mutant than in the wild-type during illumination with moderate light in a low  $\text{O}_2$  atmosphere. Since over-reduction can

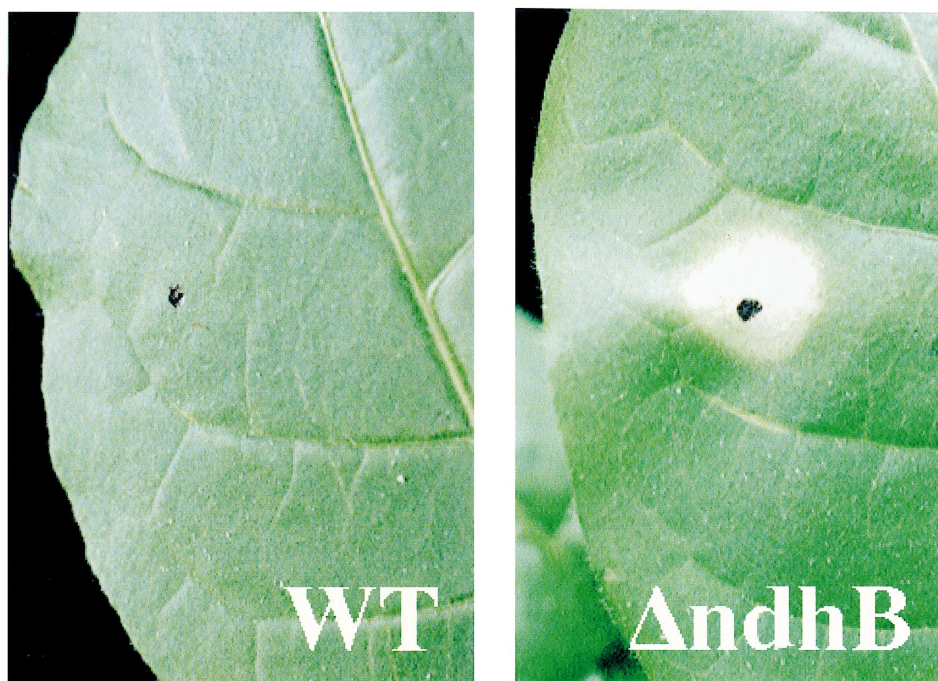


Fig. 2. Leaves exposed to repeated application of strong light. Saturating white light (20 min,  $3000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) was illuminated four times at 20-min intervals on an area measuring 1 cm in diameter on young leaves. Then the plants were kept for 48 h in a growth chamber at  $25^\circ\text{C}$  under fluorescence lamps (about  $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ). The black dot at the center is a marker for the light targeting.

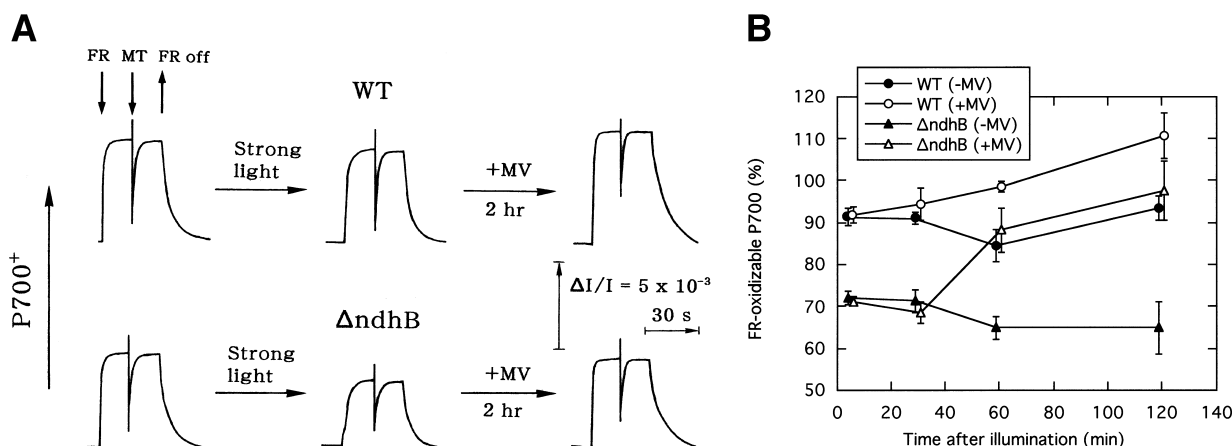


Fig. 3. Lowering of P700 oxidation level by FR ( $> 710$  nm,  $6 \text{ W m}^{-2}$ ) after light stress and its recovery in the presence of 2 mM methyl viologen (MV) in leaf discs of wild-type (WT) and  $\Delta ndhB$  plants. A: Typical traces of P700 redox changes. Measurements were taken before and 5 min after the illumination (10 min,  $3500 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ), after which leaf discs were floated on buffer A with or without methyl viologen in darkness for 2 h. During the illumination with FR, multiple turnover flash (MT) from XMT103 (Walz) was applied. B: Recovery of lowered P700 oxidation level in leaf discs by incubation with methyl viologen (MV). Steady state levels of P700 oxidation by FR ( $> 710$  nm,  $6 \text{ W m}^{-2}$ ) are expressed relative to the original steady state levels measured before the illumination with strong light ( $n=3$ ). The vertical bars represent the S.E.M.

be attained with low light illumination in low  $\text{O}_2$  conditions, this finding may coincide with the observation presented here. Our proposal is also supported by a report by Martín et al. [22], in which a high level of *ndhA* product was found in barley leaves under photooxidative conditions.

### 3.2. P700 oxidation by far-red light

To examine this proposal, redox level of stromal components after illumination of supra saturating light was monitored by redox level of P700 oxidized by FR. In an over-reduced condition, it is predicted that FR illumination would not fully oxidize P700 because of the rapid charge recombination of P700 induced by a limitation of electron acceptors at the reducing side. Indeed, a lower level of FR-oxidizable P700 was found in  $\Delta ndhB$  than in the wild-type (Fig. 3A). This situation should be distinguished from low P700 oxidation by FR reported in cyanobacteria, in which dark respiration keeps plastoquinone reduced. In such a case, where the oxidizing side of P700 is in a reduced state, multiple turnover flash (MT) can induce full oxidation of P700 [23]. However, in the over-reduced state in the reducing side of P700 shown here, even MT cannot fully oxidize P700 because of rapid charge recombination (Fig. 3A). When leaf discs were incubated with methyl viologen after the illumination by strong light, the lowered level of FR-oxidizable P700 found in  $\Delta ndhB$  was recovered within a few hours (Fig. 3B), showing that the added methyl viologen functioned as electron acceptors of PS I and prevented charge recombination of P700. Thus the lowering of the P700 oxidation level was not due to the degradation of PS I, and the results support the above assumption that a brief illumination by strong light induces over-reduction in stromal redox components.

## 4. Discussion

Illumination by strong light, induced over-reduction in stroma, which resulted in photoinhibition of PS II (Fig. 1) and eventually in irreversible chlorosis (Fig. 2). Since *Fv/Fm*, an indicator of photoinhibition, and degree of chlorosis observed

48 h after the light stress are closely correlated, it is likely that both phenomena may be caused by the same mechanism, that is, formation of active oxygen induced by over-reduction in the reducing side of PS I. Stromal over-reduction can be visualized as the lowering of FR-oxidizable P700 (Fig. 3) as a result of the charge recombination of P700. Comparisons of results between the wild-type and  $\Delta ndhB$  show that the chloroplastic NDH complex functions in lowering the photo-damage resulting from the stromal over-reduction possibly through mediating the cyclic electron transport which compensates over-reduction by supplying ATP for the Calvin cycle and adjusts the ATP/NADPH ratio.

Heber and Walker [19] proposed an additional function of cyclic electron transport in the downregulation of PS II through the formation of extra  $\Delta\text{pH}$  across the thylakoid membranes. However no difference in the formation of non-photochemical quenching, which is known to be an indicator of  $\Delta\text{pH}$  formation, was found between wild-type and  $\Delta ndhB$  leaves [8]. We also found no difference in non-photochemical quenching in any light conditions so far examined (data not shown). Thus, the main function of the cyclic electron flow mediated by the NDH complex may be the formation of ATP rather than down-regulation of PS II.

Stromal over-reduction as estimated by P700 charge recombination induced by light stress (Fig. 3) are quite reproducible, while decrease in *Fv/Fm* after the light stress (Fig. 1) is significantly variable dependent on the age of the leaves and growth stage of the plants (data not shown). Therefore over-reduction may be a direct result of light stress, and photoinhibition may be influenced not only by redox state of stroma but also other factors such as scavenging activity of active oxygen [25], activity of photorespiration [26], pool size of redox mediators in stroma [1,27] and export of the reducing equivalents to cytosol via malate and triose phosphate shuttles [28].

In natural environments, sudden changes in light conditions as shown here may happen when shade-grown plants are exposed to full sunlight due to seasonal change in the altitude of the sun or to felling of forests. Alternatively, understory

plants experience more than 10-fold changes in light intensity during short sunflecks [29]. In these conditions, the chloroplastic NDH complex is most likely to function for the protection of photosynthetic apparatus from photodamage.

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