The EMBO Journal Vol.17 No.4 pp.868–876, 1998

Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid *ndh* genes

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The plastid genomes of several plants contain homologues, termed *ndh* genes, of genes encoding subunits of the NADH:ubiquinone oxidoreductase or complex I of mitochondria and eubacteria. The functional significance of the Ndh proteins in higher plants is uncertain. We show here that tobacco chloroplasts contain a protein complex of 550 kDa consisting of at least three of the *ndh* gene products: NdhI, NdhJ and NdhK. We have constructed mutant tobacco plants with disrupted *ndhC*, *ndhK* and *ndhJ* plastid genes, indicating that the Ndh complex is dispensible for plant growth under optimal growth conditions. Chlorophyll fluorescence analysis shows that *in vivo* the Ndh complex catalyses the post-illumination reduction of the plastoquinone pool and in the light optimizes the induction of photosynthesis under conditions of water stress. We conclude that the Ndh complex catalyses the reduction of the plastoquinone pool using stromal reductant and so acts as a respiratory complex. Overall, our data are compatible with the participation of the Ndh complex in cyclic electron flow around the photosystem I complex in the light and possibly in a chloroplast respiratory chain in the dark.

Keywords: chloroplast/chlororespiration/cyclic electron flow/ndh/plastid mutant

Introduction

A longstanding question in chloroplast genetics has been the role of the *ndh* genes identified originally from sequencing studies of the tobacco (Shinozaki *et al.*, 1986) and liverwort (Ohyama *et al.*, 1986) plastid genomes. These genes were designated *ndh* on the basis of sequence similarities to genes encoding subunits of the proton-pumping NADH:ubiquinone oxidoreductase, or complex I, of the mitochondrial respiratory chain (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Fearnley and Walker, 1992). Consequently, the *ndh* genes were suggested to encode a plastidic NAD(P)H dehydrogenase analogous to complex I. However, the apparent absence of a chloroplast analogue to the NADH-oxidizing subcomplex of complex I, consisting of 19, 50 and 91 kDa proteins in *Escherichia coli* complex I (Leif *et al.*, 1995), has led to suggestions that the electron input device is quite different in the plastid Ndh complex. Possible alternative substrates for the Ndh complex include reduced ferredoxin and NADPH (Friedrich *et al.*, 1995). For NADPH, it has also been suggested that oxidation may be mediated by attachment of ferredoxin-$NADP^+$ reductase (FNR) to the Ndh complex (Guedeney *et al.*, 1996).

Although a number of the Ndh proteins have now been detected immunochemically in the thylakoid membrane of higher plants (Nixon *et al.*, 1989; Berger *et al.*, 1993; Guedeney *et al.*, 1996; Martín *et al.*, 1996), where they are found preferentially in the non-appressed stromal lamellae (Nixon *et al.*, 1989; Berger *et al.*, 1993), there has been uncertainty as to whether the Ndh proteins are subunits of a single protein complex and if so what its function may be.

Speculation on the role of the Ndh complex in higher plants has focused on two areas: as a component of a hitherto uncharacterized respiratory chain within the thylakoid membrane, akin to the chlororespiratory chain described in green algae (Bennoun, 1982), or as a pathway by which electrons can be cycled around the photosystem I (PSI) complex in so-called cyclic electron flow (reviewed by Bendall and Manasse, 1995). This latter process contributes to the trans-thylakoid proton gradient which is used to drive ATP synthesis and is thought to be an important mechanism by which plants can produce extra ATP when exposed to stress conditions such as high temperature, drought and high light (reviewed by Bendall and Manasse, 1995).

The plastid *ndh* genes have close homologues in cyanobacteria, consistent with the endosymbiotic theory for the evolution of chloroplasts. Although the cyanobacterial Ndh complex has not been isolated, it has been suggested from analysis of cyanobacterial *ndh* mutants that NADPH, NADH and reduced ferredoxin can all be oxidized by the Ndh complex (Mi *et al.*, 1995). Mutation of the cyanobacterial *ndh* genes also leads to an inhibition of both cyclic electron flow and respiration (Mi *et al.*, 1992; Yu *et al.*, 1993). At this stage, the degree of structural and functional conservation between the plastid and cyanobacterial Ndh complexes remains uncertain largely because so little is known about the plastid Ndh complex.

Here we exploit recent advances in the transformation of the chloroplast genome of tobacco (Svab *et al.*, 1990; Svab and Maliga, 1993) to generate several *ndh* null mutants. Our analyses indicate that the Ndh proteins are components of a large protein complex and that this Ndh complex is indeed functional *in vivo* but is dispensible for plant growth under optimal growth conditions. Our data also suggest a role for the Ndh complex in cyclic electron flow around PSI in the light and in chlororespiration in the dark.

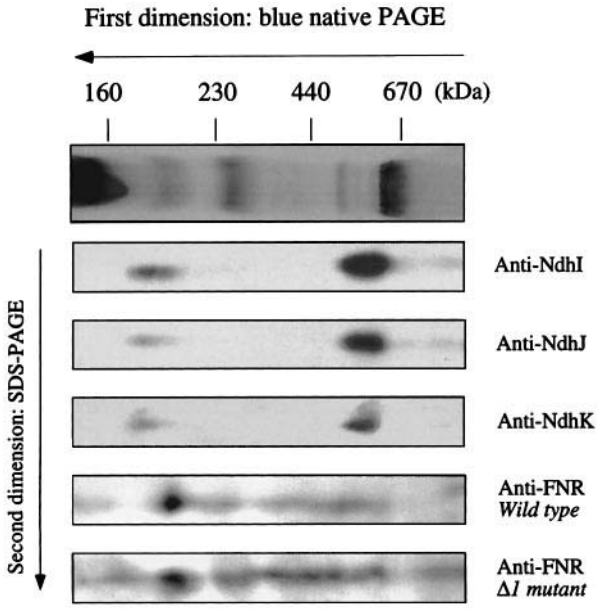

Fig. 1. The Ndh proteins form a large protein complex. Detergent-solubilized WT tobacco chloroplasts were separated by blue native PAGE in the first dimension followed by denaturing SDS–PAGE in the second dimension and immunodetection with antibodies specific for NdhI, NdhK, NdhJ and FNR. An extract from the *ndh* mutant, Δ1, was used to confirm that FNR detected in the WT blots was not part of the Ndh complex.

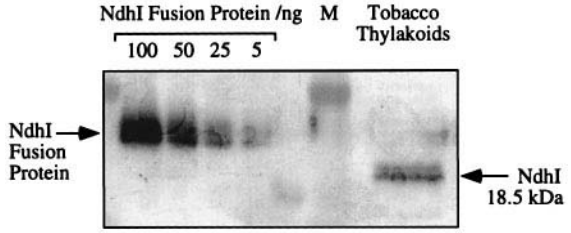

Fig. 2. Estimation of NdhI content in WT tobacco thylakoid membranes. The degree of cross-reaction obtained with thylakoid membranes containing 34 μg of chlorophyll (~240 μg of protein) was compared with that obtained from known amounts of tobacco NdhI expressed in *E.coli* as a His_{10}-tag fusion protein.
Pre-stained markers were run in lane M. Control experiments confirmed that the antiserum did not recognize the His_{10}-tag (data not shown).

Results

The Ndh proteins form a large protein complex

Northern blots have already indicated that the tobacco *ndh* genes are transcribed in the mature chloroplast (Matsubayashi *et al.*, 1987). In order to investigate whether Ndh proteins were indeed expressed in tobacco and if so whether they formed a protein complex *in vivo*, we raised antibodies against the plastid *ndhI*, *ndhJ* and *ndhK* gene products, following their overexpression in *E.coli*. Blue native polyacrylamide gel electrophoresis followed by immunodetection showed that the the NdhI, NdhJ and NdhK proteins co-migrated as part of a 550 kDa complex (Figure 1) which is similar in size to *E.coli* complex I (Leif *et al.*, 1995). A smaller complex of ~200 kDa was also detected with this technique and is probably a subcomplex formed during the isolation procedure as its abundance could be increased by raising the pH (data not shown). A similar instability has also been reported for complex I isolated from *E.coli* (Leif *et al.*, 1995). Significantly, in contrast to a recent report (Guedeney *et al.*, 1996), we found that the electron transfer component ferredoxin-$NADP^+$ reductase (FNR) did not co-purify with the Ndh complex (Figure 1).

The steady-state level of NdhI was estimated from quantitative immunoblotting to be ~33 ng in 240 μg of thylakoid protein or ~0.013% (w/w) (Figure 2). If a chlorophyll/PSII stoichiometry of 350 is assumed for tobacco thylakoids (Melis, 1991), then NdhI is present at ~1.5% of the level of PSII on a molar basis.

Plastid ndh genes are dispensible in tobacco

The functional significance of the Ndh complex was assessed in chloroplast mutants of the tobacco, *Nicotiana tabacum*, generated by biolistic transformation. The *ndhCKJ* operon (Matsubayashi *et al.*, 1987) was chosen for study as its disruption was not anticipated to affect the expression of downstream genes. Four distinct lines were generated: one each with the *ndhC*, *ndhK* or *ndhJ* coding regions disrupted by insertion of a selectable spectinomycin resistance (*aadA*) gene, and one (Δ) in which part of the *ndhCKJ* operon was deleted (Figure 3A). Southern analysis indicated that the transformants were homoplasmic for the mutations (Figure 3B), and immunoblots confirmed that NdhJ and NdhK were absent from all four lines (Figure 3C). Significantly, NdhI was also absent from thylakoid membranes isolated from these mutants, again supporting the presence of the NdhIJK proteins in a single complex. The sensitivity of detection of NdhI in immunoblotting experiments was better than 1% of the level of NdhI found in 70 μg of wild-type (WT) thylakoid membrane protein (Figure 3D), which corresponds to $<$92 pg of NdhI (Figure 3D). Membrane-bound FNR was detected in all mutants (Figure 3C). No obvious phenotype was observed in the mutants compared with WT when grown under our normal growth conditions, indicating that the plastid NdhCKJ proteins are dispensable in tobacco under the conditions studied (data not shown).

Reduction of the plastoquinone pool is inhibited in the ndh mutants

Perturbations to electron transfer within the thylakoid membrane *in vivo* were assessed in mutant, WT and WT* [a control line in which the *aadA* cassette is inserted within the *rbcL–accD* (ORF512) intergenic region of the plastid genome (Svab and Maliga, 1993)] by measuring the relative quantum yield of chorophyll fluorescence from PSII during illumination (Krause and Weis, 1991). When PSII is in the open state, the level of fluorescence is low (F_o) because the majority of light energy absorbed by PSII can be used to drive transmembrane electron transfer. When the PSII reaction centre (RC) is closed, fluorescence reaches a maximum level termed F_m.

Figure 4A shows a typical fluorescence induction curve when dark-adapted tobacco leaves are exposed to light. Upon illumination, F_m is reached quickly as the PSII RC is closed. Fluorescence is then quenched by photochemical (qP) and non-photochemical (qN) processes as the Calvin cycle is activated, CO_2 is fixed and a pH gradient is generated across the thylakoid membrane (Krause and Weis, 1991). When the actinic light is switched off after

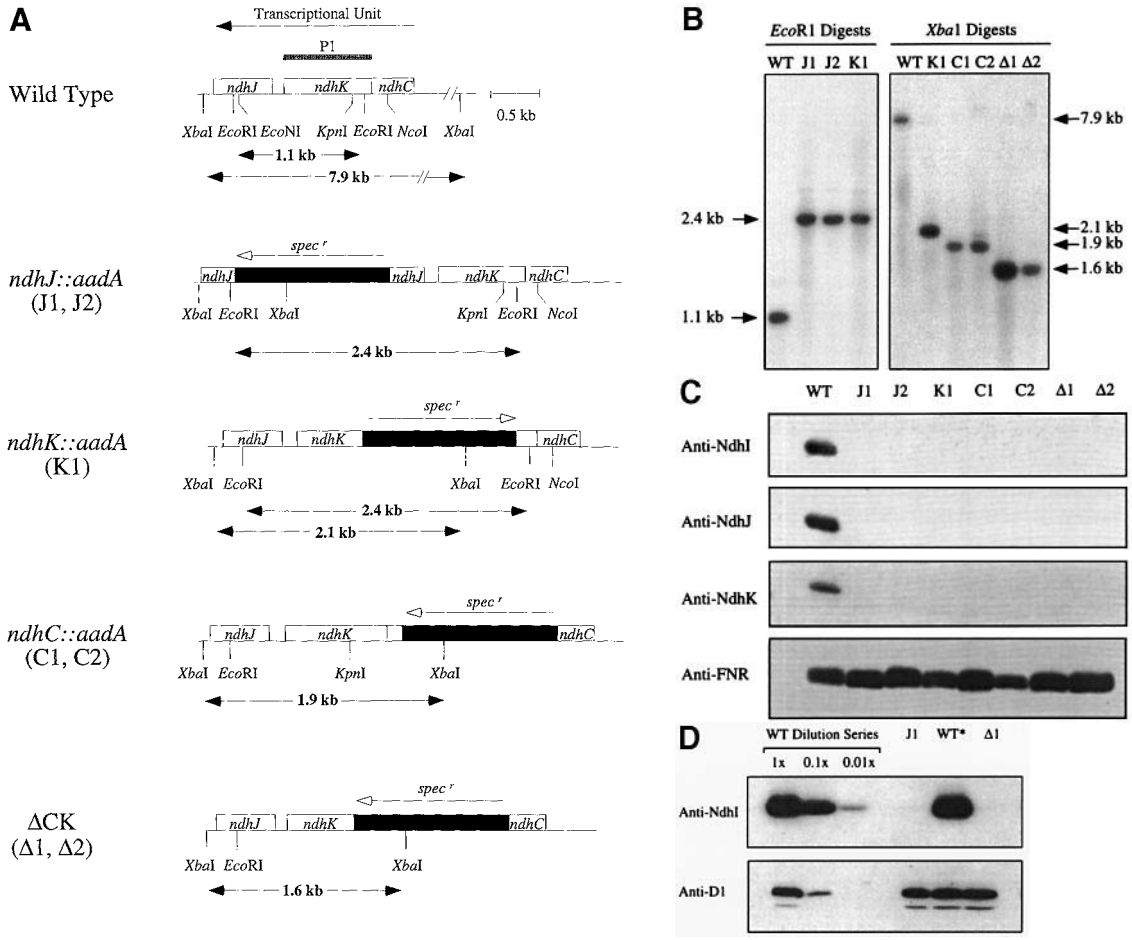

Fig. 3. Construction of plastid *ndh* mutants. (**A**) Map showing the location and orientation of the spectinomycin resistance cassette (spec^r) within the *ndhC*, *ndhK* and *ndhJ* genes (C, K, J mutants; numbers indicate independent transformants). Δ refers to lines in which the spectinomycin resistance cassette replaces part of the *ndhCK* genes. The *Xba*I site in the 1.3 kb spectinomycin resistance cassette (filled box) lies ~0.9 kb from the end containing the promoter (Svab and Maliga, 1993). (**B**) Southern analysis of WT and *ndh* mutants using a DNA probe corresponding to the coding region of NdhK (P1 in A). (**C**) Immunoblot of thylakoid membranes (containing 15 μg of chlorophyll) isolated from WT and *ndh* mutants. (**D**) Sensitivity of the immunodetection of NdhI in the *ndh* mutant, Δ1. Thylakoid membranes from the WT* control, the Δ1 mutant and serial dilutions of WT thylakoids were probed with antibodies specific for NdhI (anti-NdhI) and the D1 polypeptide of PSII (anti-D1). The undiluted samples contained 70 μg of protein.

~10–20 min illumination, fluorescence returns to the F_o level before showing a transient fluorescence increase (or apparent 'F_o rise') over a period of ~1 min consistent with dark reduction of the plastoquinone pool leading to closure of PSII RCs (Groom *et al.*, 1993; Harris and Heber, 1993; Mano *et al.*, 1995) through reverse electron flow. Treatment of the controls with far-red light, which preferentially excites PSI causing oxidation of the plastoquinone pool, leads as expected to a rapid quenching of this 'F_o rise' (Figure 4A, inset). The apparent rise in the F_o level after a light to dark transition therefore monitors dark reduction of the plastoquinone pool by stromal reductant (Mano *et al.*, 1995).

The apparent 'F_o rise' is absent in the *ndh* mutants (Figure 4B), which shows that the Ndh complex facilitates dark reduction of the plastoquinone pool. This conclusion was supported further by analysis of the redox state of the plastoquinone pool (Figure 4C) using the area above the induction fluorescence curve from F_o to F_m as a measure of the size of the oxidized plastoquinone pool (Krause and Weis, 1991). In agreement with the data shown in Figure 4A, the WT showed a transient reduction of the plastoquinone pool (Figure 4C) upon a light to dark transition. In contrast to WT and WT* which showed a more reduced plastoquinone pool 40 s after the actinic light was switched off, the plastoquinone pool in the *ndh* mutant, Δ1, remained oxidized (Figure 4D) and was similar in oxidation state to WT that had been pre-illuminated with far-red light to drive the pool oxidized (Figure 4D). The other *ndh* mutants behaved in a similar fashion to Δ1 (data not shown).

Re-reduction of P700$^+$ is slowed in the ndh mutants

Our results indicate that the chloroplast Ndh complex represents a pathway by which electrons may enter the plastoquinone pool. One possible source of reductant is that produced by PSI in the light so that the Ndh complex may participate in cyclic electron flow around PSI. A widely used indirect assay of cyclic electron transfer around PSI (Maxwell and Biggins, 1976) is to measure the rate of re-reduction of the oxidized primary electron donor in PSI, P700$^+$, in the dark following illumination by far-red light (Maxwell and Biggins, 1976; Bendall and

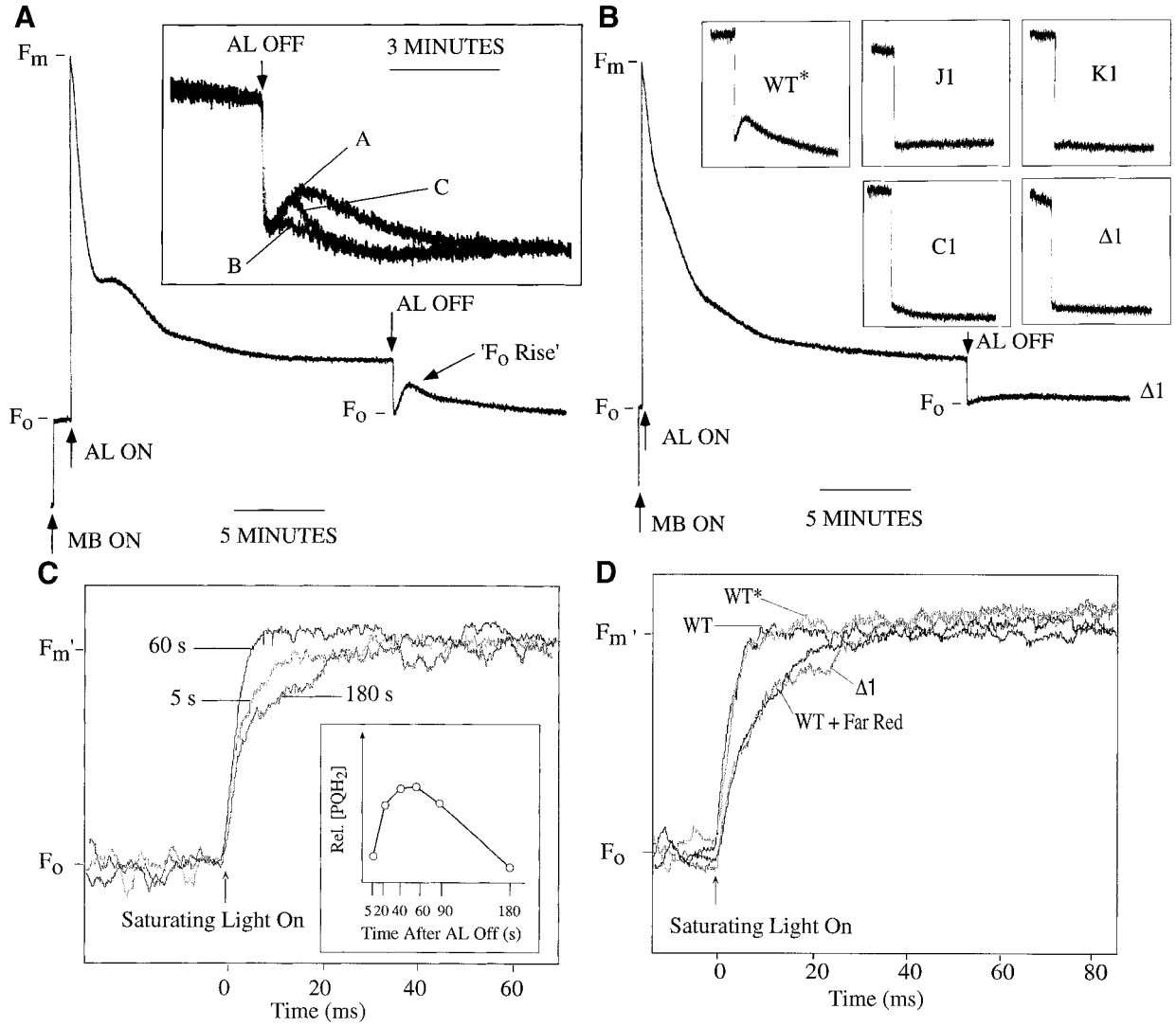

Fig. 4. Chlorophyll fluorescence induction analysis of leaves from WT and *ndh* mutants. (**A**) Fluorescence induction curve of WT tobacco. MB, measuring beam; AL, white actinic light (200 μE/m^2/s); F_m, maximum fluorescence yield; F_o, minimum fluorescence yield when PSII centres are open. Inset shows that the apparent 'F_o rise' observed upon a light to dark transition (trace A) can be quenched with far-red light (709 nm) either 10 s before the actinic light is switched off (trace B) or 30 s after (trace C). (**B**) Fluorescence induction curve of the Δ1 mutant. Insets show fluorescence transients in WT* and *ndh* mutants following light to dark transition. (**C**) Fluorescence induction curves from F_o to F_m' (where F_m' is maximal fluorescence under conditions of non-photochemical quenching) in WT during the course of the 'F_o rise', taken at various times after the actinic light has been switched off. Inset shows the relative concentration of plastoquinol in the pool estimated from the area above the fluorescence curve which is a measure of the concentration of oxidized plastoquinone (Krause and Weis, 1991). (**D**) Fluorescence induction curves from F_o to F_m' obtained 40 s after the actinic light has been switched off using the experimental conditions in (A). Far-red illumination of WT (709 nm) was initiated 10 s prior to switching off the actinic light (to drive the pool oxidized).

Manasse, 1995). Under our conditions of illumination, PSII was not excited significantly, as judged by the lack of a fast component of PSI re-reduction of $t_{1/2}$ ~10 ms (Schreiber *et al.*, 1988; Mi *et al.*, 1992); thus the rate of re-reduction of $P700^+$ in the dark is assumed to be dependent on the rate of cyclic electron flow. In WT tobacco, re-reduction occurs with a $t_{1/2}$ of ~1.3 ± 0.4 s (Figure 5), whereas in the mutants the rate was consistently slower, $t_{1/2}$ ~2.5 ± 0.7 s (mean ± SD, n = 15). It should be noted, however, that a respiratory source of electrons may also contribute to the re-reduction of $P700^+$ via the Ndh complex (see Discussion). That significant rates of re-reduction of $P700^+$ occurred under these conditions in the absence of the Ndh complex is consistent with the presence of other pathways for cyclic electron flow around PSI (Hosler and Yocum, 1985; Ravenel *et al.*, 1994) such as that mediated by reduced ferredoxin possibly catalysed by ferredoxin:plastoquinone oxidoreductase (Bendall and Manasse, 1995) or a cycle involving the PsaE protein of PSI (Yu *et al.*, 1993). The 2-fold slowing in the rate of cyclic electron transfer in the tobacco *ndh* mutants compared with WT is also consistent with studies on the *ndhF* mutant of the cyanobacterium *Synechococcus* sp. PCC 7002 (Yu *et al.*, 1993).

The Ndh complex is important for optimizing the induction of photosynthesis under conditions of water stress

The rate of linear photosynthetic electron flow involving both PSI and PSII is much faster than the rate of PSII-independent electron flow so that it is unlikely that the Ndh complex makes a significant contribution to electron

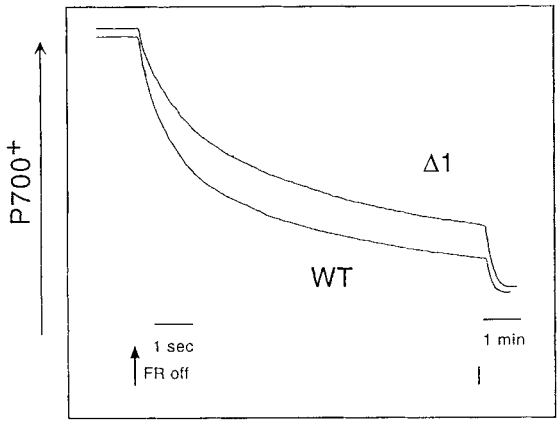

Fig. 5. Dark re-reduction of $P700^+$ in WT and Δ1 following illumination by far-red light, FR.

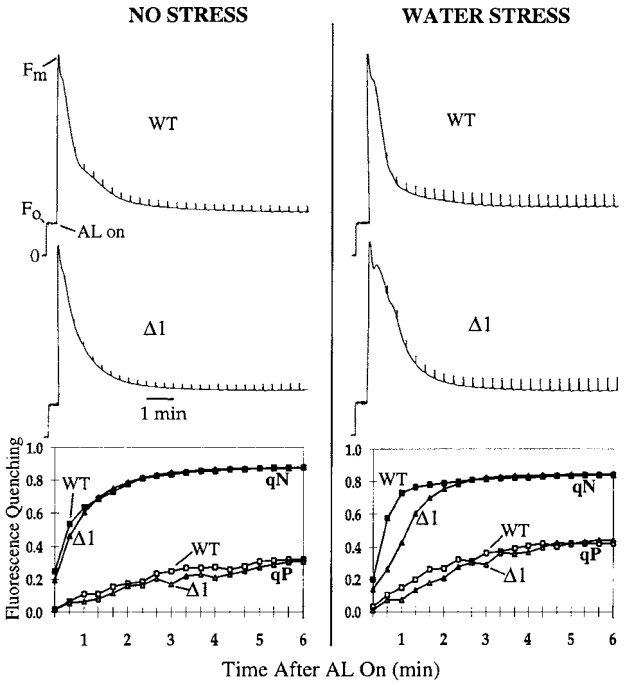

Fig. 6. Effect of water stress on induction of photosynthesis in WT and the *ndh* mutant, Δ1. Fluorescence induction curves obtained using attached leaves of either unstressed (no stress) or water-stressed plants (water stress) were used to determine the photochemical (qP) and non-photochemical (qN) quenching parameters.

flow under normal conditions of steady-state photosynthesis (Maxwell and Biggins, 1976). However, when linear electron flow is sub-optimal, such as upon the induction of photosynthesis after dark adaptation or when CO_2 availability is limited, it is possible that PSII-independent pathways may make a more significant contribution to total electron flow (Bendall and Manasse, 1995). A role for the Ndh complex was therefore examined in the WT and tobacco *ndh* mutants following water stress, a condition that is thought to promote cyclic electron transfer around PSI due to a reduction in the availability of CO_2 because of stomatal closure (Heber and Walker, 1992). The chlorophyll fluorescence assay described in Figure 4 was modified by inclusion of repetitive flashes of saturating light to distinguish between qP and qN quenching (Quick and Stitt, 1989). Changes in qP were variable between plants, with no significant differences on the average observed after stress. However, in comparison with the WT, the *ndh* mutant Δ1 consistently was compromised in its ability to quench fluorescence non-photochemically in the early stage (~2 min) of the induction process (Figure 6). As qN is determined in large part by the transmembrane pH gradient (Quick and Stitt, 1989; Heber and Walker, 1992; Horton *et al.*, 1994), this observation indicates that in the light, under conditions favouring cyclic electron flow, the Ndh complex enhances proton pumping across the thylakoid membrane. Similar results were obtained with the other *ndh* mutants (data not shown).

Discussion

Ndh proteins are part of a large protein complex of low abundance in the thylakoid membrane

The blue native gel shown in Figure 1 indicates that in tobacco chloroplasts the NdhI, J and K proteins are components of a large protein complex (designated Ndh complex) of size ~550 kDa. The sum of the molecular masses of the plastid-encoded *ndh* gene products is ~380 kDa. A 550 kDa complex is therefore of sufficient size to contain all the plastid *ndh* gene products assuming that they are present in stoichiometric amounts. The difference in mass between 380 and 550 kDa also raises the possibility of nuclear-encoded Ndh proteins, although further work is required to establish this fact as well as to confirm that 550 kDa is indeed the size of the intact Ndh complex found in the tobacco thylakoid membrane. An Ndh complex recently has been isolated from pea thylakoid membranes and is also ~550 kDa in size as estimated from gel filtration experiments (Sazanov *et al.*, 1998). The 200 kDa complex identified in Figure 1 also contains NdhIJK and is suggested to be a subcomplex generated by pH-induced fragmentation of the 550 kDa complex. Based on the analogy with *E.coli* complex I, the NdhI, J and K subunits are thought to be located within the same subcomplex connecting the membrane portion (containing NdhA–G) to the proteins involved in oxidation of reductant.

The level of expression of the NdhI protein in the mature tobacco chloroplast is ~0.01% of total thylakoid protein (Figure 2). If one assumes the presence of one copy of NdhI per Ndh complex, then this value represents a stoichiometry of approximately one Ndh complex per 50–100 PSII complexes. Similar results have been obtained for the expression of NdhK in pea (Sazanov *et al.*, 1996).

Chloroplasts contain a functional Ndh complex

The *in vivo* studies reported here provide the first evidence for the presence in higher plant chloroplasts of a functional Ndh complex, which catalyses the reduction of plastoquinone using stromal reductant. The four types of *ndh* mutants described in this work show a similar fluorescence phenotype and all lack the NdhIJK proteins. To what extent the other Ndh proteins assemble in the thylakoid membrane of these mutants awaits further analysis.

The data presented here do not allow us to identify the stromal reductant oxidized by the tobacco Ndh complex. However, a recent analysis of an isolated Ndh complex from pea indicates that, as with other complex I homologues, the reductant used by the complex is NADH

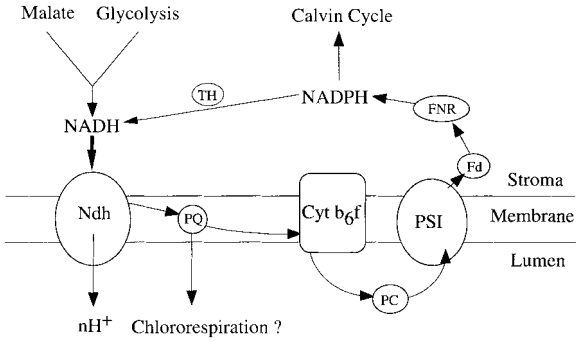

Fig. 7. Scheme showing the possible role of the Ndh complex in cyclic electron flow around PSI in the light and in chlororespiration in the dark. Fd, ferredoxin; PC, plastocyanin; PQ, plastoquinone; TH, putative transhydrogenase; FNR, ferredoxin:$NADP^+$ reductase; nH^+, an unknown number of protons pumped.

(Sazanov *et al.*, 1996, 1998). Because there are no obvious analogues so far in chloroplasts of the complex I subunits involved in the binding and oxidation of NADH, it has been widely speculated that the electron input device for the chloroplast Ndh complex deviates from that found in mitochondria and eubacteria (Friedrich *et al.*, 1995). Indeed, recent results using non-denaturing PAGE were interpreted as evidence in favour of a role for the electron transfer component ferredoxin:$NADP^+$ oxidoreductase in the Ndh complex so that the complex acted as a NADPH dehydrogenase (Guedeney *et al.*, 1996). Our conclusions based on the absence of co-migration of FNR with Ndh proteins in WT samples upon native PAGE and the similarity in migration of FNR in samples of the WT and *ndh* mutants do not support this interpretation (Figure 1). Our results do not, however, exclude the possibility that *in vivo* the Ndh complex may interact with FNR or other proteins such as ferredoxin.

A possible role for the Ndh complex in cyclic electron flow

Mutation of the *ndh* genes in cyanobacteria leads to an inhibition of both cyclic electron flow and respiration (Mi *et al.*, 1992; Yu *et al.*, 1993). Our results suggest that the plastid Ndh complex may have a similar role in higher plants. Our working model for the role of the Ndh complex in chloroplast metabolism is shown in Figure 7 and incorporates recent biochemical data which indicate that NADH is oxidized by the complex (Sazanov *et al.*, 1996, 1998).

In the light, the Ndh complex may reduce plastoquinone using stromal reductant produced indirectly by PSI (in cyclic mode) or from oxidation of metabolites such as malate imported from the cytosol (in respiratory mode). We suggest the possibility that NADH may be generated in the light directly from NADPH through the action of a putative transhydrogenase, possibly FNR, or indirectly through substrate cycles (e.g. involving $NADP^+$- and NAD^+-malate dehydrogenases). Our data do not allow us to determine definitively the source of the reductant oxidized by the Ndh complex (cyclic or respiratory), but the lack of the post-illumination reduction of the plastoquinone pool in the *ndh* mutants suggests that some of the reductant is generated by recent photosynthetic activity.

A role for the Ndh complex in cyclic photophosphorylation is also supported by its location in the stromal lamellae close to PSI (Nixon *et al.*, 1989; Berger *et al.*, 1993; Sazanov *et al.*, 1996) and the finding of elevated levels of Ndh proteins in the bundle sheath cells of C_4 plants which lack PSII and which carry out high levels of cyclic electron flow (Kubicki *et al.*, 1996). Under normal growth conditions, cyclic photophosphorylation is thought to play only a minor role in ATP production in C_3 plants such as tobacco (Bendall and Manasse, 1995), but its rate may increase under adverse environmental conditions. The lack of a dramatic visual phenotype in the *ndh* mutants grown under stress conditions (data not shown) is consistent with the ability of another cyclic pathway to compensate for loss of the Ndh complex (Ravenel *et al.*, 1994) although subtle effects of the *ndh* mutations on growth have yet to be quantified, especially under field conditions.

A role for the Ndh complex under water stress conditions

An interesting feature displayed by the *ndh* mutants was the reduced ability of the plants to quench fluorescence non-photochemically during the induction of photosynthesis under water stress conditions. The major component of non-photochemical quenching, qE, arises from the trans-thylakoid pH gradient and is thought to reflect a mechanism within the thylakoid membrane to quench excitation energy within the pigment bed which would otherwise cause overexcitation of PSII and consequent photoinhibition (reviewed by Horton *et al.*, 1994). The results presented in Figure 6 indicate that Ndh-mediated electron transport may be particularly important for the rate of formation of qE and qN, and hence for the down-regulation of PSII (Heber and Walker, 1992) upon a dark-to-light transition under conditions of water stress. This effect may be of physiological importance when plants are exposed to large fluctuations in light intensity. One interpretation of the data in Figure 6 is that under conditions of water stress and hence reduced availability of CO_2, linear electron flow is slowed and the levels of NADPH and hence stromal reductant increase. In the WT, oxidation of stromal reductant by the Ndh complex, which also pumps protons, is one pathway by which electrons cycle around PSI to generate the proton gradient needed to generate qE. In the *ndh* mutants, other cyclic electron pathways may operate but at reduced proton-pumping efficiency which therefore slows the generation of qE. Ultimately, similar levels of qE and steady-state rates of linear electron flow are achieved in the WT and *ndh* mutants. Because there are apparently multiple pathways by which electrons may cycle around PSI, it should be noted that the apparent rise in F_o after a light-to-dark transition may not always be a diagnostic test of the activity of the Ndh complex.

A possible role for the Ndh complex in the dark

In the dark, oxidation of the plastoquinone pool is probably mediated by oxygen (Diner, 1977; Harris and Heber, 1993). The Ndh complex may therefore function as a component of a respiratory chain (including plastoquinone and a putative oxidase) whose purpose will be to generate ATP in the plastid in the dark using stromal reductant (NADH) produced by, for example, plastidial glycolysis

(Plaxton, 1996). Although a chlororespiratory pathway has long been suggested, particularly from studies of the green alga, *Chlamydomonas reinhardtii* (Bennoun *et al.*, 1982), the presence of a chloroplast oxidase in higher plants has been difficult to prove using classical inhibitors (Garab *et al.*, 1989) because of the complication of the mitochondrial respiratory chain which may be in redox communication with the chloroplast plastoquinone pool (Bennoun, 1994). A role for the Ndh complex outside photosynthetic electron transfer processes is also suggested from the detection of Ndh subunits in non-photosynthetic etioplasts isolated from maize (Berger *et al.*, 1993) and pea (data not shown).

Alternatively, it is possible that the Ndh complex is a relic of an ancestral respiratory electron transfer chain and that the oxidase has been lost during evolution. The Ndh complex may have been retained because of the advantages of possessing a PSII-independent pathway in the light, under conditions where linear electron flow is inhibited, and because of the need to maintain a trans-thylakoid pH gradient in the dark to drive various cellular processes such as protein import into the lumen (Robinson and Klösgen, 1994).

A possible role for the Ndh complex in linking photosynthesis to cellular metabolism

In addition, the Ndh complex allows redox communication between the plastoquinone pool and stromal reductant, which in turn is in redox communication with the rest of the cell through metabolite shuttles (Heber, 1974). Because the redox state of the plastoquinone pool ultimately controls the relative excitation of PSI and PSII through phosphorylation of the light-harvesting chlorophyll complexes by a redox-controlled kinase (Allen *et al.*, 1981), in so-called state transitions, the Ndh complex may play a role in the metabolic control of photosynthesis in higher plants similar to that documented for *C.reinhardtii* (Bulté *et al.*, 1990). Under conditions where enhanced ATP synthesis is required but there is reduced respiratory activity because of, for instance, anaerobiosis, the Ndh complex would facilitate reduction of the plastoquinone pool driving the system into state II in the dark. Cyclic electron flow around PSI upon illumination would be stimulated, leading to enhanced synthesis of chloroplast ATP. This latter scenario also offers an alternative interpretation for the data presented in Figure 6.

In summary, we have shown that the plastid *ndh* genes code for subunits of a functional respiratory complex within the mature chloroplast. The presence in higher plants of a respiratory complex in the same membrane system as the photosynthetic electron transport chain raises a number of interesting possibilities concerning its physiological importance. The tobacco *ndh* mutants described here are potentially an excellent experimental system for future studies of the Ndh complex.

Materials and methods

Production of antisera

The coding regions of the tobacco *ndhI* [from nucleotide 120 612 to nucleotide 120 196 of the chloroplast genome, Shinozaki *et al.* (1986)] *ndhJ* (51 457–50 981) and *ndhK* genes (52 528–51 563) were amplified by PCR and cloned into pET16-b (Novagen Inc., Madison, WI) using the following pairs of oligonucleotide primers: *ndhI* (forward, 5′-GGG CTC GAG ATG CTC CCT ATG ATA ACT-3′; reverse, 5′-GGG GGA TCC TTA TTC ATT TTT TAT TTG-3′), *ndhJ* (forward, 5′-GGG CTC GAG ATG CAG GGT CGT TTG TCT-3′; reverse, 5′-GGG GGA TCC TCA ATG AGC ATC TTG TAT-3′) and *ndhK* (forward, 5′-GGG CAT ATG GTC TTA GCT CCT GAA-3′; reverse, 5′-GGG GGA TCC CTA ATT CAC TAA TTC GGG-3′). For the PCR products containing *ndhI* and *ndhJ*, novel *Xho*I and *Bam*HI sites (underlined bases in oligonucleotide sequence) were engineered into the forward and reverse primers, respectively, to facilitate cloning into pET16-b. For the *ndhK* gene, a novel *Nde*I site was incorporated into the forward primer and a *Bam*HI site into the reverse primer. Recombinant protein containing a 10 amino acid N-terminal histidine tag was expressed in *E.coli* strain BL21 (DE3) as inclusion bodies and purified on a nickel column (His.bind resin, Novagen Inc.) according to the manufacturer's protocol. Antisera were raised against the NdhI and NdhJ fusion proteins in rabbit and, together with a previously characterized rabbit NdhK antiserum (Nixon *et al.*, 1989), were affinity purified using the relevant recombinant protein attached to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions.

Preparation of chloroplast extracts

Tobacco chloroplasts were isolated as in Nixon *et al.* (1989) with modifications to exclude mitochondrial contamination (L.A.Sazanov, unpublished). Tobacco leaves were ground in a Waring blender in STE buffer (0.33 M sorbitol, 50 mM Tris, 2 mM EDTA, pH 7.5), and the homogenate filtered through muslin and cotton wool. Intact chloroplasts were collected by low speed (1500 *g*; 3000 r.p.m. for 3 min in A6.9 Kontron rotor, r_{av} 12 cm) centrifugation of the filtrate. Chloroplasts were washed a further two times with STE buffer, again pelleting at low speed to reduce mitochondrial contamination. Thylakoids were isolated from intact chloroplasts by osmotic shock (10 min on ice) in MNM buffer (20 mM MES, 15 mM NaCl, 5 mM $MgCl_2$, pH 6.0) and spun down either at 35 000 *g* (20 000 r.p.m., JA20 Beckman rotor, r_{av} 9 cm) for 20 min or in a microfuge for low volumes.

Polyacrylamide gel electrophoresis and immunodetection

Immunodetection was performed using an ECL bioluminescence immunoblotting kit (Amersham, UK) according to the manufacturer's instructions except for quantitative immunoblotting experiments (shown in Figure 2), in which case an alkaline phosphatase-conjugated secondary antibody was used (Sigma) according to Shipton and Barber (1991). Immunoreactive bands were quantified from scanned images using the 'NIH Image' public domain software (National Institutes of Health, USA) and a standard curve relating amount of NdhI fusion protein and intensity of cross-reaction. Blue native gel electrophoresis was performed according to Schägger *et al.* (1994) using solubilized extracts obtained from tobacco chloroplasts that had been subjected to 2.5% (w/v) dodecyl maltoside for 10 min on ice at a chlorophyll concentration of 1.125 mg/ml. Unsolubilized material was removed by ultracentrifugation at 4°C (20 min at 100 000 *g*) prior to electrophoresis at pH 7.0 and 4°C. For denaturing electrophoresis in the second dimension, the native gel was incubated at 20°C in 75 mM Tris pH 7.4, 4% SDS for 1 h prior to reduced SDS–12% PAGE according to Nixon *et al.* (1989). Chlorophyll concentration was determined according to Arnon (1949) and protein concentration by a dot-blot Coomassie blue staining assay using bovine serum albumin (BSA) as a standard.

Production and analysis of tobacco plastid ndh mutants

Tobacco plastid mutants were generated in *Nicotiana tabacum* cv. Petit Havana using biolistic technology (Svab and Maliga, 1993). A spectinomycin resistance cassette containing the bacterial *aadA* gene was used as a marker for transformation (Svab and Maliga, 1993). The transforming DNA plasmids are derivatives of plasmid pTB19 which contains *Bam*HI fragments Ba3 and Ba17 of the tobacco plastid genome (Shinozaki *et al.*, 1986), corresponding to a total insert size of ~12.5 kb. Plasmids containing disrupted *ndhC* (pNDHC) and *ndhK* (pNDHK) genes were constructed by blunt-end cloning of the *aadA* cassette into the *Nco*I and *Kpn*I sites, respectively, of pTB19. Plasmid pNDHJ containing a disrupted *ndhJ* gene was constructed by insertion, after blunt-ending, of the *aadA* cassette into the *Eco*NI site present in plasmid pNDH2, which is a derivative of pBluescript (Stratagene Ltd, UK) containing an ~10.1 kb *Pvu*II fragment isolated from pTB19. Partial deletion of the *ndhC* and *ndhK* genes (to give rise to the Δ strains) was achieved by replacing the 0.29 kb *Kpn*I–*Nco*I fragment within pTB19 with the *aadA* cassette to yield plasmid pΔ (Figure 3A). With plasmids pNDHC, pNDHJ, pNDHK and pΔ, 29, 28, 24 and 25 tobacco leaves,

respectively, were bombarded. In the bombarded cultures, 23, 40, seven and 15 spectinomycin-resistant shoots were obtained; of these, streptomycin resistance was displayed by 10 (pNDHC transformation), 14 (pNDHJ), two (pNDHK) and six (pΔ) lines. DNA isolation and Southern analysis were performed according to Svab and Maliga (1993) to verify that a uniform population of transformed plastid genome copies was attained in the mutant lines that were analysed further. Tobacco plants were grown in peat compost at 25°C illuminated by halogen lamps (30 μE/m^2/s) under a 17 h–7 h day–night cycle. The WT* control plant contains the spectinomycin resistance (*aadA*) cassette within the *rbcL–accD* (ORF512) intergenic region of the plastid genome (Svab and Maliga, 1993).

Measurement of chlorophyll fluorescence, the redox state of plastoquinone and P700 in leaves

Chlorophyll fluorescence was used to monitor the reduction state of plastoquinone using a PAM chlorophyll Fluorometer (Walz, Effeltrich, Germany) equipped with an emitter-detector unit 101 ED (Schreiber *et al.*, 1986). A modulated non-actinic 1.6 kHz measuring beam was used to measure fluorescence. Detached leaves from 5- to 8-week-old plants were analysed. For analysis of the apparent 'F_o rise', leaves were illuminated with actinic light of intensity between 130 and 200 μE/m^2/s for a period of ~10–20 min before switching off the actinic light. Far-red light of 709 nm and intensity 15 W/m^2 was used preferentially to excite PSI. Maximal fluorescence (F_m) was measured during a 2 s pulse of white light of intensity 1300 μE/m^2/s. Photochemical and non-photochemical quenching of fluorescence in plants exposed to an actinic light intensity of 500 μE/m^2/s was determined according to Quick and Stitt (1989) using flashes of white light (1400 μE/m^2/s, 300 ms) spaced every 20 s. The oxidation state of the plastoquinone pool as determined from the area above the fluorescence induction curve (Bennoun, 1982; Krause and Weis, 1991) was estimated using Origin 4.1 software (Microcal Software Inc., USA). Oxidation of P700 and its re-reduction were monitored by absorbance changes at 803 nm measured using an ED 800T unit attached to the PAM fluorometer (Schreiber *et al.*, 1988). Excitation was provided by a 2 min period of far-red light (5 W/m^2) using a 730 nm interference filter.

Water stress experiments

Tobacco plants were watered daily (no stress) and were stressed by withholding water for 3 days. The stress conditions were mild and did not lead to a measurable decline in either water content of the leaves (~90% before and after stress) or the fluorescence values F_v and F_m.

Acknowledgements

We thank Professor Süss (Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany) for the gift of the anti-FNR antiserum, Professor Masahiro Sugiura (Nagoya University, Japan) for the gift of plasmid pTB19 and Professor Peter Horton (University of Sheffield) for the loan of his ED-800T unit. P.B. is a recipient of a BBSRC studentship and the work was supported by grants from the BBSRC, The Royal Society (to P.J.N.) and The National Science Foundation (to P.M.).

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Received October 8, 1997; revised November 26, 1997; accepted December 1, 1997