Mol Gen Genet (1998) 258: 166–173

ORIGINAL PAPER

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Mutagenesis of the genes encoding subunits A, C, H, I, J and K of the plastid NAD(P)H-plastoquinone-oxidoreductase in tobacco by polyethylene glycol-mediated plastome transformation

Received: 15 December 1997 / Accepted: 15 January 1998

Abstract Plastids contain a NAD(P)H-plastoquinone-oxidoreductase (NDH complex) which is homologous to the eubacterial and mitochondrial NADH-ubiquinone-oxidoreductase (complex I), but the metabolic function of the enzyme is unknown. The enzyme consists of at least eleven subunits (A-K), which are all encoded on the plastid chromosome. We have mutagenized *ndhC* and *ndhJ* by insertion, and *ndhK* and *ndhA-I* by deletion and insertion, of a cassette which carried a spectinomycin resistance gene as a marker. The transformation was carried out by the polyethylene glycol-mediated plastid transformation method. Southern analysis revealed that even after repeated regeneration cycles each of the four different types of transformants had retained 1–5% of wild-type gene copies. This suggests that complete deletion of *ndh* genes is not compatible with viability. The transformants displayed two characteristic phenotypes: (i) they lack the rapid rise in chlorophyll fluorescence in the dark after illumination with actinic light for 5 min; in the wild-type this dark-rise reflects a transient reduction of the plastoquinone pool by reduction equivalents generated in the stroma; and (ii) transformants with defects in the *ndhC-K-J* operon accumulate starch, indicating inefficient oxidation of glucose via glycolysis and the oxidative pentose phosphate pathway. Both observations support the theory of chlororespiration, which postulates that the NDH complex acts as a valve to remove excess reduction equivalents in the chloroplast.

Key words NAD(P)H-plastoquinone-oxidoreductase · Complex I · Plastid transformation · Chlororespiration · *Nicotiana tabacum*

Communicated by R. G. Herrmann

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Introduction

The NADH-ubiquinone-oxidoreductase (complex I) is the first proton-pumping complex of the respiratory chain in bacteria and mitochondria (Friedrich *et al.* 1995). The enzyme is a multisubunit protein assembly with an L-shaped structure, and consists of more than 30 subunits in *Neurospora crassa* and more than 40 subunits in bovine mitochondria. Seven subunits are encoded by mitochondrial DNA and the remaining subunits by the nuclear genome (Weiss et al. 1991; Walker 1992). In *Escherichia coli,* complex I contains only 14 subunits (NUO-A-N), which are all encoded in a single operon (Weidner et al. 1993). The enzyme is therefore thought to represent a minimal form of complex I. By raising the pH from 6.0 to 7.5 in the presence of Triton X-100, *E. coli* complex I can be fragmented into three subcomplexes: (i) a peripheral NADH-oxidizing complex comprising three proteins (NUO-E, F and G); (ii) a connecting fragment consisting of four proteins (NUO-B, C, D and I); and (iii) a membrane-bound complex made up of seven subunits involved in ubiquinone reduction and proton translocation (Friedrich et al. 1995; Leif et al. 1995).

In the plastid chromosomes of the liverwort *Marchantia polymorpha* and of higher plants 11 genes homologous to genes encoding complex I of *E. coli* have been found (*ndhA-K),* and thus it was concluded that plastids contain a complex I-homologous NAD(P)H-dehydrogenase, hereafter called NDH-complex (Ohyama et al. 1988; Sugiura 1992). The deduced amino acid sequences of four NDH proteins (NdhH, I, J and K) are homologous to the subunits of the connecting fragment and seven others (NdhA-G) are equivalent to the subunits of the membrane-associated part of *E. coli* complex I. However, no polypeptides corresponding to the three

Table 1 Plasmids containing tobacco plastid DNA fragments used for the construction of mutant *ndh* genes

Plasmid	Size	Restriction sites[a]	Genes
pSB3.8	3766 bp	*Sal*I (49 833)–*Bam*HI (53 599)	*ndhCKJ*
pXS8.5	8516 bp	*Xho*I (118 604)–*Stu*I (127 120)	*ndhHAIGE*
p2038XX	2038 bp	*Xba*I (119 540)–*Xba*I (121 578)	*ndhIGE*
p3369BX	3369 bp	*Bgl*II (116 171)–*Xba*I (119 540)	*psaC-ndhD*

[a] The restriction sites are numbered according to Shinozaki et al. (1986)

subunits of the NADH-oxidizing element of the plastid enzyme have been identified so far. Moreover, the enzyme has not been purified in a form which would allow the complete characterization of all its subunits, and thus the enzymatic activity of the plastid NDH complex is unknown. While the presence in plastids of genes homologous to those encoding all subunits that make up the membrane arm of the *E. coli* complex I suggests that the NDH complex uses plastoquinone as the electron acceptor, the nature of the electron donor is still under debate. It has been proposed that reduced ferredoxin may be used or that the ferredoxin-NADP-oxidoreductase is associated with the NDH complex and serves as the entry site for NADPH (Friedrich et al. 1995; Guedeny et al. 1996). In contrast, recent activity measurements on partially purified preparations of the enzymes from barley and pea suggest that the enzyme oxidizes NADH as in bacteria and mitochondria (Cuello et al. 1995; Sazanov et al. 1996).

Another question concerns the function of the NDH complex in plastid metabolism. The subunits NdhK and NdhH are confined to the stroma thylakoids (Nixon et al. 1989; Berger et al. 1993), which are known as the thylakoid compartment where cyclic electron transport takes place (Anderson 1992), and suggests that the NDH complex is involved in this process. This view is supported by the finding that higher concentrations of NDH proteins are present in bundle sheath plastids than in mesophyll plastids of the C_4 plant *Sorghum bicolor*. In bundle sheath chloroplasts photosystem II activity is drastically reduced and so is the capacity for linear electron transport. However bundle sheath chloroplasts provide ATP for carbon fixation and contain photosystem I, the cytochrome b_6/f complex and the ATPase in comparable amounts to mesophyll plastids. Thus the NDH complex seems to be an essential component of the cyclic electron transport pathway in bundle sheath plastids (Kubicki et al. 1996)

Another possible function for the plastid NDH complex has been suggested by Bennoun (1982). According to this hypothesis, the NDH complex serves as a pressure valve to remove excess reduction equivalents in a process called chlororespiration. Surplus reduction equivalents, generated, for example, during the degradation of transitory starch in the dark, would be oxidized by the NDH complex and enter the plastoquinone pool from which they are removed by an as yet unknown quinol oxidase.

The establishment of methods allowing the transformation of plastids of higher plants (Svab et al. 1990; Golds et al. 1993; Svab and Maliga 1993) provides the opportunity to study the function of *ndh* genes directly. Since it is possible to introduce marker genes into the plastid chromosome by homologous recombination with flanking plastid DNA sequences, and even to delete genes (Maliga 1993; Allison et al. 1996), we have used the method of polyethylene glycol-mediated plastid transformation to mutagenize several *ndh* genes.

Materials and methods

Construction of mutant plasmids

The cloned plastid DNA fragments used in this study are listed in Table 1. The cassette used for transformation (*16S-aadA*) has been described by Koop et al. (1996). It contains the *16S* tobacco plastid rDNA promoter, the ribosome binding site of *rbcL* from tobacco, the *aadA* gene from the Omega interposon (Prentki et al. 1991) as the spectinomycin resistance marker, and the 3′ end of *rbcL* from *Chlamydomonas reinhardtii* as a terminator sequence. The cassette was either inserted within the reading frames of *ndh* genes (*pndhC-H* and *pndhJ-N)* or used to delete *ndhK* (*pndhCKJ-HN*) and *ndhA* and *I* (*pndhHAI-RV*) completely. In a control construct the *16S-aadA* cassette was inserted between the reading frames of *ndhE* and *psaC* (*pIGE-aad*). The exact restriction sites used for integration are described in Table 2, and the restriction maps of the mutant constructs are presented in Fig. 1. All cloning procedures were carried out using standard methods described in Sambrook et al. (1989).

Plant material

Nicotiana tabacum L. cv. Petit Havanna plants were grown from seedlings as in vitro cultures at 25° C (16 h light; 0.5–1 W/m^2, Osram L85 W/25 Universal-White fluorescent lamps). The seedlings were transferred into jars with B5 medium as reported previously (Koop et al. 1996). After a 3-week culture period, fully expanded leaves were harvested for the preparation of protoplasts.

Table 2 Mutant plasmids used for the transformation of tobacco protoplasts

Genes affected	Mutant plasmid	Type of mutation	Restriction sites[a]	Mutant series
ndhC	p*ndhC-H*	Insertion	*Hin*dIII (52 368)	52
ndhCKJ	p*ndhCKJ-HN*	Deletion/insertion	*Nde*I (51 112)–*Hin*dIII(52 368)	53
ndhJ	p*ndhJ-N*	Insertion	*Nde*I (51 112)	55
ndhHAI	p*ndhHAI-RV*	Deletion/insertion	*Eco*RV (120 818)–*Eco*RV (123 868)	54
–	p*IGE-aad*	Insertion	*Xba*I (11 954)	56

[a] The restriction sites are numbered according to Shinozaki et al. (1986)

Protoplast isolation, PEG transformation, culture procedure and selection of transformants

The isolation of protoplasts, PEG-mediated transformation and culture of protoplasts in thin alginate layers on stainless steel grids, as well as the selection of transformed calli and shoots, were carried out as reported previously (Koop and Kofer 1995; Koop et al. 1996), except that for shoot formation, the calli were placed on RMOP medium (Svab et al. 1990). In order to obtain homoplasmic lines we used a procedure in which small pieces of a fully developed leaf are placed on a petri dish containing RMOP medium with spectinomycin (500 mg/I) and streptomycin (500 mg/l) until they form shoots. After 9–12 rounds of this repetitive regeneration procedure, the shoots were transferred onto B5 medium (with 500 mg/l spectinomycin) and allowed to form roots.

Southern blot analysis

Total DNA was isolated from 100 to 150 mg of leaf material using the DNeasy kit from Qiagen (Hilden, Germany). Restriction of DNA, agarose gel electrophoresis and Southern blotting were performed using standard procedures (Sambrook et al. 1989). The blots were hybridized with α-^{32}P-labelled DNA fragments in 250 mM sodium phosphate, 7% SDS and 2.5 mM EDTA (pH 7.2) at 65° C and washed in 0.1 × SSC, 0.15% SDS at the same temperature.

Preparation of thylakoid membranes and Western blot analysis

Portions (250 to 400 mg) of leaves were homogenized in 10 ml of isolation buffer (44 mM MES-KOH, 450 mM sorbitol, 10 mM NaCl, 1 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 4 mM EDTA, 1% β-mercaptoethanol pH 5.7) in a mortar at 4° C. The homogenate was filtered through Miracloth and the plastids were sedimented at 3000 × g for 5 min. The sediment was resuspended in a small volume of isolation buffer, and then centrifuged through a Percoll step gradient (45/85%) in 2-ml Eppendorf reaction tubes at 8000 rpm in the Sorvall HB-4 rotor (Sorvall, DuPont de Nemours, Bad Homburg, Germany). The upper green band was collected, suspended in 4 volumes of isolation buffer and the plastids were sedimented again in an Eppendorf centrifuge for 5 min. The plastid pellet was resuspended in 200 to 400 µl of isolation buffer and stored at −20° C. The plastids were solubilized in sample buffer (Laemmli 1970) and then fractionated by electrophoresis through a 12% polyacrylamide gel. The proteins were then transferred to polyvinyl-iden difluoride membranes (PVDF, Immobilon P; Millipore, Eschborn, Germany) according to Kyhse-Anderson (1984). The membranes were incubated with the various antisera listed below and antigen-antibody complexes were then detected with the BM chemiluminescence blotting substrate (POD) kit from Boehringer (Mannheim, Germany).

Antibodies raised against tobacco NdhH (Kubicki et al. 1996) and spinach PSII-B (Oswald et al. 1990) were used. The antibodies against spinach ATPase and PSI-D were gifts from H. Strotmann (Heinrich Heine University, Düsseldorf, Germany) and R. Nechushtai (Hebrew University, Jerusalem, Israel), respectively.

The protein concentration were measured as described in Bensadoun and Weinstein (1976) and chlorophyll concentrations were determined according to Arnon (1949).

Fluorescence analysis

Chlorophyll fluorescence was measured with the pulse amplitude-modulated fluorometer (PAM 101/103; Walz, Effeltrich, Germany) described by Schreiber (1986) according to the protocol of van Kooten and Snel (1990). Leaf disks (8 mm in diameter) were punched from excised leaves and placed in the KS 101 suspension cuvette on wet filter paper and the cuvette was connected to the fiber optics of the 101 ED emitter/detector unit. The leaf disks were left in the dark for at least 10 min and then the weak, pulsed excitation source (650 nm, 100 kHz) was switched on and the minimal fluorescence F_0 was recorded for 30–45 s. Then, illumination with actinic light (320 µmol/m^2 per s) was started and fluorescence recorded for about 5 min. Far-red light was supplied from the 102 FR lamp (Walz) at an intensity of 14 W per m^2.

Electron microscopy

Leaf material was fixed in a buffered solution of 2.5% glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.2) for 4 h. The tissue was rinsed in cacodylate buffer and postfixed with 1% osmium tetraoxide in the same buffer. The tissue was dehydrated in a graded series of acetone; staining of the tissue block was carried out with 1% uranyl acetate in 20% acetone. The tissue samples were embedded in Spurr's resin and polymerized at 60° C. Sections were poststained with lead citrate and examined in a Zeiss EM 912 electron microscope.

Results

Transformation and Southern analysis

Three different types of constructs were generated by inserting the *16S-aadA* cassette with the spectinomycin/streptomycin maker gene into different *ndh* genes of tobacco plastid DNA (Fig. 1 and Table 2). In two constructs the cassette was inserted into the reading frames of *ndhC* and *ndhJ*, yielding transformants of the 52 and 55 series, respectively. In transformants of the 53 and 54 series, the reading frames of *ndhK* and *ndhA* and *I*, respectively, were deleted completely. The third type of transformants served as a control and contained the *16S-aadA* cassette between the reading frames of *ndhE* and *psaC* (56 series).

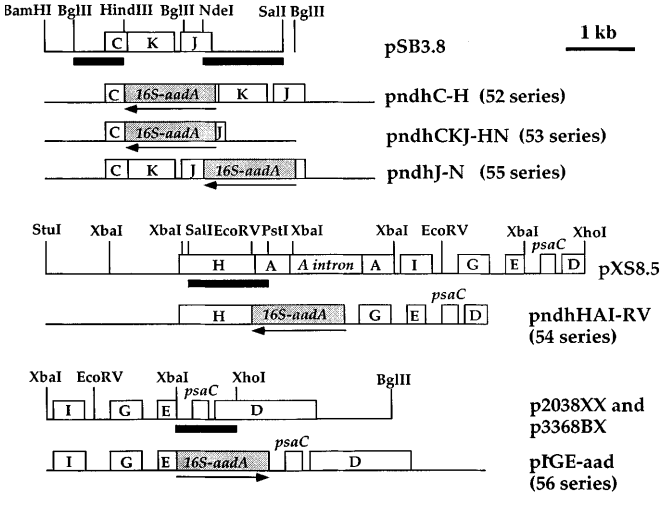

Fig. 1 Restriction maps of the plasmids used for plastid transformation. The direction of transcription of *ndhCKJ* and *ndhAIGE-psaCD* is from left to right. The direction of transcription of the *16S-aadA* cassette is shown by *arrows*. Hybridization probes are indicated as *solid bars* below the genes (52 and 53 series: 779-bp *Bgl*II-*Hind*III fragment; 55 series: 1279-bp *Nde*I-*Sal*I fragment, 54 series: 1235-bp *Sal*I-*Pst*I and 56 mutants: 936-bp *Xba*I-*Xho*I fragment)

After the transformation of protoplasts, plastid transformants were selected. Generally, the initial selection for plastid transformants is based on a green vs. a white phenotype in the presence of the antibiotics. Green calli were picked and regenerated into small plants. In order to obtain homoplasmic plants, pieces of leaf tissue of the regenerants were allowed to form shoots on selective medium. This procedure was repeated 9–12 times. During these regeneration cycles, light-green regenerants were frequently observed to be derived from independent sectors.

Southern blot analysis of two independently regenerated transformants from each series revealed that in all transformants the mutant constructs had inserted by homologous recombination (Fig. 2). The same is true for the control construct (data not shown). However, with none of the mutant constructs were we able to obtain complete elimination of the wild-type gene copies. A dilution series of the wild type provides an estimate of the relative amounts of the introduced mutant construct and the remaining wild-type gene copies (Fig. 2). The sensitivity of the method allows the detection of gene copies present down to a level of 1–2% of total plastid chromosomes in the preparation. While mutant copies are the predominant species, in all transformants wild-type gene copies were retained at a level of about 1–5%.

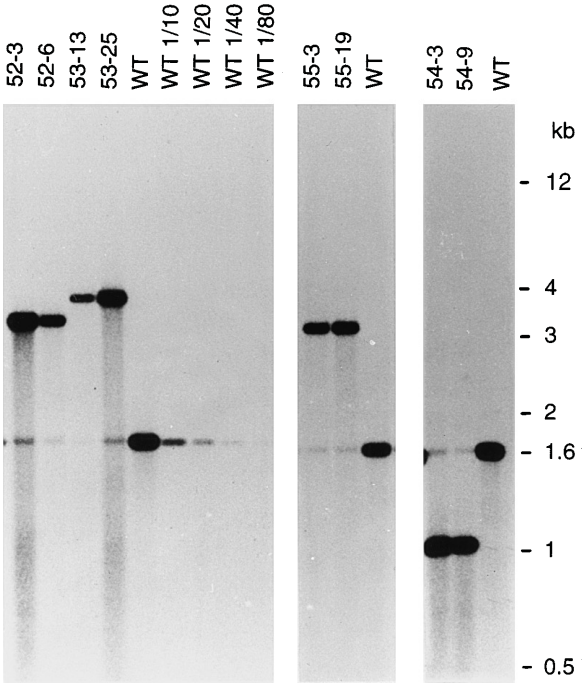

Fig. 2 Southern analysis of wild type and two mutant plants from each series. Total DNA (0.5–1 μg) was cut with *Bgl*II (52, 53 and 55 transformants) or *Xba*I (54 transformants). The blots were hybridized with the probes indicated in Fig. 1

Western analysis

The immunoblot analysis of the transformants (Fig. 3) demonstrates that the concentration of NdhH is drastically reduced in the transformants of the 52, 53, 54 and 55 series, but not in a plant that had been transformed with the control construct (56-21). As expected from the results of the Southern analysis, faint signals from *NdhH* are still visible in some mutants (e.g. 52-3, 53-13, 53-25 and 54-9), indicating expression of the wild-type gene. In contrast, subunits of both photosystems and the ATPase were found in concentrations comparable to wild type in most transformants. However, transformants 52-6 and 53-25, which also showed a lower chlorophyll/protein ratio and reduced growth (data not shown), contain significantly lower levels of PSI-D and PSII-B.

Fluorescence analysis

Determination of chlorophyll fluorescence parameters provides a convenient measure of the redox state of the plastoquinone pool (Krause and Weis 1991). Wild-type and transformed plants were analysed by recording their fluorescence induction curves with the PAM fluorometer. Both wild type and mutants displayed the typical rapid rise in fluorescence (Fig. 4). In general the transformants showed a slight increase in the level of minimal fluorescence F_o and a higher level of steady-state fluorescence, but the level of variable fluorescence F_v was only altered slightly, from 0.82 in the wild type to 0.71–0.80 in the transformants (data not shown). A significant difference between the wild type and the transformants was observed after the end of actinic illumination. In the wild type, fluorescence increased transiently, for about 2–4 min above the F_o level, while in the transformant 53-13 this rise was absent (Fig. 4). For better resolution, the transient dark-rise was recorded at a higher sensitivity (Fig. 5). In transformants of the 52 and 53 series, the transient dark-rise was almost completely abolished,

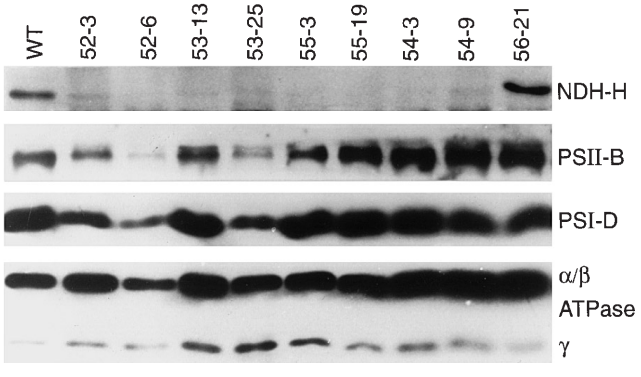

Fig. 3 Western analysis of wild type and transformed plants (5 μg protein per lane). The blots were incubated with antisera against NdhH, PSII-B, PSI-D and ATPase subunits α, β, and γ respectively, as described in Materials and methods

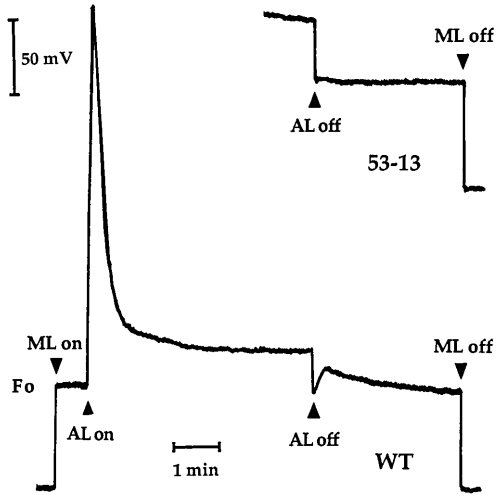

Fig. 4 Fluorescence induction curves of the wild type and the 53–13 transformant. The induction curve was recorded as described in Materials and methods. F_0, minimal fluorescence; ML, measuring light; AL, actinic light

while transformants of the 55 and 54 series displayed small transient increases in fluorescence at the end of actinic illumination. The control transformant (56-21) showed the transient dark-rise typical of the wild type, albeit at a lower level. In the wild type far red light at the end of actinic illumination (Fig. 5, WT + FR) resulted in only a slight increase in fluorescence above the F_o level (see Discussion).

Electron microscopy

Leaves from cultured plants grown on a day/night cycle of 8/16 h were harvested after 2 h of illumination and then analysed by electron microscopy. No significant differences in the stroma-grana thylakoid membrane structure were observed; however, while wild-type chloroplasts were completely devoid of starch reserves, transformants of the 52, 53 and 55 series contained starch grains (Fig. 6). Transformants of the 54 series resembled the wild type and no starch grains were found (data not shown).

Discussion

So far the NDH complex in chloroplasts has not been taken into account in the classical scheme of photosynthetic electron transport because the enzyme is present at substoichiometric amounts when compared to both photosystems and to the cytochrome b_6/f complex.

Our experiments indicate, however, that the NDH proteins are essential for plant growth and that the total deletion of respective genes is not compatible with viability. From Southern analysis of plants with defective *ndh* genes we infer that the level of wild-type *ndh* gene copies cannot be reduced below 1–5%. Interestingly, this level is the same for all four types of transformants. If we assume that a plastid without a functional *ndh* gene copy is not viable and that a typical chloroplast of a mature leaf contains about 100 copies of the chromosome (Bendich 1987), this level would reflect the presence of at least one to five copies of a wild-type chromosome in a single chloroplast. The faint signals for NdhH in the Western blots and the small amplitude of the dark-rise in some transformants indicates that even this small number of gene copies is sufficient for expression and assembly of some functional NDH complexes.

Two of our transformants (52-6 and 53-25) contain reduced amounts of chlorophyll and of both photosystems. They grow poorly, which indicates that the number of wild-type *ndh* copies is at the minimum, and that the mutagenesis has caused severe problems in plastid energy metabolism. It should be noted that all experiments were carried out on plants that had been grown in tissue culture in the presence of 2% sucrose in the medium. Preliminary experiments with plants grown in soil show that reduction of the number of *ndh* gene copies leads to leaf bleaching and yellow pigmentation in young leaves and to a drastic reduction in plant growth. Also,

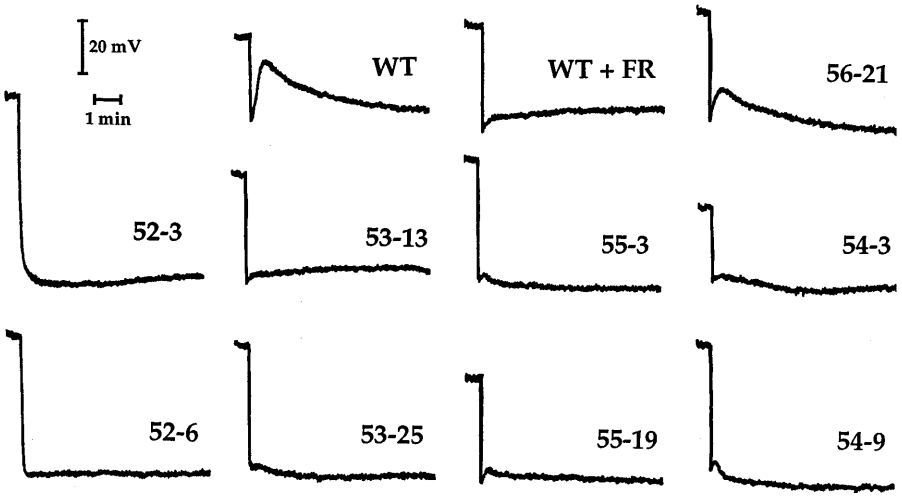

Fig. 5 Higher-resolution scans of the dark-rise of fluorescence of the wild type and two transformants from each series, as well as of a plant transformed with the control construct (56-21). In a control experiment with the wild type, far-red light was applied immediately at the end of actinic illumination (WT + FR)

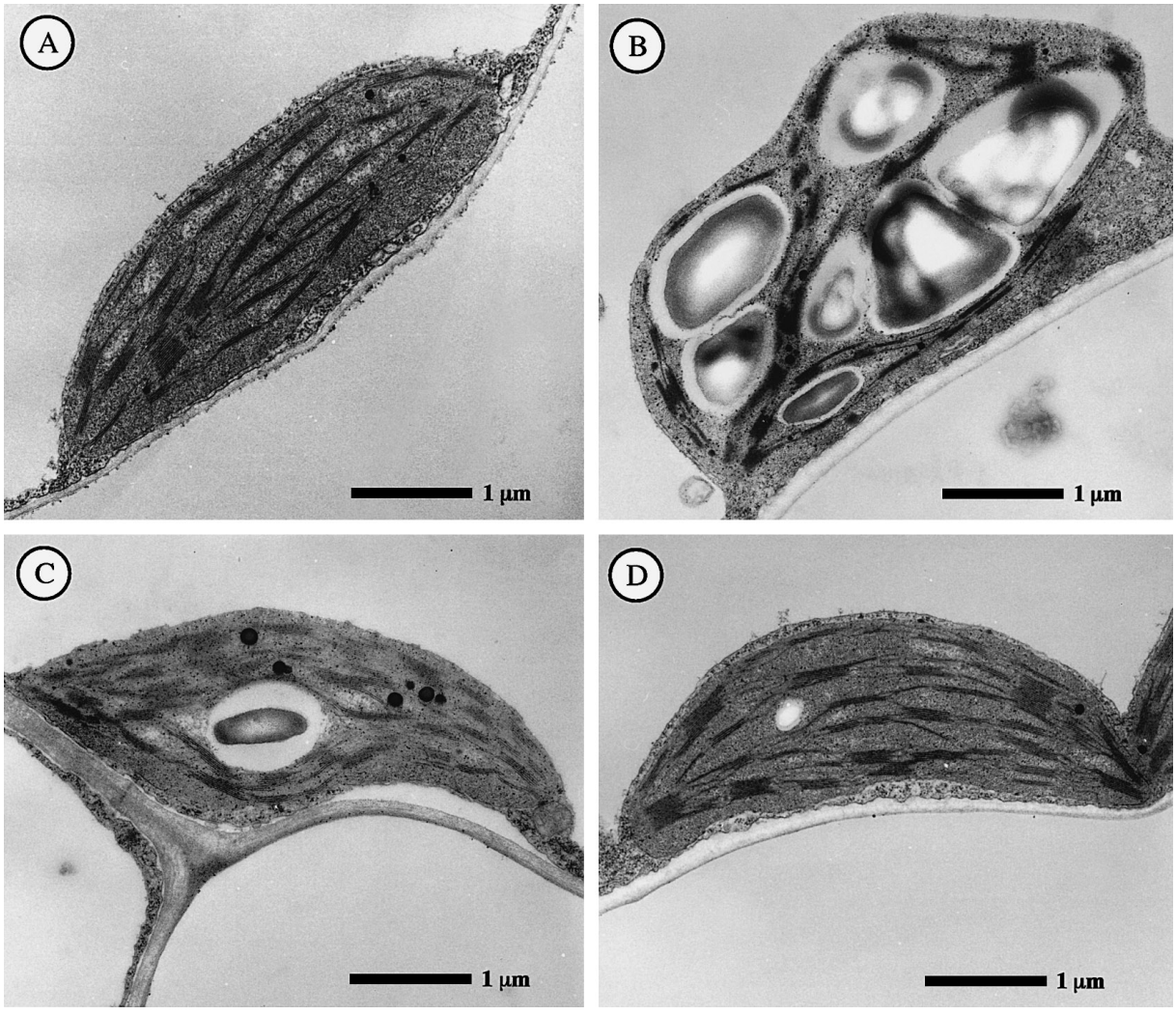

Fig. 6 A–D Electron micrographs of chloroplasts of the wild type (**A**) and the transformants 52-6 (**B**), 53-13 (**C**) and 55-19 (**D**)

abnormalities in flower development and reduced fertility were observed (manuscript in preparation).

An analysis of chlorophyll fluorescence of the transformants demonstrates that the plants exhibit a characteristic phenotype. After the end of actinic illumination, fluorescence in the wild type, but not in the transformants, increases for a short period. This dark-rise reflects a transient reduction of plastoquinone and can be quenched by activating photosystem I with far-red light, whereby electrons are removed from the plastoquinone pool. The experiments described in this paper clearly demonstrate that the transient dark-rise in fluorescence requires a functional NDH complex. Presumably this process serves the oxidation of excess NADPH produced during the preceding period of illumination with actinic light. In accordance with this explanation, studies with intact spinach chloroplasts have shown that the dark-rise can be enhanced by supplying dihydroxyacetone phosphate (DHAP) to the medium; DHAP is converted to 3-phosphoglyceric acid and NADPH inside the chloroplast (Mano et al. 1995).

Another distinct phenotype of the transformed plants is the accumulation of starch in the mutants of the 52, 53 and 55 series. This observation strongly supports the hypothesis of chlororespiration (Bennoun 1982). During the day, assimilatory starch is deposited in the chloroplast, and is degraded and exported during the night. The metabolism of the end product of sucrose degradation, glucose-1-phosphate, can occur via glycolysis or the oxidative pentose phosphate pathway (OxPPP; Ap Rees 1985). Oxidation via the OxPPP yields two molecules of NADPH, while oxidation via glycolysis yields two molecules of NADH only if it proceeds to 1,3-diphosphoglyceric acid. The NADPH produced may be used for other biosynthetic processes within the plastid, but if surplus reduction equivalents are produced they need to be oxidized in order to keep the cycle operating. Thus it is conceivable that starch degradation is blocked when the NDH complex is inactivated by mutation.

In our experiments six different *ndh* genes were affected by the mutations. Since it is not possible to de-

termine the number of wild-type gene copies still present in a single chloroplast, it is difficult to correlate the effect of a specific mutant construct with the structure of the NDH complex. Moreover, since the genes *ndhCKJ* and *ndhHAI* are part of large operons (Matsubayashi et al. 1987; Kanno and Hirai 1993), it is likely that not only the deletion but also the disruption of the reading frames by the insertion of the *16S-aadA* cassette causes a mutant phenotype. Therefore transformants of the 52 and 53 series show the strongest effects on the dark-rise of chlorophyll fluorescence and on starch accumulation. In contrast, mutagenesis of *ndhJ* (55 series), which is the last gene of the *ndhCKJ* operon, may have led to a partially functional NDH complex as indicated by a small dark-rise observed in this series. Surprisingly, plastids of the transformants of the 54 series also display a small dark-rise in fluorescence and contain no starch. Since this mutation (deletion of subunit NdhI of the peripheral fragment and NdhA of the membrane arm) should cause the most severe effect, these transformants probably have found a way to circumvent the deletion. A possible mechanism by which this might have occured is an increase in the total number of chromosome copies per chloroplast. Indeed, plastids from these mutants seem to contain an increased number of weakly staining areas which are believed to contain DNA (manuscript in preparation)

Conclusion

Our experiments demonstrate the suitability of plastome transformation for the analysis of plastid gene functions in higher plants. We have shown that mutagenesis of *ndh* genes affects photosynthetic electron flow. Also, carbon metabolism in the chloroplast is affected. This may make it necessary to modify the generally accepted scheme of photosynthetic electron transport. The experiments indicate that one function of the NDH complex lies in the oxidation of surplus reduction equivalents, supporting the view that the enzyme plays a role in chlororespiration. At least 1 to 5% of wild-type plastome copies were present in all transformants, even after prolonged culture in the presence of selection pressure. This underlines the importance of the NDH complex for plant growth and metabolism

Acknowledgements This research was supported by the Deutsche Forschungsgemeinschaft by grants to H. U. K. (A2/SFB 184) and K. S. (A2/SFB 189)

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