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

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Received March 18, 2002; Accepted May 3, 2002

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 that 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 tabaccum

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 as 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.2–6) Only ndhCKJ operon disruptants generated by the polyethylene glycol (PEG) -mediated method by Kofer et al. grew slowly,3) in contrast to the normal phenotype of other ndhCKJ operon disruptants generated by particle bombardment methods by Burrows et al.2) 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 disrupt- ed. 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.9) 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, F_s, 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,\textsuperscript{2-4,6} because the latter NDH-dependent plastoquinone reduction lasted only a few minutes.

Materials and Methods

Plants. All experiments were done with \textit{Nicotiana tabacum} cv. Xanthi cultivated on fertilized soil in a growth chamber at 28°C under fluorescent lamps (about 100 μmol of quanta m\textsuperscript{-2} s\textsuperscript{-1}, 16 h of light, 8 h of dark). Transformant lines ΔndhB (ndhB gene disruptant) and the 4Y26 control were reported previously.\textsuperscript{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 ΔndhKJ and ΔndhCKJ 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_m, was brought about by a 1-s pulse of saturating white light. The nomenclature of van Kooten and Snel\textsuperscript{11} was used for indices of chlorophyll fluorescence.

Plasmid constructs. The chimeric aadA gene was described previously.\textsuperscript{6} The 3.6-kb EcoRV-SalI fragment of tobacco chloroplast DNA containing the ndhCKJ operon (sites at nucleotides 53483 and 49841 of the chloroplast genome, Shinozaki \textit{et al.}\textsuperscript{12}) was cloned into pBluescript SK(+) (Stratagene). The CiaI site between the \textit{Bam}HI and the \textit{EcoRI} sites within the multiple cloning site of pBluescript SK(+) was eliminated by double digestion with \textit{Bam}HI and \textit{EcoRI}, and the plasmid was subsequently blunt-ended and self-ligated, giving pndhCKJ. The plasmid pΔndhKJ for disruption of the \textit{ndhK} and \textit{ndhJ} genes was generated by replacement of the \textit{Xhol}—\textit{CiaI} fragment of pndhCKJ with a chimeric aadA gene. Similarly, plasmid pΔndhCKJ for disruption of \textit{ndhC}, K, and J genes was generated by replacement of the \textit{Not}I—\textit{CiaI} fragment with a chimeric aadA gene.

Chloroplast transformation. Tobacco plants were grown aseptically on agar-solidified Murashige-Skoog medium\textsuperscript{13} containing 30 g l\textsuperscript{-1} sucrose. The particle bombardment method used for chloroplast transformation was described previously.\textsuperscript{5,6} Transformed calli and shoots were selected on RMOP medium\textsuperscript{6} containing spectinomycin dihydrochloride (500 mg l\textsuperscript{-1}). Spectinomycin-resistant shoots were rooted on agar-solidified Murashige-Skoog medium containing 30 g l\textsuperscript{-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 cetyltrimethylammonium bromide method\textsuperscript{19} and PCR was done with the following primers: ndhC-FF, 5'-GGATAAAGAAACCAACCTTTCCG-3'; ndhK-F, 5'-CAAGACTCTCTAGTTATGGC-3'; ndhJ-RR, 5'-CTCATTGGTTTATCATCTATGG-3'; Pr02, 5'-TTCAAGCTTCAATATAGCTTCTTTC-3'.

Results

Generation of ΔndhKJ and ΔndhCKJ

Constructs of pΔndhKJ for disruption of \textit{ndhK} and J genes and pΔndhCKJ for disruption of \textit{ndhC}, K, and J genes were generated by insertion of a chimeric aadA gene into the \textit{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 pΔndhKJ (ΔndhKJ) and one spectinomycin-resistant line from pΔndhCKJ (ΔndhCKJ). 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 \textit{et al.}\textsuperscript{2} On immunoblotting analysis, the NDH-H subunit was not detected in any of our ndh disruptants. A similar phenomenon was reported previously,\textsuperscript{2,6} 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,
NAD(P)H Dehydrogenase Disruptants

Fig. 1. Construction of ndh Disruptants ΔndhKJ and ΔndhCKJ.
(a) The ΔndhKJ chloroplast genome had a spectinomycin-resistance gene (chimeric aadA) between XhoI and ClaI sites, and ndhK and ndhJ genes were disrupted. The ΔndhCKJ chloroplast genome had a chimeric aadA gene between the NcoI and ClaI sites, and ndhC, K, and J genes were disrupted. (b) PCR products from wild-type tobacco and ΔndhKJ chloroplast genomes. The primers ndhK-F and ndhJ-RR were used (see text for details). As ΔndhKJ had only a 1.8-kb band, ΔndhKJ was homoplasmic. (c) PCR products from wild-type tobacco and ΔndhCKJ chloroplast genomes. The primers ndhC-FF and ndhJ-RR were used (see text for details). As ΔndhCKJ had only a 2.6-kb band, ΔndhCKJ was homoplasmic. (d) Detection of chimeric aadA gene with its specific primer, Pr02 and primers ndhC-FF and ndhK-F (see text for details). The 1.7-kb fragment from ΔndhKJ was amplified with the primers ndhK-F and Pr02, and the 2.5-kb fragment from ΔndhCKJ was amplified with the primers ndhC-FF and Pr02. Both ΔndhKJ and ΔndhCKJ had the chimeric 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 μmol 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 ndhCKJ operon disruptants ΔndhKJ and ΔndhCKJ.

we measured Fm 15 min after exposure to suprasaturating light (3,000 μmol m⁻² s⁻¹ for 20 min). As in previous observations with ΔndhB,¹⁰ such light lowered Fm in ΔndhKJ and ΔndhCKJ compared with wild-type controls (data not shown). These results confirmed that photoinhibition of photosystem II was caused by severe light stress in all ndh disruptants.

In addition, we found an increase in the Fv level measured under weak light conditions (10 μmol m⁻² s⁻¹) in ndh disruptants after light stress (1200 μmol 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 Fv was suppressed by transient darkness or far-red light illumination (data not shown), suggesting that the increase in Fv level reflected the reduction of the plastoquinone pool.

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 μmol m⁻² s⁻¹) was calculated as 1-qP. Fv / Fm, representing photoinhibition of PS II, also was monitored in complete darkness. We used ΔndhB as a representative 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-
Table 1. Recovery from Over-reduction after Light Stress

<table>
<thead>
<tr>
<th>Time</th>
<th>(1-qp) × 100a</th>
<th>Fv/Fm b</th>
</tr>
</thead>
<tbody>
<tr>
<td>4Y26 ΔndhB</td>
<td>4Y26 ΔndhB</td>
<td></td>
</tr>
<tr>
<td>Before stress</td>
<td>1.1 ± 0.4</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>1h after</td>
<td>2.6 ± 0.9</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>6h after</td>
<td>2.5 ± 1.1</td>
<td>0.79 ± 0.00</td>
</tr>
<tr>
<td>24h after</td>
<td>1.7 ± 0.3</td>
<td>0.80 ± 0.01</td>
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Leaves were illuminated at 2,000 μmol m⁻² s⁻¹ for 4 min. Values are the means ± standard deviations of three independent measurements.

aFo level was measured in complete darkness, and from the difference between steady-state fluorescence (Fv measured under weak light condition) under room light (10 μmol m⁻² s⁻¹) and Fo, photochemical quenching qp was calculated as (Fm - Fs measured under weak light conditions) / (Fm - Fo). The value of 1qp reflects the redox state of plastoquinone pool.
bFo and Fm were measured in complete darkness, 15 min after light was extinguished.

Fig. 3. Post-illumination Reduction of the Plastoquinone Pool. After 10 min of illumination (1,200 μmol of m⁻² s⁻¹; AL1), Fs was measured under weak light (10 μmol of m⁻² s⁻¹; AL2).

Discussion

Phenotype of ndhCKJ operon disruptants

Ndh disruptants ΔndhKJ and ΔndhCKJ 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. 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 ndhB 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. 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 of chlorophyll fluorescence mediated by NDH. 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. 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), which can produce more ATP than linear electron flow and is more important under ATP-consuming stress; i.e., illumination with strong light will use photorespiration, which will require more ATP than for CO2 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-
characterize chloroplastic redox-regulation in detail.

Acknowledgments

We thank Dr. H. Mi, Shanghai Institute of Plant Physiology, for useful discussion. We also thank Dr. H. Koiwa, Purdue University, for critical reading of the manuscript. This work was supported in part by a Grant-in-Aid for Basic Research C (No.136440646) from the Ministry of Education, Science, and Culture, Japan to T.E.A.T. was supported by a fellowship from the Japanese Society for the Promotion of Science.

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