Implication du complexe Ndh dans le transfert cyclique des électrons autour du PS I.

Nous avons montré lors du chapitre précédent que les plantes transplastomiques dont le complexe Ndh n'est pas fonctionnel présentent un retard de croissance et une inhibition de l'assimilation photosynthétique dans des conditions où la disponibilité en CO_2 est limitée. Nous avons émis l'hypothèse que le complexe Ndh est impliqué dans des processus bioénergétiques capables de fournir de l'ATP, probablement via les photophosphorylations cycliques. Cet apport en ATP supplémentaire serait particulièrement utile dans des conditions, comme les conditions photorespiratoires, où la demande en ATP est forte.

Dans la suite de notre travail, nous avons cherché à mettre en évidence un rôle du complexe Ndh dans le transfert cyclique des électrons autour du PSI. Nous avons utilisé l'antimycine A, un composé connu pour inhiber le transfert cyclique et la fixation du CO_2 sur des chloroplastes isolés (Herber et al., 1978; Woo et al., 1983) ou des protoplastes (Furbank et Horton, 1987). Nous avons étudié l'effet de l'antimycine A en traitant des disques foliaires de tabac débarrassés de leur épiderme inférieur (voir l'article joint Joët et al. (2001) Plant Physiology, 125: 1919-1929). Nos expériences ont permis de montrer que l'inhibition des capacités photosynthétiques par l'antimycine A est dépendante des conditions photorespiratoires et donc de la demande en ATP. Le mutant déficient en complexe Ndh est beaucoup plus sensible à l'antimycine A que les plantes sauvages, la photosynthèse étant également inhibée dans des conditions non photorespiratoires où la demande en ATP est faible. De ces expériences, nous avons conclu à l'existence de deux voies de transfert cyclique des électrons autour du PSI *in vivo*. L'une de ces voies impliquerait le complexe Ndh et l'autre voie, sensible à l'antimycine A, impliquerait la FQR.

Toutefois, jusqu'à présent de faibles activités de transfert cyclique des électrons autour du PSI ont été détectées *in vivo* par les techniques de spectrométrie photoacoustique ou par

l'étude des cinétiques de re-réduction du P_{700}^{+} (Herbert et al., 1990 ; Malkin et al., 1992 ; Havaux, 1991). Certains auteurs ont proposé que le transfert cyclique des électrons est contrôlé par l'état rédox des transporteurs d'électrons entre les deux photosystèmes ou par l'état rédox du pool stromal de NADPH (Arnon et Chain, 1979 ; Ziem-Hanck et al., 1980 ; Takahama et al., 1981 ; Hosler et Yocum, 1987). Nous avons émis l'hypothèse que ce type de régulation pouvait intervenir *in vivo*, l'activité chlororespiratoire étant susceptible de contrôler l'état rédox des transporteurs d'électrons entre les deux photosystèmes. Pour tester cette hypothèse, nous avons étudié l'effet de faibles concentrations en oxygène sur l'induction de la photosynthèse (voir l'article joint Joët el al. (1998) G. Garab (ed.), Photosynthesis : Mechanisms and effects, Vol III, 1967-1970) et sur les stockages photochimiques liés à l'activité du transfert cyclique des électrons autour du PS I (voir l'article joint Joët et al., soumis). Ces mesures nous ont permis de montrer de fortes activités de transfert cyclique en conditions de microaérobie et de confirmer l'implication du complexe Ndh dans une des voies de transfert cyclique.

A. Increased sensitivity of photosynthesis to antimycin A induced by inactivation of the chloroplast *ndhB* gene. Evidence for a participation of the NADH-dehydrogenase complex to cyclic electron flow around photosystem I

Joët et al. (2001) *Plant Physiology*, **125** : 1919-1929.

Increased Sensitivity of Photosynthesis to Antimycin A Induced by Inactivation of the Chloroplast *ndhB* Gene. Evidence for a Participation of the NADH-Dehydrogenase Complex to Cyclic Electron Flow around Photosystem I[1]

Thierry Joët, Laurent Cournac, Eva M. Horvath[2], Peter Medgyesy[3], and Gilles Peltier*

Commissariat à l'Energie Atomique, Cadarache, Laboratoire d'Ecophysiologie de la Photosynthèse, Département d'Ecophysiologie Végétale et Microbiologie, Bât. 161, F–13108 Saint-Paul-lez-Durance, France (T.J., L.C., G.P.); and Biological Research Center, Hungarian Academy of Science, P.O. Box 521, H–6701 Szeged, Hungary (E.M.H., P.M.)

Tobacco (*Nicotiana tabacum* var Petit Havana) *ndhB*-inactivated mutants (*ndhB*$^-$) obtained by plastid transformation (E.M. Horvath, S.O. Peter, T. Joët, D. Rumeau, L. Cournac, G.V. Horvath, T.A. Kavanagh, C. Schäfer, G. Peltier, P. Medgyesy-Horvath [2000] Plant Physiol 123: 1337–1350) were used to study the role of the NADH-dehydrogenase complex (NDH) during photosynthesis and particularly the involvement of this complex in cyclic electron flow around photosystem I (PSI). Photosynthetic activity was determined on leaf discs by measuring CO_2 exchange and chlorophyll fluorescence quenchings during a dark-to-light transition. In the absence of treatment, both non-photochemical and photochemical fluorescence quenchings were similar in *ndhB*$^-$ and wild type (WT). When leaf discs were treated with 5 μM antimycin A, an inhibitor of cyclic electron flow around PSI, both quenchings were strongly affected. At steady state, maximum photosynthetic electron transport activity was inhibited by 20% in WT and by 50% in *ndhB*$^-$. Under non-photorespiratory conditions (2% O_2, 2,500 μL L^{-1} CO_2), antimycin A had no effect on photosynthetic activity of WT, whereas a 30% inhibition was observed both on quantum yield of photosynthesis assayed by chlorophyll fluorescence and on CO_2 assimilation in *ndhB*$^-$. The effect of antimycin A on *ndhB*$^-$ could not be mimicked by myxothiazol, an inhibitor of the mitochondrial cytochrome bc_1 complex, therefore showing that it is not related to an inhibition of the mitochondrial electron transport chain but rather to an inhibition of cyclic electron flow around PSI. We conclude to the existence of two different pathways of cyclic electron flow operating around PSI in higher plant chloroplasts. One of these pathways, sensitive to antimycin A, probably involves ferredoxin plastoquinone reductase, whereas the other involves the NDH complex. The absence of visible phenotype in *ndhB*$^-$ plants under normal conditions is explained by the complement of these two pathways in the supply of extra-ATP for photosynthesis.

During oxygenic photosynthesis of C_3 plants, both photosystem II (PSII) and photosystem I (PSI) cooperate to achieve $NADP^+$ reduction using water as an electron donor and generate a trans-membrane proton gradient driving ATP synthesis. Although $NADP^+$ reduction is recognized to be dependent on the activity of both photosystems through electron transport reactions of the "Z" scheme (Hill and Bendall, 1960; Redding et al., 1999), it has early been reported from studies on isolated thylakoids that ATP could be produced by the sole PSI through cyclic electron transfer reactions (Arnon, 1959). The cyclic electron flow around PSI has been extensively studied in thylakoids and/or chloroplasts of C_3 plants (for review, see Fork and Herbert, 1993; Bendall and Manasse, 1995). This mechanism has been suggested to provide ATP for a variety of cellular processes, including stress adaptation (Havaux et al., 1991) and CO_2 fixation (Furbank and Horton, 1987; Herbert et al., 1990). During photosynthetic CO_2 fixation, both NADPH and ATP are used to regenerate ribulose-1,5-bisphosphate and allow functioning of the photosynthetic carbon reduction cycle (Calvin cycle). In the absence of Q cycle, when one NADPH is produced by linear electron transport reactions, four H^+ are released in the lumen. If we consider that translocation of three H^+ is required for the synthesis of one ATP (Hangarter and Good, 1982), the ATP to NADPH ratio produced during linear electron transport would be around 1.33. However in C_3 plants, the ATP to NADPH ratio required for CO_2 fixation has been reported to vary from 1.5 to 1.66, depending on the activity of photorespiration (Osmond, 1981). Insufficient ATP consequently would be synthesized for carbon reduction (Heber and Walker, 1992) and different mechanisms, including cyclic electron flow around PSI, have been proposed to fulfill this func-

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[2] Present address: Department of Genetics, Trinity College, University of Dublin, Dublin 2, Ireland.

[3] Present address: Department of Biology, National University of Ireland, Maynooth, County Kildare, Ireland.

* Corresponding author: e-mail gilles.peltier@cea.fr; fax 33–4–42256265.

tion. A central question is the possible involvement of the Q-cycle, a cyclic electron flow inside the cytochrome (cyt) b_6/f complex (Mitchell, 1975, 1977) able to translocate additional H^+ and therefore provide extra ATP. However, the obligatory character or the flexibility of the Q-cycle during CO_2 fixation remains a matter of debate (Davenport and McCarty, 1984; Ort, 1986; Heber and Walker, 1992; Cramer et al., 1996). Other mechanisms, like cooperation with mitochondrial respiration (Krömer, 1995; Hoefnagel et al., 1998) and Mehler reactions (also known as water-water cycle) (Schreiber and Neubauer, 1990) have also been suggested to re-equilibrate the chloroplastic ATP to NADPH ratio by generating extra-ATP, but their contribution during CO_2 fixation remains to be established.

Cyclic electron transfer reactions around PSI have been early reported to be inhibited by antimycin A (Tagawa et al., 1963). Most studies concluding to an involvement of cyclic electron flow during photosynthesis in C_3 plants have been based on the effect of this compound on photosynthetic reactions such as photophosphorylation (Cleland and Bendall, 1992), rereduction of $P700^+$ (Scheller, 1996), CO_2-dependent O_2 evolution (Furbank and Horton, 1987), $^{14}CO_2$ fixation (Heber et al., 1978; Woo, 1983), or chlorophyll fluorescence (Ivanov et al., 1998). It was suggested that inhibition of photosynthetic reactions by antimycin A was related to the involvement of an antimycin A-sensitive ferredoxin plastoquinone reductase activity in cyclic reactions (Moss and Bendall, 1984; Cleland and Bendall, 1992). The actual efficiency of cyclic electron flow in vivo during photosynthesis of C_3 plants is still unclear (Heber et al., 1995a). Photoacoustic measurements, which allow a direct and quantitative measurement of energy storage by cyclic electron flow around PSI in vivo, have been used to show the existence of cyclic electron transfer reactions in C_4 plants, algae, and cyanobacteria (Herbert et al., 1990). However, until now, this technique failed to show significant cyclic activity in C_3 plants (Herbert et al., 1990; Malkin et al., 1992). For the unicellular alga *Chlamydomonas reinhardtii*, Ravenel et al. (1994), by studying the effect of antimycin A and of different inhibitors on photoacoustic measurements, proposed that two pathways are operating in vivo around PSI. One pathway was shown to be sensitive to antimycin A, whereas the other would involve a NAD(P)H dehydrogenase activity (Ravenel et al., 1994). The existence of an antimycin A-insensitive cyclic electron pathways around PSI was also proposed in C_3 plants from experiments performed in vitro (Hosler and Yocum, 1987; Scheller, 1996).

The plastid genome of higher plants contains genes encoding subunits homologous to the proton-pumping NADH: ubiquinone oxidoreductase, a component of the mitochondrial respiratory chain (Ohyama et al., 1986; Shinozaki et al., 1986). An NADH-dehydrogenase complex (NDH) containing some *ndh* gene products recently has been purified from pea and barley thylakoid membranes (Sazanov et al., 1998; Quiles et al., 2000). To elucidate the function of the plastidial NDH complex in C_3 plants, *ndh* genes were inactivated by chloroplast transformation of tobacco (*Nicotiana tabacum* var Petit Havana) in different laboratories. Inactivation of *ndhB*, *ndh*C, *ndh*K, and *ndh*J genes revealed that the NDH complex is dispensable for plant growth under standard conditions (Burrows et al., 1998; Shikanai et al., 1998; Horvath et al., 2000). The absence of a transient postillumination increase in chlorophyll fluorescence in all NDH-inactivated plastid transformants led to conclude that the NDH complex is involved in the dark reduction of the plastoquinone (PQ) pool, this phenomenon being considered as an after effect of cyclic electron flow around PSI (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Horvath et al. (2000) recently reported an enhanced growth retardation in $ndhB^-$ inactivated plants when grown under controlled conditions of decreased air humidity. Under such conditions, moderate stomatal closure lowers internal CO_2 concentration, thus increasing the activity of photorespiration. It was proposed by the authors that the NDH complex is involved, via the activity of cyclic electron flow around PSI, in the production of extra-ATP necessary to fulfill the higher ATP demand occurring under photorespiratory conditions.

The aim of the present work is to further study the physiological function of the plastidial NDH complex in plants. For this purpose, we investigated the effect of antimycin A on *ndhB* inactivated plants (Horvath et al., 2000). We observe an increased sensitivity to antimycin A of $ndhB^-$ mutants, this effect being dependent on the photorespiration rate. We conclude to the existence of two cyclic electron transport pathways operating in vivo around PSI, both of these pathways participating to the supply of extra-ATP for photosynthesis.

RESULTS

Chlorophyll fluorescence was measured during dark to light transitions on stripped tobacco leaf discs of WT and $ndhB^-$. In the dark, the nonactinic modulated light allows to determine the F_0 fluorescence level (Fig. 1). Maximal efficiency of PSII was 0.78 ± 0.02 and was similar in both WT and $ndhB^-$. Upon illumination (230 μmol photons m^{-2} s^{-1}), the chlorophyll fluorescence level transiently increased in both WT and $ndhB^-$ and then rapidly decreased due to both photochemical and non-photochemical quenchings. Saturating pulses were used to evaluate photochemical (qP) and non-photochemical (qN) quenching values (Fig. 2). Under illumination, chlorophyll fluorescence induction was similar in WT and $ndhB^-$ (Figs. 1A and 2A). However, a significant difference between the WT and $ndhB^-$ was observed

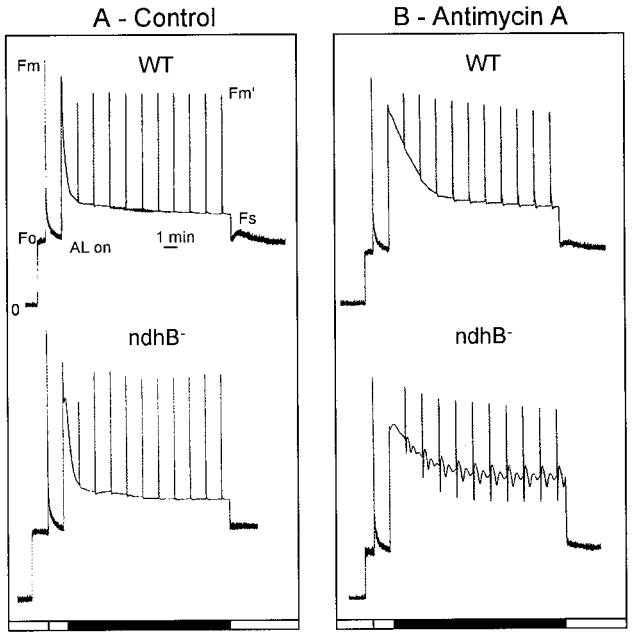

Figure 1. Effect of antimycin A on chlorophyll fluorescence induction curves measured on stripped leaf discs of WT and *ndhB*$^-$ tobacco plants. A, WT and *ndhB*$^-$ in the absence of treatment. B, WT and *ndhB*$^-$ treated with 5 μM antimycin A. Light intensity was 230 μmol photons m^{-2} s^{-1} during the actinic light period (shown by a black box on the *x* axis).

when turning the light off (Fig. 1A). In the WT, a transient increase in the fluorescence level was observed before reaching the F_0 level, this effect being absent in *ndhB*$^-$. This confirms recent work reporting that the postillumination fluorescence rise is absent in *ndh* inactivated mutants (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). Figure 2A shows that qP rapidly increased during the first 2 min of illumination in both WT and *ndhB*$^-$ before reaching progressively a plateau. On the other hand, qN transiently increased after switching on the light and then decreased to a plateau. No significant differences could be detected in qP and qN values between WT and *ndhB*$^-$.

After treatment with antimycin A (Fig. 1B) the maximal efficiency of PSII was not altered (F_v/F_m = 0.78 ± 0.02 in both WT and *ndhB*$^-$), but the chlorophyll fluorescence transient observed following illumination was strongly affected. The fluorescence level F_s of *ndhB*$^-$ remained at a higher value than the WT and quite noticeably, saturating pulse induced strong oscillations of F_s in *ndhB*$^-$ leaves (Fig. 1B). The period of the oscillations was between 20 and 30 s. Quenching analysis was performed during oscillations by illuminating the sample with saturating pulses. Determination of qN and qP values clearly show that oscillations in F_s (Fig. 3A) are due to changes in qP, qN values remaining remarkably stable (Fig. 3B).

As shown in Figure 2B, the transient increase in qN was suppressed by antimycin A in both WT and *ndhB*$^-$, and the qN value progressively reached a level close to that measured in Figure 2A. The establishment of qP was delayed by the antimycin A treatment in WT, but qP finally reached a plateau close to that measured in the absence of antimycin A. The effect of antimycin A was more drastic on *ndhB*$^-$. At steady state, differences between fluorescence induction curves of WT and *ndhB*$^-$ treated by antimycin A (Fig. 1B) were mainly explained by differences in qP values, qN values being less affected (Fig. 2B). Similar effects were observed at high light intensity (1,250 μmol photons m^{-2} s^{-1}; data not shown).

Antimycin A is known to inhibit cyclic electron transport in chloroplasts (Woo, 1983) but is also a potent inhibitor of the cytochrome bc_1 complex in mitochondria. To determine whether the effect of

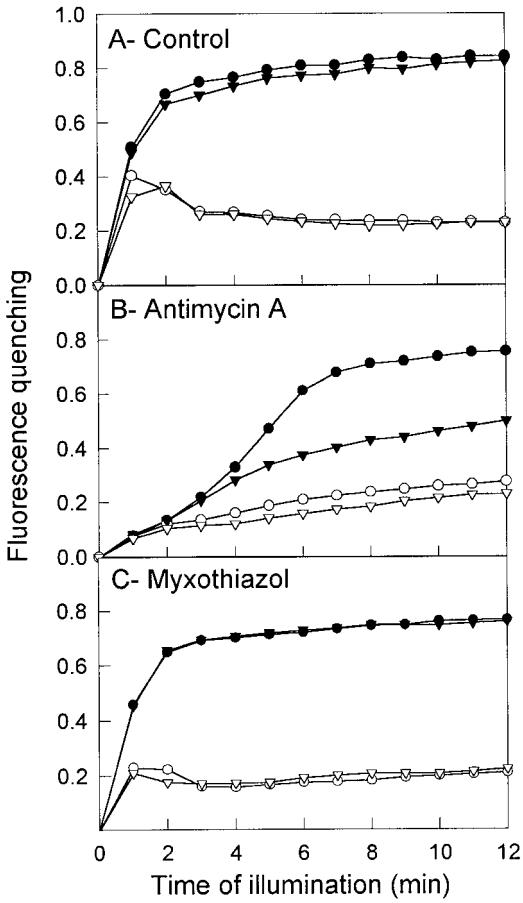

Figure 2. Effect of antimycin A and myxothiazol on photochemical (qP) and non-photochemical (qN) quenchings values during a light to dark transition in WT and *ndhB*$^-$ tobacco. Fluorescence quenchings were measured on stripped leaf discs: A, control; B, treated with 5 μM antimycin; C, treated with 10 μM myxothiazol. WT, ● and ○; *ndhB*$^-$ mutant, ▼ and ▽. Light intensity was 230 μmol photons m^{-2} s^{-1}. Fluorescence levels F_t, F_m, $F_{m'}$, F_0, and $F_{0'}$ were measured during illumination and were used to determine photochemical (qP, black symbols) and non-photochemical (qN, white symbols) quenchings.

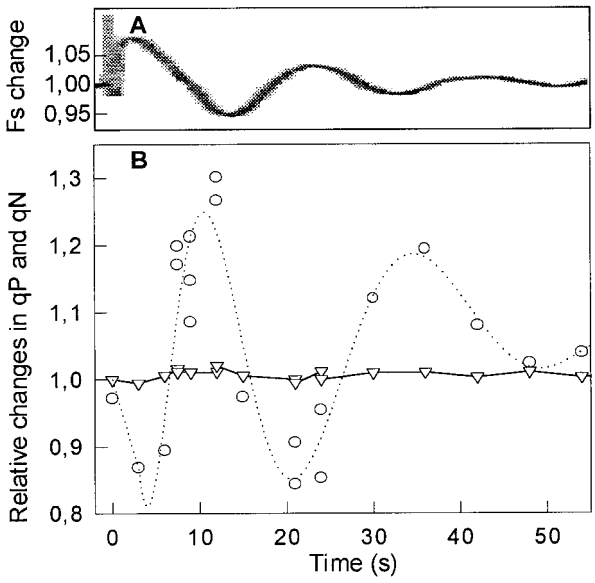

Figure 3. Quenching analysis of light pulse-induced chlorophyll fluorescence oscillations in antimycin A-treated *ndhB*$^-$ leaves. A, Oscillations of F_s induced by a saturating light pulse. B, qP (○) and qN (▽) values were determined during oscillations of F_s by illuminating the sample at various times by a second saturating light pulse. F_s, qP, and qN values are expressed relatively to initial values. Experimental conditions are similar to the experiment shown in Figure 1B.

antimycin A could be attributed to an effect on chloroplasts or mitochondria, we used myxothiazol, another cytochrome bc_1 inhibitor that inhibits the mitochondrial complex by interacting with cytochrome *b* at a different site (von Jagow and Engel, 1981; Thierbach and Reichenbach, 1981). We found that, in contrast to the effect of antimycin A, myxothiazol had no significant effect on qP and a slight inhibitory effect on qN induction curves, this effect being similar in WT and *ndhB*$^-$ (Fig. 2C).

The light saturation of the photosynthetic electron transport was determined at steady state (Fig. 4). In the absence of treatment, both WT and *ndhB*$^-$ leaf discs showed similar light saturation curves. In WT, the antimycin A treatment decreased the maximum photosynthetic electron transport activity by approximately 15%. This decrease was much more pronounced in *ndhB*$^-$ leaf discs (approximately 50%). At high-light intensities, the effect of myxothiazol on WT photosynthesis was similar to the effect of antimycin A (approximately 15% inhibition). However, in contrast to antimycin A, myxothiazol did not generate an additional effect on *ndhB*$^-$ leaf discs photosynthesis (Fig. 4B). It is interesting that at low and medium light intensities (less than 500 μmol photons m^{-2} s^{-1}), myxothiazol had no significant effect on the photosynthetic electron transport activity (Fig. 4B), whereas an inhibitory effect of antimycin A was observed on WT samples under these conditions (Fig. 4A). We checked that under our experimental conditions both antimycin A and myxothiazol inhibited the cyt *bc* pathway of mitochondrial respiration. Respiration rates were measured as CO_2 production in the dark on control leaf discs and on leaf discs treated with respiratory inhibitors (Fig. 5). Antimycin A or myxothiazol alone inhibited the respiration rate by respectively 34% and 16%. Salicyl hydroxamic acid (SHAM), an inhibitor of the alternative oxidase, had almost no effect on respiration. Simultaneous addition of myxothiazol and SHAM or antimycin A and SHAM inhibited the respiration rate by 73% and 83%, respectively, thus showing the participation of the alternative oxidase pathway. Note that when added in the presence of antimycin A, myxothiazol, or alone, SHAM had no effect on chlorophyll fluorescence induction curves (data not shown). As antimycin A and myxothiazol similarly inhibited mitochondrial respiration, we conclude that the additional effect of antimycin A on *ndhB*$^-$ leaf discs compared with the WT is not related to a mitochondrial inhibition but rather linked to the inhibition of a chloroplast process.

The effect of antimycin A on photosynthesis was investigated under different photorespiratory conditions, by simultaneously measuring at steady-state

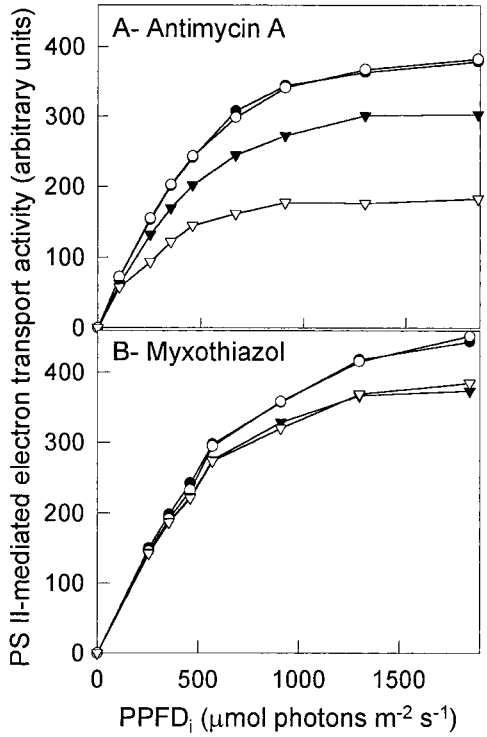

Figure 4. Photosynthetic electron flow estimated as ($\Delta F/F_{m'} \times PPFD_i$) as a function of light intensity (incident PPFD) in stripped leaf discs treated with antimycin A or myxothiazol. Stripped tobacco leaf discs were treated with 5 μM antimycin or 10 μM myxothiazol. ●, WT control; ○, *ndhB*$^-$ control; ▼, WT treated with 5 μM antimycin A (A) or 10 μM myxothiazol (B); ▽, *ndhB*$^-$ treated with 5 μM antimycin A (A) or 10 μM myxothiazol (B).

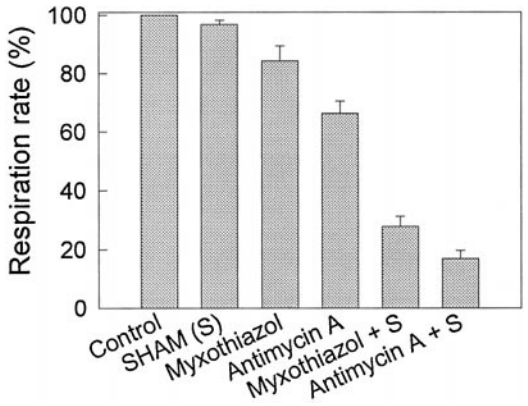

Figure 5. Inhibition of tobacco leaf discs respiration by antimycin A (5 μM), myxothiazol (10 μM), and SHAM (0.6 mM). WT tobacco leaf discs were stripped and treated with inhibitors. Respiration rates were measured by following CO_2 production in the dark. Values are the average of three independent measurements. Vertical bars represent SEs.

PSII-mediated electron transport activity and CO_2 assimilation. In air, relative inhibition by antimycin A of PSII activity was approximately 17% for the WT and 33% for ndhB$^-$ leaves (Fig. 6A). Similar effects of antimycin A were observed on CO_2 assimilation, although differences between WT and *ndhB*$^-$ appeared less obvious. Under non-photorespiratory conditions (2% [v/v] O_2, 2,500 μL L^{-1} CO_2), where the ATP demand is decreased (Osmond, 1981), photosynthetic activity of WT leaves was almost unaffected by antimycin A (less than 3% inhibition at the steady state; Fig. 6B). In contrast, in ndhB$^-$ leaves relative inhibition by antimycin A was similar to that observed in air (approximately 25% inhibition).

DISCUSSION

Involvement of the NDH Complex in Cyclic Electron Flow around PSI

We observed a sensitivity of photosynthesis to antimycin A in tobacco leaves (monitored either by chlorophyll fluorescence or gas exchange measurements), which was increased in *ndhB*$^-$ mutants. Sensitivity of the electron transport to antimycin was highest at saturating light intensity and so was the difference between WT (20% inhibition) and mutant (50% inhibition). The additional inhibitory effect observed in *ndhB*$^-$ mutants was not observed when using myxothiazol, thus showing that it is related to an inhibition of plastidial rather than mitochondrial reactions.

In addition to its well-known effect on cyclic electron flow, antimycin A has been reported to affect qN (Oxborough and Horton, 1987; Ivanov et al., 1998). In our experiments, antimycin A induced a significant delay in the establishment of qN, but the steady-state level was virtually not affected and no differences were observed between WT and *ndhB*$^-$ even at high intensities, when qN reaches its maximal values. In antimycin-treated leaves, F_s values at steady state remained higher in mutants than in WT, whereas the qN values were comparable. This higher F_s in mutants was then not related to variations in qN, but attributable to a more reduced state of the plastoquinone pool, indicating a less efficient functioning of electron acceptor reactions after PSI (Calvin cycle, etc.). We conclude that the simultaneous inhibition by antimycin A of cyclic electron flow around PSI and of NDH activity by gene inactivation leads to a reduced ability to use reducing power on the acceptor side of PSI.

Previous studies, based on the disappearance of the transient postillumination rereduction of PQ in *ndh*-inactivated mutants (Burrows et al., 1998; Cournac et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) or on a decrease of the P700$^+$ reduction rate in the dark (Burrows et al., 1998) already concluded to an involvement of the NDH complex in intersystem chain reduction and therefore to its potential implication during cyclic electron transport. It appears from our experiments that the NDH activity is involved in cyclic electron transport together with the antimycin-sensitive pathway. Since photosynthesis is only slightly inhibited by antimycin A in WT, we conclude that the NDH-mediated pathway has a sufficient efficiency to compensate for the antimycin-sensitive pathway to a large extent.

The existence of different cyclic electron pathways around PSI has previously been suggested in the literature. In spinach thylakoids, Hosler and Yocum (1985) reported the insensitivity to antimycin A of cyclic photophosphorylations measured in the presence of ferredoxin and NADP$^+$. Based on photoacoustic measurements performed in vivo in *C. reinhardtii* cells, Ravenel et al. (1994) observed that antimycin A and *N*-ethyl-maleimide could inhibit PSI energy storage in vivo when added together, these compounds having no effect when added alone. More recently, based on P700$^+$ rereduction measurements performed in barley thylakoids, Scheller (1996) proposed the existence of an antimycin-insensitive cyclic electron transport around PSI. The involvement of the NDH complex in cyclic electron flow in association with other pathways was shown in cyanobacteria (Mi et al., 1992; Yu et al., 1993) and recently suggested in higher plants from in vitro experiments performed on broken chloroplasts (Endo et al., 1998). Based on a differential sensitivity to antimycin A of PQ reduction in the WT and in a *ndhB*$^-$ mutant, these authors concluded to the existence of two pathways, one of them involving the NDH complex. All of the evidences obtained in C_3 plants are based on experiments performed on in vitro systems. Our study, performed on leaves, clearly shows the importance of cyclic pathways during photosynthesis in C_3 plants in vivo.

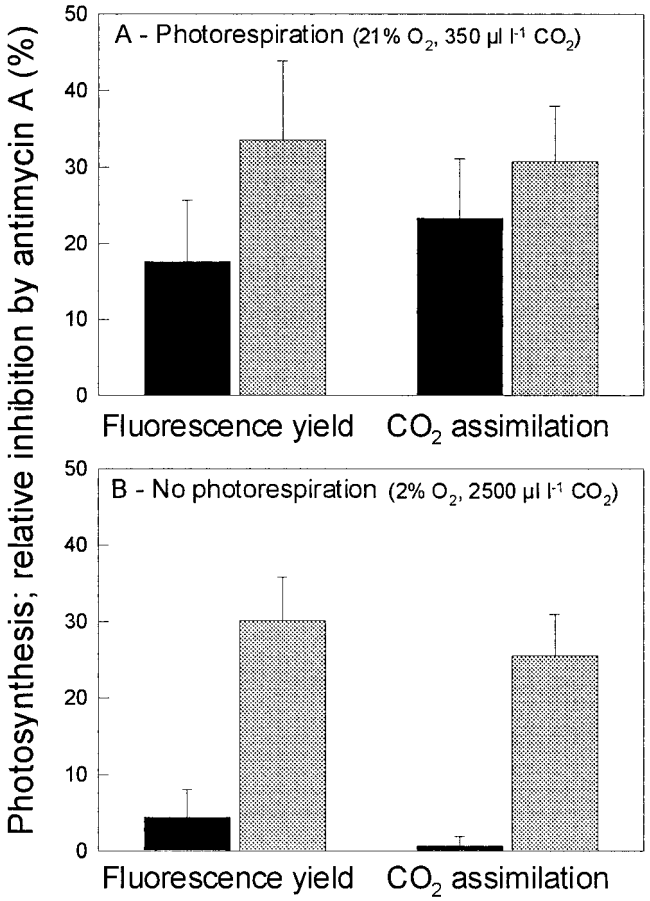

Figure 6. Inhibition of photosynthesis by antimycin A measured on stripped leaf discs of WT (black square) and *ndhB*$^-$ (gray square) mutant. Photosynthesis was measured by following the chlorophyll fluorescence yield ($\Delta F/F_{m'}$) or by measuring CO_2 assimilation rates. A treatment with 5 μM antimycin A was performed under a light intensity of 100 μmol photons m^{-2} s^{-1}. Values are the average of eight independent measurements performed on four independent experiments. Vertical bars represent SEs.

Antimycin A was found to induce strong damped oscillations of the fluorescence yield in response to saturating pulse. This effect, observed in *ndhB*$^-$ mutant leaves but not in WT, was attributed to variations in the redox level of Q_A, since no variation in qN could be detected during oscillations. Oscillations in chlorophyll fluorescence yield and in O_2 evolution rates have been previously reported to be induced by rapid changes in light intensity or gas composition (Slovacek et al., 1980). They have been proposed to result from an imbalance between ATP production (by either linear or cyclic electron transport) and ATP consumption (by photosynthetic carbon reduction or oxidation cycle) processes occurring in response to rapid changes in light intensity or gas composition (Furbank and Horton, 1987; Horton and Nicholson, 1987; Veljovic et al., 1990). Based on the effect of antimycin A on O_2 evolution and chlorophyll fluorescence observed in barley protoplasts during light transitions, and particularly on the fact that antimycin A increased the frequency of oscillations, it was concluded that cyclic electron flow is involved in the ATP balance during the early phase of illumination (Quick and Horton, 1985; Furbank and Horton, 1987). In our conditions, inactivation of the NDH complex induced strong oscillations of chlorophyll fluorescence in antimycin A-treated leaves, therefore suggesting that the NDH complex is involved in the supply of ATP for photosynthesis.

The differential effect of antimycin A on WT and *ndhB*$^-$ could be observed using antimycin A concentrations as low as 1 μM (data not shown), whereas optimal effects were obtained at 5 μM. At medium light intensities (less than 500 μmol photons m^{-2} s^{-1}) inhibition of photosynthetic electron transport by antimycin A was approximately 15%. The inhibition of photosynthesis by antimycin A measured using in vitro systems such as thylakoids or chloroplasts generally reported in the literature is much more important, generally between 50% and 80% (Tagawa et al., 1963; Mills et al., 1978; Woo, 1983; Moss and Bendall, 1984; Cleland and Bendall, 1992). We observed a much more important inhibition of the photosynthetic electron transport activity in *ndhB*$^-$ plants (approximately 50%). One possible explanation for the hypersensitivity of in vitro systems to antimycin A may be the lability of the NDH complex during organelle isolation procedures (Guedeney et al., 1996; Sazanov et al., 1998) or dysfunctioning of the NDH complex under in vitro conditions. It is interesting that Hosler and Yocum (1985) reported particular conditions where photophosphorylation measured in spinach thylakoids membranes system with ferredoxin and $NADP^+$ was not sensitive to antimycin A.

Our experiments highlight the importance of cyclic electron transport during photosynthesis in C_3 plants in vivo. This point has been a matter of debate in the last decade given that direct measurements of cyclic electron flow such as those given by photoacoustic experiments failed to detect any significant activity in C_3 plants (Heber and Walker, 1992; Bendall and Manasse, 1995). Using photoacoustic measurements on peas, Malkin et al. (1992) measured a weak cyclic activity, saturating at low-light intensity. In the same way, based on measurements of P700 rereduction, chlorophyll fluorescence, and light scattering on spinach, Heber et al. (1995a) concluded to low cyclic activity in C_3 plants, mainly restricted to the control of PSII, taking part in the complex machinery that acts to protect the photosynthetic apparatus against photo-inhibition. In contrast, and in line with our present data, a recent study by Cornic et al. (2000) based on $P700^+$ rereduction and light scattering measurements, performed on pea and spinach leaves, concluded that cyclic electron flow around PSI participates to the ATP supply during photosynthesis. The apparent discrepancy between conclusions based on photoacoustic measurements and those ob-

tained using antimycin A and/or mutations is an intriguing question that remains to be answered.

ATP Supply, Cyclic Electron Flow, and Photorespiration

At low-light intensity and under non-photorespiratory conditions, antimycin A had no significant effect on the steady-state photosynthesis rate of WT. Under such conditions, the ATP to NADPH ratio required for CO_2 fixation is only 1.5 (Osmond, 1981) and the NDH complex pathway likely provides sufficient extra-ATP to reach optimal photosynthesis rates. Under the same conditions, the significant effect of antimycin A observed in the *ndhB*$^-$ mutant (25% inhibition rate), which was not mimicked by myxothiazol, shows that under the lowest ATP demand, a minimal activity of cyclic electron flow is required. As a consequence, we conclude that the Q-cycle is not able to fully satisfy the ATP demand in these conditions. Under photorespiratory conditions, like in air, needs for extra-ATP are increased (Osmond, 1981). In such conditions, antimycin A significantly inhibited electron transport in WT and an enhanced effect was observed in *ndhB*$^-$ mutants. This likely reflects the fact that the sole NDH-mediated pathway is unable to fully satisfy the ATP demand. These interpretations are consistent with data previously reported on the same tobacco *ndhB*$^-$ mutant by Horvath et al. (2000). These authors reported significant growth retardation when growing *ndhB*$^-$ plants under CO_2 limitation occurring in response to a moderate water limitation or abscisic acid spraying. These conditions induce stomatal closure and consequently reduce internal CO_2 concentration, thus stimulating the photorespiration rate (Cornic and Briantais, 1991; Lawlor, 1995). We therefore conclude, in agreement with Horvath et al. (2000), that the NDH complex is involved in extra-ATP supply under conditions where photorespiration is high. The NDH complex recently was proposed to be involved in photoprotection (Endo et al., 1999). The sensitivity of a *ndhB*$^-$ mutant to photo-inhibition was explained by an involvement of the NDH complex in the control of electron flow through PSII, which may be mediated by pH changes. Noticeably, photorespiration has been proposed to protect C_3 plants from photooxidation and to prevent photo-inhibition (Heber et al., 1995b; Kozaki and Takeba, 1996). Therefore, a possible role of the NDH complex in producing extra-ATP necessary to sustain high photorespiration rates should also be considered to explain the higher sensitivity of *ndhB*$^-$ to photo-inhibition.

Involvement of Other Mechanisms for Extra-ATP Supply and H^+ Requirement for ATP Synthesis

One of the central questions concerning the debate about the extra-ATP supply for photosynthesis is how the cytochrome $b_{6/f}$ complex mediates the oxidation of plastoquinol. In case the Q-cycle would be obligatory during the "Z" scheme, electron shuttled back to the plastoquinone pool through the Mitchellian Q-cycle would increase the H^+ gradient and in turn form more ATP (Davenport and McCarty, 1984; Rich, 1991). If we consider that three H^+ are needed to synthesize one ATP, there would be no need for other mechanisms to supply extra-ATP for CO_2 fixation (Rich, 1988). However, if the H^+/ATP ratio is four, as reported by Kobayashi et al. (1995) and Rumberg et al. (1990), other mechanisms of extra ATP supply would be needed. Another thing to consider is that, assuming that both Suc and starch are the predominant end products of photosynthesis, there is an additional cost for CO_2 fixation of 0.17 mol ATP per mol CO_2 fixed, consumed in the formation of glycosidic bonds (Furbank et al., 1990). Results presented here provide evidence that an input from cyclic electron transport is essential to fully satisfy the ATP requirements of C_3 plants. This is in accordance with recent studies of Cornic et al. (2000) concluding to the involvement of both cyclic electron flow around PSI and of the Q-cycle for the supply of ATP.

At high-light intensity, maximum photosynthetic electron transport rates measured in WT were inhibited by approximately 20% indistinctly by antimycin or myxothiazol. This effect, which is clearly related to a mitochondrial inhibition, might be explained by a cooperation between chloroplasts and mitochondria to achieve maximal photosynthetic rates. Two mechanisms of interaction can be proposed to explain such a dependency. In the first one, Gly decarboxylation, occurring in mitochondria during the photosynthetic carbon oxidation cycle (or photorespiration), produces NADH. Inhibition of the mitochondrial respiratory chain, by preventing NADH oxidation, might explain such an inhibition. In the second one, part of reducing equivalents produced in the chloroplast during photosynthesis might be shuttled to mitochondria. After mitochondrial conversion to ATP, shuttling back to chloroplasts might participate to re-equilibrate the chloroplastic ATP to NADPH ratio. Such a mechanism was proposed by Krömer (1995), based on the inhibition of photosynthesis by mitochondrial inhibitors like oligomycin in protoplasts. Whatever the mechanism involved in this interaction, it is interesting to note that the mitochondrial contribution is almost undetectable at low light intensity (below 400 μmol photons m^{-2} s^{-1}). We therefore propose that mitochondrial contribution to ATP supply, if it occurs, acts as an ultimate mechanism, which may be used when other mechanisms such as Q-cycle, cyclic pathways are already fully engaged in ATP production.

CONCLUSION

In conclusion, differences in steady-state photosynthetic activities could be observed between WT and

ndhB$^-$ mutants when treating leaves with antimycin A, an inhibitor of cyclic electron flow around PSI. These effects are interpreted by the existence of two independent cyclic electron pathways around PSI, one pathway being sensitive to antimycin A and the other, insensitive to antimycin A, involving the plastidial NDH complex. Under non-photorespiratory conditions (CO_2-enriched air), each pathway would be able to support the extra-ATP demand of photosynthetic CO_2 fixation. Under photorespiratory conditions, like in air, the antimycin A-sensitive pathway would be able to provide sufficient extra-ATP, whereas the NDH-dependent pathway alone would be limiting CO_2 assimilation. Under high photorespiration rate (occurring for instance when stomata close in response to a water limitation) both antimycin A-sensitive pathway and NDH complex are needed to re-equilibrate the chloroplastic ATP to NADPH ratio, thus explaining why *ndhB*$^-$ mutants grow more slowly than WT in response to a water shortage (Horvath et al., 2000).

MATERIALS AND METHODS

Plant Material and Preparation of Leaf Samples

Wild-type tobacco (*Nicotiana tabacum* var Petit Havana) and *ndhB*-inactivated mutants (Horvath et al., 2000) were grown on compost in a phytotron (25°C day/20°C night; 12-h photoperiod) under a light fluence of 350 μmol photons m^{-2} s^{-1} supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered using a half-diluted nutritive solution (Hoagland and Arnon, 1950). Leaf discs (12-mm diameter) were sampled from 5- to 8-week-old plants. After stripping the lower epidermis, leaf samples were kept in the dark on a moist paper filter in a close Petri dish until use. Stripped tobacco leaf discs were soaked in Petri dishes containing water and inhibitors. Times of incubation were respectively 20 and 90 min for photosynthesis and respiration measurements. Inhibitors were added diluted in methanol (maximal final methanol concentration was 0.5% [v/v]). Control leaf discs were soaked in Petri dishes containing water and methanol.

Chlorophyll Fluorescence Measurements

Stripped leaf discs were deposited on a wet filter and placed under a watch glass. Chlorophyll fluorescence was measured using a pulse modulated amplitude fluorometer (PAM-2,000, Heinz-Walz, Effeltrich, Germany). The optic fiber of the fluorometer was in contact with the watch glass. Non-actinic modulated light (655-nm maximum emission, 600 Hz) was used to determine the chlorophyll fluorescence level F_0. Maximum chlorophyll fluorescence level (F_m) was measured following a saturating pulse (0.8-s duration) of white light (10,000 μmol photons m^{-2} s^{-1}). For determination of qP and qN, leaf discs were exposed to actinic light and pulsed every 60 s by a 10,000-μmol photons m^{-2} s^{-1} saturating pulse (0.8-s duration) according to Quick and Stitt (1989). The maximal efficiency of PSII was determined as F_v/F_m (Kitajima and Butler, 1975). Apparent PSII activity under illumination, reflecting the electron transport rate of the photosynthetic chain, was estimated from quantum yield measurement as:

$$(F_{m'}-F_s)/F_{m'} \times PFFD_i \text{ (Genty et al., 1989)}$$

Photosynthetic CO_2 Fixation Measurements

CO_2 exchange measurements were performed using a LICOR LI-6,262 analyzer in a differential mode on stripped tobacco leaf discs kept on a moist paper filter in a homemade chamber. Chlorophyll fluorescence was measured simultaneously using a PAM-2,000 fluorometer as described above. A LICOR LI-610 portable Dew Point Generator was used to generate moist air (75% relative humidity) at a flow rate of 2 mL s^{-1}. A gas mixer (SEMY Engineering, Montpellier, France) was used to generate gas mixtures with various O_2 and CO_2 concentrations. Unless specified, CO_2 concentration was 350 μL L^{-1} and O_2 concentration was 20%. O_2 concentration was monitored by an O_2 analyzer OXOR 6 N (MAIHAK, Hamburg, Germany) and CO_2 concentration using an infrared gas analyzer (LI-6,262, LI-COR, Lincoln, NE).

Respiration Measurements

Five leaf discs (10-mm diameter) were placed on a wet filter paper in the sample chamber of a close gas circuit connected to a UNOR 6 N (MAIHAK) CO_2 analyzer. Respiration was measured at room temperature (20°C) as the CO_2 production rate in the dark.

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B. Increased sensitivity of photosynthesis to anaerobic conditions induced by targeted inactivation of *ndhB* gene

Joët el al. (1998) G. Garab (ed.), *Photosynthesis : Mechanisms and effects*, **Vol III**, 1967-1970.

INCREASED SENSITIVITY OF PHOTOSYNTHESIS TO ANAEROBIC CONDITIONS INDUCED BY TARGETED INACTIVATION OF THE CHLOROPLAST *ndh*B GENE

[1]Thierry Joët, [2]Zoran Cerovic, [1]Dominique Rumeau [1]Laurent Cournac, [1]Geneviève Guedeney, [3]Eva Horváth, [3]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]BRC, Hungarian Academy of Sciences, H-6701 Szeged, Hungary

Keywords : alternative electron transport - cyclic electron transfer - NAD(P)H-dehydrogenase - chl fluorescence induction - Mehler reaction - plastid transformation

1. Introduction

The plastid genome of higher plants has been reported to contain 11 *ndh* genes which have been recently shown to encode a NADH dehydrogenase (NDH) complex (1, 2). The NDH complex has been suggested to be involved in the reduction of plastoquinones occurring during cyclic electron flow around PSI (3) or during chlororespiration. Inactivation of *ndh* genes has been achieved by plastid transformation in tobacco (4). Inactivation of *ndh*C, *ndh*K and *ndh*J genes revealed that this complex is functional and catalyzes the post-illumination reduction of the PQ pool (4). However, a possible participation of the NDH complex during light reactions and particularly an involvement in cyclic electron flow was not shown by the authors. It has been previously reported that cyclic electron flow was stimulated under anaerobic conditions (5) due to an adequate redox poise of the NAD(P)/NAD(P)H ratio (6). Also, O_2, by accepting electrons during Mehler reactions, could drain electrons out of the cyclic electron pathway (7). We hypothesized that differences in photosynthetic activity might be observed in *ndh*-disrupted plants in anaerobiosis conditions where cyclic electron flow is expected to be stimulated. Targeted inactivation of the plastidic *ndh*B gene was achieved by plastid transformation of tobacco. Analysis of homoplasmic transformants showed that *ndh*B inactivation leads to a disappearance of the NDH complex. Although no difference in chlorophyll fluorescence was observed between control and inactivated ndhB transformants (ndhB$^-$) during a dark to light transition performed under aerobic conditions, we observed a significant reduction of electron transfer activity under anaerobic conditions in ndhB$^-$ leaves.

2. Procedure

Tobacco leaf discs (10 mm Ø) were sampled from 5 to 8 week-old plants grown in phytotron under a light intensity of 350 µE m^{-2} s^{-1}. After stripping the lower epidermis (limiting gas diffusion), leaf discs were placed in the sample compartment of a modified experimental device for chlorophyll fluorescence and blue-green fluorescence measurements (8). The pulsed excitation light was delivered by a high-power xenon flash lamp (L4633, Hamamatsu) while the actinic light was provided by an array of 7 red light emitting diodes (LED) (HLMP-8150, Hewlett Packard). The

maximum chlorophyll fluorescence level (F_m) was measured under a 0.8 s saturating pulse of white light (800 $\mu E.m^{-2}.s^{-1}$). Photochemical and non-photochemical fluorescence quenchings were determined according to (9). CO_2-free air or pure nitrogen were flushed over the sample at a rate of 45 $L.h^{-1}$.

The *ndh*B gene was inactivated 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 plastids transformants (10). Physical mapping and Southern hybridation were performed to confirm the presence of the mutation in the transformants. Control lines were generated by inserting the selectable markers alone.

3.Results

Chlorophyll fluorescence was measured during a dark to light transition under aerobic (CO_2-free air) and anaerobic (N_2) conditions on stripped leaves of wild-type (WT), control and ndhB$^-$ tobacco.

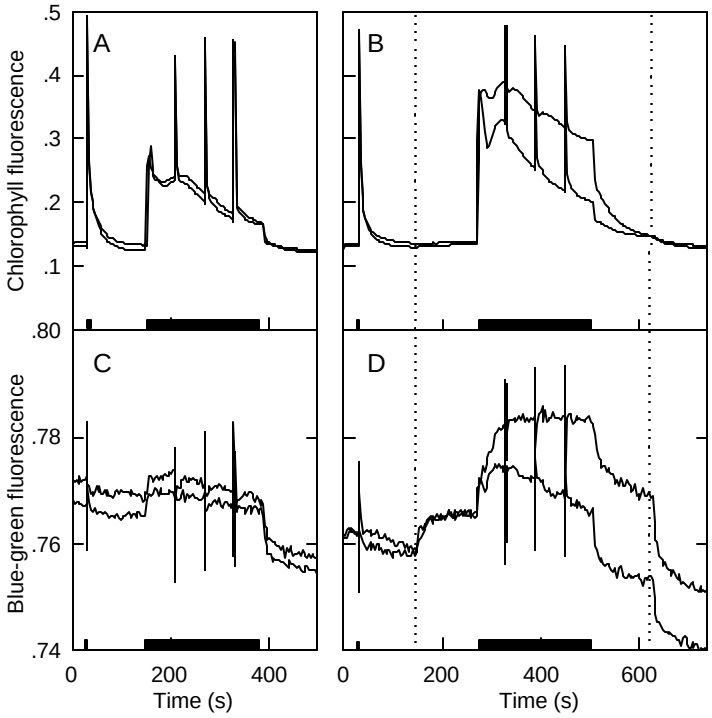

Figure 1. Chlorophyll fluorescence (A,B) and blue-green fluorescence (C,D) measured in tobacco leaf discs during a dark to light transition in the presence of CO_2-free air (A,C) or N_2 (B,D). Control and ndhB$^-$ stripped leaf discs were subjected to anaerobic conditions as indicated by dotted lines. Light intensity was 21 $\mu E.m^{-2}.s^{-1}$ (−−). Control (**thick line**), ndhB$^-$ (thin line). In (C) and (D), a 0.04 offset in the blue-green fluorescence value was applied in controls.

Control transformants and WT showed similar behaviour in all of the experiments described here (not shown). Before induction, a saturating flash was used to determine Fv/Fm the optimal quantum yield of PS II (9) which was found to be around 0.76 and was similar in control and ndhB$^-$. Under aerobic conditions, chlorophyll fluorescence transients were similar in control and ndhB$^-$ (Fig. 1A) while under anaerobic conditions a marked difference was observed (Fig 1B). Saturating flashes were used to determine photochemical and non-photochemical quenchings (Fig. 2).

Under anaerobic conditions, qP rapidly reached a plateau during the first minute of illumination, whereas qN progressively increased during at least 4 minutes. Fig. 2 shows that the difference in fluorescence transients between control and ndhB$^-$ is mainly due to a difference in qP values. Measurements of blue green fluorescence (BGF) were performed in the same experiment to determine changes in the redox state of the NAD(P)/NAD(P)H pool (8). Under aerobic conditions, BGF slightly increased upon illumination reflecting a reduction of the NAD(P) pool (Fig. 1C). No difference was observed between control and ndhB$^-$. In the dark, N_2-flushing induced a similar increase in BGF level in both samples (Fig. 1D). Illumination induced a much stronger increase of BGF in ndhB$^-$ than in control leaves, although the initial increase rate was identical. Quite interestingly, the BGF level was stable during illumination in ndhB$^-$ while it decreased in the control.

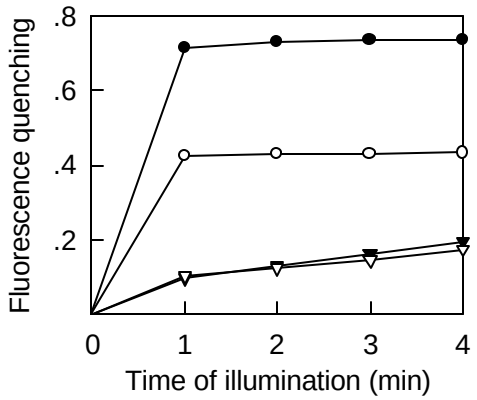

Figure 2. Effect of anaerobiosis on evolution of photochemical (qP) and non-photochemical (qN) quenchings during a dark to light transition in control and ndhB$^-$ tobacco leaf discs. Samples were flushed with N_2 for 2 min before the light was switched on. Ft, Fm, Fm', F0 and F_0' were measured during illumination and were used to determine photochemical quenching (qP) in control (●) and ndhB$^-$ (○), and non-photochemical quenching (qN) in control (♦) and ndhB$^-$ (∇). Light intensity was 25 µE.m^{-2}.s^{-1}.

Effective PS II quantum yield under illumination was estimated from (Fm'-Ft)/Fm' (9) and was measured at different light intensities (Fig. 3A). PS II quantum yield was almost stable in both control and ndhB$^-$ under aerobic conditions. However, under anaerobic conditions, PS II efficiency of both control and ndhB$^-$ gradually decreased, the decrease being much more pronouced in ndhB$^-$. Apparent PS II activity, deduced from the fluorescence yield and estimated from (Fm'-Ft)/Fm' x PPFDi is shown in Fig. 3B. This graphical representation allows to visualize the light saturation of PS II activity. Under anaerobic conditions, photosynthetic electron transport activity of ndhB$^-$ reached a plateau, which was about 40% lower than in the control.

4. Discussion

While no difference in fluorescence induction was observed between control and ndhB$^-$ under aerobic conditions, a clear difference was revealed under anaerobiosis, which was due to a reduced electron transport activity in ndhB$^-$. Under anaerobic conditions (N_2), electron acceptors should be less available, both the Calvin cycle and Mehler reaction activity being strongly reduced (lack of CO_2 and O_2). However, some electron transport activity saturating at low light was observed, suggesting the presence of an electron acceptor. The acceptor might be O_2 produced at PS II, nitrite or an reutilization of NAD(P)H by a low level of Calvin cycle activity. This electron transport activity was more important in control than it ndhB$^-$ (Fig. 3B), At the same time, the redox state of the NAD(P)/NAD(P)H pool initially increased at a similar rate both in ndhB$^-$ and control, but reached a much higher value in ndhB$^-$, suggesting that NAD(P)H is recycled more efficiently in the control.

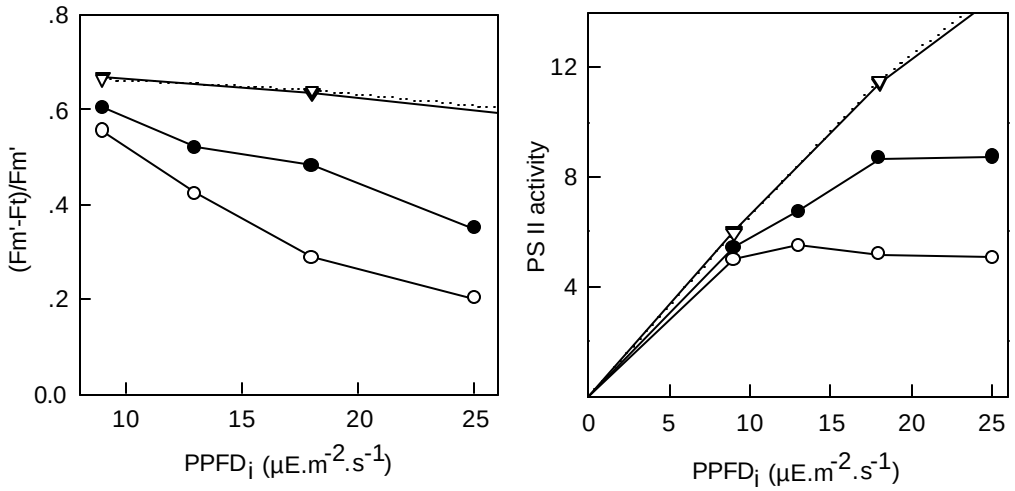

Figure 3. A : PS II quantum yield estimated from ((Fm'-Ft)/Fm') as a function of light (incident photosynthetic photon flux density, $PPFD_i$) in stripped tobacco leaf discs. For anaerobic conditions leaves were flushed with N_2 in the dark during 2 min before light was turned on. Photosynthetic activity was measured 4 min after turning on the light. Anaerobic conditions: control (♦), ndhB$^-$ (●); Control and ndhB- under CO_2-free air (∇;○). **B :** Photosynthetic flow estimated from ($\Delta F/Fm'$ x $PPFD_i$) as a function of light intensity. Anaerobic conditions: Control (♦);ndhB$^-$ (●);Control and ndhB- under CO_2-free air (∇;○).

We interpret these data through an involvement of the NDH complex in cyclic electron flow around PS I. When O_2 is lacking, cyclic electron flow activity would be necessary to activate the reutilization of NAD(P)H, may be through the establisment of a proton gradient. In the presence of O_2, this activation would be achieved by pseudocyclic activity (Mehler reaction), explaining the absence of difference between control and ndhB$^-$. We conclude that the NDH complex is involved in cyclic electron flow. However, under aerobic conditions, pseudocyclic electron flow could replace cyclic electron flow activity which might explain the lack of phenotype in *ndh* inactivated plants.

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C. Cyclic electron flow around PS I in C_3 plants: *in vivo* control by the redox state of chloroplasts and involvement of the Ndh complex

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Cyclic Electron Flow around Photosystem I in C_3 Plants. In Vivo Control by the Redox State of Chloroplasts and Involvement of the NADH-Dehydrogenase Complex

Thierry Joët, Laurent Cournac, Gilles Peltier, and Michel Havaux*

Commissariat à l'Energie Atomique/Cadarache, Département d'Ecophysiologie Végétale et de Microbiologie, Laboratoire d'Ecophysiologie de la Photosynthèse, Unité Mixte de Recherche 163 Centre National de la Recherche Scientifique/Commissariat à l'Energie Atomique, Univ-Méditerranée/Commissariat à l'Energie Atomique 1000, F–13108 Saint-Paul-lez-Durance, France

Cyclic electron flow around photosystem (PS) I has been widely described in vitro in chloroplasts or thylakoids isolated from C_3 plant leaves, but its occurrence in vivo is still a matter of debate. Photoacoustic spectroscopy and kinetic spectrophotometry were used to analyze cyclic PS I activity in tobacco (*Nicotiana tabacum* cv Petit Havana) leaf discs illuminated with far-red light. Only a very weak activity was measured in air with both techniques. When leaf discs were placed in anaerobiosis, a high and rapid cyclic PS I activity was measured. The maximal energy storage in far-red light increased to 30% to 50%, and the half-time of the P_{700} re-reduction in the dark decreased to around 400 ms; these values are comparable with those measured in cyanobacteria and C_4 plant leaves in aerobiosis. The stimulatory effect of anaerobiosis was mimicked by infiltrating leaves with inhibitors of mitochondrial respiration or of the chlororespiratory oxidase, therefore, showing that changes in the redox state of intersystem electron carriers tightly control the rate of PS I-driven cyclic electron flow in vivo. Measurements of energy storage at different modulation frequencies of far-red light showed that anaerobiosis-induced cyclic PS I activity in leaves of a tobacco mutant deficient in the plastid Ndh complex was kinetically different from that of the wild type, the cycle being slower in the former leaves. We conclude that the Ndh complex is required for rapid electron cycling around PS I.

During oxygenic photosynthesis, photosystem (PS) II and PS I cooperate to achieve a linear electron flow from H_2O to $NADP^+$ and to generate a transmembrane proton gradient driving ATP synthesis. However, ATP can also be produced by the sole PS I through cyclic electron transfer reactions (Arnon, 1959). This mechanism enables the generation of a proton gradient across the thylakoid membrane without NADP reduction by rerouting electrons of reduced PS I acceptors toward the intersystem carriers. Cyclic and linear electron transfers share a common sequence of electron carriers, namely the plastoquinone (PQ) pool, cytochrome b_6/f complex, and plastocyanin (for review, see Fork and Herbert, 1993; Bendall and Manasse, 1995). This alternative electron flow has been shown to occur in vivo in cyanobacteria (Carpentier et al., 1984), in algae (Maxwell and Biggins, 1976; Ravenel et al., 1994), and in bundle sheath cells of C_4 plants (Herbert et al., 1990; Asada et al., 1993). In cyanobacteria, cyclic electron flow around PS I has been shown to provide extra ATP for different cellular processes, e.g. adaptation to salt stress conditions (Jeanjean et al., 1993). In the bundle sheath cell chloroplasts of C_4 plants, PS II is low or undetectable (Woo et al., 1970) and ATP supply is totally dependent upon PS I-mediated cyclic electron transport (Leegood et al., 1981).

In C_3 plants, PS I-driven cyclic electron flow has been studied mainly in vitro on isolated chloroplasts or thylakoids with addition of artificial cofactors or reduced ferredoxin (Bendall and Manasse, 1995). Under those conditions, the redox poise was proposed to play an important role in the regulation of the rate of cyclic electron flow (Arnon and Chain, 1975; Heber et al., 1978; Fork and Herbert, 1993), with neither full reduction of the chloroplast electron transport chain (Ziem-Hanck and Heber, 1980) nor excessive oxidation allowing cyclic electron flow to occur in vitro. In intact leaves, PS I-mediated cyclic electron flow in far-red light was analyzed indirectly by measuring the light-scattering signal at 535 nm, which reflects changes in the trans-thylakoid pH gradient (Heber et al., 1992, 1995; Cornic et al., 2000). Cyclic electron transport around PS I can also be estimated indirectly by measuring the re-reduction rate of the oxidized primary electron donor in PS I (P_{700}^+) after switching off the far-red light (Maxwell and Biggins, 1976; Asada et al., 1992). It was observed that this rate measured in leaves of C_3 plants (e.g. Burrows et al., 1998) was considerably much slower than that measured in the green alga *Chlamydomonas reinhardtii* (Maxwell and Biggins, 1976; Ravenel et al., 1994) or in

* Corresponding author; e-mail michel.havaux@cea.fr; fax 33–4–42256265.

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cyanobacteria (Mi et al., 1992), suggesting a very slow recycling of electrons around PS I in vivo. The existence of cyclic electron transport in vivo in C_3 plants has also been questioned by photoacoustic measurements in far-red light (Herbert et al., 1990), which allow a direct and quantitative measure of energy storage (ES) by cyclic electron flow around PS I (for review, see Malkin and Canaani, 1994). This method has confirmed the existence of cyclic electron transfer reactions in C_4 plants, algae, and cyanobacteria (Herbert et al., 1990), but failed to show significant cyclic activity in C_3 plant leaves (Herbert et al., 1990; Havaux et al., 1991; Malkin et al., 1992).

New biochemical and genetic data support, however, the idea that cyclic electron flow around PS I occurs in vivo in C_3 plants. The plastid genome of higher plants contains *ndh* genes encoding peptides homologous to subunits of the proton-pumping NADH:ubiquinone oxidoreductase, a component of the mitochondrial respiratory chain (Ohyama et al., 1986; Shinozaki et al., 1986), and an NADH-dehydrogenase complex (Ndh) has been purified from pea (*Pisum sativum*) and barley (*Hordeum vulgare*) thylakoid membranes (Sazanov et al., 1998; Quiles et al., 2000). Inactivation of some *ndh* genes using plastid transformation of tobacco (*Nicotiana tabacum* cv Petit Havana) demonstrated the existence of a functional Ndh complex and its involvement in the transient nonphotochemical reduction of the PQ pool after a light to dark transition (Burrows et al., 1998; Cournac et al., 1998; Shikanai et al., 1998). Based on the study of chlorophyll fluorescence kinetics and the effects of inhibitors such as antimycin on tobacco leaf discs of an Ndh-less tobacco mutant, it was recently suggested that the Ndh complex could be involved in a PS I cyclic electron pathway operating in vivo in C_3 plants (Joët et al., 2000).

The apparent discrepancy between those results and the absence of measurable cyclic activity in vivo remains to be elucidated. Cyclic PS I activity is usually measured under very special conditions (PS I excitation by far-red light, PS II inhibition by 3-[3,4-dichlorophenyl]-1,1-dimethylurea [DCMU]) in which linear electron flow is diminished or even abolished. We assumed that the adequate redox poise supposedly required for cyclic electron flow in vivo is not achieved under those experimental conditions. In the present study, we have used photoacoustic spectroscopy and kinetic spectrophotometry to monitor cyclic electron transport around PS I in C_3 plants. A rapid electron cycling around PS I was induced in vivo by increasing the reduction level of the stromal NADP pool and of the intersystem electron carriers using anaerobic conditions or respiration inhibitors. The high cyclic activity of PS I thus obtained was different in wild-type (WT) tobacco and in a mutant lacking the Ndh complex, demonstrating the involvement of the Ndh complex in cyclic PS I activity.

RESULTS

Cyclic Electron Transport around PS I in Different Organisms

PS I-mediated cyclic electron flow was monitored in vivo in *Synechocystis* sp., maize (*Zea mays*) and tobacco using the photoacoustic technique (Fig. 1A). This technique measures the conversion of light energy to heat in an absorbing sample and hence the storage of light energy as chemical energy (photochemical ES; see "materials and Methods"; Malkin and Canaani, 1994). Figure 1A shows a typical in vivo photothermal signal generated by *Synechocystis* sp. cells deposited on a nitrocellulose filter and irradiated with modulated far-red light. Addition of a strong nonmodulated far-red light to the modulated light beam saturates PS I photochemistry, causing a noticeable rise in the photoacoustic signal. Thus, the comparison of the actual and maximal heat-emission signals provides a measure of the amount of absorbed light energy that was stored in intermediates of the photochemical processes (Malkin and Canaani, 1994). ES measured in the cyanobacterium *Synechocystis* sp. PCC 6803 under such conditions was close to 18%. As far-red light is almost exclusively absorbed in PS I, the measured ES is specifically related to the PS I function, reflecting ES in photochemical

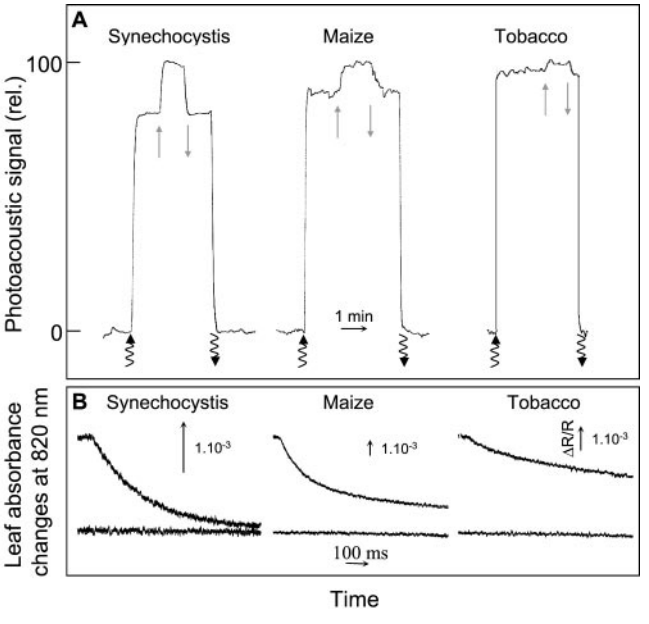

Figure 1. A, Photoacoustic signals generated by filter-deposited *Synechocystis* sp. cells and by water vacuum-infiltrated leaf discs of maize and tobacco, in wavelengths of measuring light absorbed predominantly by PS I (>715 nm; 10 Hz; 30 W m^{-2}). Upward-pointing arrows and downward-pointing arrows respectively indicate saturating far-red light (320 W m^{-2}) on and off. Thin wavy arrows represent the modulated measuring light. B, Dark re-reduction of P_{700}^+ after a far-red light period was monitored on the same samples and was expressed as Δ reflectance/reflectance (ΔR/R). Data expressing the half-time ($t_{1/2}$) of the dark P_{700}^+ reduction are means ± SD of six experiments.

products associated with the cycling of electrons around PS I (Canaani et al., 1989; Herbert et al., 1990). In higher plants, photoacoustic measurements must be conducted on water- or buffer-infiltrated leaves to eliminate the oxygen-evolution-related component of the photoacoustic signal (Malkin et al., 1992). In infiltrated leaves of maize (a C_4 plant), ES in far-red light (approximately 15%) was close to the activity measured in *Synechocystis* sp. In contrast, only a very small fraction (<5%) of the absorbed far-red light was used for photochemistry in infiltrated leaf discs of tobacco, a C_3 plant. This confirms a previous photoacoustic study of Herbert et al. (1990) who showed that C_3 plants exhibit no significant ES in far-red light.

Leaf absorbance measurements at 820 nm, which reflect changes in the redox state of the PS I reaction center (P_{700}), were also performed on the samples used for the photoacoustic measurements. P_{700} was oxidized by far-red light, and its subsequent reduction in the dark by stromal reductants was recorded (Fig. 1B). The half-time ($t_{1/2}$) of the dark re-reduction of P_{700}^+ after a period of far-red light was about 178 ± 12 ms in the cyanobacterium and 196 ± 29 ms in maize leaves. In tobacco, the re-reduction rate was considerably much slower, the $t_{1/2}$ value being close to 1,157 ± 113 ms. The latter value indicates a slow re-reduction of P_{700}^+, which can be attributed to a slow kinetics of electron donation from the stroma to the intersystem electron transport chain and/or a small pool of electron donors in the stroma after the far-red light illumination (Mi et al., 1992). In tobacco, it is possible that this pool is oxidized in far-red light and is regenerated very slowly in the dark.

Induction of Cyclic PS I Activity in C_3 Plants by Anaerobiosis

Assuming that the redox poise is a key factor in the induction of cyclic electron flow in C_3 plants, ES and the P_{700} dark reduction were measured under anaerobiosis, a condition known to reduce both the intersystem electron transport chain (Harris and Heber, 1993) and the stromal NADP pool (Joët et al., 1998). Anaerobiosis was reached in situ by placing tobacco leaf discs between two plastic wrap films impermeable to gas exchanges. Under those conditions, mitochondrial respiration consumes molecular O_2 in the gas phase of the leaf sample. This was checked by placing a leaf disc between a Clark-type O_2 electrode and a plastic film (data not shown). Oxygen was quickly consumed within 25 to 30 min, finally reaching the level obtained by flushing nitrogen in the chamber. We followed the changes in chlorophyll fluorescence emission (F_o and F_m levels) by tobacco leaf discs placed between two plastic films (Fig. 2). As previously reported by Harris and Heber (1993), obtention of anaerobiosis resulted in a noticeable increase in the dark level (F_o) of chlorophyll fluorescence (around +35%), indicating a partial reduction

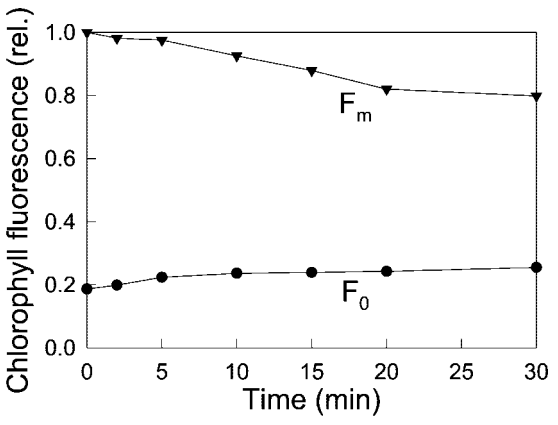

Figure 2. Changes in chlorophyll fluorescence of a film-enclosed leaf disc. The dark chlorophyll fluorescence F_o (●) was monitored by rapidly switching the nonactinic modulated measuring light on and off. The maximal fluorescence level F_m (▼) was determined by applying an 800-ms flash of saturating white light to the dark-adapted leaf discs.

of the PQ pool. At the same time, the maximal fluorescence level (F_m) progressively decreased. The latter effect probably reflects a transition from state 1 to state 2, which modified the light energy distribution between PS II and PS I in response to the reduction of the PQ pool via lateral movement of a fraction of the light-harvesting complexes II (Allen, 1992). This was confirmed by measuring 77 K chlorophyll fluorescence spectra of tobacco leaf discs in air or in anaerobiosis. The ratio between the F730 fluorescence peak (corresponding to PS I-associated pigments) and the F685 peak (PS II-associated pigments) was 3.49 ± 0.27 for leaf discs dark-adapted in air and 4.84 ± 0.19 for leaf discs placed between two plastic films, indicating that the latter leaves underwent a transition to state 2 in which light energy is redistributed in favor of PS I. However, we cannot exclude that part of the F_m quenching involves also other factors such as nonphotochemical fluorescence quenching related to a trans-thylakoidal ΔpH.

Photoacoustic measurements of ES in far-red light were conducted in tobacco leaves subjected to anaerobiosis. Figure 3A shows that anaerobiosis led to a considerable increase in ES by cyclic PS I activity from less than 5% to approximately 25%. The effect of anaerobiosis was also measured on the rate of P_{700} reduction after far-red light (Fig. 3B). In air, $t_{1/2}$ was about 1,157 ± 114 ms whereas anaerobic conditions drastically decreased the $t_{1/2}$ value to about 393 ± 89 ms, indicating a marked acceleration of the electron donation to P_{700}. The photoacoustic photothermal signal generated by tobacco leaf discs was measured at different fluence rates of far-red light, and Figure 4 shows the plot of ES versus the fluence rate. In air, ES was very low at any fluence rate of far-red light. In contrast, ES measured in anaerobiosis was much higher and significantly decreased with increasing far-red light fluence rate, indicating a progressive

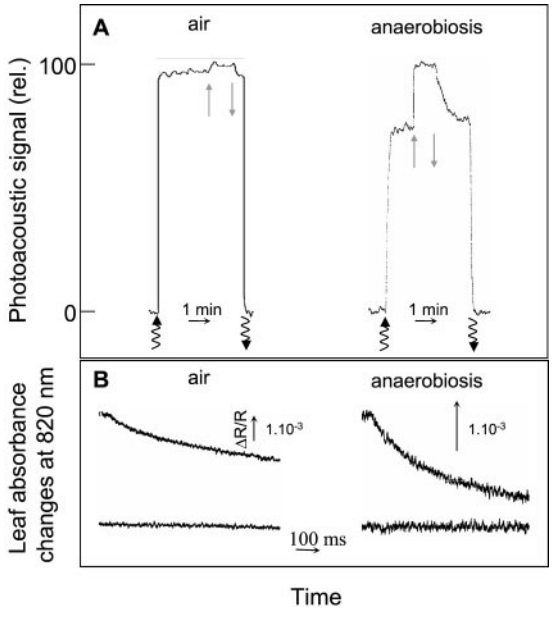

Figure 3. A, Photoacoustic signals (arbitrary units) generated by buffer vacuum-infiltrated (aerobiosis) or plastic film-enclosed (anaerobiosis) tobacco leaf discs were measured in wavelengths of measuring light absorbed predominantly by PS I (>715 nm; 10 Hz; 30 W m^{-2}). B, Dark re-reduction of P_{700}^+ after a far-red light period was measured on buffer vacuum-infiltrated (aerobiosis) and plastic film-enclosed (anaerobiosis) tobacco leaf discs. Data expressing the half-time ($t_{1/2}$) of the P_{700}^+ dark reduction are means ± SD of six experiments.

light saturation of cyclic PS I activity. The inset of Figure 4 represents the plot of ES^{-1} versus light fluence rate, which is linear (Havaux et al., 1989). The extrapolation of this plot to a fluence rate of zero gives an estimate of the maximal efficiency of photochemical ES. This maximal ES was close to 8% in aerobic conditions whereas it was increased to 30% to 50% in anaerobic conditions. ES measured in anaerobiosis was not inhibited by DCMU, thus confirming that ES measured in far-red light is specifically related to PS I photochemistry (Fig. 4).

Mechanisms by which anaerobiosis leads to a stimulation of PS I-mediated cyclic activity were then investigated. First, the stimulatory effect of anaerobiosis on the P_{700}^+ re-reduction rate was mimicked by inhibiting mitochondrial respiration either with myxothiazol or salicyl hydroxamic acid (SHAM; Table I). The use of two inhibitors is necessary to inhibit both the cytochrome respiratory pathway and the alternative oxidase pathway. Via chloroplast-mitochondria interactions, inhibition of respiration increased the reducing power in the chloroplasts leading to a rapid electron donation from the stroma to the intersystem chain, as did anaerobiosis. We observed the same phenomenon using antimycin A and SHAM (Table I), although antimycin A is also a potent inhibitor of the ferredoxin-dependent pathway of cyclic electron flow around PS I. This indicates that the latter pathway is not limiting for P_{700} re-reduction under our experimental conditions and it can be compensated by the other pathways of nonphotochemical reduction of intersystem electron carriers. It is interesting that P_{700}^+ re-reduction was also faster in leaves infiltrated with propyl gallate, a potent inhibitor of the newly discovered plastid terminal oxidase (Cournac et al., 2000). This protein has been recently overexpressed in tobacco leaves and was clearly shown to be involved in the dark oxidation of the PQ pool and to be sensitive to propyl gallate in planta (T. Joët, B. Genty, E.M. Josse, M. Kuntz, L. Cournac, and G. Peltier, unpublished data). This shows that electron flow to P_{700} can be enhanced by either increasing nonphotochemical reduction of PQ or decreasing its oxidation, suggesting that the reduction state of the PQ pool play a central role in the induction of cyclic electron flow.

Cyclic electron flow around PS I was searched in various C_3 plant species exposed to anaerobiosis. In air, ES was very low or even undetectable, whereas anaerobiosis caused a strong increase in ES in all species tested (Table II). As a consequence the phenomenon reported for tobacco is not restricted to this species but can be considered as a general response of C_3 plants to anaerobic conditions.

Anaerobiosis-Induced Cyclic Electron Transport around PS I in a Ndh-Less Tobacco Mutant

To further characterize PS I cyclic electron flow in C_3 plants, we performed photoacoustic and 820-nm absorbance measurements on leaves of a tobacco mutant lacking the plastid Ndh complex (Horvath et al., 2000). In Figure 5A, the reciprocal of ES measured in the WT and the mutant under anaerobic conditions was plotted versus the far-red light fluence rate. When photoacoustic measurements were performed

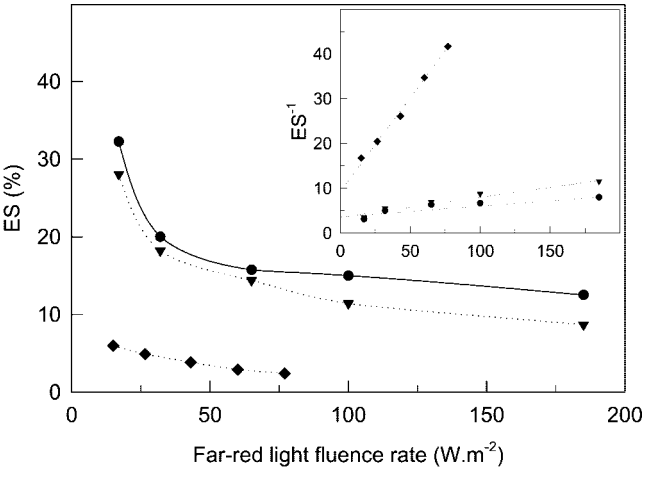

Figure 4. Plot of ES versus the fluence rate of the measuring far-red light (>715 nm, 10 Hz) for tobacco leaf discs under aerobic (◆) or anaerobic conditions (●). The effect of 50 μM DCMU under anaerobiosis is also shown (▼). Inset, Plot of the reciprocal of ES versus light fluence rate.

Table I. *Effects of various chemicals on the half-time ($t_{1/2}$) of the dark reduction of P_{700}^+ after far-red illumination (6.5 W m^{-2}; >715 nm)*

The measurements were performed in air, unless specified otherwise. Data are means ± SD of six experiments.

Treatment	P_{700} Re-Reduction
	$t_{1/2}$, ms
Control	1,157 ± 114
DCMU (20 μM)	1,135 ± 148
Antimycin A (5 μM)	1,118 ± 78
Myxothiazol (5 μM)	1,253 ± 233
SHAM (0.8 mM)	1,100 ± 117
Myxothiazol (5 μM) + SHAM (0.8 mM)	397 ± 95
Antimycin A (5 μM) + SHAM (0.8 mM)	440 ± 128
Propyl gallate (1 mM)	387 ± 85
Anaerobiosis	393 ± 89

using far-red light modulated at a low frequency of 10 Hz, no significant difference in ES was found between the WT and the Ndh-less mutant. This shows that ES by PS I cyclic electron flow can occur in the absence of the Ndh complex, via alternative electron transfer pathways. No significant difference was detected between WT and Ndh-deficient mutant on the basis of the half-time of the P_{700} re-reduction under anaerobiosis (Fig. 5B). However, increasing the modulation frequency to 22 Hz revealed a noticeable difference between the two genotypes (Fig. 5A). The linear plot of the mutant had a much steeper slope than the plot of the WT, indicating that PS I-driven cyclic electron transport was more rapidly saturated with increasing far-red light intensity in the Ndh-deficient mutant. The frequency dependence of ES in far-red light was analyzed in more detail in Figure 6. In WT leaves placed in anaerobiosis, a progressive decrease in ES was observed with increasing frequency of the modulated light. A similar frequency dependence of ES by cyclic PS I activity was previously observed in *C. reinhardtii* (Canaani et al., 1989). ES depends on the modulation frequency because this parameter reflects energy stored in photochemical products that decay with a time constant larger than the modulation frequency of excitation (Malkin and Canaani, 1994). Thus, at very low frequency, ES reflects long-lived intermediates. More

Table II. *Photoacoustically measured energy storage (%) by PS I cyclic activity in leaves of various C_3 plants illuminated with far-red light (30 W·m^{-2}; 10 Hz) in air and in anaerobiosis*

nd, Not detected.

C_3 Plant Species	Air	Anaerobiosis
B. napus	6	42
Barley	5.5	28
Arabidopsis	nd	17
Potato	nd	37
P. koreana × *P. trichocarpa* Torr. & Gray	9	41
A. pseudoplatanus	8	18

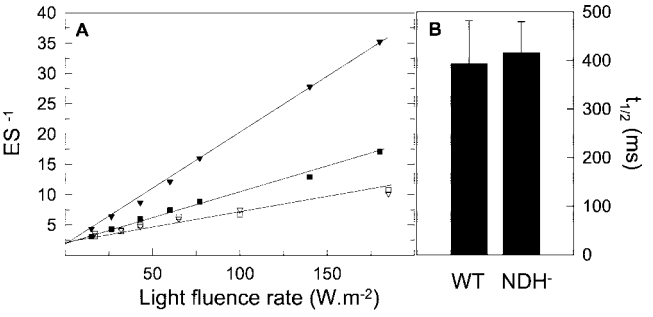

Figure 5. A, Plot of the reciprocal of ES versus the fluence rate of the measuring far-red light (>715 nm) under anaerobic conditions. ES was measured in WT (□, ■) and Ndh-less mutant (▽, ▼) at 10 Hz (□, ▽) and 22 Hz (■, ▼). B, Dark re-reduction of P_{700}^+ after a far-red light period was measured on WT and Ndh-less mutant tobacco leaf discs under anaerobiosis. Data expressing the half-time ($t_{1/2}$) of the P_{700}^+ dark reduction are means ± SD of 12 experiments.

precisely, at a given frequency of modulation f, ES corresponds to photochemical products of electron transport that persist for $1/2\pi f$ seconds after excitation (Canaani et al., 1989). Considering a maximal ES value of about 20% (at modulation frequencies close to zero), we estimated that the frequency corresponding to the half-value of the maximal ES was around 26 Hz in the WT. From this value, an apparent reaction half-time of 6 ms can be calculated for the limiting step of the reaction responsible for the measured ES. It is striking that ES measured in the Ndh-deficient mutant decreased much more rapidly with the modulation frequency, and the half-value of the maximal ES value was reached at around 12 Hz. In this case, the photochemical reaction responsible for ES in far-red light has a half-time of 13 ms. As expected, ES was low at any frequency in both WT and Ndh-less mutant leaves in air. The data of Figure 6 show that cyclic electron transfers around PS I in WT

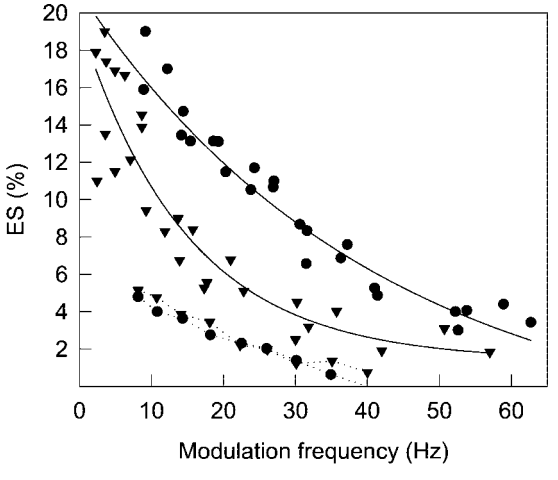

Figure 6. Plot of ES versus the frequency of the modulated light for WT (●) and Ndh-less mutant leaves (▼). ES was measured under aerobic (dotted lines) or anaerobic conditions (full lines) using far-red modulated light (>715 nm; 40 W m^{-2}). The experimental points are the results of three independent experiments.

tobacco and in the Ndh-deficient mutant are kinetically different, the electron cycle being much slower in the latter one.

DISCUSSION

Rapid Cyclic Electron Flow around PS I in Vivo in C_3 Plants

In this study, cyclic electron flow around PS I was triggered in vivo by placing tobacco leaf discs in anaerobiosis. The maximal ES level measured in tobacco leaves in far-red light was equivalent to that measured in cyanobacteria and C_4 plants. The frequency dependence of ES measured in anaerobiosis showed that the frequency corresponding to one-half of the maximal ES was about 26 Hz, indicating that the rate constant of the photochemical reaction responsible for the ES was about 6 ms. This is to compare to the 2 ms previously observed in *C. reinhardtii* (Canaani et al., 1989). To the best of our knowledge, this is the first time that such a high and rapid cyclic electron flow around PS I is measured in vivo in C_3 plants. Moreover, anaerobiosis-induced cyclic PS I activity was observed in several C_3 plant species, indicating that this phenomenon is a general feature of C_3 plants (Table II). The half-time of P_{700}^+ re-reduction measured in tobacco leaf discs was also accelerated in anaerobiosis from 1,200 ms (in air) to 400 ms which is very close to the $t_{1/2}$ reported for isolated thylakoids under anaerobiosis (Scheller, 1996). This value corresponds to a rapid electron donation to P_{700} which is of the same magnitude of that measured in *Synechocystis* sp. and maize (respectively close to 180 and 200 ms) or that reported for *C. reinhardtii* (220 ms; Ravenel et al., 1994) and other unicellular algae (150 ms; Maxwell and Biggins, 1976). Our results are in accordance with previous studies performed by Heber et al. (1978) who observed a significant increase in the light scattering of spinach leaves illuminated with far-red light by flushing with nitrogen. Since this signal is indirectly related to the thylakoid pH gradient, these authors proposed that PS I-mediated cyclic activity was stimulated by anaerobiosis.

Involvement of the Ndh Complex in Cyclic Electron Flow

The rapid cyclic electron transport around PS I in anaerobiosis is related to the Ndh complex activity. The cyclic PS I activity in Ndh-less mutant was kinetically different from that of the WT as shown by the more abrupt decrease of ES with increasing frequency. This indicates a slower cycling of electrons in the absence of functional Ndh complex. The photochemical reaction responsible for the measured ES in far-red light had a calculated half-time of approximately 13 ms, which is much higher than the value measured in the WT (approximately 6 ms). It is interesting that PS I-mediated cyclic electron flow was not inhibited in Ndh-less mutant leaves of tobacco at very low frequencies of modulated light (<10 Hz) indicating the existence of cyclic electron pathways independent of the Ndh complex. Those alternative pathways could be the antimycin-sensitive pathway (Cleland and Bendall, 1992; Joët et al., 2000) or a pathway involving alternative NAD(P) H dehydrogenases (Corneille et al., 1998; Cournac et al., 1998). The rapid disappearance of ES with increasing frequency in the Ndh-less mutant shows that these additional pathways are slow and that the predominant pathway in the WT is dependent on the Ndh complex. It should incidentally be noted that the photoacoustic method revealed differences in kinetics of cyclic electron flow whereas the P_{700}^+ re-reduction rate measured in the dark was equivalent in the WT and the Ndh-less mutant. This can be explained by the fact that the photoacoustic technique measures the energy stored during the complete electron cycle in far-red light whereas the P_{700} reduction rate in the dark reflects electron donation to P_{700} from stromal donors. This rate depends not only on the kinetics of electron transfer from stromal donors to the intersystem chain but also on the size of the stromal electron donor pool. Thus, the $t_{1/2}$ value is expected to depend on the experimental conditions (Mi et al., 1992). It has been shown in cyanobacteria that $t_{1/2}$ measured after continuous far-red light represents the donation from respiratory donors, and depletion of respiratory donors in dark-starved cells resulted in a very slow reduction of P_{700}^+ (Mi et al., 1992). Then, $t_{1/2}$ cannot be taken as an absolute value of the turnover time of P_{700} during cyclic electron transport around PS I. Rather, it should be considered as an indicator of the potential capacity of electron donation to P_{700} from stroma electron donors. In tobacco, re-reduction of oxidized P_{700} was possibly determined mainly by the re-generation of stromal electron donors after switching off the far-red light. This phenomenon was probably more limiting for the P_{700} reduction rate than the absence of Ndh, thus explaining why the WT and the Ndh-deficient mutant could not be distinguished on the basis of the P_{700} reduction kinetics. In contrast, in the photoacoustic experiments, the frequency dependence of ES allows the kinetics of the electron cycle to be analyzed and provides information on the lifetime of the intermediates that limit the energy-storage reaction (Malkin and Canaani, 1994; Malkin, 1996). Also, it cannot be excluded that some phenomena needed for cyclic electron flow are deactivated in the dark and are thus not observable in the P_{700} redox change experiments. Further studies are probably required to confirm our interpretations.

The involvement of the Ndh complex in cyclic electron transport around PS I is thus demonstrated in this study, confirming a number of previous reports that have hypothesized the role of the Ndh complex

in cyclic electron transport via measurements of nonphotochemical reduction of the PQ pool in the dark (Burrows et al., 1998; Shikanai et al., 1998; Cournac et al., 1998). It was recently shown that photosynthesis of the Ndh-less mutant leaves was highly sensitive to antimycin A, and it was concluded to the participation of the Ndh complex in cyclic electron flow (Joët et al., 2000). Cyclic electron flow via the NADPH pool has already been described in the cyanobacterium *Synechocystis* sp., where lesions in the *ndh* genes were observed to cause a strong slowdown of the P_{700} turnover in far-red light (Mi et al., 1992, 1995) and a marked inhibition of ES in far-red light (Jeanjean et al., 1998). At last, an NAD(P) H dehydrogenase activity involved in PS I cyclic activity has also been suggested in *C. reinhardtii* where several independent pathways may coexist in vivo (Ravenel et al., 1994). It should be pointed out that the involvement of Ndh in cyclic electron transport around PS I can be direct by allowing rapid recycling of electrons from NADPH to the PQ pool or indirect as a redox poise regulator (compare with below).

The Redox Poise Controls Cyclic PS I Activity in C_3 Plants

It is likely that PS I-mediated cyclic electron transport is controlled by the atmospheric O_2 concentration in C_3 plants via changes in the redox state of intersystem electron carriers and/or stromal reductants. The PQ pool was found to be partially reduced in tobacco leaves exposed to anaerobiosis in plastic wrap films. In a previous study, the NADPH to NADP ratio, as measured in vivo by the blue-green fluorescence emission, was observed to increase noticeably in anaerobiosis (Joët et al., 1998). A control of PS I-driven cyclic electron transfer by the reduction state of intersystem electron carriers and through the $NADP^+$ to NADPH ratio was previously suggested from in vitro data obtained on isolated C_3 chloroplasts (Arnon and Chain, 1975, 1979; Slovacek et al., 1980; Takahama et al., 1981; Hosler and Yocum, 1985, 1987) and from light scattering measurements on leaves (Heber et al., 1978). In those experiments, it was shown that cyclic activity is modulated by varying the O_2 partial pressure to modify the redox poise of the intersystem electron carriers (Arnon and Chain, 1975, 1979; Scheller, 1996). This suggests that adequate redox poise was not achieved in leaves placed in air and illuminated with far-red light, thus explaining why PS I-mediated cyclic activity was not detectable in vivo in C_3 plants.

Adequate redox poise was also induced in air either by inhibiting mitochondrial oxidases using myxothiazol or antimycin and SHAM or by inhibiting a chloroplastic PQ oxidation pathway using propyl gallate. In the former case, the cytosolic NAD(P) H, which cannot be oxidized by mitorespiration, is rerouted toward chloroplasts because of the existence of redox interactions between mitochondria and plastid (for review, see Hoefnagel et al., 1998), hence stimulating nonphotochemical reduction of the intersystem electron carriers (Gans and Rebeillé, 1990). In leaves treated with propyl gallate, inhibition of a plastid oxidase involved in nonphotochemical oxidation of the PQ pool, led to an over-reduction of the intersystem electron carriers. Those mechanisms, by which the PQ pool is nonphotochemically reduced and subsequently reoxidized in the dark using molecular oxygen as a terminal acceptor, are commonly described as chlororespiration (Bennoun, 1982; Peltier et al., 1987; Nixon, 2000). From our data, we conclude that the entire chlororespiratory electron transfer chain, i.e. the nonphotochemical reduction as well as the nonphotochemical oxidation of the PQ pool, may control the redox poise of intersystem electron transport chain in vivo in C_3 plants, which in turn controls PS I-mediated cyclic electron flow.

Anaerobic conditions were used in this study as an experimental trick that allows measurement of cyclic electron transport in far-red light. Under physiological conditions, the adequate redox state of the PQ pool may be achieved by the PS II activity, especially when PS I acceptors are not fully available. Under our experimental conditions, however, cyclic electron flow is measured in far-red light, which cannot stimulate PS II activity, and cyclic electron flow is then artificially activated in anaerobiosis by simultaneous stimulation of nonphotochemical PQ reduction and inhibition of PQ oxidation by the plastid oxidase. The situation described here in higher plant chloroplasts is close to that occurring in cyanobacteria where respiration and photosynthesis electron transfer chains share the PQ pool in common (Scherer, 1990). It is interesting that Schubert et al. (1995) reported a stimulation of PS I-driven cyclic electron flow, estimated by the photochemical ES in far-red light and by the P_{700}^+ dark reduction in the cyanobacterium *Fremyella diplosiphon* when treated with KCN, a well-known inhibitor of cytochrome c oxidase.

Transition to the light state 2, which is activated when the intersystem redox carriers are reduced (Allen, 1992), took place in tobacco leaves exposed to anaerobiosis. During transition to state 2, the cytochrome b_6/f complex was shown to accumulate in the stroma lamellae of maize and *C. reinhardtii* (Vallon et al., 1991) where both PS I and the Ndh complex are located (Horvath et al., 2000; Sazanov et al., 1998). This could possibly facilitate PS I cyclic activity. One may then suppose that reduction of the PQ pool can indirectly favor cyclic electron flow via state-transition-related migration of cyt b_6f to the vicinity of PS I. It is interesting that inhibitors of mitochondrial respiration were also reported to induce reduction of the PQ pool and transition to state 2 in *C. reinhardtii* (Bulté et al., 1990).

One intriguing question is why the adequate redox poise for cyclic electron transport requires specific conditions in C_3 plants and is naturally observed in

other types of plants under far-red light conditions. Under normal conditions, it is possible that nonphotochemical reduction of the PQ pool is not sufficient to achieve the adequate redox poise of intersystem electron carriers. Cyclic electron flow will be triggered by a reduction of the PQ pool mediated either by an imbalance in chlororespiration activity between nonphotochemical reduction and oxidation of PQs or by PS II activity. This could be the case for example during induction of photosynthesis or in high light, where the intersystem electron carriers are partly reduced. The Calvin cycle uses more ATP than NADPH so that a high photosynthetic rate will lead to an increased NADPH to ATP ratio (Osmond, 1981). It has been suggested that one function of cyclic electron transfer around PS I is to synthesize extra ATP to adjust the NADPH to ATP ratio (Bendall and Manasse, 1995). Our observation that PS I cyclic activity is controlled by the redox poise of the chloroplasts is consistent with this function: An accumulation of NADPH will trigger the cyclic electron flow, thus compensating the ATP deficit.

MATERIALS AND METHODS

Plant Material and Preparation of Leaf Samples

WT tobacco (*Nicotiana tabacum* cv Petit Havana) and *ndhB*-inactivated mutant (see Horvath et al., 2000) were grown on compost in a phytotron (25°C day/20°C night) under a photon flux density of 350 μmol photons m^{-2} s^{-1} (photoperiod, 12 h) supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered with one-half-strength Hoagland nutritive solution. Leaf samples were taken from plants aged 5 to 8 weeks. Potato (*Solanum tuberosum* L. cv Desirée), Arabidopsis cv Colombia, barley (*Hordeum vulgare* L. cv Plaisant), and *Brassica napus* cv Orphée were cultivated under the same conditions, whereas poplar (*Populus koreana* × *Populus trichocarpa Torr. & Gray* cv Peace) was grown in a greenhouse. Samples from *Acer pseudoplatanus* were harvested outside.

Anaerobiosis was induced by placing leaf discs (12 mm in diameter) between two plastic films of barrier food wraps (Saran, Dow Chemical, Midland, MI) and keeping them in the dark for 60 min before photoacoustic or absorbance measurements. Because of respiration, O_2 was rapidly depleted in the leaf discs, as controlled with an O_2 electrode at 25°C.

Treatments with Inhibitors

DCMU treatment was performed on whole leaves that were infiltrated with 50 μM DCMU via their petiole through the transpiration flux for 5 h. PS II inhibition by DCMU was checked by chlorophyll fluorescence yield measurements with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). For other treatments, tobacco leaf discs were stripped by removal of lower epidermis and soaked for 90 min in petri dishes containing distilled water and various inhibitors. The inhibitors were added diluted in methanol (maximal final methanol concentration was 0.5%). Control leaf discs were soaked in petri dishes containing water and 0.5% (v/v) methanol.

Photoacoustic Measurements of Photochemical ES

ES by cyclic electron flow around PS I was measured in vivo using the photoacoustic technique (Herbert et al., 1990; Havaux et al., 1991; Ravenel et al., 1994). The photoacoustic spectrometer is described in Ravenel et al. (1994). Leaf discs, placed in the photoacoustic chamber, were illuminated with modulated far-red light (>715 nm). The far-red light fluence rate was measured with a LI-COR radiometer (Li-185B/Li-200SB, LI-COR, Lincoln, NE). PS I photochemistry was saturated with a strong background far-red light (>715 nm, 320 W m^{-2}). ES was calculated from the amplitude of the maximal photothermal photoacoustic signal (Apt^+ measured when the strong far-red light was added to the modulated measuring light) and the actual photothermal signal amplitude (Apt):

$$ES = (Apt^+ - Apt)/Apt^+ \quad (1)$$

The plastic wrap film is impermeable to O_2 and, therefore, abolishes the gas-exchange-related photoacoustic signal that can appear at low modulation frequencies. When the plastic film was not used, leaf discs were soaked for 3 h in an osmoticum buffer (25 mM phosphate buffer, pH 7.0, 200 mM sorbitol, 10 mM KCl, and 2 mM $MgCl_2$) to eliminate the photobaric component of the photoacoustic signal as described by Malkin et al. (1992). *Synechocystis* sp. cells were filtered under pressure through an MF-Millipore filter (cellulose nitrate/acetate, SS type, 3-μm pore size, Millipore, Bedford, MA). The cells deposited on the filter (diameter, 1.2 cm) were then placed in the photoacoustic cell for measurement.

Redox State of P700

Changes in the redox state of the reaction center P_{700} of PS I were monitored via leaf absorbance changes at around 820 nm (Schreiber et al., 1988). A Walz PAM-101 system connected to an ED-800-T emitter/detector unit (Walz) was used in the reflection mode (Schreiber et al., 1988). The rate of P_{700} re-reduction was measured with a storage oscilloscope (Tektronix 5111A, Tektronix, Guernsey, Channel Islands) after a period of far-red light (6.5 W m^{-2}, >715 nm).

Room Temperature and Low Temperature Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence spectra were recorded in liquid nitrogen (77 K) with leaf discs dark-adapted for 30 min using a bifurcated light guide connected to a Perkin-Elmer LS50B spectrofluorometer (Perkin-Elmer, Beaconsfield, UK). The wavelength of the excitation light beam was 440 nm.

Room temperature chlorophyll fluorescence was measured with a PAM-2000 chlorophyll fluorometer (Walz). F_o was excited with a dim red light modulated at 600 Hz. Fm was induced by a 800-ms pulse of intense white light.

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