Post-illumination Reduction of the Plastoquinone Pool in Chloroplast Transforms in which Chloroplastic NAD(P)H Dehydrogenase was Inactivated

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We reported previously that an ndhB gene disruptant, ΔndhB, had the same phenotype as wild-type tobacco plants under normal growth conditions. Two other groups have reported conflicting phenotypes with each other for ndhCKJ operon disruptants. Here, we generated two transformants in which the ndhCKJ operon was disrupted, and found that new transformants had the same phenotype as ΔndhB. After illumination with visible light, all ndh disruptants had higher levels of steady-state fluorescence than wild-type controls when measured under weak light, suggesting that reduction of the plastoquinone pool in ndh disruptants was greater than those in wild-type controls. The weak light itself could not reduce the plastoquinone much, so the reduction in the plastoquinone in the mutant was due to electron donation from stromal reductants generated during illumination with the strong light. These results supported the hypothesis that NAD(P)H dehydrogenase prevents overreduction in chloroplasts and suggested that chlororespiratory oxidase did not function under low light or in the dark.

Key words: chloroplast transformation; cyclic electron transport; NAD(P)H dehydrogenase; Nicotiana tabacum

NAD(P)H dehydrogenase (NDH) in chloroplasts of higher plants is the homolog of eubacterial and mitochondrial complex I in the respiratory chain. Since the discovery of ndh genes in chloroplast genomes,1 the function of chloroplastic NDH has been studied extensively. These studies found that NDH is encoded by 11 subunit genes in the chloroplast genome and possibly by yet unidentified subunit genes in the nuclear genome. NDH functions as NAD(P)H:plastoquinone oxidoreductase in thylakoid membranes, but the physiological role of NDH in higher plants is still unknown.

To investigate NDH function in higher plants, several groups generated ndh disruptants using the chloroplast transformation technique, and most of these disruptants, including an ndhB disruptant, had phenotypes similar to those of wild-type controls under nonstressed growth conditions.2-4 Only ndhCKJ operon disruptants generated by the polyethylene glycol (PEG) -mediated method by Kofer et al. grew slowly,5 in contrast to the normal phenotype of other ndhCKJ operon disruptants generated by particle bombardment methods by Burrows et al.6 These contradictory results have been discussed extensively but as yet no consensus has not been reached.7,8

The NDH complex consists of a hydrophilic subcomplex and a hydrophobic subcomplex. On the basis of our earlier studies, we suspected that the hydrophilic subcomplex could have NADPH oxidation activity by itself9 and that disruptants of the hydrophilic subcomplex would have a phenotype different from that of ΔndhB, in which the NDH-B subunit in the hydrophobic subcomplex was disrupted. Therefore, another aim in generating ndhCKJ disruptants ΔndhKJ and ΔndhCKJ was to examine this hypothesis. Although the NDH-C subunit is contained in the hydrophobic subcomplex, NDH-K and -J subunits are contained in the hydrophilic subcomplex. We reported previously that ΔndhB caused severer photoinhibition than the wild-type control after illumination with suprasaturating light,10 and Horváth et al.10 reported that photosynthesis efficiency declines in another ndhB disruptant under humidity stress, suggesting the physiological importance of NDH during stress. Here, we found that ΔndhKJ and ΔndhCKJ as well as ΔndhB were susceptible to photoinhibition, and the level of post-illumination...
reduction of the plastoquinone pool, which was estimated by increases in the steady-state fluorescence, F5, level of chlorophyll fluorescence measured under weak light, increased in all \( ndh \) disruptants as compared to control plants. This kind of post-illumination reduction seemed to be different from the previously reported post-illumination reduction found in the wild type but not in the \( ndh \)-less mutants,\(^3\) because the latter \( NDH \)-dependent plastoquinone reduction lasted only a few minutes.

**Materials and Methods**

*Plants.* All experiments were done with *Nicotiana tabacum* cv. Xanthi cultivated on fertilizer soil in a growth chamber at 28°C under fluorescent lamps (about 100 \( \mu \text{mol of quanta m}^{-2} \text{s}^{-1} \), 16 h of light, 8 h of dark). Transformant lines \( \Delta ndhB \) (\( ndhB \) gene disrupted) and the 4Y26 control were reported previously.\(^6\) The 4Y26 line has gained spectinomycin resistance by insertion of a chimeric \( aadA \) gene in a nondestructive way. We used the line 4Y26 as a control to verify the effects of aminoglycoside 3'-adenylytransferase, the product of the \( aadA \) gene. The lines \( \Delta ndhKJ \) and \( \Delta ndhCKJ \) are described below. For measurement of chlorophyll fluorescence, young leaves (60 to 80 mm long) from plants 7 or 8 weeks old were used.

**Measurement of chlorophyll fluorescence.** Chlorophyll fluorescence was measured with a PAM-2000 portable fluorometer (Walz, Effeltrich, Germany). The maximum yield of chlorophyll fluorescence, \( F_0 \), was brought about by a 1-s pulse of saturating white light. The nomenclature of van Kooten and Snell\(^3\) was used for indices of chlorophyll fluorescence.

**Plasmid constructs.** The chimeric \( aadA \) gene was described previously.\(^6\) The 3.6-kb *EcoRV-Sall* fragment of tobacco chloroplast DNA containing the \( ndhCKJ \) operon (sites at nucleotides 53483 and 49841 of the chloroplast genome, Shinozaki et al.\(^1\)) was cloned into pBluescript SK(+) (Stratagene). The *Clal* site between the *BamHI* and *EcoRI* sites within the multiple cloning site of pBluescript SK(+) was eliminated by double digestion with *BamHI* and *EcoRI*, and the plasmid was subsequently blunt-ended and self-ligated, giving pndhCKJ. The plasmid p\( \Delta ndhKJ \) for disruption of the \( ndhK \) and \( ndhJ \) genes was generated by replacement of the *XhoI-* *Clal* fragment of pndhCKJ with a chimeric \( aadA \) gene. Similarly, plasmid p\( \Delta ndhCKJ \) for disruption of \( ndhC \), \( K \), and \( J \) genes was generated by replacement of the *NotI-* *Clal* fragment with a chimeric \( aadA \) gene.

**Chloroplast transformation.** Tobacco plants were grown aseptically on agar-solidified Murashige-Skoog medium\(^3\) containing 30 g \( \text{L}^{-1} \) sucrose. The particle bombardment method used for chloroplast transformation was described previously.\(^6\) Transformed calli and shoots were selected on RMOP medium\(^4\) containing spectinomycin dihydrochloride (500 mg \( \text{L}^{-1} \)). Spectinomycin-resistant shoots were rooted on agar-solidified Murashige-Skoog medium containing 30 g \( \text{L}^{-1} \) sucrose and shoot regeneration from leaves was repeated on the same selective medium to obtain homoplasmic plants.

**Genomic PCR analysis.** Total cellular DNA was isolated by the cetlytrimethylammonium bromide method\(^5\) and PCR was done with the following primers:

- \( ndhC-FF, \) 5'-GGATAAAGAAACCCACCTTTTCG-3';
- \( ndhK-F, \) 5'-CAAGACTCTCTAGTTTATGGC-3';
- \( ndhJ-RR, \) 5'-CTCATTGTTACACTTATTGG-3';
- Pr02, 5'-TTCAAAGCTTCAGAATAGCCTTCTT-3'.

**Results**

**Generation of \( \Delta ndhKJ \) and \( \Delta ndhCKJ \)** Constructs of p\( \Delta ndhKJ \) for disruption of \( ndhK \) and \( J \) genes and p\( \Delta ndhCKJ \) for disruption of \( ndhC \), \( K \), and \( J \) genes were generated by insertion of a chimeric \( aadA \) gene into the \( ndhCKJ \) operon (Fig. 1a). To transform the chloroplast genome, we introduced these constructs into tobacco leaves by the particle bombardment method. We obtained two spectinomycin-resistant lines from p\( \Delta ndhKJ \) (\( \Delta ndhKJ \)) and one spectinomycin-resistant line from p\( \Delta ndhCKJ \) (\( \Delta ndhCKJ \)). To obtain homoplasmic lines, we repeated shoot regeneration from leaf sections of their transformants. After this selection, total DNA was extracted from these transformants by the CTAB method and we confirmed the insertion of a chimeric \( aadA \) gene (Fig. 1d) and homoplasmicity by genomic PCR (Fig. 1b, c).

All \( ndh \) disruptants had growth rates identical to the growth rate of wild-type control under normal growth conditions (Fig. 2) as reported previously by Burrows et al.\(^7\) On immunoblotting analysis, the NDH-H subunit was not detected in any of our \( ndh \) disruptants. A similar phenomenon was reported previously,\(^2\) suggesting that lack of any one subunit prevented assembly of the other NDH subunits and led to their degradation.

These findings indicated that disruption of the hydrophilic subunits NDH-K and NDH-J as well as of the hydrophobic subunits NDH-B and NDH-C, resulted in total inactivation.

**Post-illumination reduction of plastoquinone** To characterize each transformant in more detail,
Fig. 1. Construction of \textit{ndh} Disruptants \textit{AndhKJ} and \textit{AndhCKJ}.
(a) The \textit{AndhKJ} chloroplast genome had a spectinomycin-resistance gene (chimeric \textit{aadA}) between \textit{Xhol} and \textit{ClaI} sites, and \textit{ndhK} and \textit{ndhJ} genes were disrupted. The \textit{AndhCKJ} chloroplast genome had a chimeric \textit{aadA} gene between the \textit{NcoI} and \textit{ClaI} sites, and \textit{ndhC}, \textit{K}, and \textit{J} genes were disrupted. (b) PCR products from wild-type tobacco and \textit{AndhKJ} chloroplast genomes. The primers \textit{ndhK-F} and \textit{ndhJ-RR} were used (see text for details). As \textit{AndhKJ} had only a 1.8-kb band, \textit{AndhKJ} was homoplasmic. (c) PCR products from wild-type tobacco and \textit{AndhCKJ} chloroplast genomes. The primers \textit{ndhC-FF} and \textit{ndhJ-RR} were used (see text for details). As \textit{AndhCKJ} had only a 2.6-kb band, \textit{AndhCKJ} was homoplasmic. (d) Detection of chimeric \textit{aadA} gene with its specific primer, \textit{Pr02} and primers \textit{ndhC-FF} and \textit{ndhK-F} (see text for details). The 1.7-kb fragment from \textit{AndhCKJ} was amplified with the primers \textit{ndhK-F} and \textit{Pr02}, and the 2.5-kb fragment from \textit{AndhCKJ} was amplified with the primers \textit{ndhC-FF} and \textit{Pr02}. Both \textit{AndhKJ} and \textit{AndhCKJ} had the chimeric \textit{aadA} gene in the expected region of their chloroplast genome.

Fig. 2. Growth of Control and Transformed Plants.
All plants were cultivated on fertilized soil in a growth chamber at 28°C under fluorescent lamps (about 100 \textmu mol of quanta m\(^{-2}\) s\(^{-1}\), 16 h of light, 8 h of dark) for 6 weeks. We could not find any phenotypic difference between nontransformed plants of 4Y26 and \textit{ndhCKJ} operon disruptants \textit{AndhKJ} and \textit{AndhCKJ}.

we measured \(F_m\) 15 min after exposure to suprasaturating light (3,000 \textmu mol m\(^{-2}\) s\(^{-1}\) for 20 min). As in previous observations with \textit{AndhB},\textsuperscript{10} such light lowered \(F_m\) in \textit{AndhKJ} and \textit{AndhCKJ} compared with wild-type controls (data not shown). These results confirmed that photoinhibition of photosystem II was caused by severe light stress in all \textit{ndh} disruptants.

In addition, we found an increase in the \(F_\text{r}\) level measured under weak light conditions (10 \textmu mol m\(^{-2}\) s\(^{-1}\)) in \textit{ndh} disruptants after light stress (1200 \textmu mol m\(^{-2}\) s\(^{-1}\) for 10 min) (Fig. 3), but such drastic increase was not observed in 4Y26 or wild-type controls. This increase in \(F_\text{r}\) was suppressed by transient darkness or far-red light illumination (data not shown), suggesting that the increase in \(F_\text{r}\) level reflected the reduction of the plastoquinone pool.

\textit{Recovery kinetics}
To investigate how long the light-induced reduction of the plastoquinone pool lasted, we monitored the process of re-oxidation of the pool after light stress in terms of chlorophyll fluorescence (Table 1). The reduction of the plastoquinone pool under weak light (10 \textmu mol m\(^{-2}\) s\(^{-1}\)) was calculated as 1-qP. \(F_\text{r}/F_\text{m}\), representing photoinhibition of PS II, also was monitored in complete darkness. We used \textit{AndhB} as a representative \textit{ndh} disruptant because no differences were observed among our disruptants in any of the above experiments. Reduction of the plastoquinone pool under weak light increased within 10 min (Fig. 3), and this high level was maintained for sever-
al hours. In contrast, \( F_r/F_m \) measurements showed that cells recovered from photoinhibition by between 1 and 6 h, suggesting that recovery from photoinhibition was dependent of re-oxidation of the plastoquinone pool.

**Discussion**

**Phenotype of ndhCKJ operon disruptants**

*Ndh* disruptants \( \Delta ndhKJ \) and \( \Delta ndhCKJ \) showed no specific phenotypic abnormalities compared with wild-type controls grown either in vitro or in soil under normal growth conditions, consistent with the phenotype of *ndhCKJ* operon disruptants described by Burrows et al.\(^2\). In addition, although we did not interrupt *ndhH* gene expression in any of our *ndh* disruptants, we could not detect the NDH-H subunit in any of them by immunoblotting. Therefore, we proposed that disruption of any of the NDH subunits (at least *ndhH* and *ndhCKJ* operons) prevented assembly of the complex or subcomplex of NDH and led to NDH subunit degradation; if so, therefore almost all *ndh* disruptants would have similar phenotypes. We suspected that transformants generated Kofer et al.\(^3\) had unexpected mutations when transformation or repeated regenerations were done.

**Post-illumination reduction of plastoquinone after light stress**

In this study, we found a novel kind of post-illumination reduction of plastoquinone in *ndh* disruptants, different from the previously known increase in chlorophyll fluorescence mediated by NDH.\(^2\) The novel increase in chlorophyll fluorescence was observed in weak light after exposure to stronger light, and the plastoquinone pool remained reduced for several hours. We supposed that this plastoquinone reduction was associated with the accumulation of reduced species such as NADPH or ferredoxin, and that such stromal over-reduction contributed to the reduction of plastoquinone via as yet uncharacterized reductases other than NDH or via nonenzymatic redox equilibrium between the stroma and plastoquinone pool in thylakoid membranes. Joët et al.\(^6\) also reported more reduced plastoquinone in an *ndhB*-defective tobacco mutant than in wild-type controls under anaerobic conditions with moderate light. The mechanism by which stromal reductants accumulate in *ndh* disruptants is unclear. One possible explanation is that NDH may participate in cyclic electron flow around PS I (for review, see Shikanai and Endo\(^2\)), which can produce more ATP than linear electron flow and is more important under ATP-consuming stress;\(^6\) i.e., illumination with strong light will use photorespiration, which will require more ATP than for CO\(_2\) fixation. Thus, *ndh* disruptants would cause ATP shortage and consequent NADPH surplus under light conditions, and an NADPH surplus may cause accumulation of reduced species in the stroma, this accumulation will reduce plastoquinone levels by unidentified enzymes such as ferredoxin-quinone oxidoreductase or nonenzymatic redox equilibrium.

Our results suggested that safety valves such as terminal oxidase in the thylakoid membrane or envelope oxidase or other stromal enzymes to discharge extra reducing power do not operate. These *ndh* disruptants would be useful for future studies to charac-
terize chloroplastic redox-regulation in detail.

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