Obtention et caractérisation de transformants chloroplastiques dépourvus de complexe Ndh: implication du complexe dans la réduction non photochimique du pool de PQ.

Le séquençage du génome chloroplastique de différentes plantes supérieures (Ohyama et al., 1986 ; Shinozaki et al., 1986 ; Hiratsuka et al., 1989) a révélé l'existence de onze gènes (gènes *ndh*) homologues aux gènes codant pour des sous-unités des complexe I mitochondriaux et bactériens (Friedrich et al., 1995). La démonstration de la fonctionnalité des gènes *ndh* par la mise en évidence des ARNm et polypeptides correspondants, a rapidement conduit à suggérer l'existence d'un complexe analogue au complexe I dans le chloroplaste.

Afin d'établir le rôle du complexe Ndh chloroplastique, nous avons adopté une approche visant à inactiver la biosynthèse d'une des sous-unités codées par un des gènes *ndh* porté par le plastome. Cette démarche expérimentale procède de la transformation chloroplastique, une technique relativement récente chez les végétaux supérieurs, et mise au point chez *Nicotiana tabacum* (Svab et al., 1990). Deux techniques de transformation ont été utilisées : celle de « biolistique », où des tissus foliaires sont bombardés avec des billes de tungstène sur lesquelles est fixé l'ADN vecteur (Svab et Maliga, 1990) et celle dite « au PEG (polyethylène glycol) » où des protoplastes en présence de l'ADN sont traités par une solution de PEG modifiant les membranes et favorisant l'entrée de l'ADN dans le chloroplaste (O'Neill, 1993). La transformation chloroplastique procédant de la recombinaison homologue, on utilise un plasmide porteur du gène d'intérêt et contenant de part et d'autre des régions flanquantes homologues à celles de la zone d'intégration. La sélection des plantes transplastomiques

s'effectue à l'aide de marqueurs de résistance, la pression de sélection permettant d'atteindre un état d'homoplasmie où toutes les copies du génome chloroplastique de l'ensemble des chloroplastes (de l'ensemble des cellules) sont recombinantes. Deux marqueurs de sélection sont couramment utilisés. Il s'agit soit du gène *aad*A dont le produit, l'aminoglycoside adényl transférase, détoxique la spectinomycine ou la streptomycine (Svab et Maliga, 1993), soit du gène *16SrDNA* présentant une mutation ponctuelle qui rend son produit l'ARN ribosomique 16S, insensible à la spectinomycine et/ou à la streptomycine (O'Neill et al.,1993). Le marqueur *aad*A est dit "dominant", et les transformants sélectionnés doivent subir plusieurs cycles différenciation/dédifférenciation (régénération) sur le milieu de sélection avant d'atteindre l'homoplasmie (O'Neill et al.,1993). Le marqueur alternatif *16SrDNA* est considéré comme un marqueur « récessif » et son utilisation permet d'atteindre immédiatement l'homoplasmie (O'Neill et al., 1993); cet avantage expérimental est limité car l'emploi de ce marqueur ne pourra être envisagé que pour des modifications affectant la zone proximale du gène *16SrDNA* sur le plastome. En collaboration avec le laboratoire de Peter Medgyesy (Biological Research Center, Szeged, Hongrie), qui avait mis au point cette technique de transformation, nous avons choisi d'inactiver le gène *ndh*B, qui n'est distant que de 3,6 kb du gène *16SrDNA*. Une construction contenant une partie du gène *ndh*B modifié par l'addition d'une paire de bases dans sa séquence provocant ainsi un décalage du cadre de lecture et engendrant plusieurs codons stop dès le début de la séquence et le gène *16SrDNA* portant la mutation conférant la résistance à la spectinomycine a été réalisée. Cette construction a été introduite dans des protoplastes par traitement au PEG. Après sélection, des transformants homoplasmiques ont été obtenus. La caractérisation biochimique et moléculaire des mutants (voir l'article joint Horvath et al. (2000) Plant Physiology, 123 : 1337-1349) a permis de montrer que le complexe Ndh est absent des membranes thylacoïdiennes. D'autre part, des mesures de fluorescence de la chlorophylle (voir l'article joint Cournac et al. (1998) G. Garab (ed.), Photosynthesis : Mechanisms and effects, Vol III, 1877-1882) ont permis de démontrer que le complexe Ndh, fonctionnel *in vivo*, est directement impliqué dans la réduction non photochimique du pool de PQ. Les plants de tabac mutants présentent des caractéristiques de croissance et des capacités photosynthétiques similaires aux plantes de type sauvage dans la plupart des conditions testées. Par contre, dans des conditions de stress hydrique modéré, un écart de croissance est observé entre les plantes sauvages et les plantes témoins. Nous avons fait l'hypothèse que cette différence phénotypique était observé dans des conditions où la disponibilité interne en CO_2 est faible et où une forte activité photorespiratoire augmente la demande métabolique en ATP. Le complexe Ndh serait

impliqué dans un mécanisme, tel que le transfert cyclique des électrons, capable de fournir de l'ATP supplémentaire.

Parallèlement à nos expériences, plusieurs laboratoires ont utilisé les techniques de transformation du génome chloroplastique en vue d'obtenir des plantes transplastomiques dont le complexe Ndh ne serait pas fonctionnel. Ainsi, Kofer et al. (1998) ont procédé à l'inactivation individuelle des gènes *ndh A,C,H,I,,J,K* appartenant à deux opérons polycistroniques contenant d'une part *ndh C,K,J* et d'autre part *ndh H,A,I,G,E, psaC, ndh D.* En utilisant la cassette de sélection aadA, ces auteurs ne sont pas parvenus à atteindre l'homoplasmie et ont conclu que le complexe Ndh était indispensable à la survie des plantes. Burrows et al. (1998) ont également procédé à l'inactivation individuelle de chaque gène de l'opéron *ndh C,K,J.* En utilisant la même cassette de sélection aadA, ces auteurs ont obenus l'homoplasmie et des mutants dont le complexe Ndh n'était pas fonctionnel. Ces auteurs concluent que le complexe Ndh n'est pas indispensable à la survie des plantes et proposent que Kofer et al. (1998) ont également atteint l'homoplasmie sans s'en rendre compte à cause de contaminations par des pseudogènes *ndh* nucléaires lors de l'analyse Southern (Maliga et Nixon, 1998). Enfin, Shikanai et al. (1998) ont inactivé le gène *ndhB* en utilisant la cassette de sélection aadA. Ces auteurs ont atteints l'homoplasmie et ont conclu, comme les autres équipes et la notre, à l'implication du complexe dans les mécanismes de réduction non photochimique du pool de PQ.

A. Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure

Horvath et al. (2000) *Plant Physiology* **123**, 1337-1349.

Targeted Inactivation of the Plastid *ndhB* Gene in Tobacco Results in an Enhanced Sensitivity of Photosynthesis to Moderate Stomatal Closure[1]

Eva M. Horváth, Stefan O. Peter[2], Thierry Joët, Dominique Rumeau, Laurent Cournac, Gabor V. Horváth, Tony A. Kavanagh, Christian Schäfer[3], Gilles Peltier, and Peter Medgyesy*

Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H–6701 Szeged, Hungary (E.M.H., G.V.H., P.M.); Botanisches Institut, Universität Bayreuth, D–95440 Bayreuth, Germany (S.O.P., C.S.); Commissariat à l'Energie Atomique Cadarache, Département d'Ecophysiologie Végétale et Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, F–13108 Saint-Paul-lez-Durance, France (T.J., D.R., L.C., G.P.); and Department of Genetics, Trinity College, University of Dublin, Dublin 2, Ireland (T.A.K.)

The *ndh* genes encoding for the subunits of NAD(P)H dehydrogenase complex represent the largest family of plastid genes without a clearly defined function. Tobacco (*Nicotiana tabacum*) plastid transformants were produced in which the *ndhB* gene was inactivated by replacing it with a mutant version possessing translational stops in the coding region. Western-blot analysis indicated that no functional NAD(P)H dehydrogenase complex can be assembled in the plastid transformants. Chlorophyll fluorescence measurements showed that dark reduction of the plastoquinone pool by stromal reductants was impaired in *ndhB*-inactivated plants. Both the phenotype and photosynthetic performance of the plastid transformants was completely normal under favorable conditions. However, an enhanced growth retardation of *ndhB*-inactivated plants was revealed under humidity stress conditions causing a moderate decline in photosynthesis via stomatal closure. This distinctive phenotype was mimicked under normal humidity by spraying plants with abscisic acid. Measurements of CO_2 fixation demonstrated an enhanced decline in photosynthesis in the mutant plants under humidity stress, which could be restored to wild-type levels by elevating the external CO_2 concentration. These results suggest that the plastid NAD(P)H:plastoquinone oxidoreductase in tobacco performs a significant physiological role by facilitating photosynthesis at moderate CO_2 limitation.

Comparative analyses of the completely sequenced plastid genomes of such taxonomically distant plant species as liverwort (Ohyama et al., 1986), tobacco (*Nicotiana tabacum*; Shinozaki et al., 1986), and rice (Hiratsuka et al., 1989) has revealed a set of genes showing a surprising homology to subunits of the mitochondrial NADH dehydrogenase complex. This set of *ndh* genes proved to contain at least 11 members (Fearnley et al., 1989; Videira et al., 1990; Dupuis et al., 1991; Masui et al., 1991; Pilkington et al., 1991; Arizmendi et al., 1992), which are represented in all vascular plant divisions (Meng et al., 1986; Maier et al., 1995; Neyland and Urbatsch, 1996). The deduced amino acid sequence of the subunits of this plastid NAD(P)H dehydrogenase (NDH) complex shows significant homology with that of the corresponding subunits of the bacterial proton-pumping NADH: ubiquinone oxidoreductase and with the appropriate subunits of mammalian, fungal, and plant mitochondrial complex I (Fearnley and Walker, 1992; Weidner et al., 1993; Rasmusson et al., 1998). Subunits forming the highly conserved NADH-binding unit of complex I are apparently absent in the plastid NDH complex (Friedrich et al., 1995). However, this module might correspond to the additional, still uncharacterized (and presumably nuclear-encoded) subunits detected in the plastid NDH complex by biochemical methods (Quiles and Cuello, 1998; Sazanov et al., 1998b).

Plastid *ndh* genes are transcribed (Matsubayashi et al., 1987; Kanno and Hirai, 1993), the mRNAs are edited (Freyer et al., 1995; Maier et al., 1995), and the protein products of these genes are located in the stromal thylakoid membranes (Nixon et al., 1989; Berger et al., 1993; Kubicki et al., 1996). Expression of the various genes under different developmental and environmental conditions has been studied primarily in monocotyledonous plants (Kubicki et al., 1996; Martín et al., 1996; Catalá et al., 1997; Fischer et al., 1997). On the basis of western-blot analyses these investigators suggested that NDH proteins are primarily expressed in tissues of limited photosynthetic capacity. However, the expres-

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[2] Present address: Institute of Plant Sciences, Eidgenössische Technische Hochschule Center LFW C33, Universitätstrasse 2, CH–8092 Zurich, Switzerland.

[3] Present address: Molekulare Pflanzenphysiologie, Fachbereich 2: Biologie/Chemie, Universität Bremen, Postfach 33 04 40, D–28334 Bremen, Germany.

* Corresponding author; e-mail pmedgyesy@matavnet.hu; fax 36–62–433434.

sion of the *ndhD* gene in tobacco, measured as the extent of RNA editing creating the start codon, was restricted to chloroplasts and was highest in young, illuminated, photosynthetically active leaves (Hirose and Sugiura, 1997). Light activation of the thylakoidal NDH activity has also been recently demonstrated (Teicher and Scheller, 1998).

The longstanding question of the function of this putative NAD(P)H:plastoquinone (PQ) oxidoreductase in plastids has recently been tackled by targeted inactivation of several of the plastid-encoded *ndh* genes (*ndhA*, *B*, *C*, *H*, *I*, *J*, and *K*) in tobacco (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). A common feature of these NDH-inactivated plastid transformant plants is the absence of a transient increase in postillumination chlorophyll fluorescence, the presence of which is interpreted as a dark reduction of the PQ pool (Groom et al., 1993; Feild et al., 1998). On the basis of these results it was concluded that the NDH complex is functional in tobacco chloroplasts, mediating donation of electrons from a stromal reductant to the PQ pool in the dark (Burrows et al., 1998; Endo et al., 1998; Kofer et al., 1998; Sazanov et al., 1998a; Shikanai et al., 1998). These plastid transformant plants showed the normal characteristics of steady-state photosynthesis and, although water stress seemed to delay non-photochemical fluorescence quenching during induction of photosynthesis in certain mutants (Burrows et al., 1998), no NDH-specific phenotype was observed under normal or various stress conditions (Burrows et al., 1998; Sazanov et al., 1998a; Shikanai et al., 1998). In one laboratory the primary regenerants showed various abnormalities but neither linkage with the inactivated *ndh* genes nor cytoplasmic inheritance of these traits was demonstrated (Kofer et al., 1998). Therefore, the role of the NDH complex in the light reactions of photosynthesis and its physiological role in higher plants has remained hypothetical, and the various controversial conclusions are a matter of extensive discussion (Koop et al., 1998; Maliga and Nixon, 1998; Nixon and Maliga 1999; Roldán, 1999).

In the present study we have produced tobacco plastid transformants in which the *ndhB* gene was translationally inactivated. Preliminary data obtained on chlorophyll fluorescence transients under illumination showed marked differences between *ndhB*-inactivated and wild-type plants under anaerobic conditions (Cournac et al., 1998; Joët et al., 1998). Whereas this observation demonstrated the functioning of NDH complex during photosynthesis, since it was based on anaerobic conditions lacking both CO_2 and O_2, it was difficult to predict what natural physiological conditions might reveal a role for NDH. We show that under conditions that do not block but moderately inhibit photosynthesis by CO_2 limitation, the lack of NDH activity results in an enhanced growth retardation of *ndhB*-inactivated tobacco plants in comparison with the wild type.

RESULTS

Targeted Inactivation of the Plastid *ndhB* Gene in Tobacco

The *ndhB* is the only gene of the plastid *ndh* family located in the inverted repeat (IR_A and IR_B) region of the tobacco plastid genome (Shinozaki et al., 1986) and is most probably a part of the *rps12*(3′)-*rps7-ndhB-trnL* transcription unit (Matsubayashi et al., 1987; Kanno and Hirai, 1993). Inactivation of *ndhB* was accomplished by creating translational stop codons in the coding region of the gene. The pSSH1 plastid transformation plasmid contains a nightshade (*Solanum nigrum* L.) plastid DNA fragment and possesses mutations conferring spectinomycin and streptomycin insensitivity (Kavanagh et al., 1994, 1999). In the 7.8-kb inverted repeat region covered by the insert there is a 2.4% nucleotide sequence divergence between tobacco and nightshade plastid DNA (Kavanagh et al., 1999). The insert spans the first 732 nucleotides of *ndhB* (Wakasugi et al., 1998), which are identical in tobacco and nightshade. A single C was introduced into codon 206 of *ndhB* by oligonucleotide-directed mutagenesis of the plasmid in a region of the gene showing no editing site in tobacco (Freyer et al., 1995). This additional nucleotide generated a *Sma*I site and, in addition to a frame shift, stop codons (Fig. 1).

The mutant plasmid (pSSH1M) was introduced into tobacco protoplasts by polyethylene glycol treatment (O'Neill et al., 1993). Putative plastid transformant colonies were selected on the basis of their green color in a medium containing spectinomycin. The distinction between transformed and non-transformed tobacco plastids was facilitated, in addition to their insensitivity to streptomycin, by diagnostic RFLP differences in respect of the restriction enzymes *Sma*I, *Bam*HI, *Xho*I,

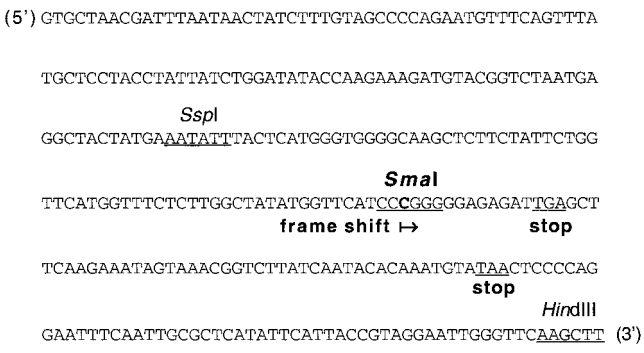

Figure 1. Translational inactivation of the *ndhB* gene in the pSSH1 plastid transformation plasmid. The pSSH1 plasmid insert spans the first 732 bp of the *ndhB* gene. An additional C-G bp was introduced into codon 206 of *ndhB* by oligonucleotide-directed mutagenesis. The resulting plasmid was called pSSH1M. This additional nucleotide generated a diagnostic *Sma*I site and, in addition to a frame shift, all three stop codons (only two of which are shown). A 300-bp portion of the *ndhB* coding region (identical in tobacco and nightshade) adjacent to the *Hind*III cloning site is shown as it appears in IR_A (5′–3′ direction, strand A), from position 143,798 in the tobacco plastid genome. Selected restriction enzyme sites are also shown.

and *Aat*II (Kavanagh et al., 1999). The selected lines comprised only 20% spontaneous spectinomycin-resistant mutants. On average one plastid transformant callus was selected in 10^4 viable colonies (or in 10^5 protoplasts treated). Each primary regenerant and its seed progeny was completely homoplasmic for the resistance markers. The polymorphic DNA regions (revealed as RFLPs between the donor and the recipient plastid DNA) were shown to be homoplasmic in all but one of the transformants demonstrating complete intraorganellar plastid DNA segregation after transformation (for representative *Sma*I patterns, see Fig. 3). A compilation of the RFLP and genetic markers in all of the 18 transformants revealed a high-frequency co-integration of the non-selected markers (Fig. 2). Nevertheless, the integration of the homeologous nightshade plastid DNA was mediated by multiple recombination events (Fig. 2). A schematic interpretation of the postulated recombination events following transformation of tobacco plastids with the pSSH1M plasmid in the individual transformants has been published elsewhere (Kavanagh et al., 1999). Because of the remarkably high recombination frequency in the 113-bp homologous peripheral region located between the *Sma*I site and the pUC19 vector (Fig. 2), one-half of the plastid transformants possessed the mutated (and putatively inactivated) *ndhB* gene.

Molecular and Biochemical Analysis of the Inactivation of the NDH Complex

Homoplasmy of selected transformants for the mutation inactivating the *ndhB* gene was also verified by Southern hybridization and PCR analysis. Probing of *Sma*I-digested plastid DNA by a plastid DNA probe covering the diagnostic *Sma*I site revealed both the site-specificity and homoplasmy of the introduced mutation (Fig. 3). This was further verified by *Sma*I digestion of PCR products generated using isolated plastid DNA as template and primers flanking the diagnostic *Sma*I site on the plastid genome (Fig. 4). In the subsequent investigations, unless stated otherwise, both wild-type tobacco and a double-resistant tobacco plastid transformant (possessing the full nightshade insert of the original pSSH1 plasmid) were used as controls. Furthermore, in most of the investigations two types of *ndhB*-inactivated transformant were used: number 1.2 was resistant to both streptomycin and spectinomycin, whereas number 3.3 contained only the spectinomycin-insensitivity mutation.

The fate of the NDH complex in the *ndhB*-inactivated plants was investigated by protein analysis. Fractioned chloroplast protein extracts were analyzed by PAGE and Coomassie Blue staining. No obvious difference was observed between the protein patterns of control (noninactivated transformant) and *ndhB*$^-$ plants (Fig. 5). As has been previously demonstrated (Burrows et al., 1998; Sazanov et al., 1998b) the NDH complex has a very low abundance in the thylakoid membrane. Attempts to produce a recombinant NDH-B polypeptide were not successful probably due to the high overall hydrophobicity of the NDH-B protein (Fearnley and Walker, 1992). Therefore, antibodies raised against NDH-H were used for western-blot analysis of chloroplast proteins. Immunostaining with the NDH-H antiserum confirmed the localization of NDH pro-

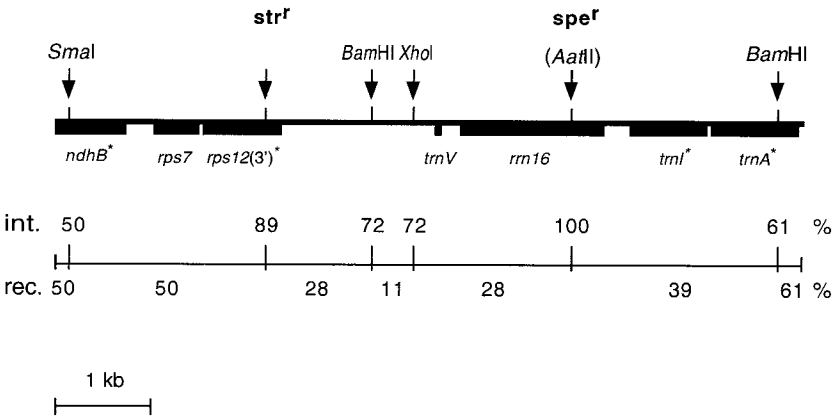

Figure 2. Distribution of co-integration and recombination frequencies in the targeted region following transformation of tobacco plastids with the pSSH1M plasmid. The 7.8-kb donor insert of the pSSH1M plasmid is shown at the top of the figure. Arrows mark the location of the specific resistance and RFLP sites scored (brackets indicate the absence of the wild-type restriction enzyme site). Asterisks mark intron-containing genes. At the lower part of the figure the line is sectioned to show the major intervals between the donor-type marker sites investigated in the plastid transformants. The frequency of co-integration (int.) of the individual non-selected markers with the selected spectinomycin resistance locus is shown above the line. A 100% value represents the total number of spectinomycin-resistant transformants possessing a donor marker. The observed recombination frequency (rec.) in the individual internal sections, calculated as a percentage of the transformants recombined in the particular interval, is shown below the line. A 100% value represents the total number of transformants possessing a recombination event.

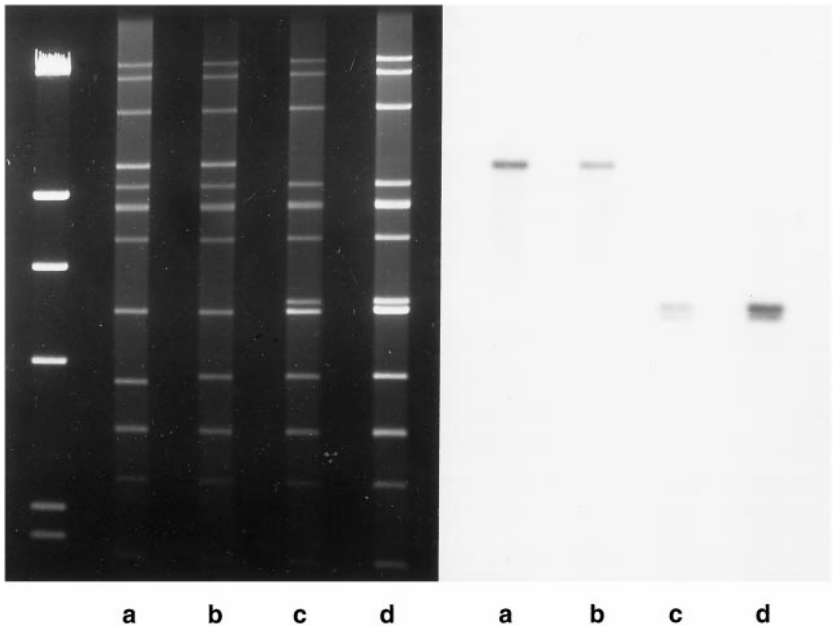

Figure 3. Site-specific inactivation of the *ndhB* gene in the plastid transformants. Gel electrophoresis of *Sma*I-digested plastid DNA of wild-type tobacco (a) and several plastid transformants (b–d) distinguishes a noninactivated transformant (b) from those possessing the inactivated *ndhB* gene (c–d). The smaller of the new, inactivation-specific fragments (5.68 and 5.45 kb) comigrates with the unchanged fragment number 9. On the left a *Hin*dIII digest of λ DNA is also shown (fragment sizes: 23.13, 9.42, 6.56, 4.36, 2.32, and 2.03 kb). Southern hybridization with a plastid DNA probe spanning the region containing the diagnostic restriction site in the 11.13-kb *Sma*I fragment number 4 reveals both the site-specificity and homoplasmy of the introduced mutation.

teins in the stroma lamellae of thylakoid membranes (Nixon et al., 1989; Berger et al., 1993; Kubicki et al., 1996) in control plants (Fig. 5). However, the western-blot analysis detected no NDH-H protein in the *ndhB*$^-$ plastid transformant investigated (Fig. 5), indicating that no functional NDH complex can be assembled. This conclusion is supported by similar observations in other tobacco plastid transformants where the inactivation of one of the *ndh* genes resulted in disappearance of the other NDH subunits investigated, even if they were encoded by separate transcription units (Burrows et al., 1998; Kofer et al., 1998).

Growth and Photosynthetic Performance of the NDH-Inactivated Transformants under Normal Conditions

Plastid transformants with a wild-type or an inactivated *ndhB* gene showed no visible NDH-specific phenotype under standard growth conditions either when cultured in vitro (autotrophically or on sugar-containing medium) or grown in soil in the greenhouse. Several of the primary regenerants showed morphological abnormalities (e.g. slow growth, distorted leaves, and poor pollen production) typical of chromosomal aneuploidy detected regularly in a certain percentage of tobacco plants regenerated from cell culture (Thanh et al., 1988). These morphological deviations did not show maternal inheritance and were not observed in the progeny obtained after pollination with wild-type tobacco. Transformant plants derived from three crosses with wild-type tobacco were analyzed in detail. No developmental deviation was observed in *ndhB*$^-$ plants compared with wild type from seed germination to seed set, including aging.

Measurements of photosynthetic CO_2 fixation or O_2 evolution in plants grown either in vitro or in soil showed no significant difference in steady-state photosynthetic rates between controls and *ndhB*$^-$ mutants. The in vivo light dependence curves of photosynthetic oxygen evolution were similar in control and *ndhB*$^-$ plants, indicating that neither the maximum efficiency nor the light-saturated capacity were affected in intact leaves under normal conditions (data not shown). Chlorophyll fluorescence measurements performed during dark-light-dark transitions indicated no difference in photochemical and non-photochemical quenching processes between control and *ndhB*$^-$ plants (Cournac et al., 1998). In these fluorescence measurements, following light extinction, a transitory and slow increase in the fluorescence level, before reaching the F_0 level, was observed in control plants. This phenomenon, generally ascribed to the dark reduction of the PQ pool by stromal reductants like NADPH or NADH (Groom et al., 1993), was not observed in *ndhB*$^-$ transformants (Cournac et al., 1998), indicating that stromal reductants in the dark do not reduce the PQ pool in the absence of the *ndhB* gene product. This impaired non-photochemical PQ reduction is apparently a standard feature of *ndh* gene-inactivated mutants (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) and is the primary basis for the conclusion that the NDH complex is functional in chloroplasts (see the introduction). However the absence of any obvious phenotype in our *ndhB*$^-$ mutants, as was reported for other *ndhB*$^-$ mutants (Shikanai et al., 1998), supports the conclusion that under favorable growth conditions NDH function is dispensable. All these data prompted an extensive

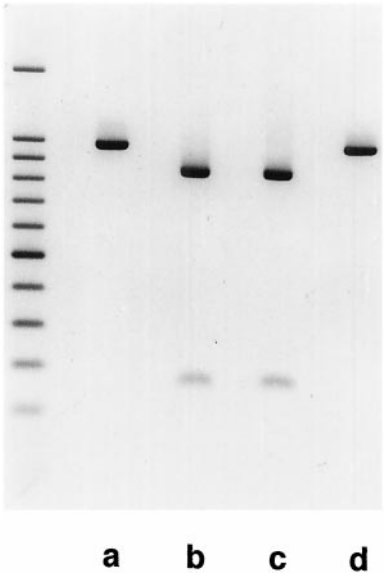

Figure 4. Homoplasmy of the plastid DNA population in the *ndhB*-inactivated transformants. Gel electrophoresis of *Sma*I-digested PCR product of wild-type tobacco (a), two *ndhB*-inactivated transformants (b and c), and a noninactivated transformant (d). The priming sites were located to cover the 5′ end of *ndhB* and flank the diagnostic *Sma*I site. The primer located inside the *ndhB* gene is outside the targeted plastid DNA region. The primers amplify a product of 966 bp, which is cut into 814- and 152-bp fragments by *Sma*I if the mutation introduced into *ndhB* is present. On the left a 100-bp DNA ladder is also shown (fragment sizes: 1,500 and 1,000–100 bp). The complete and correct cleavage of the PCR product in the *ndhB*-inactivated transformants reveals both the homoplasmy and site-specificity of the introduced mutation.

search for potential NDH function-specific stress conditions.

A Decrease in Air Humidity Generates a Discriminating Phenotype in NDH-Inactivated Transformants

We have investigated the effect of various stress conditions that have been proposed to stimulate cyclic electron transport activity (Heber and Walker, 1992; Fork and Herbert, 1993). The effect of a moderately long light stress (5 h, 700 μmol m^{-2} s^{-1}) on light dependence curves of photosynthetic O_2 evolution were measured in in vitro-grown *ndhB*-inactivated and noninactivated transformants. Neither the maximum efficiency nor the light-saturated capacity of photosynthetic O_2 evolution were differentially affected in intact leaves (data not shown). The effect of light stress on soil-grown plants was investigated in growth chambers. No significant difference was observed in photosynthesis performance (measured as whole-plant CO_2 fixation) of *ndhB*-inactivated and noninactivated transformants grown under an illumination of 1,200 μmol m^{-2} s^{-1} (16-h day) for 2 weeks (data not shown).

The effect of water stress (by severely limiting or withholding water) was also investigated on control and *ndhB*$^-$ plants grown under normal phytotron conditions. Under such stress conditions both types of plants showed a similarly strong (gradual or immediate) inhibition of vegetative development, in addition to a similar degree of wilting, yellowing, and withering of the older leaves. However, natural drought typically involves not only a soil water deficiency but also a dry atmosphere. Therefore, in another experiment we investigated the effect of low air humidity (30 relative %) on well-watered plants in a growth chamber. A few days of growth under these conditions surprisingly resulted in a remarkable growth difference between wild-type and *ndhB*$^-$ plants (for a representative pair of plants, see Fig. 6). After a 5-d-long growth period in low air humidity both fresh and dry weights of *ndhB*$^-$ plants (19.04 $\pm$ 2.05 and 1.38 $\pm$ 0.10 g, respectively) were almost 20% lower than those of wild-type plants (22.83 $\pm$ 1.16 and 1.70 $\pm$ 0.03 g, respectively). The enhanced growth retardation of *ndhB*$^-$ plants was visible primarily as a reduced growth of the young, expanding leaves (Fig. 6). No other phenotypic difference between the mutant and wild-type plants was detected in this experiment. The increased sensitivity to air humidity of mutants with a non-functional NDH

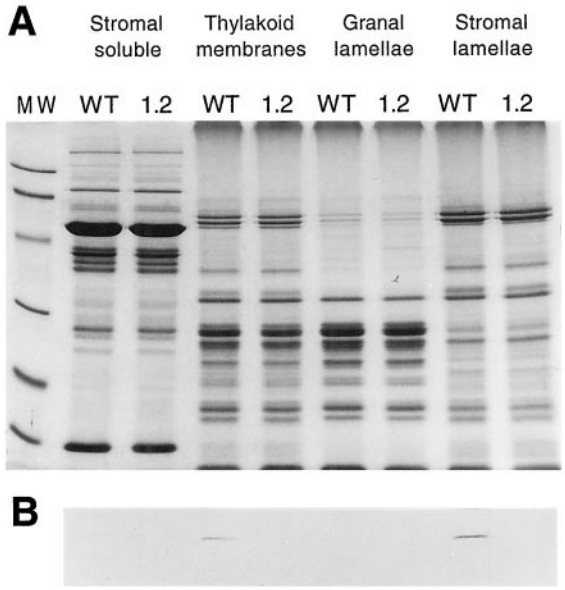

Figure 5. Absence of the NDH-H subunit in the *ndhB*$^-$ plastid transformants. Separation of protein fractions (20 mg of protein per lane) derived from purified chloroplasts by fully denaturating PAGE reveals no obvious difference after Coomassie Brilliant Blue staining between wild-type (WT) and *ndhB*$^-$ (1.2) tobacco plants. Western hybridization of the separated protein fractions electrotransferred onto nitrocellulose membranes by an anti-NDH-H antibody detects the protein in total thylakoid membranes and the stroma lamellae of the wild type. The absence of detectable NDH-H protein in the *ndhB*$^-$ mutant indicates that no functional NDH complex can be assembled. Molecular mass markers: 96, 66.2, 45, 31, 21.5, and 14.4 kD.

Figure 6. Enhanced growth delay of the *ndhB*$^{-}$ plastid transformants under humidity stress. Well-watered wild-type (left) and *ndhB*$^{-}$ (right) plants were grown in low air humidity (30% and 40% relative humidity during the day and the night, respectively), following a month of growth under normal conditions (60% relative humidity). A visible growth difference was developed in less than a week under humidity stress. The development of freshly expanding leaves was specifically hindered in *ndhB*$^{-}$ plants.

complex prompted an analysis of gas exchange under humidity stress.

Enhanced Reduction of Photosynthesis under Conditions Generating Moderate Stomatal Closure in NDH-Inactivated Transformants

Measurement of total CO_2 exchange by intact soil-grown plants was recorded continuously in computer-controlled growth chambers. The vigorous development of young wild-type tobacco plantlets in normal air humidity (60 relative %) was manifested as a steady day-by-day increase in the levels of whole-plant CO_2 fixation, whereas under conditions of low air humidity (30 relative %) photosynthetic development was considerably delayed (data not shown). Under normal growth conditions both wild-type and *ndhB*$^{-}$ plants displayed similar photosynthetic activity. In contrast, after a transition to low air humidity *ndhB*$^{-}$ plants showed a reduced photosynthesis that was up to 20% lower than that of wild-type plants (Fig. 7). These results indicated a primary role of humidity stress in triggering the cascade of events leading to a differential decline in photosynthesis and, concomitantly, growth. Therefore, the direct role of stomatal closure in generating the mutant-specific phenotype was investigated. Stomatal closure was induced in well-watered plants grown in normal air humidity by spraying with abscisic acid (ABA). A moderate treatment (spraying with a 10-μM ABA solution every 2nd d) resulted in a clearly visible growth difference between wild-type and mutant plants under normal phytotron conditions (Fig. 8). After a 2-week-long ABA treatment both fresh and dry

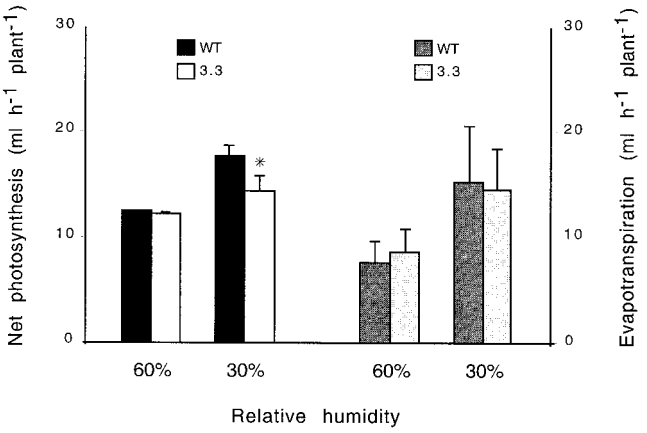

Figure 7. Differential reduction in photosynthesis of wild-type and NDH-inactivated plants grown in low air humidity. Photosynthesis and evapotranspiration of 5-week-old wild-type and *ndhB*$^{-}$ plants are shown in normal (60 relative %) and low (30 relative %) air humidity on the day preceding and following the humidity transition, respectively. The whole-plant photosynthesis and evapotranspiration values recorded in one experiment were the sum of four to eight plants of the same type grown in one computer-controlled growth chamber recording CO_2 consumption and water vapor condensation. On the left net photosynthesis calculated for one plant is displayed as the mean ± SD of four independent experiments. On the right evapotranspiration calculated for one plant is displayed as the mean ± SD of four independent experiments. Asterisk indicates significant differences from controls ($P < 0.05$). Low air humidity generated a difference in photosynthesis of *ndhB*$^{-}$ and wild-type plants, demonstrating an enhanced sensitivity of the *ndhB*$^{-}$ transformants to humidity stress. Evapotranspiration of *ndhB*$^{-}$ and wild-type plants showed a similar response, indicating that their stomata responded non-differentially to low air humidity.

Figure 8. ABA treatment provokes the mutant-specific stress phenotype under normal growth conditions. Wild-type (left) and *ndhB*$^{-}$ (right) plants grown for a month under normal phytotron conditions were subsequently sprayed with 10 μM ABA solution. The growth difference was developed during 2 weeks of spraying of the leaves every 2nd d. The development of freshly expanding leaves was specifically hindered in *ndhB*$^{-}$ plants.

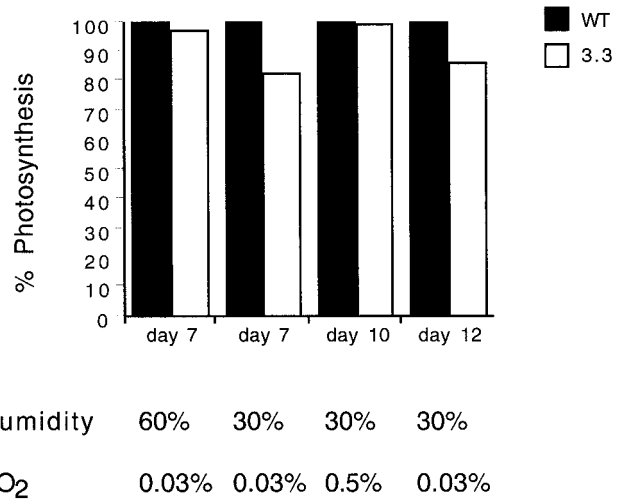

Figure 9. Elevated external CO_2 concentration complements the differential photosynthesis reduction generated by humidity stress. Five-week-old wild-type and *ndhB*$^-$ plantlets were grown under normal conditions in computer-controlled growth chambers recording CO_2 exchange. The effect of humidity stress (caused by decreasing the relative air humidity from 60% to 30%) and the additional effect of elevated CO_2 concentration (from ambient to 0.5%) was tested. Whole-plant photosynthesis in one chamber during illumination was recorded as the CO_2 consumption of six plants of the same type. Relative photosynthesis values are displayed on the *y* axis. For ease of comparison, photosynthesis of wild-type plants was taken to be 100%. The mean wild-type absolute photosynthesis values (from left to right) were the following: 15.55, 18.06, 54.26, and 31.51 mL^{-1} h^{-1} $plant^{-1}$. The relative decrease in whole-plant photosynthesis in NDH-inactivated plants was fully compensated during the transitory elevation of the CO_2 level. This result pinpoints limitation of CO_2 availability as a direct cause of the differential reduction in photosynthesis in wild-type and *ndhB*$^-$ transformant plants under humidity stress conditions.

weights of *ndhB*$^-$ plants (18.40 ± 1.20 and 0.93 ± 0.11 g, respectively) were almost 25% lower than those of wild-type plants (23.84 ± 3.60 and 1.25 ± 0.27 g, respectively). Similar to the effect of humidity stress, the differential phenotype appeared primarily as a reduced growth of the young, expanding leaves of *ndhB*$^-$ plants (Fig. 8). It was notable that a strong ABA treatment (daily spraying with 20 μM ABA) resulted in a strong but non-differential growth inhibition of both wild-type and mutant plants, similar to that caused by water stress (data not shown). These results suggested that a moderate increase in stomatal resistance is an important intermediary in the process leading to a decline in photosynthesis in low air humidity. Therefore, we measured stomatal conductance changes occurring during the transition from high (75 relative %) to low (30 relative %) air humidity on the basis of gas exchange measurements on attached leaves. These investigations demonstrated a moderate and similar decline in leaf conductance in *ndhB*$^-$ mutants (from 22.73 ± 7.29 to 16.05 ± 6.13 mmol m^{-2} s^{-1}) and the wild type (from 23.61 ± 7.30 to 17.08 ± 5.58 mmol m^{-2} s^{-1}). In line with these results whole-plant evapotranspiration rates of *ndhB*$^-$ and wild-type plants measured in growth chambers showed a similar increase after a transition to low air humidity (Fig. 7). We have concluded from these observations that reduced CO_2 availability, due to the (non-differential) stomatal response to low air humidity, is the principle factor generating the mutant-specific phenotype.

Our conclusion that a decline in internal CO_2 concentration is the key element in the cascade of events leading to the differential inhibition of photosynthesis was further investigated by photosynthesis measurements on plants simultaneously grown in low air humidity and elevated external CO_2 concentration. Low air humidity triggered a greater decrease in the photosynthetic capacity of *ndhB*$^-$ plants in comparison with that of wild-type plants. However, increasing the ambient CO_2 level to 0.5% resulted in the disappearance of the difference in photosynthesis levels while markedly increasing overall levels in both types of plant (Fig. 9). When the ambient CO_2 concentration was returned to normal, the differential effect of low air humidity on photosynthesis levels re-appeared. The above data strongly support the view that the differential effect of low air humidity on NDH-inactivated plants is implemented by a differential sensitivity of photosynthesis to limiting CO_2 availability. Our observations also demonstrate that the lack of a functional NDH complex is primarily manifested at a level of CO_2 limitation that does not strongly inhibit the growth and photosynthetic development of wild-type plants.

DISCUSSION

The low abundance of the chloroplast NDH complex (Burrows et al., 1998; Sazanov et al., 1998b) has hampered investigations into its molecular and physiological role. However, the recent application of plastid transformation techniques, which permit targeted inactivation of individual *ndh* genes, has greatly facilitated these investigations (see the introduction). In these experiments insertional mutagenesis or deletion of the gene was achieved via site-specific integration of a dominant selectable marker gene (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Our experiments demonstrate that the *ndhB* gene can also be efficiently inactivated using a different strategy: translational inactivation by replacement of the wild-type plastid *ndhB* gene with a frame-shifted mutant produced by site-directed mutagenesis. In this approach a single nucleotide change was introduced into a cloned copy of the *ndhB* gene, which was located on the same DNA fragment several kilobase pair distant from a binding-type antibiotic insensitivity mutation in the *rrn16* gene. The latter gene was then used to select for plastid transformants in which both mutant genes had replaced their wild-type counterparts on the plastid genome. In our experiments the mutated *ndhB* gene was located close to the end of the trans-

forming DNA (0.1 kb from the junction with vector DNA) and at a distance of 5 kb from the spectinomycin insensitivity mutation used for selection purposes. Nevertheless, in 50% of the transformants the unselected mutation was co-integrated with the selectable marker and yielded a homozygous population of *ndhB*-inactivated plastid DNA, indicating high local recombination frequencies near the vector-insert junction. In homeologous plastid transformation experiments in *Nicotiana* sp. this phenomenon routinely results in recombination/integration frequencies up to 10 times higher than expected at the ends of the plastid DNA insert (Kavanagh et al., 1999). An additional remarkable observation was that our experiments revealed transformation efficiencies typical of those found in homologous plastid DNA transformations (Golds et al., 1993; O'Neill et al., 1993), despite the 2.4% nucleotide sequence divergence between tobacco and nightshade plastid DNA in the transformed region. This observation indicates the prevalence of a RecA-type homeologous recombination mechanism in higher plant plastids (for discussion, see Kavanagh et al., 1999) and suggests that plastid transformation vectors directed to this region do not need to be species specific, at least for species that show a similarly low degree of nucleotide sequence divergence.

The absence of any obvious specific phenotype in the *ndhB*-inactivated transformants when grown under favorable conditions supports earlier conclusions concerning the dispensability of NDH function (Burrows et al., 1998; Shikanai et al., 1998). In other experiments various abnormalities of *ndh*-inactivated primary regenerants were detected (Kofer et al., 1998), but since their linkage with the inactivated gene was not demonstrated, tissue culture effects cannot be excluded as a plausible explanation of the morphological deviations (Maliga and Nixon, 1998). We have shown that under conditions where air humidity is decreased or when plants are sprayed with ABA, photosynthesis is more significantly reduced in *ndhB*-inactivated transformants than in wild-type plants, and this effect on photosynthesis causes a corresponding reduction in biomass. This phenotypic difference was suppressed when ambient CO_2 concentration was increased, thus showing that it is likely mediated by stomatal closure triggered either by low air humidity or by ABA treatment (Downton et al., 1988; Robinson et al., 1988; Willmer and Fricker, 1996). Since no differences in transpiration rates were observed in either whole plants or leaves of controls and *ndhB*$^-$ mutants in response to changes in air humidity, we conclude that stomatal regulation was not affected in the mutant plants. Therefore, low internal CO_2 concentration resulting from partial stomatal closure is likely responsible for the observed phenotypic difference. In previous investigations *ndh*$^-$ mutants have been reported to display a reduced non-photochemical quenching of fluorescence during photosynthesis induction under water stress conditions (Burrows et al., 1998). However, no distinctive visible phenotype was observed by the authors in response to water stress (Burrows et al., 1998). We also observed that in conditions where stomatal closure is pronounced, which occurred in response either to a severe limitation in water supply or to spraying with a high ABA concentration, there was no phenotypic difference. This can be explained by the fact that under such conditions net photosynthesis and growth are so strongly inhibited in both types of plants that the presence or absence of a functional NDH complex has no discernible effect. This is in line with the observations that water stress decreases photosynthetic assimilation of CO_2 by metabolic inhibition (Tezara et al., 1999) and that fully functional photosynthesis is required for the humidity dependence of CO_2 assimilation to be manifested (Stitt et al., 1991). We conclude from our experiments that moderate inhibition of photosynthesis by CO_2 limitation can trigger a phenotypic difference between wild-type and *ndh*-inactivated plants.

Several processes are part of the photosynthetic controls that coordinate the synthesis of ATP and NADPH with their rate of use in carbon metabolism (Foyer et al., 1990; Heber and Walker, 1992). Metabolic demands can often require that light-dependent ATP production be increased relative to NADP reduction. Inhibition of the linear electron flow can occur if there is an imbalance between the stoichiometry of ATP/NADPH production and consumption. A common feature of the NDH-inactivated tobacco plants is the disappearance of a dark transient increase in fluorescence after illumination (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). This phenomenon has been ascribed to a dark reduction of the PQ pool by stromal reductants (Groom et al., 1993) and has been considered to be an after-effect of a light-dependent process, i.e. cyclic electron transport around photosystem I (Burrows et al., 1998; Shikanai et al., 1998). This auxiliary electron flow may modulate the ATP to NAD(P)H ratio by participating in the redox control of the PQ pool. However, different pathways of cyclic electron flow around photosystem I have been suggested to occur in chloroplasts, which are considered likely to involve the NDH complex or a ferredoxin:PQ oxidoreductase activity (Ravenel et al., 1994; Endo et al., 1997). Also, the recovery of the postillumination fluorescence increase in NDH-inactivated plants under certain stress or developmental conditions (Sazanov et al., 1998a; Shikanai et al., 1998) supports the existence of alternative pathways involved in non-photochemical PQ reduction. The absence of a phenotypic difference between wild-type and *ndhB*-inactivated plants when grown under normal conditions suggests that the alternative mechanisms can generate sufficient extra ATP. In contrast, under conditions where CO_2 availability decreases due to moderate stomatal closure, a differ-

ential phenotype is observed. Under such conditions photorespiratory activity is increased due to competition between CO_2 and O_2 at the Rubisco catalytic site. It has been reported that the requirement for ATP is increased during photorespiration (Osmond, 1981). Therefore, we propose that the alternative pathways involved in the production of extra ATP are not efficient enough to fulfill the higher ATP demand of active photosynthesis when photorespiration is operating at a high rate. This would explain why the phenotypic differences between wild-type and NDH-inactivated plants are observed only under conditions that result in moderate CO_2 limitation.

Field-grown plants typically experience extensive periods during which the evaporative demand exceeds the water supply. These conditions can occur in the absence of severe soil water deficiency, e.g. because of fluctuating water inputs from rainfall or irrigation in a dry atmosphere. Under such conditions, the sensitive response of stomata to humidity as the environmental evaporative demand changes provides an efficient means by which tissue water deficits can be avoided. As a consequence, photosynthetic tissues will be subjected to partial stomatal closure for extensive time periods. Under these conditions, in which photosynthesis is limited by CO_2 availability, extra ATP production through an NAD(P)H:PQ oxidoreductase-dependent pathway may confer a selective advantage of sufficient magnitude to explain the conservation of plastid *ndh* genes during the course of evolution.

MATERIALS AND METHODS

Plasmid Construction

The pSSH1 plasmid (Kavanagh et al., 1999) contains a 7.8-kb *Hin*dIII fragment cloned from a black nightshade (*Solanum nigrum*) plastid double mutant (McCabe et al., 1989; Kavanagh et al., 1994). The pSSH1 mutations confer spectinomycin and streptomycin insensitivity and are located in the *rrn16* and the *rps12*(3′) genes, respectively. A 732-bp initial portion of the *ndhB* gene is located at one end of the cloned cpDNA insert. The nucleotide sequence of this region of *ndhB* is identical in both tobacco (*Nicotiana tabacum*) (Shinozaki et al., 1986; GenBank accession no. Z00044) and nightshade (Kavanagh et al., 1999; EMBL accession no. Y18934). Oligonucleotide-directed mutagenesis of the *ndhB* gene was performed using the in vitro mutagenesis system (Altered Sites II, Promega, Madison, WI). The *Hin*dIII fragment from pSSH1 was cloned into the pAlter-1 vector and was mutagenized using the following mutagenic oligonucleotide: 5′-AATCTCTCCCCCGGGATGAACCATA-3′.

Plastid Transformation

Tobacco (*N. tabacum* L. cv Petit Havana) was maintained as shoot cultures on agar-solidified Murashige and Skoog medium (Murashige and Skoog, 1962) in the light (50 μmol m^{-2} s^{-1}, 16-h day, 25°C). Polyethylene glycol-mediated plastid transformation was performed as described (O'Neill et al., 1993). The selective medium contained 1,000 mg L^{-1} spectinomycin dihydrochloride. Plants were regenerated from the resistant colonies, and leaf calli and seedlings were tested for their resistance as described (Cséplő, 1994; Medgyesy, 1994). In the resistance tests spectinomycin dihydrochloride and streptomycin sulfate were used separately at 1,000 mg L^{-1} each.

Plastid DNA Analysis

Chloroplasts were isolated from aseptically grown plants according to Bookjans et al. (1984). Lysis of chloroplasts, the purification of DNA, and the RFLP analysis followed standard protocols (Sambrook et al., 1989). Nonradioactive Southern hybridization was performed using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). *Sma*I-digested plastid DNA separated by horizontal agarose slab-gel electrophoresis and visualized by ethidium bromide staining was denatured and bound to positively charged nylon membranes (Hybond N+, Amersham, Arlington Heights, IL) according to standard protocols. The probe was a 483-bp *Nco*I-*Bsr*GI fragment of tobacco plastid DNA, which covered the diagnostic *Sma*I site. The nucleotide sequence of oligonucleotide primers that were used for PCR analysis of plastid transformants together with their position in IR_B within the tobacco plastid genome is as follows: 5′-ACGTCAGGAGTCCATTGATGA-3′ (98,495–98,515) and 5′-CGAAACAAACGAAAAGGAAAG-3′ (99,459–99,439). Efficient amplification was achieved using approximately 20 ng of plastid DNA in a 20-μL reaction using the PCR System of Fermentas (Vilnius, Lithuania) and the following cycle parameters: 94°C, 30 s; 55°C, 30 s; 72°C, 30 s; 25 cycles.

Preparation of Antibody against NDH-H

The tobacco *ndhH* gene that extends from nucleotide 123,672 to 124,910 (Shinozaki et al., 1986; GenBank accession no. Z00044) was PCR-amplified using *Pfu* polymerase (Stratagene, La Jolla, CA) and cloned by blunt-end ligation into the *Sma*I site of pGEX4-T3 (Pharmacia Biotech, Uppsala). The recombinant plasmid was transformed into the *Escherichia coli* strain DH5α (Gibco-BRL, Cergy Pontoise, France). The resulting clones were sequenced to ensure in-frame fusion of *ndhH* with the glutathione-*S*-transferase gene and to avoid clones that contained PCR-generated mutations. Overexpression of the GST−NDH-H fusion protein was induced with 50 μM isopropylthio-β-galactoside for 5 h at 37°C. Bacterial cultures were pelleted by centrifugation (2,000*g*, 10 min) and resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM Na_2-EDTA, and 100 mM NaCl. Fusion protein was extracted from inclusion bodies by standard procedure (Sambrook et al., 1989) and separated by SDS-PAGE. The relevant band was excised from the gel and the protein was electroeluted. Antiserum was raised against the fusion protein in rabbit (Bioenvirotech, Marseille, France).

Preparation of Thylakoid Membranes

Intact chloroplasts were isolated and purified from leaves using discontinuous Percoll (Pharmacia Biotech) gradients as described (Rumeau et al., 1996). Chloroplasts were osmotically lysed in MNM solution containing 20 mM MES (2-[*N*-morpholino]-ethanesulfonic acid), pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$, and centrifuged for 20 min at 35,000*g*. The supernatant fraction comprised stromal soluble proteins. Stromal and grana lamellae were separated following a stacking step carried out as described by Sazanov et al. (1998b). Briefly, thylakoid membranes were allowed to stack for 1 h and then solubilized by adding *n*-dodecyl-β-D-maltoside dropwise to 1% (w/v) with constant stirring. After incubation (30 min) insoluble material was removed by centrifugation at 1,000*g* for 2 min, and the different fractions were recovered by differential centrifugation. Grana thylakoids were recovered by centrifugation at 10,000*g* for 30 min and stroma thylakoids by centrifugation at 150,000*g* for 1 h.

PAGE and Immunodetection

Denaturing SDS-PAGE was performed as described by Laemmli (1970) using 13% (w/v) acrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or electrotransferred onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and probed with NDH-H antibodies. Immunocomplexes were detected using alkaline phosphatase-conjugated antibodies.

O_2 Evolution Measurements in Plants Cultured in Vitro

Seedling-derived plants were grown in vitro as described (Peter et al., 1999). The leaf discs were collected from plantlets grown under 60 μmol m^{-2} s^{-1} photon flux density (PFD), illuminated by cool-white fluorescence tubes. The effect of light stress was analyzed by exposing the culture vessels to a PFD of approximately 700 μmol m^{-2} s^{-1}. The light was provided by low-voltage (12-V/50-W) multimirror halogen lamps (Precise, General Electric, Fairfield, CT). Three heat filters (Tempax, Schott, Cologne, Germany) and a water filter (10-cm height, 13°C) were placed between the culture vessel and the light source to minimize any temperature increase in the vessel. Light dependence curves of net oxygen evolution were measured at 25°C, approximately 2% (v/v) CO_2 (carbonate/bicarbonate buffer), with a leaf disc oxygen electrode (LD2, Hansatech, King's Lynn, UK) and a pulse-amplitude-modulation fluorometer (PAM, Walz, Effeltrich, Germany). The leaf discs were exposed to alternating light periods (7.5 min) and dark periods (5 min), and the PFD was raised at each light period up to 400 μmol m^{-2} s^{-2}. Gross photosynthesis was calculated from the difference of O_2 evolution rate in the light and in the consecutive dark period.

Leaf Conductance Measurements in Soil-Grown Plants

CO_2 and H_2O exchange of attached leaves of soil-grown plants under humidity transition was measured in an air-tight chamber. Relative humidity (75% and 30%) was set by generating moist air using a portable dew point generator (LI-610, LI-COR, Lincoln, NE) at a flow rate of 2 mL s^{-1}. The moist air was drawn into a leaf clip (PLC model, Ppsystem, Hotchin, UK), equipped with leaf ventillation, thermistor air temperature measurement, and infrared sensor leaf temperature measurement. A gas mixer (SEMY Engineering, Montpellier, France) was used to generate gas mixture with a defined CO_2 and O_2 concentration. CO_2 and O_2 concentrations were measured using an infrared gas analyzer (LI-6262, LI-COR, Lincoln, NE) and an oxygen analyzer (OXOR 6N, Maihak, Hamburg, Germany). CO_2 and H_2O exchange was measured by monitoring air humidity and CO_2 concentration changes in air between the inlet and outlet of the chamber. Standard calculations were used to determine stomatal conductance (Farquhar and Sharkey, 1982).

Whole-Plant Photosynthesis and Growth Measurements

Three-week-old in vitro-grown seedlings were potted into soil and grown for an additional 2 weeks in a phytotron or a growth chamber before using a stress condition. The standard conditions in the phytotron were 16-h light (250–350 μmol m^{-2} s^{-1} PFD, Osram HQI-T/DV lamps, 30°C–32°C), 8-h dark (22°C), 50% to 60% relative humidity. The plants were supplied six times a day by nutrient solution (one-half-diluted Hoagland salts; Hoagland and Arnon, 1950) in an excess amount resulting in over-dipping from the soil. ABA treatment was performed by spraying 15 to 25 μL of ABA solution (10 μM) on the lower surface of all leaves of a plant every 2nd d. The computer-controlled $C_2$3A system (Fabreguettes et al., 1994) consists of air-tight twin growth chambers suitable for comparative investigation of two sets of plants. The continuously adjusted and recorded parameters in the chambers (the standard values are indicated in brackets) were the following: CO_2 concentration (0.034%), O_2 concentration (16%), air humidity (60 relative %), evapotranspiration, temperature (day/night: 30°C/25°C). Illumination (16-h day) was provided by metal halogen lamps (Powerstar HQI-T/D, Osram, Munich), offering 250 and 350 μmol m^{-2} s^{-1} PFD at the level of upper leaves of young and mature (non-flowering) plants, respectively. Water was supplied normally four times a day as nutrient solution (one-half-diluted Hoagland salts; Hoagland and Arnon, 1950) in controlled amounts 20% to 50% more than the daily water loss by evapotranspiration. In the case of extended water stress by limited watering, after some days pure water was used to avoid salt accumulation. Humidity stress was achieved by specifying 30% and 40% relative humidity during day and night, respectively. The measurements of net photosynthesis and dark respiration were based on the quantitative balance of CO_2 injection and trapping, respectively, maintaining a constant CO_2 concentration (measured by an infrared gas analyzer) in the chambers (Fabreguettes et al., 1994). Air humidity was measured using, in addition to the in-built humidity detector, a portable humidity detector (Hydrodig 2010, Tecnic Instruments, Marseilles, France) at the level of leaves. Evapotranspiration was measured by

weighing the collected condensed water vapor. Six plants normally were grown in each chamber. The use of large growth chambers for whole-plant photosynthesis measurements does not easily allow statistical evaluation of individual plants, therefore the experiments were repeated to test their reproducibility.

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B. Non-photochemical reduction of intersystem electron carriers in chloroplasts of higher plants and algae

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NON-PHOTOCHEMICAL REDUCTION OF INTERSYSTEM ELECTRON CARRIERS IN CHLOROPLASTS OF HIGHER PLANTS AND ALGAE

[1]Laurent Cournac, [1]Geneviève Guedeney, [1]Thierry Joët, [1]Dominique Rumeau, [2]Gwendal Latouche, [2]Zoran Cerovic, [3]Kevin Redding, [4]Eva Horvath, [4]Peter Medgyesy, [1]Gilles Peltier

[1]CEA Cadarache, DEVM Bat 161, 13108 Saint-Paul-lez-Durance, France
[2]CNRS-LURE Bat 203, Centre Universitaire Paris-Sud, 91405 Orsay, France
[3]University of Geneva, 30, quai Ernst-Ansermet, CH-1211, Switzerland
[4]BRC, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

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1. Introduction

Besides reactions involved in the « Z » scheme of photosynthesis, additional electron transport pathways, such as cyclic electron flow around PS1 or chlororespiration, have been reported in algal and in higher plant chloroplasts (1-4). These pathways generally involve a reduction of the intersystem electron transport chain (plastoquinones and/or the cyt b_6f complex) by stromal components such as NAD(P)H or reduced ferredoxin. Different electron carriers have been proposed to mediate the non-photochemical reduction of inter-system electron transport chain. The discovery of chloroplast genes showing homologies with genes encoding subunits of the mitochondrial complex I (*ndh* genes), was taken as an evidence for the existence of a NADH dehydrogenase complex I in chloroplasts. Since, the existence of such a complex has been reported from non-denaturing gel electrophoresis (5) and recently, the NDH complex has been purified and partially characterized from pea chloroplasts (6). Inactivation of chloroplast *ndh* genes by plastid transformation has been undertaken in order to assess the role of the NDH complex during photosynthesis. However, if the disruption of *ndh* genes allowed to show that the NDH complex is functional *in vivo* and is involved in the dark re-reduction of the PQ pool after a period of illumination (7), a participation of the NDH complex during photosynthesis was not shown. Surprisingly, no clear phenotype was observed in inactivated transformants (7), suggesting that the NDH complex has a marginal role during photosynthesis. Other mediators have been proposed to be involved in the non-photochemical reduction of inter-system electron carriers : these include the putative ferredoxin-quinone-oxidoreductase activity (FQR), FNR (4) and more recently a NAD(P)H-PQ oxidoreductase activity characterized in potato chloroplasts that would be different from the NDH complex, from FNR, and from FQR (8).

Interestingly, although chlororespiration and cyclic electron flow around PS1 have been extensively studied in the green alga *Chlamydomonas*, sequencing of the chloroplast genome of different *Chlamydomonas* species has revealed the absence of *ndh* genes (9). One possibility would be that *ndh* genes have been transferred to the nuclear genome during the time course of evolution, but the high hydrophobicity of some *ndh* gene products makes this possibility rather unrealistic. Another possibility would be that in green algae like *Chlamydomonas*, a different mechanism is involved in the non-photochemical reduction of the PQ pool.

In the present paper, we have studied the nature of the electron carriers(s) involved in the non-photochemical reduction of the intersystem electron transport chain in higher plant chloroplasts and in *Chlamydomonas.* Targeted inactivation of the *ndh*B gene has been achieved by plastid transformation of tobacco protoplasts and the ability of exogenous NAD(P)H to donate electrons to the intersystem electron transport chain was studied in transformed plants. In *Chlamydomonas* cells, interaction between stromal components and the PQ pool was studied by measuring H_2 production in WT cells and O_2 exchange in PS1-deficient mutants. Results are discussed in relation to the existence of different pathways of PQ reduction in chloroplasts.

2. Material and Methods

2.1 Chlorophyll fluorescence measurements - chlorophyll fluorescence was measured on tobacco chloroplasts at 25°C using a pulse modulated amplitude fluorimeter (PAM 101-103, Walz, Effeltrich, Germany) as described in (8). Anaerobiosis was achieved by addition of glucose (20 mM) and glucose oxidase (2 $mg.ml^{-1}$) to the chloroplast suspension about 15 min before measurements. Blue green fluorescence measurements were made on a new version of the pulse modulated fluorometer described in (10).

2.2 Mass spectrometric measurement of gas exchange - algal cultures were harvested and 1.5 ml of the suspension was placed in the measuring chamber of the mass spectrometer. The experimental procedure for O_2 exchange assays has been described in (11). For measurements of H_2 evolution, cultures were subjected to anaerobiosis by sparging the sample with N_2, closing the chamber and leaving algae consume residual O_2 from the medium. When O_2 concentration was undetectable (< 0.2 µM), cultures were incubated for 30 min before starting measurements.

2.3 Plant material - *Chlamydomonas* strains deleted in *psaA* and *psa*B genes (*psa*AΔ and *psa*BΔ) were made as described earlier (12). The *Chlamydomonas* MUD2 mutant resistant to myxothiazol was generously supplied by Dr. P. Bennoun. Double mutants were produced by sexual crosses. Targeted inactivation of the *ndh*B gene was achieved in tobacco (Petit Havana) by replacing the gene with a mutant version possessing a translational stop in the coding region. The mutated gene was physically linked to selectable markers (spectinomycin and streptomycin resistance) located on the same plastid DNA fragment derived from a *Solanum nigrum* plastid mutant. Plastid transformants were produced via polyethylene glycol-mediated transformation of tobacco protoplasts, and selection for the binding-type antibiotic resistances was used to generate single-cell derived homoplasmic primary regenerates of plastid transformants (13). Physical mapping and Southern hybridation were performed to

confirm homoplasmy and the presence of the mutation in transformants. Control lines were generated by inserting the selectable markers alone.

3. Results

When *Chlamydomonas* cells are placed under anaerobic conditions, the chloroplast enzyme hydrogenase is induced and produces H_2 by using reduced ferredoxin as an electron donor. This reaction can function in the absence of PS2 activity (for instance in DCMU-treated algae), electrons being supplied to PS1 by the intersystem electron transport chain. This supplies a convenient experimental model to study *in vivo* reactions involved in the non-photochemical reduction of PQ. In normal conditions, H_2 production is transitory, being slowed by the generation of a thylakoid proton gradient and followed by activation of Calvin cycle CO_2 fixation. In the presence of uncouplers (2µM FCCP), H_2 production can be sustained, as proton gradient vanishes and CO_2 fixation, which needs ATP, is prevented. In the absence of DCMU, H_2 was produced at PS1 and O_2 was produced by PS2 (Fig. 1A). In the presence of DCMU, O_2 evolution was stopped and H_2 was still produced at a consequent rate (about 500 nmoles. $min^{-1}.mg^{-1}$chlorophyll). H_2 production measured in the presence of DCMU was insensitive to rotenone or to low concentrations of piericidin A, well-known inhibitors of complex I, thereby indicating that a complex I-like enzyme was likely not involved in this reaction. On the other hand, we found that H_2 production in the presence of DCMU was inhibited by N-ethylmaleimide (NEM - Fig. 1D) a sulfhydryl group reagent, although this compound did not affect PS2-driven H_2 production (Fig. 1C).

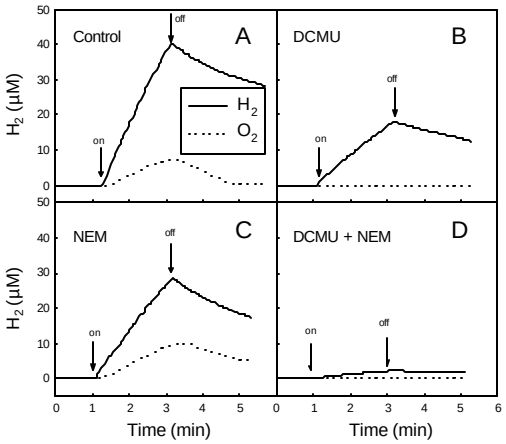

Fig 1 : Light-driven H_2 production by *Chlamydomonas* cells under anaerobic conditions. FCCP (2µM) was added before measurements. A : control ; B : in the presence of 10 µM DCMU ; C : in the presence of 1 mM NEM ; D : in the presence of 10 µM DCMU and 1 mM NEM.

In order to better characterize reactions involved in the reduction/oxidation of the PQ pool, we investigated O_2 exchange in *Chlamydomonas* mutants deleted in PS1. In such mutants, CO_2 fixation does not occur, but a PS2-dependent electron flow has been characterized by mass spectrometry (11), electrons produced at

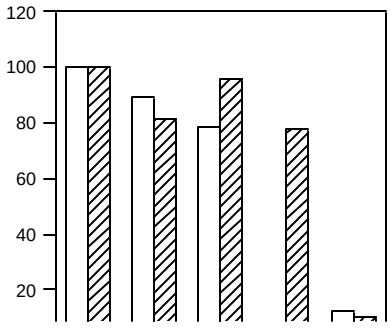

Fig 2 : Effect of mitochondrial inhibitors (SHAM : 0.4 mM; Mx : 2 µM myxothiazol; AntA : 2 µM antimycin A) on PS2-driven O_2 exchange in *Chlamydomonas* mutants *psa*BΔ (□) and *psa*BΔ MUD2 ( ).

PS2 being used for O_2 reduction. We found that the PS2-dependent electron flow was inhibited by DCMU, thus indicating the involvement of the PQ pool, and was unaffected in a mutant lacking the cyt b_6/f complex (not shown). Quite interestingly, the PS2-dependent pathway was sensitive to the presence of inhibitors of mitochondrial respiration (Fig. 2). Simultaneous addition of myxothiazol (an inhibitor of the mitochondrial cytochrome bc_1 complex) and of salicyl hydroxamic acid (SHAM - an inhibitor of the alternative oxidase) inhibited the PS2-dependent electron flow, whereas myxothiazol or SHAM alone did not, as is classicaly observed for inhibition of mitochondrial respiration. The inhibition was not observed in a mutant resistant to myxothiazol (MUD2), unless myxothiazol was replaced by an other inhibitor of the cytochrome oxidase pathway such as antimycin A. This clearly shows that this PS2-dependent flow needs ongoing mitochondrial respiration.

In order to investigate the nature of the interaction between chloroplasts and mitochondria, we monitored redox state variations of the NAD(P)H pool by measuring light-induced changes in blue-green fluorescence. In PS1 deficient mutants, the light-induced reduction of NAD(P)H is very small when compared to what occurs in WT (Fig. 3). When mitochondrial activity was blocked by addition of myxothiazol and SHAM, both the dark level of NAD(P)H reduction and the light-dependent increase were enhanced. The inhibition of PS2 activity likely results from the reduction of the PQ pool by soluble reductants that accumulate when respiration is blocked. In the absence of PS1 and of a complex I-like enzyme (which may function in a reverse mode), $NAD(P)^+$ reduction by PQH_2 is not likely to occur. NAD(P)H accumulation in the light in presence of myxothiazol and SHAM would then be explained by a competition between NAD(P)H and PS2 for the reduction of PQ, O_2 uptake occurring in thylakoid membranes (chlororespiration).

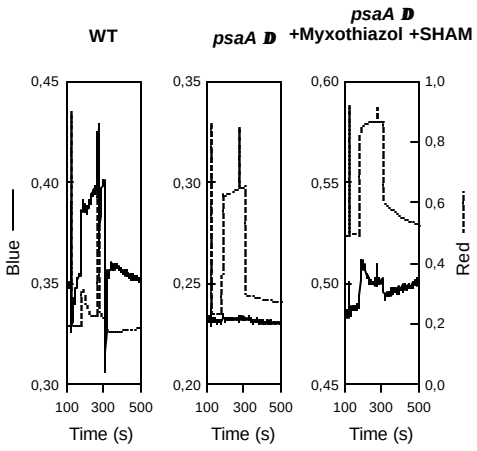

Fig 3: Evolution of chlorophyll ('Red') and NAD(P)H ('Blue') fluorescences during dark-light-dark transitions in WT and PS1-deficient *Chlamydomonas* mutants (*psa*AΔ). On the right figure, cells were incubated in the presence of myxothiazol (4 µM) and SHAM (0.8 mM) before measurements. Light saturating flashes were applied 1 min before and 2 min after light onset.

In higher plant chloroplasts, a NDH complex encoded by plastidial genes has been characterized (5-7). In order to determine the role of the NDH complex during photosynthesis, targeted inactivation of a plastidial *ndh* gene (*ndh*B) was carried out by plastid transformation of tobacco protoplasts. *Ndh*B inactivated tranformants ($ndhB^-$) grew normally and could not be distinguished from WT controls. Western analysis showed that the *ndh*H gene product was absent from thylakoid membranes of $ndhB^-$ (not shown) indicating that the NDH complex disappeared in response to *ndh*B inactivation as previouly reported for other *ndh* genes (7). Chlorophyll fluorescence measurements showed no obvious differences between WT and $ndhB^-$ during a dark to

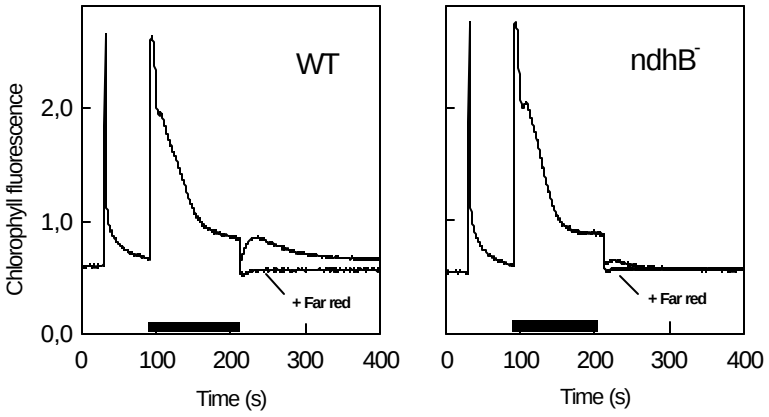

Fig 4 : Chlorophyll fluorescence measurement during a dark to light transition in control and ndhB⁻ tobacco leaves. Light intensity during the light period (–) was 280 µE. $m^{-2}.s^{-1}$. Far red light was switched on after turning the actinic light off in experiments indicated by the arrow.

light transition (Fig. 4). However, in the dark period following illumination, the re-reduction of the PQ pool (which is reverted in the presence of far-red light) was almost completely suppressed in ndhB⁻, thereby indicating that the NDH complex is functional *in vivo*.

Non-photochemical reduction of the PQ pool was studied in tobacco chloroplasts by measuring the effect of an exogenous supply of NADH or NADPH on the chlorophyll fluorescence level measured under non-actinic light (8). Under anaerobic conditions, the NADH (or NADPH)-induced fluorescence rise results from the non-photochemical reduction of the PQ pool (8). In order to determine whether the NDH complex is involved in this reaction, this experiment was performed using chloroplasts prepared from ndhB⁻ leaves. Fig. 5 (A,B) clearly shows that the NADH-induced reduction of the PQ pool is not diminished in ndhB⁻, and seems even enhanced.

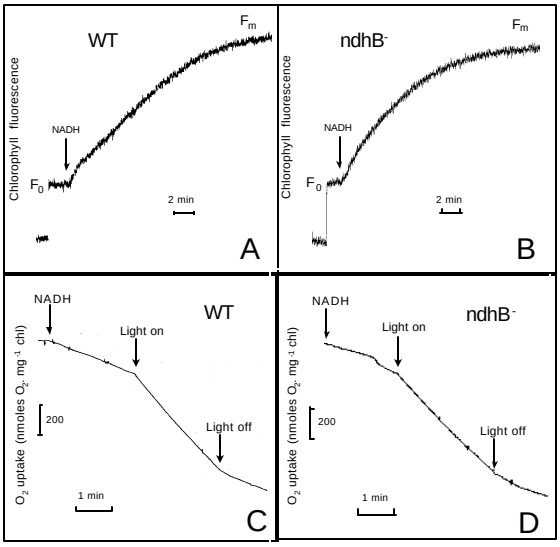

Fig 5 (A,B) : Effect of 2mM NADH addition on the chlorophyll fluorescence level measured in dim modulated light on tobacco chloroplasts. Measurements were performed under anaerobic conditions (in the presence of glucose, glucose oxidase and catalase) using chloroplasts prepared from control (A) and ndhB⁻ (B). **(C,D)** : Light-induced O_2 uptake measured in control (C) and ndhB⁻ (D) tobacco chloroplasts. O_2 uptake measurements were performed in the presence of DCMU (10µM), MV (500µM) and NADH (2mM), catalase (1000 units.ml^{-1}) and superoxide dismutase (500 units.ml^{-1}).

Non-photochemical reduction of intersystem electron carriers was also investigated by measuring light-induced O_2 uptake in the presence of DCMU and methyl viologen (MV). In these conditions, PS2 cannot reduce the PQ pool and light-induced O_2 uptake reflects the ability of PS1 to reduce O_2 using intersystem electron donors. Fig. 5 (C,D) clearly shows that this reaction was not affected in ndhB⁻ chloroplasts. These two experiments show that the

NDH complex is not involved in the NADH-dependent reduction of intersystem electron carriers measured in the chloroplast preparation used here. This is in agreement with the conclusion of a previous study performed on potato chloroplasts, where a NAD(P)H-PQ oxidoreductase activity sensitive to NEM and diphenylene-iodonium, but insensitive to rotenone was characterized (8).

4. Discussion

We conclude from these experiments that different pathways of non-photochemical reduction of the PQ pool take place in chloroplasts. In *Chlamydomonas*, the existence of a significant light-dependent H_2 production in DCMU-treated cells as well as the existence of strong interactions between mitochondrial activity and photosynthetic electron transport activity in PS1-deficient mutants show that electrons can enter the PQ pool from stromal donors. Properties of the H_2 production measured in the presence of DCMU (insensitivity to rotenone and piericidin A) argue against the participation of a NDH complex that would be similar to complex I. We rather conclude to the involvement of another NAD(P)H-PQ oxidoreductase activity, which on the basis of the sensitivity to NEM and the insensitivity to rotenone might be similar to that previously reported in potato chloroplasts (8). This would be consistent with the absence of *ndh* genes in the chloroplast genome of *Chlamydomonas*.

In tobacco, inactivation of the plastidial *ndh*B gene leads to a suppression of the dark re-reduction of the PQ pool, which confirms previous report showing that the plastidial NDH complex is functional *in vivo* (7). However, addition of exogenous NADH to tobacco chloroplasts provokes a reduction of the PQ pool which is unaffected in ndhB$^-$ transformants. We conclude that NDH activity might be lost during chloroplast isolation, and that another pathway of PQ reduction using NAD(P)H (and probably similar to the NAD(P)H-PQ oxidoreductase activity previously characterized in potato chloroplasts - see ref. 8) is present. The existence of different pathways of PQ reduction would explain why *ndh* inactivated transformants grow normally and do not show apparent phenotype.

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