Implication de la protéine PTOX dans l'oxydation non photochimique du pool de PQ

Si le complexe Ndh est impliqué dans la réduction non photochimique du pool de PQ, l'existence d'une chaîne chlororespiratoire implique qu'une oxydase terminale intervienne dans l'oxydation non photochimique du pool de PQ. A ce titre, nous nous sommes interressés à PTOX, une protéine chloroplastique homologue aux oxydases alternatives mitochondriales, dont il a été récemment montré qu'elle était impliquée dans la biosynthèse des caroténoïdes (Carol et al., 1999 ; Wu et al., 1999).

L'étude de mutants de *Chlamydomonas reinhardtii* dépourvus de PS I a permis de montrer l'implication de PTOX dans l'oxydation du pool de PQ par l'oxygène moléculaire (voir article joint Cournac et al. (2000) Phil Trans R Soc London B 355 : 1447-1454). Nous avons ensuite cherché à caractériser la fonction de PTOX au sein des chloroplastes des végétaux supérieurs. Pour ce faire, nous avons entrepris de surexprimer la protéine PTOX d'*Arabidopsis thaliana* dans le tabac (voir article joint Joët et al., soumis). La caractérisation de plantes transgéniques surexprimant PTOX, par des mesures de fluorescence de la chlorophylle et d'échange d'oxygène, nous a permis de montrer que PTOX est impliquée dans l'oxydation non photochimique du pool de PQ et utilise l'oxygène moléculaire comme accepteur terminal d'électrons. A l'obscurité le complexe Ndh et PTOX sont connectés au pool de PQ formant les deux éléments d'une chaîne de transfert d'électrons chlororespiratoire. L'effet du propyl gallate sur les plantes sauvages a permis de montrer un rôle similaire pour la protéine PTOX native au sein des chloroplastes de tabac.

A. Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration

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Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration

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Besides electron transfer reactions involved in the 'Z' scheme of photosynthesis, alternative electron transfer pathways have been characterized in chloroplasts. These include cyclic electron flow around photosystem I (PS I) or a respiratory chain called chlororespiration. Recent work has supplied new information concerning the molecular nature of the electron carriers involved in the non-photochemical reduction of the plastoquinone (PQ) pool. However, until now little is known concerning the nature of the electron carriers involved in PQ oxidation. By using mass spectrometric measurement of oxygen exchange performed in the presence of ^{18}O-enriched O_2 and *Chlamydomonas* mutants deficient in PS I, we show that electrons can be directed to a quinol oxidase sensitive to propyl gallate but insensitive to salicyl hydroxamic acid. This oxidase has immunological and pharmacological similarities with a plastid protein involved in carotenoid biosynthesis.

Keywords: chlororespiration; quinol oxidase; chloroplast; oxygen; *Chlamydomonas*

1. INTRODUCTION

During photosynthesis, two photosystems (PS II and PS I), coupled through an electron transfer chain, transform light energy to chemical energy. Besides this main electron transport pathway, called the 'Z' scheme of photosynthesis, alternative pathways such as cyclic electron transport around PS I (Arnon 1955; Heber & Walker 1992; Ravenel *et al*. 1994) and a respiratory chain called chlororespiration (Bennoun 1982; Peltier *et al*. 1987) have been identified in thylakoid membranes. Recent work has supplied some clues on the molecular properties of electron carriers involved in alternative pathways. First, a NAD(P)H dehydrogenase complex (Ndh), encoded by plastidial *ndh* genes, has been characterized in thylakoid membranes (Guedeney *et al*. 1996; Sazanov *et al*. 1998). Inactivation of *ndh* genes by plastid transformation was simultaneously performed by different laboratories (Burrows *et al*. 1998; Shikanai *et al*. 1998; Kofer *et al*. 1998; Cournac *et al*. 1998). It was shown that the Ndh complex is involved in the non-photochemical reduction of plastoquinones (PQ) occurring in the dark after a period of illumination and it was further suggested that this complex is involved in cyclic electron flow around PS I and in chlororespiration. Although not characterized at a molecular level, the existence of other activities, such as ferredoxin quinone reductase activity (Bendall & Manasse 1995; Endo *et al*. 1998) or non-electrogenic NAD(P)H dehydrogenase activity—different from the Ndh complex and involved in PQ reduction (Corneille *et al*. 1998)—have been reported in thylakoids.

If the nature of electron carriers involved in non-photochemical reduction of the PQ pool appears better understood, the nature of electron carriers involved in plastoquinol oxidation remains a subject of controversy. Recently, a homologue to mitochondrial alternative oxidase has been simultaneously characterized in *Arabidopsis* thylakoid membranes by two different laboratories (Carol *et al*. 1999; Wu *et al*. 1999). This enzyme, which is encoded by the nuclear gene *immutans*, has been shown to be essential during carotenoid biosynthesis and it was assumed that it might catalyse plastoquinol oxidation and be involved in chlororespiration. In contrast, based on experiments performed *in vitro*, Casano *et al*. (2000) recently proposed a chlororespiration model in which plastoquinol oxidation would be achieved by a plastidial peroxidase, H_2O_2 being used as an electron acceptor.

In order to elucidate the nature of the chlororespiratory oxidase, we have used photosynthetic mutants of the green alga *Chlamydomonas* and performed mass spectrometric measurements. Mass spectrometry, using ^{18}O-labelled O_2,

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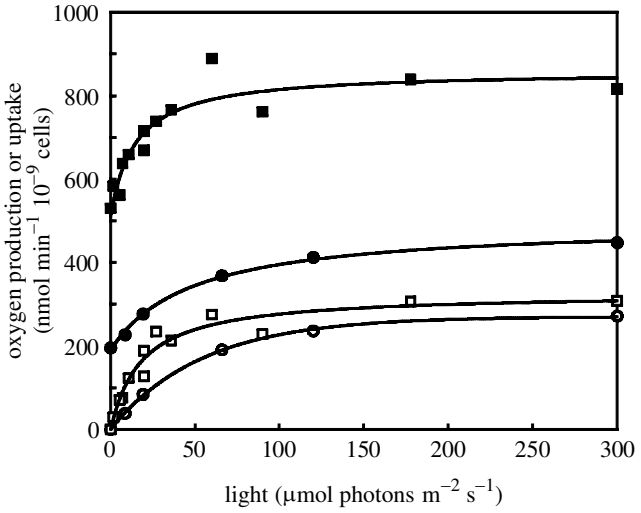

Figure 1. Photosynthetic oxygen evolution measured in intact *Chlamydomonas* cells. Measurements were performed in two independent PS-I-deficient strains showing different chlorophyll contents. Circles, mutant strain *psaAΔ*1, 0.44 mg chlorophyll 10^{-9} cells. Squares, mutant strain *psaBΔ*7, 2.3 mg chlorophyll 10^{-9} cells. Rates of PS II oxygen production (deduced from $^{16}O_2$ enrichment of the medium) and of oxygen uptake (deduced from $^{18}O_2$ depletion of the medium) are plotted versus the illumination intensity. Open circles, *psaAΔ*1 production; closed circles, *psaAΔ*1 uptake; open squares, *psaBΔ*7 production; closed squares, *psaBΔ*7 uptake.

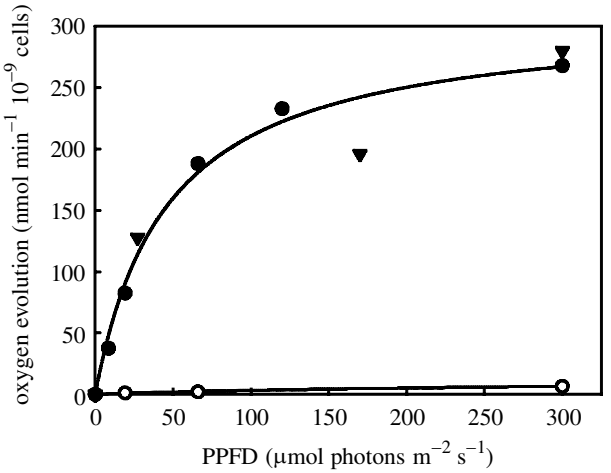

Figure 2. Effect of DCMU and loss of the cyt b_6f complex on the PS-II-driven O_2 production measured in intact *Chlamydomonas* cells using $^{18}O_2$ to label dissolved oxygen and monitoring $^{16}O_2$ (produced by PS II) and $^{18}O_2$ (taken up) with mass spectrometry. Closed circles, mutant strain *psaAΔ* deficient in PS I; open circle, mutant strain *psaAΔ* in the presence of 10 μM DCMU; closed triangles, double mutant *psaAΔ petAΔ* deficient in PS I and in the cyt b_6f complex.

is a powerful way to determine whether electrons produced at PS II (measured as unlabelled O_2 from water photolysis) are diverted towards O_2 or to another electron acceptor. By performing such measurements in *Chlamydomonas* preparations lacking either the PS I complex or the cytochrome (cyt) b_6f complex, we show that electrons provided by PS II can be diverted at a significant rate towards a chloroplast quinol oxidase. Based on the similarity of immunological (Cournac *et al*. 2000) and pharmacological properties between the *immutans* encoded plastid terminal oxidase (PTOX) in *Arabidopsis* and the plastoquinol oxidizing activity in *Chlamydomonas*, we propose the involvement of a quinol oxidase in chlororespiration.

2. EXPERIMENTAL PROCEDURES

Chlamydomonas reinhardtii cells were grown on a tris–acetate–phosphate medium (TAP). Algal cultures were maintained at 25 °C under continuous agitation and low illumination (about 1 μmol photons m^{-2} s^{-1}). The wild-type strain used in this work was isolated as a *mt*$^+$ segregant of a cross between two strains isogenic to the 137c strain (Harris 1989). The original deletions of *psaA* and *psaB* (chloroplast genes which encode essential subunits of PS I) were made in this strain as previously reported (Fischer *et al*. 1996). Marker recycling allowed subsequent transformations to delete the chloroplast *petA* gene, which encodes for an essential subunit of cyt b_6f (Cournac *et al*. 2000).

Prior to thylakoid isolation, the cells were harvested, centrifuged (600 *g*, 5 min) and washed once with 15 mM HEPES-KOH, pH 7.2. After centrifugation in the washing medium (600 *g*, 5 min), the pellet (around 5×10^8 cells) was resuspended in 10 ml buffer A (0.3 M sorbitol, 50 mM HEPES-KOH, pH 7.8, 2 mM EDTA, 5 mM $MgCl_2$) supplemented with 1% bovine serum albumin (BSA). Thylakoids were obtained through disruption in a French press chamber of the cells at 5000 psi in buffer A + 1% BSA (two runs). After disruption, broken or intact cells and heavy parts were discarded by centrifugation (600 *g*, 3 min). The supernatant was then centrifuged at 3000 *g*. The pellet (thylakoid fraction) was resuspended in 300–500 μl buffer A (without BSA) and stored on ice until used in the experiments. Oxygen exchange assays were conducted in buffer A without BSA.

Thylakoid membranes were resuspended in buffer A up to 1.5 ml in the measuring chamber. For measuring O_2 exchange on whole cells, algal cultures were harvested in exponential growth phase, centrifuged, washed and resuspended in buffer A. One and a half millilitres of the suspension was placed in the measuring chamber: a Clarke electrode-type thermostated and stirred cylindrical vessel (Hansatech, Norfolk, UK) fitted onto a mass spectrometer connecting device. Dissolved gases were directly introduced in the ion source of the mass spectrometer (model MM 14-80, VG instruments, Cheshire, UK) through a Teflon membrane as described in Cournac *et al*. (1993). For O_2 exchange measurements, the sample was sparged with N_2 to remove $^{16}O_2$, and $^{18}O_2$ (95% ^{18}O isotope content, Euriso-Top, Les Ulys, France) was then introduced to achieve an O_2 concentration in solution close to that in equilibrium with normal air. Light was supplied by a fibre-optic illuminator (Schott, Main, Germany) and neutral filters were used to vary light intensity. Unless specified, experiments shown here were performed at 300 μmol photons m^{-2} s^{-1} incident light. All gas exchange measurements were performed at 25 °C. The chloroplastic extracts were used as quickly as possible after extraction.

The portion of the *Arabidopsis immutans* cDNA coding for the entire mature peptide (PTOX) was PCR-amplified and inserted in the *Escherichia coli* expression vector pQE31 (Qiagen, Courtaboeuf, France) as described elsewhere (Cournac *et al*. 2000; Josse *et al*. 2000). The recombinant membrane protein PTOX which possesses a 6 His-tag was

Table 1. *Effect of electron acceptors on oxygen exchange*

(Measured in intact cells or thylakoids of the *psa AΔ* and *psaAΔ petAΔ* strains. *E*, photosynthetic O_2 evolution; δU, light-induced oxygen uptake (uptake in the light − uptake in the dark).)

	psaAΔ				*psaAΔ petAΔ*			
	nmol O_2 min^{-1} mg^{-1} chlorophyll				nmol O_2 min^{-1} mg^{-1} chlorophyll			
	intact cells		thylakoids		intact cells		thylakoids	
treatment	*E*	δU	*E*	δU	*E*	δU	*E*	δU
control	520	560	120	130	570	510	140	170
FeCN	620	640	670	80	550	590	170	160
DCBQ	980	10	900	0	1100	30	930	10

produced in *E. coli*. After induction, cells were lysed and membranes were recovered upon centrifugation at 100 000 *g* for 1 h. Pelleted membranes were resuspended in tris–HCl 0.2 M, pH 7.5, sucrose 0.75 M. Oxygen consumption was measured in a Clark O_2 electrode chamber (Hansatech). A typical assay contained 100 g membrane protein in the following buffer: tris–maleate 50 mM, pH 7.5, KCl 10 mM, $MgCl_2$ 5 mM, EDTA 1 mM, decyl-plastoquinone 0.2 mM.

3. RESULTS

PS-I-deficient algae obtained by inactivation of *psaA* or *psaB* genes were illuminated in the presence of ^{18}O-labelled O_2, and O_2 exchange was determined by mass spectrometry by following concentration changes in $^{18}O_2$ and $^{16}O_2$. As previously reported in nuclear mutants deficient in PS I (Peltier & Thibault 1988) or in plastid mutants (Cournac *et al*. 1997), significant O_2 evolution by PS II was measured, this phenomenon being accompanied by a simultaneous stimulation of O_2 uptake (figure 1). In these conditions, no change in the apparent respiration rate was observed, since light-dependent O_2 production and light-stimulated O_2 uptake are of the same amplitude. Light-dependent oxygen evolution was measured in different PS-I-deficient mutants. The maximal (light-saturated) activity was variable when expressed on a chlorophyll basis (from 120–600 nmol O_2 min^{-1} mg^{-1} chlorophyll), but was more constant when normalized to the cell number (250–350 nmol O_2 min^{-1} 10^{-9} cells) or to the protein amounts (8–13 nmol O_2 min^{-1} mg^{-1} protein), probably reflecting differences in chlorophyll contents between strains. Figure 1 shows O_2 exchange data in two strains with different chlorophyll contents. Comparable rates of maximal electron transfer activity were reached by both strains, but strains with higher chlorophyll contents were found to be more efficient at low light intensities. Note that the maximum rate of O_2 evolution in PS-I-deficient mutants represented about 10% of the maximal O_2 production rate measured in wild-type cells (not shown). The PS-II-dependent O_2 production was previously reported to be strongly affected by inhibition of mitochondrial respiration (Peltier & Thibault 1988; Cournac *et al*. 2000). However, we found that the light-driven activity of PS II was unaffected by the increase in respiration consecutive to acetate addition (data not shown) or by the level of basal respiration observed in different mutant strains (see figure 1). In contrast, the PS-II-dependent activity was found to vary during the algal cell cycle. Maximal activity was present during exponential growth, but severely decreased during the stationary phase (data not shown).

DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), an inhibitor blocking photosynthetic electron transfer between Q_A (the primary quinone acceptor of PS II) and Q_B (the secondary quinone acceptor, which exchanges with the PQ pool), strongly inhibited the PS-II-driven O_2 evolution (figure 2). Also, the PS-II-dependent electron flow was observed in the absence of the cyt b_6f complex in a *Chlamydomonas* double mutant *psaAΔ petAΔ* lacking both PS I and cyt b_6f (figure 2). Similar results were obtained in the single mutant (*petAΔ*) deficient in cyt b_6f or in the presence of 1 μM dibromothymoquinone (DBMIB), a cyt b_6f inhibitor (not shown). We conclude from these data that the PQ pool, but not the cyt b_6f complex, is involved in the PS-II-dependent pathway.

In order to determine the maximal PS II activity present in thylakoids of PS-I-deficient mutants, we measured photosynthetic O_2 evolution in the presence of artificial electron acceptors like 1,5-dichlorobenzoquinone (DCBQ) or potassium ferricyanide (FeCN) (table 1). In the presence of DCBQ, PS II activity was increased, indicating that PS II was not limiting the electron transport activity. In parallel, the light stimulation of O_2 uptake was completely suppressed. A similar effect was observed in whole cells and in a double mutant lacking PS I and the cyt b_6f complex (table 1). An increase in O_2 evolution was also observed in thylakoids of PS-I-deficient mutants when using FeCN as an electron acceptor. This effect was accompanied by a *ca*. 40% diminution of the light-induced stimulation of O_2 uptake (table 1). However, FeCN had no significant effect on the PS-II-dependent O_2 evolution in intact cells, which is explained by the fact that this compound cannot enter intact cells. Interestingly, FeCN has no significant effect on O_2 exchange rates measured in thylakoids from the *Chlamydomonas* strain lacking both PS I and the cyt b_6f complex (*psaAΔ petAΔ*, table 1).

A gene (*immutans*) encoding a plastid protein (PTOX) showing a high homology with the mitochondrial alternative oxidase, was recently discovered in *Arabidopsis thaliana* (Carol *et al*. 1999; Wu *et al*. 1999). As it was not easy to assay oxidase activity in *Arabidopsis* chloroplasts,

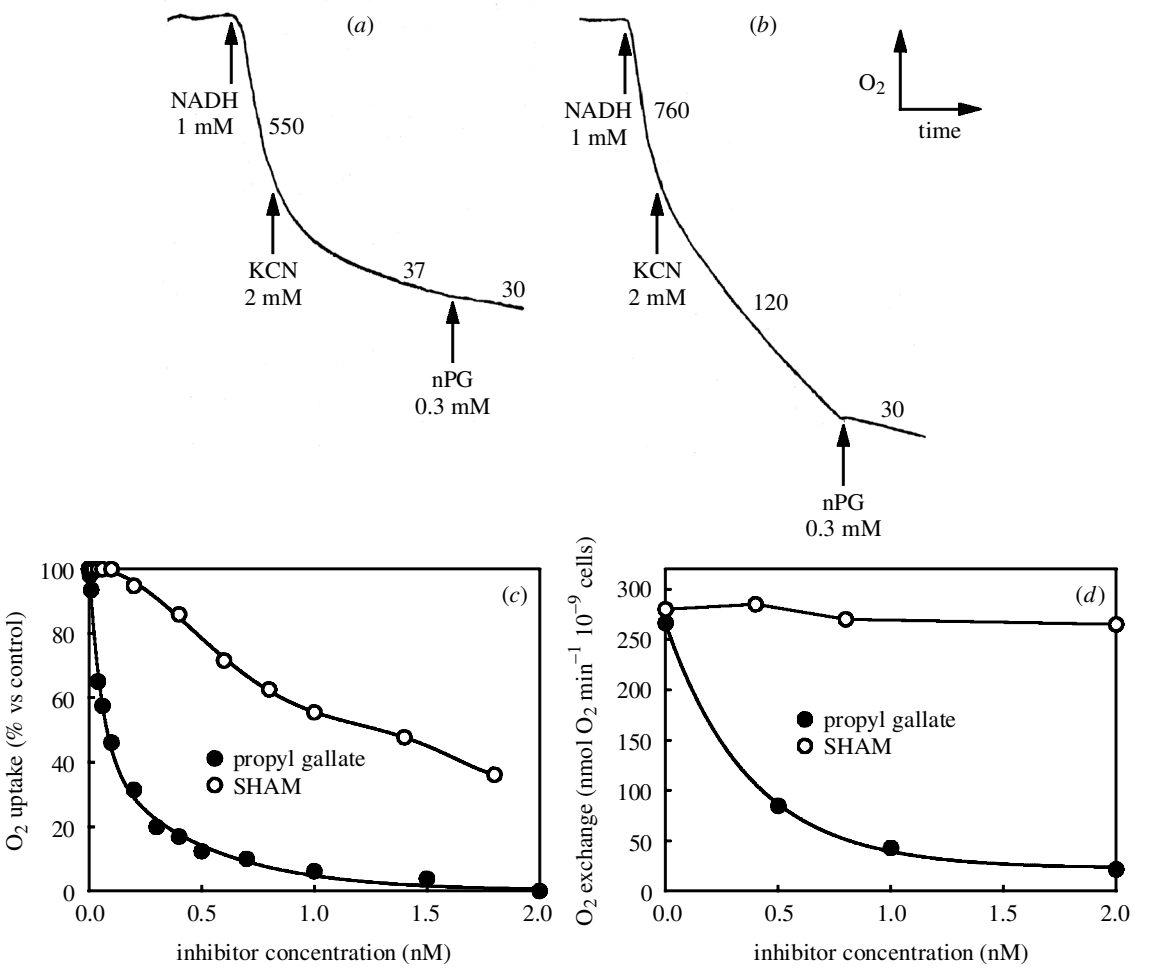

Figure 3. (*a*,*b*) Effects of sequential addition of KCN and propyl gallate on O_2 uptake induced by addition of NADH in membranes of *E. coli* from (*a*) control cells and (*b*) cells expressing the PTOX protein. O_2 uptake are given in nmol min^{-1} mg $protein^{-1}$. (*c*) Sensitivity of the PTOX-induced O_2 uptake to propyl gallate and SHAM in membranes of *E. coli*. (*d*) Sensitivity of PS-II-driven O_2 exchange to propyl gallate and SHAM in *Chlamydomonas* mutants deficient in PS I.

due to the low abundance of PTOX in chloroplasts and to the possible occurrence of mitochondrial cross-contamination, PTOX was produced as a recombinant protein in *E. coli*. After induction of the chimeric gene, the oxidase activity of membrane preparations was assayed by adding NADH and measuring oxygen consumption. KCN (1 mM) was used to inhibit oxygen consumption due to the cytochrome oxidase pathway (Josse *et al.* 2000). Expression of PTOX in *E. coli* membranes conferred a significantly higher cyanide-resistant oxygen consumption (figure 3*a*,*b*). Propyl gallate and salicylhydroxamic acid (SHAM) are well-known inhibitors of the mitochondrial alternative oxidase. The PTOX-dependent and cyanide-resistant oxidase activity was sensitive to propyl gallate (figure 3*a*–*c*), but at least ten times less sensitive to SHAM (figure 3*c*). The PS-II-dependent activity of PS-I-inactivated mutants showed comparable sensitivity to propyl gallate and was insentive to SHAM up to 2 mM (figure 3*d*).

4. DISCUSSION

(a) *Characteristics of photosynthetic electron transport in PS-I-deficient mutants*

In agreement with previous findings (Peltier & Thibault 1988; Cournac *et al.* 1997; Redding *et al.* 1999), results shown in this paper show that significant electron transport activity occurs from PS II to O_2 in PS-I-deficient *Chlamydomonas* mutants. Based on the effect of DCMU and on measurements performed in strains lacking the cyt b_6f complex, we conclude that the electron flow between PS II and molecular O_2 involves the thylakoid PQ pool, but not the cyt b_6f complex. Due to its electronic requirements and to its insensitivity to relative oxygen species (ROS) scavengers, PQ oxidation has been concluded to involve an enzymatic process reducing molecular O_2 into water (Cournac *et al.* 2000). As demonstrated here using an artificial electron acceptor for PS II (DCBQ), the activity of oxidase limits PS-II-dependent O_2 evolution in the absence of PS I. This explains why the maximal rates of O_2 evolution in PS-I-deficient cells are five to 20 times lower than that in wild-type cells, where PS I and cyt b_6f cooperate to reoxidize the PQ pool. However, light saturation curves of PS II activity indicate that PS-II-driven electron transport is limited by chlorophyll content at low light, and by oxidase content at high light. This suggests that oxygen uptake is not directly dependent on chlorophyll and is not related to chlorophyll photo-oxidation, further supporting the involvement of an enzymatic process in plastoquinol oxidation.

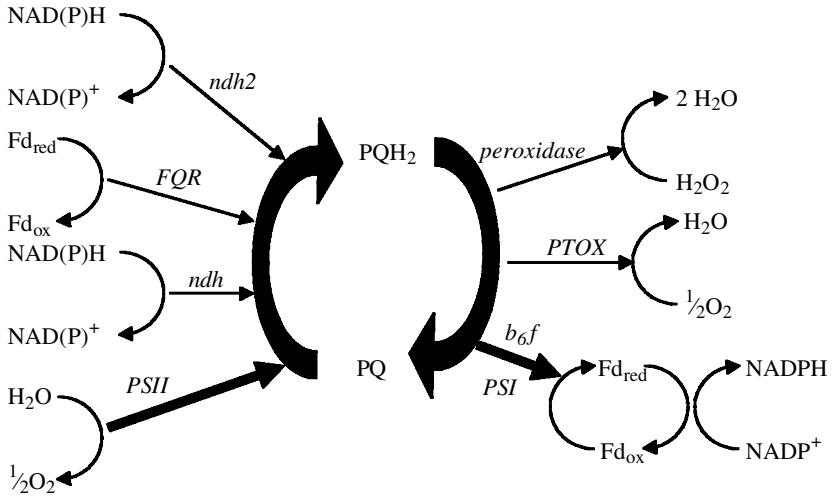

Figure 4. Schematic representations of the different plastoquinone (PQ) reduction and plastoquinol (PQH_2) oxidation pathways now evidenced in thylakoid membranes. Fd_{red}, reduced ferredoxin; Fd_{ox}, oxidized ferredoxin; ndh, complex I-like NAD(P)H dehydrogenase; ndh2, alternative NADH dehydrogenase; FQR, ferredoxin–quinone reductase; PTOX, plastid terminal oxidase (the quinol oxidase described in this paper).

Interestingly, we found that FeCN could accept electrons from PS II in PS-I-deficient strains containing cyt b_6f, but not in cyt b_6f-depleted strains. This shows that FeCN can interact with the intersystem photosynthetic electron transport chain, probably at the level of cyt *f* as previouly reported (Wood & Bendall 1976). This also indicates that the cyt b_6f complex of PS-I-deficient mutants keeps the ability to oxidize plastoquinol and to compete efficiently with the quinol oxidase.

The influence of various inhibitors has given us clues as to the nature of the chloroplast oxidase involved in this plastoquinol oxidation. Cyanide, which has been reported to impair chlororespiration (Buchel & Garab 1995; Bennoun 1982; Peltier *et al.* 1987) or cyanobacterial quinol oxidases (Howitt & Vermaas 1998; Buchel *et al.* 1998), had no effect unless very high concentrations were used (Cournac *et al.* 2000). The absence of effect of FeCN on plastoquinol oxidation in the cyt b_6f-deleted strain (table 1) also precludes the involvement of a soluble transporter such as soluble cytochromes, since FeCN can interact with such cytochromes, as shown in mitochondria (Hoefnagel *et al.* 1995).

(b) *Similarities between the* Chlamydomonas *plastoquinol oxidase and PTOX*

In plant mitochondria, quinol oxidation can be accomplished either by the cyt bc_1 complex (cyanide-sensitive pathway), or directly to molecular O_2 through an alternative oxidase (cyanide-insensitive pathway). Alternative oxidases have been reported to be inhibited by compounds such as SHAM or propyl gallate (Siedow 1980). We found that propyl gallate, but not SHAM, inhibited the PS II-to-O_2 electron flow in *C. reinhardtii* mutants deficient in PS I. Interestingly, Berthold (1998) reported the existence of different mutant forms of the *Arabidopsis thaliana* mitochondrial alternative oxidase that are resistant to SHAM but remain sensitive to propyl gallate, thus showing that sensitivity to these two inhibitors is separable.

Recently, two laboratories simultaneously reported the existence, in *Arabidopsis thaliana*, of a gene (*immutans*) coding for a plastid protein (PTOX) showing homology with mitochondrial alternative oxidases (Carol *et al.* 1999; Wu *et al.* 1999). Based on the phenotype of mutants affected in the *immutans* gene, it was concluded that PTOX is involved in carotenoid biosynthesis, more particularly in phytoene desaturation. The authors proposed a model in which PTOX would catalyse reoxidation of plastoquinol to PQ, using O_2 as a terminal acceptor. We have shown that PTOX, when expressed in *E. coli*, confers a KCN-insensitive quinol oxidase activity. In this assay, the plastid oxidase PTOX is sensitive to propyl gallate and much less sensitive to SHAM. Interestingly, PTOX appears to be more resistant to both inhibitors than mitochondrial alternative oxidase (Berthold 1998). Figure 3 indicates that both PTOX and the *Chlamydomonas* plastoquinol oxidase have similar sensitivities towards propyl gallate. Both activities show resistance towards SHAM, but PTOX appears significantly more sensitive. Differences in SHAM sensitivity can be explained by different hypotheses.

(i) The oxidases are not exactly the same, and the *Chlamydomonas* type is more resistant to SHAM.
(ii) The O_2 uptake in *E. coli* membranes is more sensitive to SHAM than in thylakoids, some modifications of its properties being induced by the expression system (a chimeric gene in a bacterial context).

Based on similar effects of inhibitors on PTOX and PS-II-driven electron flow, we conclude that the enzyme responsible for plastoquinol oxidation in *Chlamydomonas* is closely related to PTOX. This conclusion is further supported by immunological data (Cournac *et al.* 2000).

(c) *Oxygen, reactive oxygen species and chlororespiration*

We have concluded from our experiments that the major part of chloroplast O_2 uptake is due to the activity of a quinol oxidase that uses molecular O_2 as an electron acceptor and is sensitive to propyl gallate but insensitive to cyanide. Such a sensitivity to inhibitors appears contradictory to the involvement in chlororespiration of a cyanide-sensitive oxidase, as concluded by different authors (Bennoun 1982; Peltier *et al.* 1987; Buchel & Garab 1995). On the other hand, the use of molecular O_2 as a terminal acceptor is not consistent with the model of chlororespiration recently proposed by Casano *et al.* (2000). Indeed, based on experiments performed on an

in vitro reconstructed system, these authors proposed that plastoquinol oxidation was achieved by a plastid peroxidase using H_2O_2 as a terminal acceptor.

We cannot exclude at this stage the existence of different pathways of non-photochemical oxidation of plastoquinols, one involving a quinol oxidase and the other a peroxidase. According to Casano *et al.* (2000), the participation of a peroxidase might explain the cyanide sensitivity through an inhibition of superoxide dismutase. These different pathways might be differentially regulated depending on the environmental conditions. One might expect that the peroxidase pathway, provided that its existence is confirmed *in vivo*, would be associated with conditions generating ROS such as stress or senescence. On the other hand, PTOX would be involved in reactions occurring during the early biogenesis of chloroplasts (see Carol *et al.* 1999). This would be consistent with the higher plastoquinol oxidation activity observed during active phases of division. In this respect, it would be interesting to determine whether the peroxidase pathway is triggered during phases of senescence or in stress conditions.

It seems now likely that just as the non-photochemical PQ reduction pathways are diverse, so too are the chloroplastic O_2 (or ROS) uptake pathways (figure 4). Unravelling the molecular basis of these activities and their physiological significance will be an exciting task for the future.

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REFERENCES

Arnon, D. I. 1955 Conversion of light into chemical energy in photosynthesis. *Nature* **184**, 10–21.

Bendall, D. S. & Manasse, R. S. 1995 Cyclic photophosphorylation and electron transport. *Biochim. Biophys. Acta* **1229**, 23–38.

Bennoun, P. 1982 Evidence for a respiratory chain in the chloroplast. *Proc. Natl Acad. Sci. USA* **79**, 4352–4356.

Berthold, D. A. 1998 Isolation of mutants of the *Arabidopsis thaliana* alternative oxidase (ubiquinol: oxygen oxidoreductase) resistant to salicylhydroxamic acid. *Biochim. Biophys. Acta* **1364**, 73–83.

Buchel, C. & Garab, G. 1995 Evidence for the operation of a cyanide-sensitive oxidase in chlororespiration in the thylakoids of the chlorophyll *c*-containing alga *Pleurochloris meiringensis* (Xanthophyceae). *Planta* **197**, 69–75.

Buchel, C., Zsiros, O. & Garab, G. 1998 Alternative cyanide-sensitive oxidase interacting with photosynthesis in *Synechocystis* PCC6803. Ancestor of the terminal oxidase of chlororespiration? *Photosynthetica* **35**, 223–231.

Burrows, P. A., Sazanov, L. A., Svab, Z., Maliga, P. & Nixon, P. J. 1998 Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid *ndh* genes. *EMBO J.* **17**, 868–876.

Carol, P., Stevenson, D., Bisanz, C., Breitenbach, J., Sandmann, G., Mache, R., Coupland, G. & Kuntz, M. 1999 Mutations in the *Arabidopsis* gene *immutans* cause a variegated phenotype by inactivating a chloroplast terminal oxidase associated with phytoene desaturation. *Plant Cell* **11**, 57–68.

Casano, L. M., Zapata, J. M., Martin, M. & Sabater, B. 2000 Chlororespiration and poising of cyclic electron transport—plastoquinone as electron transporter between thylakoid NADH dehydrogenase and peroxidase. *J. Biol. Chem.* **275**, 942–948.

Corneille, S., Cournac, L., Guedeney, G., Havaux, M. & Peltier, G. 1998 Reduction of the plastoquinone pool by exogenous NADH and NADPH in higher plant chloroplasts—characterization of a NAD(P)H-plastoquinone oxidoreductase activity. *Biochim. Biophys. Acta* **1363**, 59–69.

Cournac, L., Dimon, B. & Peltier, G. 1993 Evidence for ^{18}O labeling of photorespiratory CO_2 in photoautotrophic cell cultures of higher plants illuminated in the presence of $^{18}O_2$. *Planta* **190**, 407–414.

Cournac, L., Redding, K., Bennoun, P. & Peltier, G. 1997 Limited photosynthetic electron flow but no CO_2 fixation in *Chlamydomonas* mutants lacking photosystem I. *FEBS Lett.* **416**, 65–68.

Cournac, L., Guedeney, G., Joet, T., Rumeau, D., Latouche, G., Cerovic, Z., Redding, K. Horvath, E., Medgyesy, P. & Peltier, G. 1998 Non-photochemical reduction of intersystem electron carriers in chloroplasts of higher plants and algae. In *Photosynthesis: mechanism and effects* (ed. G. Garab), pp. 1877–1882. Dordrecht, The Netherlands: Kluwer.

Cournac, L., Redding, K., Ravenel, J., Rumeau, D., Josse, E.-M., Kuntz, M. & Peltier, G. 2000 Electron flow between PS II and oxygen in chloroplasts of PS I deficient algae is mediated by a quinol oxidase involved in chlororespiration. *J. Biol. Chem.* (In the press.)

Endo, T., Shikanai, T., Sato, F. & Asada, K. 1998 NAD(P)H dehydrogenase-dependent, antimycin A-sensitive electron donation to plastoquinone in tobacco chloroplasts. *Plant Cell Physiol.* **39**, 1226–1231.

Fischer, N., Stampacchia, O., Redding, K. & Rochaix, J.-D. 1996 Selectable marker recycling in the chloroplast. *Mol. Gen. Genet.* **251**, 373–380.

Guedeney, G., Corneille, S., Cuine, S. & Peltier, G. 1996 Evidence for an association of *ndh* B, *ndh* J gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex. *FEBS Lett.* **378**, 277–280.

Harris, E. H. 1989 *The* Chlamydomonas *sourcebook. A comprehensive guide to biology and laboratory use.* San Diego, CA: Academic Press.

Heber, U. & Walker, D. A. 1992 Concerning a dual function of coupled cyclic electron transport in leaves. *Plant Physiol.* **100**, 1621–1626.

Hoefnagel, M. H., Millar, A. H., Wiskich, J. T. & Day, D. A. 1995 Cytochrome and alternative respiratory pathways compete for electrons in the presence of pyruvate in soybean mitochondria. *Arch. Biochem. Biophys.* **318**, 394–400.

Howitt, C. A. & Vermaas, W. F. J. 1998 Quinol and cytochrome oxidases in the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochemistry* **37**, 17 944–17 951.

Josse, E.-M., Simkin, A. J., Gaffé, J., Laboure, A.-M., Kuntz, M. & Carol, P. 2000 A plastid terminal oxidase associated with carotenoid desaturation during chromoplast differentiation. *Plant Physiol.* (Submitted.)

Kofer, W., Koop, H. U., Wanner, G. & Steinmuller, K. 1998 Mutagenesis of the genes encoding subunits A, C, H, I, J and K of the plastid NAD(P)H-plastoquinone-oxidoreductase in tobacco by polyethylene glycol-mediated plastome transformation. *Mol. Gen. Genet.* **258**, 166–173.

Peltier, G. & Thibault, P. 1988 Oxygen-exchange studies in *Chlamydomonas* mutants deficient in photosynthetic electron transport: evidence for a photosystem II-dependent oxygen uptake *in vivo*. *Biochim. Biophys. Acta* **936**, 319–324.

Peltier, G., Ravenel, J. & Verméglio, A. 1987 Inhibition of a respiratory activity by short saturating flashes in *Chlamydomonas*: evidence for a chlororespiration. *Biochim. Biophys. Acta* **893**, 83–90.

Ravenel, J., Peltier, G. & Havaux, M. 1994 The cyclic electron pathways around photosystem-I in *Chlamydomonas reinhardtii* as determined *in vivo* by photoacoustic measurements of energy storage. *Planta* **193**, 251–259.

Redding, K., Cournac, L., Vassiliev, I. R., Golbeck, J. H., Peltier, G. & Rochaix, J. D. 1999 Photosystem I is indispensable for photoautotrophic growth, CO_2 fixation, and H_2 photoproduction in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* **274**, 10 466–10 473.

Sazanov, L. A., Burrows, P. A. & Nixon, P. J. 1998 The plastid *ndh* genes code for an NADH-specific dehydrogenase: isolation of a complex I analogue from pea thylakoid membranes. *Proc. Natl Acad. Sci. USA* **95**, 1319–1324.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K. & Yokota, A. 1998 Directed disruption of the tobacco *ndh* B gene impairs cyclic electron flow around photosystem I. *Proc. Natl Acad. Sci. USA* **95**, 9705–970 9.

Siedow, J. N. 1980 Alternative respiratory pathway: its role in seed respiration and its inhibition by propyl gallate. *Plant Physiol.* **65**, 669–674.

Wood, P. M. & Bendall, D. S. 1976 The reduction of plastocyanin by plastoquinol-1 in the presence of chloroplasts. A dark electron transfer reaction involving components between the two photosystems. *Eur. J. Biochem.* **61**, 337–344.

Wu, D. Y., Wright, D. A., Wetzel, C., Voytas, D. F. & Rodermel, S. 1999 The *immutans* variegation locus of *Arabidopsis* defines a mitochondrial alternative oxidase homolog that functions during early chloroplast biogenesis. *Plant Cell* **11**, 43–55.

Discussion

J. Barber (*Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, UK*). Have you estimated the stoichiometric level of the PQ oxidase and the NAD complex in normal chloroplasts relative to the major complexes such as PS I, PS II and cyt b_6f?

G. Peltier. Sazanov *et al.* (1996) have estimated that the Ndh complex of pea chloroplasts represented less than 0.2% of total thylakoid membrane proteins (about one complex every 100 photosynthetic chains). It is therefore clearly a minor component of thylakoid membranes compared with the major complexes such as PS I, PS II or cyt b_6f. We have not yet estimated the amounts of the chlororespiratory oxidase present in thylakoid membranes. However, one may speculate that, like the Ndh complex, it represents a minor component of thylakoid membranes. This probably explains why these enzymes have not been discovered earlier.

K. Niyogi (*Department of Plant and Microbial Biology, University of California, Berkeley, USA*). Have you found any evidence for reverse electron flow through the Ndh complex?

G. Peltier. No, we have no evidence for this occurrence of reverse electron flow through the Ndh complex. Initially, the inhibition of the PS-II-dependent O_2 evolution by respiratory inhibitors observed in intact *Chlamydomonas* cells from PS-I-deficient mutants was interpreted by the generation of NAD(P)H through an energy-dependent reverse electron transfer occurring through a putative chloroplast Ndh complex and a transfer of reducing equivalents from the chloroplast to the mitochondria (Peltier & Thibault 1988). However, as shown here, and as recently published by Cournac *et al.* (2000), PS-II-dependent O_2 evolution could be measured in chloroplasts from PS-I-deficient mutants and was insensitive to respiratory inhibitors. We have concluded from these data that the PS-II-dependent O_2 evolution observed in PS-I-deficient mutants is due to a diversion of electrons towards a chloroplast oxidase. The inhibition of the PS-II-dependent electron flow by respiratory inhibitors would be explained by a competition between PS II and stromal donors for the reduction of the PQ pool. Moreover, it now seems clear that the plastid genome of most unicellular algae lacks *ndh* genes. In *Chlamydomonas*, non-photochemical reduction of the PQ pool is probably achieved by a non-electrogenic enzyme (for a review, see Cournac *et al.* 2000). This argues against the existence of a reverse electron flow, which would be only possible with an electrogenic complex. In higher plants, such a possibility cannot be excluded, since the Ndh complex is probably electrogenic, but no evidence for such a mechanism has been obtained until now.

C. H. Foyer (*Department of Biochemistry and Physiology, IACR-Rothamsted, UK*). The role of the alternative oxidase in the mitochondrial electron transport chains is considered to be prevention of over-reduction of the PQ pool and hence uncontrolled electron drainage to oxygen. Would you consider that a possible role of the chloroplast oxidase is to prevent over-reduction of the PQ pool and hence photoinhibition?

G. Peltier. Such a role should be considered. It is clear from our experiments that in PS-I-deficient *Chlamydomonas* mutants electrons can be diverted towards the chloroplast oxidase. Whether this reaction occurs *in vivo* in the presence of active PS I remains to be answered. One may speculate that in conditions where PS I is partially inhibited, for instance during introduction of photosynthesis, where electron acceptors are lacking, or during low temperature photoinhibition, diversion towards the oxidase may prevent over-reduction of the PQ pool.

A. Laisk (*Department of Plant Physiology, Tartu University, Estonia*). Is chloroplast Ndh a proton translocating enzyme? The background of my question is that with G. Edwards we measured quantum yields of C_4 plants and found them to be 15% higher than possible considering the known efficiency of cyclic electron transport. The discrepancy could be resolved with the assumption that proton-translocating Ndh participates in the cyclic electron flow in C_4-plant bundle-sheath chloroplasts.

G. Peltier. Based on the homology between plastid Ndh genes and bacterial genes encoding subunits of the NADH dehydrogenase complex, it seems likely that the chloroplast Ndh complex involved in chlororespiration and cyclic electron flow around PS I is a proton-translocating enzyme. In C_4 plants, Kubicki *et al.* (1996) have reported strong expression of Ndh genes in bundle-sheath chloroplasts. Possibly, the participation of such a proton-translocating complex to cyclic electron flow around PS I may explain increases in quantum yields.

H. C. P. Matthijs (*Department of Microbiology, University of Amersterdam, The Netherlands*). Professor Badger asked about the role of Ndh 1 in PS I cyclic, and pointed to the fact that Ndh 1, in addition to a role in PS I cyclic,

may be directly linked to CO_2 uptake. To this I added that in a Ndh-1-less mutant of the cyanobacterium *Synechocystis* which cannot grow in low CO_2 condition, growth on low CO_2 can be restored after (NaCl) stress. In this stress, PS I cyclic activity increases two- to three-fold, flavodoxin and FNR induction up to 20–30 times. This shows an intimate relationship between PS I cyclic and CO_2 uptake (Jeanjean *et al.* 1998).

G. Peltier. Our recent studies on Ndh-inactivated mutants (Horvath *et al.* 2000), have shown a role of the Ndh complex during photosynthesis under low CO_2 concentration, for instance during a stomatal closure induced by water limitation. Our interpretation is that under such conditions the requirement of photosynthetic CO_2 fixation for ATP is higher. To fix one CO_2, an ATP–NADPH ratio of 1.5 is needed under non-photorespiratory conditions, but under photorespiratory conditions this ratio increases up to 1.65. We proposed that cyclic electron flow around PS I mediated by the Ndh complex is a putative CO_2 concentrating mechanism similar to that occurring in cyanobacteria or algae. In this respect, the existence in the chloroplast genome of an open reading frame encoding a protein sharing homologies with a cyanobacterial and *Chlamydomonas* protein involved in CO_2 concentrating mechanisms is rather intriguing. However, until now, such a mechanism has not been evidenced in higher plant chloroplasts.

Additional References

Cournac, L., Redding, K., Ravenel, J., Rumeau, D., Josse, E.-M., Kuntz, M. & Peltier, G. 2000 Electron flow between PS II and oxygen in chloroplasts of PS I deficient algae is mediated by a quinol oxidase involved in chlororespiration. *J. Biol. Chem.* (In the press.)

Horvath, E. M., Peter, S. O., Joët, T., Rumeau, D., Cournac, L., Horvath, G. V., Kavanagh, T. A., Schäfer, C., Peltier, G. & Medgyesy, P. 2000. Targeted inactivation of the plastid *ndhB* gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. *Plant Physiol.* **123**, 1337–1350.

Jeanjean, R., Bedu, S., Havaux, M., Matthijs, H. C. P. & Joset, F. 1998 Salt-induced photosystem I cyclic electron transfer restores growth on low inorganic carbon in a type 1 NAD(P)H dehydrogenase deficient mutant of *Synechocystis* PCC6803. *FEMS Microbiol. Lett.* **167**, 131–137.

Kubicki, A., Funk, E., Westhoff, P. & Steinmüller, K. 1996 Differential expression of plastome-encoded *ndh* genes in mesophyll and bundle-sheath chloroplasts of the C_4 plant *Sorghum bicolor* indicates that the complex I-homologous NAD(P)H-plastoquinone oxidoreductase is involved in cyclic electron transport. *Planta* **199**, 276–281.

Peltier, G. & Thibault, P. 1988 Oxygen-exchange studies in *Chlamydomonas* mutants deficient in photosynthetic electron transport: evidence for a photosystem II-dependent oxygen uptake *in vivo*. *Biochim. Biophys. Acta* **936**, 319–324.

Sazanov, L. A., Burrows, P. & Nixon, P. J. 1996 Detection and characterization of a complex I-like NADH-specific dehydrogenase from pea thylakoids. *Biochem. Soc. Trans.* **24**, 739–743.

B. Involvement of a plastid terminal oxidase in both chlororespiratory and photosynthetic electron transport chains: evidence from overexpression in tobacco

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Involvement of a Plastid Terminal Oxidase in Plastoquinone Oxidation as Evidenced by Expression of the *Arabidopsis thaliana* Enzyme in Tobacco*

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Chlororespiration has been defined as a respiratory electron transport chain in interaction with photosynthetic electron transport involving both non-photochemical reduction and oxidation of plastoquinones. Different enzymatic activities, including a plastid-encoded NADH dehydrogenase complex, have been reported to be involved in the non-photochemical reduction of plastoquinones. However, the enzyme responsible for plasquinol oxidation has not yet been clearly identified. In order to determine whether the newly discovered plastid oxidase (PTOX) involved in carotenoid biosynthesis acts as a plastoquinol oxidase in higher plant chloroplasts, the *Arabidopsis thaliana PTOX* gene (*At-PTOX*) was expressed in tobacco under the control of a strong constitutive promoter. We showed that At-PTOX is functional in tobacco chloroplasts and strongly accelerates the non-photochemical reoxidation of plastoquinols; this effect was inhibited by propyl gallate, a known inhibitor of PTOX. During the dark to light induction phase of photosynthesis at low irradiances, At-PTOX drives significant electron flow to O_2, thus avoiding over-reduction of plastoquinones, when photosynthetic CO_2 assimilation was not fully induced. We proposed that PTOX, by modulating the redox state of intersystem electron carriers, may participate in the regulation of cyclic electron flow around photosystem I.

In photosynthetic organisms like photosynthetic bacteria or cyanobacteria, photosynthesis and respiration operate in close interaction within the same membranes where they share some electron transport components such as the plastoquinone (PQ)[1] pool (1). In chloroplasts, the existence of a respiratory electron transport chain (chlororespiration) in interaction with photosynthesis has been suggested (2, 3), and this activity has been proposed to originate from the cyanobacterial ancestor of chloroplasts (1). Chlororespiration would involve non-photochemical reduction of the PQ pool and subsequent oxidation by a plastoquinol terminal oxidase. Non-photochemical reduction of PQs is a well established phenomenon that likely occurs during cyclic electron transfer reactions around photosystem I (PS I). A plastid-encoded NADH dehydrogenase (Ndh) complex showing homologies with bacterial complex I has been characterized in thylakoid membranes from higher plants (4–7). Inactivation of some *ndh* genes using plastid transformation of tobacco showed the involvement of the Ndh complex in non-photochemical reduction of PQs (8, 9). It was proposed that the Ndh complex participates in both chlororespiration and cyclic electron transfer around PS I (8–11). A role of the Ndh complex in cyclic electron flow around PS I was recently confirmed by photoacoustic measurements performed in tobacco *ndh* mutants (12). It should be noted that alternate activities, such as a putative ferredoxin-PQ reductase (FQR) (13, 14) or a Ndh-2 type activity may also be involved in these processes (15, 16).

Nevertheless, the involvement of a plastid terminal oxidase in chlororespiration has been the subject of controversy during the last decade (2, 3, 16, 17). Initially, the existence of chlororespiration was based mainly on the effect of respiratory inhibitors such as cyanide and CO on the redox state of the PQ pool (2). However, such effects can be alternatively explained by an inhibition of mitochondrial respiration and the existence of redox interactions between chloroplasts and mitochondria (16–20). Recently, the study of an *Arabidopsis* mutant (*immutans*) showing a variegated phenotype led to the identification of a protein involved in carotenoid biosynthesis (21, 22). Based on sequence homology with mitochondrial alternative oxidases, this protein was suggested to act as a plastid terminal oxidase (PTOX) (21, 23). Expression of *Arabidopsis* PTOX (At-PTOX) in *Escherichia coli* conferred a cyanide-resistant O_2 uptake sensitive to propyl gallate, a known inhibitor of alternative oxidases (24, 25). In PS I-less mutants of the green algae *Chlamydomonas reinhardtii*, a limited but significant electron flow from photosystem II (PS II) to molecular O_2 was measured. Based on the effects of inhibitors (insensitivity to KCN and CO and sensitivity to propyl gallate) on this process and on the detection of a thylakoid protein that cross-reacted with an antibody raised against PTOX, it was proposed that the chlororespiratory O_2 uptake is because of a *Chlamydomonas* homologue of PTOX (24). In higher plants, an involvement of PTOX in PQ oxidation has not been experimentally evidenced. Based on the study of a reconstituted system, Casano *et al.* (6) proposed that a peroxidase using hydrogen peroxide as an electron acceptor may be involved in chlororespiration.

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[1] The abbreviations used are: PQ, plastoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Ndh, NADH dehydrogenase complex; PS I and II, photosystem I and II, respectively PTOX, plastid terminal oxidase; WT, wild type; MES, 2-(*N*-morpholino)ethanesulfonic acid; At-PTOX, *A. thaliana* PTOX; RT, reverse transcriptase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

To get further insight into the function of PTOX and in particular to determine whether this protein can achieve quinol oxidation in chloroplasts, tobacco plants constitutively expressing At-PTOX have been generated. We show that At-PTOX facilitates the oxidation of reduced PQs using O_2 as a terminal acceptor.

EXPERIMENTAL PROCEDURES

Plant Material—Tobacco plants (*Nicotiana tabacum* var. *petit havana*) were grown on compost in a phytotron (25 °C day/20 °C night; 12-h photoperiod) under an irradiance of 300 μmol photons·m^{-2}·s^{-1} supplied by quartz halogen lamps (HQI-T 400W/DV, Osram, Germany). Plants were watered with a half-diluted Hoagland's nutritive solution.

Production of Transgenic At-PTOX Tobacco Plants Overexpressing At-PTOX—The *Arabidopsis thaliana* PTOX cDNA (GenBank™ accession number AJ004881) was used as a template for PCR amplification using the primers 5′-CCGCTCGAGCCTGACGGAGATGGCGGCGATTTCAGG-3′ and 5′-CCCGAGCTCTTATTAACTTGTAATGGATTTCTTGAGGC-3′, respectively, containing an *Xho*I and an *Sac*I restriction site at the 5′ and 3′ end. The amplified fragment started 9 bp upstream to the coding sequence of the At-PTOX cDNA and contained two stop codons (the start codon and two stop codons are underlined). After digestion, the amplified fragment was introduced in a sense orientation into a plant binary vector (pKYLX71). Expression of At-PTOX was driven by a double sequence of the cauliflower mosaic virus ^{35}S-labeled constitutive promoter (26). The recombinant plasmid was introduced by electroporation into *Agrobacterium tumefaciens* (strain C58), which was used for tobacco transformation employing the standard leaf disc transformation method (27). Two independent transformation experiments were carried out, and six transformants were recovered on a kanamycin-selective medium (100 mg·liter^{-1}). Two independent transgenic lines ($PTOX_1^+$ and $PTOX_2^+$), overexpressing high amounts of PTOX, were selected and were self-pollinated. The T1 generation was used for further experiments.

Preparation of Osmotically Lysed Chloroplasts for O_2 Exchange and Chlorophyll Fluorescence Measurements—Leaves were harvested at the end of the night period, and intact chloroplasts were isolated at 4 °C on a Percoll gradient according to a modification of the method described by Mills and Joy (51). Approximately 30 g of leaves were ground in a blender for 2 s in 100 ml of medium A containing 330 mM sorbitol, 50 mM Tricine-NaOH, pH 7.8, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM ascorbic acid, and 5 mM dithiothreitol. After filtration through 250- and 60-μm nylon net, followed by centrifugation (2000 × *g*, 3 min), the crude extract was resuspended in medium A (dithiothreitol-free) and layered onto a Percoll step gradient formed with two layers of medium A containing 90 and 40% (v/v) Percoll, respectively. After centrifugation in a swing out rotor at 3,500 × *g* for 15 min, intact chloroplasts were recovered from the 40:90% Percoll interphase, washed with 60 ml of medium A, pelleted at 2,000 × *g* for 3 min, and osmotically lysed by resuspension in 10 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride for 30 min. Lysed chloroplasts were diluted at a final concentration of 200 μg of chlorophyll·ml^{-1} in 30 mM Hepes-KOH buffer, pH 7.5, containing 0.3 M sorbitol, 5 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $NaHPO_4$, 50% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride. Aliquots of the chloroplast preparation were stored at −20 °C. For O_2 exchange and chlorophyll fluorescence measurements, aliquots were resuspended in 30 mM Hepes-KOH buffer, pH 7.5, containing 0.3 M sorbitol, 5 mM NaCl, and 10 mM $MgCl_2$. $(F_m - F_0)/F_m$ measured on chloroplasts samples was 0.7 (±0.02, 6 experiments). Anaerobiosis was achieved by addition of glucose (20 mM) and glucose oxidase (2 mg·ml^{-1}) to the chloroplast suspension. Reactive oxygen species generated by the glucose oxidase activity were scavenged by adding superoxide dismutase (500 units·ml^{-1}) and catalase (1,000 units·ml^{-1}).

Mass Spectrometric O_2 Exchange Measurements—For mass spectrometric measurements of O_2 exchange, osmotically lysed chloroplasts (20 μg of chlorophyll·ml^{-1}) were placed in the measuring chamber (1.5-ml reaction volume). The sample was sparged with N_2 to remove $^{16}O_2$, and $^{18}O_2$ (95% ^{18}O isotope content, Euriso-Top, Les Ulys, France) was then introduced to reach an O_2 concentration close to the equilibrium with air. Dissolved gases were introduced into the ion source of the mass spectrometer (model MM 14–80, VG Instruments, Cheshire, UK) through a Teflon membrane. Light was supplied by a fiber optic illuminator (Schott, Main, Germany) supplying a light intensity of 150 μmol photons·m^{-2}·s^{-1}. All gas exchange measurements were performed at 25 °C. The use of $^{18}O_2$ allowed the *in vivo* determination of O_2 evolution by PS II (originating from the photolysis of water which is not enriched) in the presence of O_2 consuming processes.

Chlorophyll Fluorescence Measurements in Chloroplasts and Leaves—Chlorophyll fluorescence was measured at 25 °C using pulse-modulated fluorimeters (PAM 101–103 and PAM 2000, Walz, Effeltrich, Germany for chloroplasts and leaves, respectively). The maximal chlorophyll fluorescence level (F_m) was measured under a 0.8–1-s saturating pulse (about 8,000–10,000 μmol photons·m^{-2}·s^{-1}) in dark-adapted leaves, on which the basal fluorescence (F_0) was recorded before the pulse. The maximal photochemical yield of PS II was determined as $(F_m - F_0)/F_m$. Fluorescence levels F_m, F_s (fluorescence in the light), $F_{m'}$ (maximal fluorescence in the light, using a saturating pulse), and $F_{0'}$ (basal fluorescence of light-adapted leaves, recorded after rapid reoxidation of the PQ pool using far-red light) were used to calculate PS II photochemical yield ($F_{m'} - F_s/F_{m'}$), non-photochemical quenching ($qN = 1 - F_{m'}/F_m$), and photochemical quenching ($qP = F_{m'} - F_s/F_{m'} - F_{0'}$) under different irradiances (28). Apparent photosynthetic electron transport rates (μmol electrons·m^{-2}.s^{-1}) were estimated as $(F_{m'} - F_s)/F_{m'} \times PFFD_i \times LA \times 0.5$, where $PFFD_i$ is the incident photosynthetic photon flux density; LA is the leaf absorbance (0.84), and 0.5 the factor accounting for the light partition between the two photosystems.

For chlorophyll fluorescence measurements in stripped leaf discs, leaf samples were placed on a wet paper filter at 25 °C in ambient air. Chlorophyll fluorescence measurements on attached leaves were performed using the gas exchange cuvette of a Licor gas exchange system (LI-6400, Li-Cor Inc, Lincoln, NE) to control leaf temperature (25 °C) and gas atmosphere. Illumination was provided by a homemade red (663 nm) LEDs source.

Inhibitor Treatment of Leaf Discs—Leaf discs were sampled from 5- to 8 week-old plants. After stripping the lower epidermis, leaf discs were soaked in water for 60 min. 2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB, 50 μM final concentration) or propyl gallate (1 mM final concentration) was added diluted in methanol (maximal final methanol concentration was 0.5%). Control leaf discs were soaked in water containing methanol. It should be noted that the DBMIB concentration (50 μM) used for leaf discs was much higher than the concentration generally used to obtain specific inhibition of the cytochrome b_6/f complex on isolated thylakoids (1 μM). Despite this relatively high concentration necessary to obtain an effect in leaf discs, we checked that DBMIB did not act as an electron acceptor.

Photosynthetic CO_2 Fixation Measurements on Attached Leaves—Net CO_2 exchange measurements were performed on attached leaves using a portable gas exchange system (LI-6400, Li-Cor Inc, Lincoln, NE) and a homemade red (663 nm) LEDs source. Leaf temperature was maintained at 25 °C, and leaf vapor pressure deficit was maintained around 0.8 kPa. Various O_2 and N_2 concentrations were provided by mixing pure gases. O_2 concentration was measured using a paramagnetic O_2 analyzer (MAIHAK, Hamburg, Germany). This mixing system was also used for fluorescence measurements in attached leaves. Quantum yield of CO_2 fixation in air and under non-photorespiratory conditions (O_2 1.5% (v/v); CO_2 750 μl·liter^{-1}) were calculated from the slope of the linear portion of the light response curve (5 measurements at irradiances between 40 and 80 μmol photons·m^{-2}·s^{-1} and 10 measurements at irradiances between 10 and 100 μmol photons·m^{-2}·s^{-1} for air and non-photorespiratory conditions, respectively).

Electrophoresis and Western Analysis on Chloroplast Fractions—Intact chloroplasts were isolated and purified from leaves using discontinuous Percoll (Amersham Biosciences) gradients as described previously (29). Chloroplasts were osmotically lysed in a solution containing 20 mM MES, pH 6.0, 15 mM NaCl, and 5 mM $MgCl_2$ and centrifuged for 20 min at 35,000 × *g*. Stroma lamellae and grana membranes were separated following a stacking step carried out as described previously (4).

To prepare total insoluble proteins, tobacco leaves (1 g fresh weight) were frozen in liquid nitrogen and ground to a fine powder with a chilled pestle and mortar. The powder was resuspended in a 5-ml extraction buffer (50 mM Tris-HCl, pH 8.0) containing 50 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. After 30 min of stirring (4 °C) and centrifugation (40,000 × *g* for 20 min), the pellet was resuspended in the same buffer containing 1% SDS. After 30 min of stirring (4 °C) and centrifugation (40,000 × *g* for 20 min), proteins contained in the supernatant were precipitated with acetone (80% final concentration).

Denaturing SDS-PAGE was performed as described by Laemmli (30) using 13% acrylamide (w/v) gels. Proteins were transferred onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell) and were probed with the purified anti-At-PTOX serum (24). Immunocomplexes were detected using the chemiluminescence Western blotting kit (Amersham Biosciences).

Transgene Transcript Analysis—RT-PCR analysis of *At-PTOX* transcripts was carried out as described previously (25) by amplifying 363

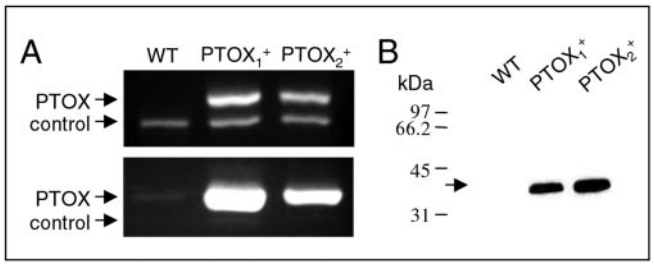

FIG. 1. **At-PTOX transcripts and protein levels in transgenic tobacco lines (PTOX$^+$).** *A*, mRNA levels were determined by RT-PCR amplification of total cellular RNA (*upper panel*) and subsequently re-amplified by a second PCR (*lower panel*). PCR products were separated by electrophoresis on a 1.5% (v/v) agarose gel and visualized by ethidium bromide staining. Total mRNA amounts were standardized beforehand based on fluorescence intensities observed in an ethidium bromide containing RNA gel. Amplification of globin mRNA (added to the RT reaction mix) was used as a control for the RT-PCR to ensure that each sample was reverse-transcribed and amplified equally. *B*, Western analysis was performed on total insoluble leaf proteins (20 μg of proteins) from WT and two PTOX$^+$ transgenic lines, using an anti-At-PTOX antibody.

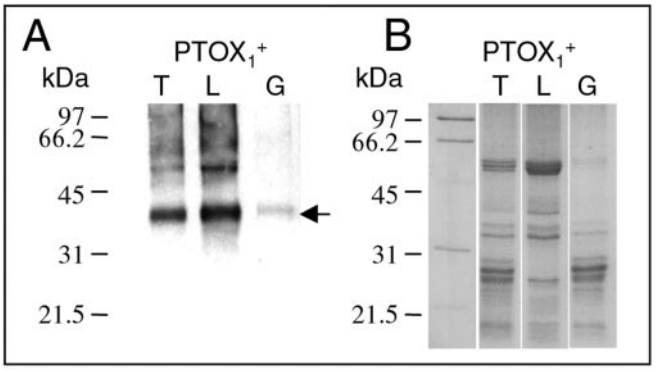

FIG. 2. **Membrane localization of At-PTOX in PTOX$^+$ tobacco chloroplasts.** *A*, Western analysis using an anti-At-PTOX antibody, performed on different membrane fractions prepared from tobacco chloroplasts (5 μg of proteins). *B*, separation of protein fractions (5 μg of proteins) derived from purified chloroplasts by fully denaturing PAGE revealed by Coomassie Brilliant Blue staining. *T*, thylakoid membrane proteins; *L*, stroma lamellae proteins; *G*, grana proteins.

nucleotides of the *At-PTOX* RNAs using the following primers: 5′-GTG CAY TTT GCI GAR AGC TGG AAT G-3′ and 5′-TCA TYG TIT TIC AAT GIT CTG CIT CRT CAT CTC-3′, where Y = C + T, R = A + G, and I = deoxyinosine. The amplification consisted of 30 cycles of 30 s at 94 °C, 20 s at 45 °C, and 20 s at 72 °C. The control was the rabbit globin mRNA (supplied by Invitrogen).

Protein and Chlorophyll Determination—Protein content was determined using a modified Lowry method (Sigma). Chlorophyll content was measured according to the method of Lichtenthaler and Wellburn (31).

RESULTS

Expression of At-PTOX in Tobacco—Transgenic tobacco plants expressing the *At-PTOX* cDNA sequence under the control of the doubled constitutive ^{35}S promoter of the cauliflower mosaic virus were generated by *Agrobacterium*-mediated transformation. Two lines, PTOX$_1^+$ and PTOX$_2^+$, showing a particularly strong expression of the transgene were selected among six transformant lines and were further studied (Fig. 1*A*). Note that although no signal was observed in WT tobacco (Fig. 1*A*, *upper panel*), a faint band was detected after re-amplification (Fig. 1*A*, *lower panel*). We checked that amplified RT-PCR fragments, including the faint band amplified in WT tobacco (Fig. 1*A*), cross-hybridized with the At-PTOX probe by Southern analysis (data not shown). Antibodies raised against At-PTOX were used to characterize At-PTOX expression in tobacco transgenic lines using Western analysis. Both transformant lines showed large amounts of a 41-kDa band corresponding to At-PTOX in total insoluble leaf proteins (25), whereas no signal was observed in wild type (Fig. 1*B*). In both lines, At-PTOX was targeted to the chloroplasts, thanks to the presence of an N-terminal transit peptide (22), and was found to be associated with thylakoid membranes, essentially stroma lamellae, with only small amounts being found in grana (Fig. 2). Subsequent experiments were performed on both PTOX$_1^+$ and PTOX$_2^+$ lines and yielded similar results.

Because PTOX has been reported previously (21, 22) to be involved in carotenoid biosynthesis, the pigment content of transgenic plants was analyzed. High pressure liquid chromatography measurements did not reveal any significant difference in chlorophyll or carotenoid content in WT and PTOX$^+$ extracts (data not shown). In addition, after transfer to high light conditions, similar amounts of xanthophyll cycle carotenoids (violaxanthin, zeaxanthin, and antheraxanthin) were found in both plants. PTOX$_1^+$ and PTOX$_2^+$ plants did not show any particular phenotype, and growth was comparable with WT plants when cultivated under normal conditions (not shown).

Expression of At-PTOX Suppresses the Post-illumination F_0 Fluorescence Increase—When intact WT leaves were illuminated for a few minutes and then placed in the dark, a transient increase in the F_0 chlorophyll fluorescence level occurred (Fig. 3*A*) (see Refs. 32 and 33). The post-illumination fluorescence transient was absent in PTOX$^+$ leaves, and the F_0 fluorescence level rapidly decreased after switching off the light (Fig. 3*B*). As reported previously, the fluorescence increase was absent in Ndh-less mutants (Fig. 3*C*), but interestingly the fluorescence signal decreased more slowly that in PTOX$^+$. The absence of a post-illumination chlorophyll fluorescence increase in Ndh-less mutants was interpreted as the involvement of the Ndh complex in the re-reduction of the PQ pool occurring in the dark after a period of illumination (8, 9, 34). This experiment suggests that like the Ndh complex At-PTOX was able to modulate the redox state of PQ in the dark, most likely by oxidizing reduced plastoquinones. In agreement with this interpretation, when leaf discs were treated with propyl gallate, a potent inhibitor of PTOX (24), a reversal of the loss of the F_0 fluorescence rise was observed (data not shown). Subsequent experiments were designed to characterize the role of At-PTOX in PQ oxidation.

Involvement of At-PTOX in the Dark Oxidation of the PQ Pool—In the experiment described in Fig. 4, chlorophyll fluorescence changes were measured in dark-adapted leaves in response to a saturating light pulse. During a pulse, PS II primary electron acceptors were fully reduced, and chlorophyll fluorescence rapidly reached a maximum level (F_m). After the light pulse, the chlorophyll fluorescence level decreased in the dark, and this decay was related to the reoxidation of PS II primary acceptors (Q_A) in redox equilibrium with the PQ pool. The fluorescence decay was clearly biphasic. The fast phase was similar in WT and PTOX$^+$. On the other hand, the slowly decreasing phase was much faster in PTOX$^+$ than in WT, indicating that PQs were more efficiently reoxidized in transgenic plants. Addition of propyl gallate severely slowed down the fluorescence decay, which came close to that observed in WT leaves (Fig. 4*B*). On the other hand, cyanide (KCN 1 mM) had no significant effect on the fluorescence decay measured in PTOX$^+$ (data not shown). In order to check that PS II acceptors were more reduced in WT than in PTOX$^+$ during the fluorescence decay shown on Fig. 4*A*, a control experiment was performed by flashing a second light pulse 4 s after the first pulse (Fig. 5). Under such conditions, because no non-photochemical

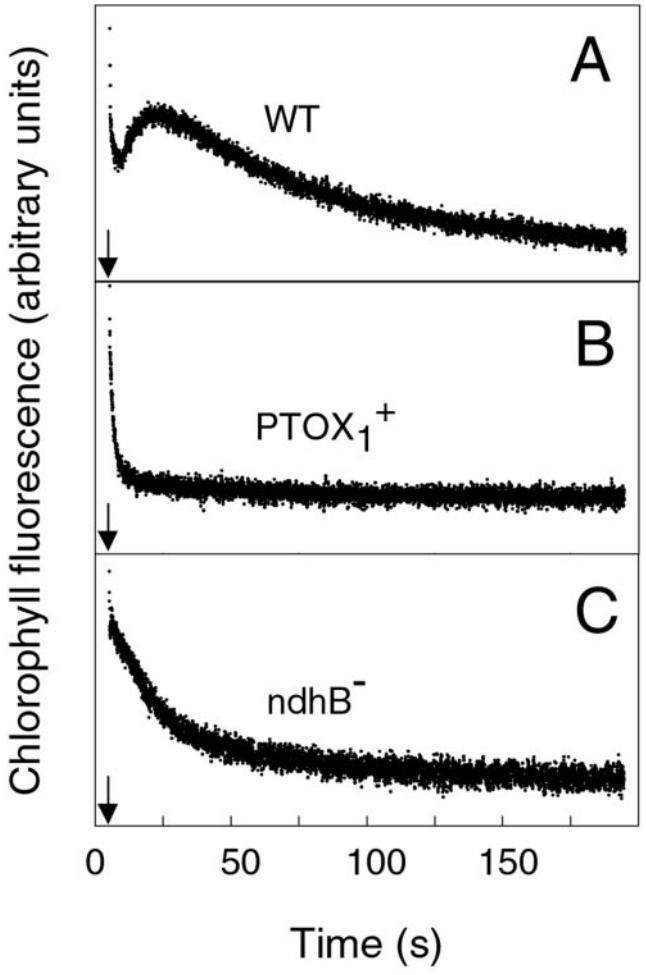

FIG. 3. **Apparent "F_0 rise" of chorophyll fluorescence measured following a light to dark transition in tobacco leaf discs.** Light was switched off when indicated (↓) after a 10-min period of actinic illumination (100 μmol photons. $m^{-2}{\cdot}s^{-1}$). *A*, WT; *B*, $PTOX_1^+$, transgenic tobacco expressing At-PTOX; *C*, *ndhB*$^-$, tobacco plastid transformant inactivated in the *ndhB* gene, and lacking the Ndh complex.

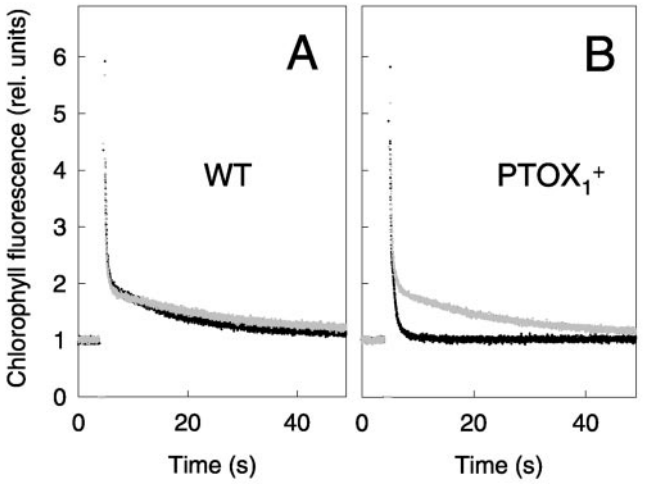

FIG. 4. **Chlorophyll fluorescence decay following a saturating pulse of white light measured on dark-adapted tobacco leaf discs.** Leaf discs, either treated (*gray tracing*) or untreated (*black tracing*) with 1 mM propyl gallate were placed in the dark for 1 h. *A*, WT; *B*, $PTOX_1^+$. Experimental chlorophyll fluorescence values have been normalized to the F_0 value. The maximal photochemical yield of PS II was 0.823 ± 0.013 in WT and 0.820 ± 0.012 in $PTOX^+$ (20 experiments).

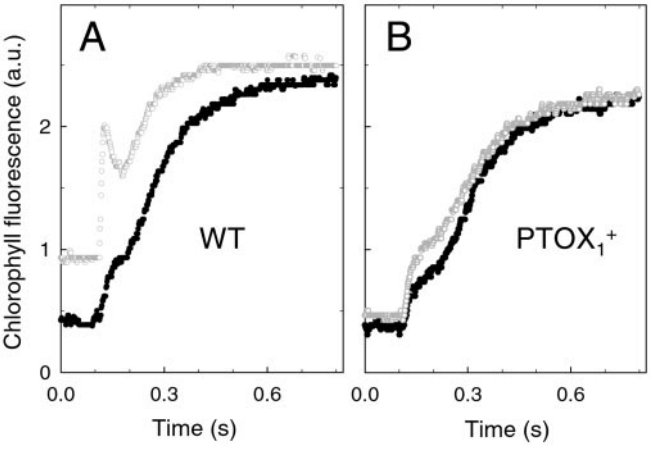

FIG. 5. **Chlorophyll fluorescence measurements in response to a high light intensity (350 μmol photons·$m^{-2}{\cdot}s^{-1}$) in tobacco leaf discs either dark-adapted (*black tracing*) or 4 s following a saturating white light pulse (*gray tracing*).** *A*, WT; *B*, $PTOX_1^+$.

quenching of F_m occurred, the upper area delimited by the fluorescence induction curve reflected the relative pool size of electron acceptors of PS II, mainly the PQ pool (2, 35). Fig. 5*A* shows that in WT leaves, 4 s after the first pulse, PS II acceptors are more reduced than in dark-adapted leaves. In contrast, the redox state of PS II acceptors measured in $PTOX^+$ leaves 4 s after a pulse illumination was close to that measured in dark-adapted leaves (Fig. 5*B*). We concluded from these experiments that At-PTOX was functional in transgenic tobacco leaves and was able to oxidize efficiently reduced PQs following their reduction by a saturating light pulse. We found that propyl gallate slightly (but in a reproducible manner) affected the slow phase of the fluorescence decay measured in WT leaves (Fig. 4*A*), possibly indicating the contribution of a putative tobacco PTOX in PQ oxidation.

At-PTOX Is Active in Thylakoids and Used Molecular O_2 as a Substrate—The activity of PTOX on PQ oxidation was then investigated in chloroplast preparations. Addition of exogenous NADH to osmotically lysed chloroplasts isolated from WT leaves increased the apparent F_0 chlorophyll fluorescence level measured under low non-actinic light, indicating an increase in the redox state of the PQ pool (Fig. 6*A*). Note that in chloroplast preparations, NADH-induced PQ reduction was not mediated by the Ndh complex (Ndh-1), which likely inactivated during the extraction procedure, but rather by an alternative (Ndh-2 like) activity (15, 16, 34). Under aerobic conditions, the NADH-induced fluorescence increase was significantly slower in $PTOX^+$ than in WT chloroplasts (Fig. 6*B*). Addition of propyl gallate increased the chlorophyll fluorescence level in $PTOX^+$ chloroplasts, whereas no significant effect could be detected in WT chloroplasts. Removing O_2 from the sample strongly increased the chlorophyll fluorescence signal in a similar manner in both WT and $PTOX^+$ chloroplasts, and the F_m level corresponded to a full reduction of PQs being rapidly reached (Fig. 6, *A* and *B*). This experiment showed that At-PTOX was functional in isolated tobacco chloroplasts and that the redox state of the PQ pool resulted from a competition between reduction by NADH and oxidation by PTOX.

Mass spectrometric measurements of O_2 exchange were then performed on chloroplast preparations using $^{18}O_2$, to determine which electron acceptor was used during PTOX-mediated PQ oxidation (Fig. 7). In the absence of either cytochrome b_6/f or PS I, an electron flow from PS II to O_2 involving PTOX occurred in *Chlamydomonas* cells (36). When tobacco chloroplasts were treated with DBMIB (a potent inhibitor of the cytochrome b_6/f

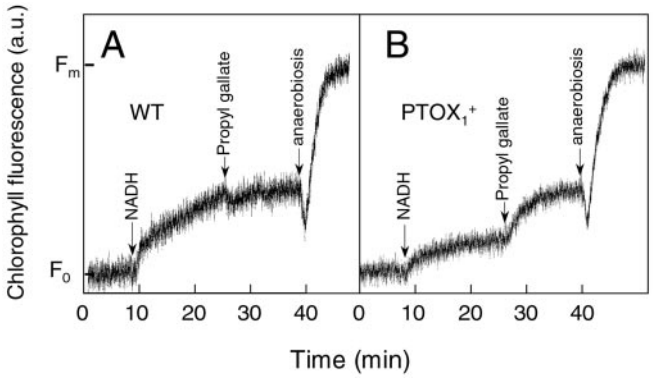

FIG. 6. **Effect of exogenous addition of NADH on the chlorophyll fluorescence level measured in non-actinic modulated light on osmotically lysed tobacco chloroplasts prepared from WT or PTOX$_1^+$ leaves.** When indicated (↓), 2 mM NADH or propyl gallate (1 mM) were added. Anaerobic conditions were obtained by addition of glucose, glucose oxidase, and catalase.

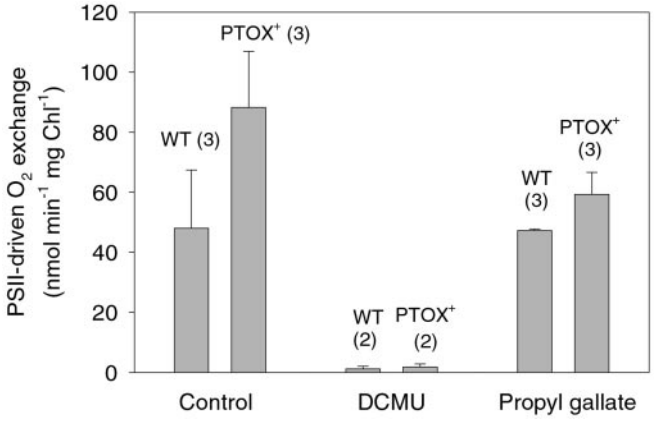

FIG. 7. **Mass spectrometric measurements of light-dependent O_2 exchange in chloroplasts isolated from WT and PTOX$_1^+$ leaves.** O_2 exchange was measured in the presence of $^{18}O_2$ under a light intensity of 150 μmol photons·m^{-2}·s^{-1}. Measurements were performed in the presence of DBMIB (1 μM final concentration). 3-(3,4-Dichlorophenyl)-1,1-dimethylurea or propyl gallate were added at final concentrations of 25 and 8 μM, respectively. Numbers of independent experiments are indicated in *brackets*.

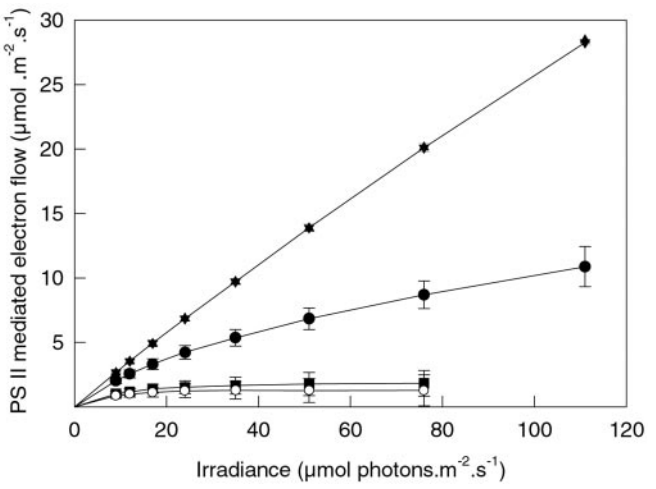

FIG. 8. **Effect of DBMIB on PS II-mediated electron flow measured in stripped leaf discs from WT and PTOX$^+$.** ▲, WT control in water; ▼, PTOX$^+$ control in water; ■, WT treated with 50 μM DBMIB; ●, PTOX$^+$ treated with 50 μM DBMIB; ○, PTOX$^+$ treated with 50 μM DBMIB and 1 mM propyl gallate. Note that because values for WT control and PTOX$^+$ control are very close, corresponding symbols are superimposed. Results obtained for PTOX$_1^+$ and PTOX$_2^+$ were grouped, and *error bars* represent S.D. (six experiments).

complex) and illuminated, simultaneous O_2 production by PS II and O_2 uptake were observed using $^{18}O_2$ and mass spectrometry. PS II activity was higher in PTOX$^+$ than in WT (Fig. 7). Addition of 25 μM of the PS II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea fully suppressed the electron transfer activity, showing the involvement of the PQ pool in both cases. Treatment by propyl gallate largely suppressed the difference in electron transfer activity observed between WT and PTOX$^+$, showing that this difference was likely to be due to the activity of the oxidase. In these experiments, the PS II-mediated electron flow was balanced by a simultaneous increase in the O_2 uptake rate, thus supporting the view that, as in the case of *Chlamydomonas* PTOX, At-PTOX is a true quinol oxidase, using O_2 as an electron acceptor and releasing H_2O as a final product (24).

Measurement of a PS II-mediated Electron Flow to At-PTOX in Leaves—Similar fluorescence experiments were performed in stripped leaf discs treated with DBMIB, measuring chlorophyll fluorescence in order to probe PS II activity (Fig. 8). In the absence of inhibitor, similar electron flow rates were observed at low irradiances both in WT and transgenic leaf discs. In WT leaf discs, DBMIB strongly inhibited linear electron flow (90% inhibition at 75 μmol photons·m^{-2}·s^{-1}), whereas in transgenic leaf discs inhibition by DBMIB was much less pronounced (60% inhibition at 75 μmol photons·m^{-2}·s^{-1}). The DBMIB-insensitive electron flow observed in PTOX$^+$ was inhibited by propyl gallate (Fig. 8) and reached basal rates measured in WT discs treated with propyl gallate. This experiment showed that in leaves placed under low light intensity, when linear electron flow to PS I was inhibited, a significant part of PS II-driven electron flow (about 35% of the maximal electron flow to PS I) can be directed toward PTOX and O_2.

Involvement of PTOX during Photosynthesis—We were then interested to determine whether the activity of At-PTOX, which can be evidenced either in the dark (Figs. 3 and 4) or in the light in the absence of functional electron transfer to PS I (Fig. 8), could be observed in the light during normal conditions of photosynthesis. During a dark to light induction of photosynthesis, typical variations in chlorophyll fluorescence were observed (37). Under low light intensity, these variations reflected changes in the electron transfer rate occurring during the activation of photosynthesis. The transient increase in fluorescence commonly observed in WT during the induction phase reflected the transient accumulation of plastoquinols due to the initial absence of PS I electron acceptors. In fact, an activation of the PS I acceptor side and of Calvin cycle enzymes was generally required to initiate CO_2 assimilation and further reoxidize NADPH. This transient was almost completely abolished in PTOX$^+$ at the lowest irradiance (8 μmol photons·m^{-2}·s^{-1}, Fig. 9*A*), indicating a highly efficient plastoquinol oxidation before the activation of PS I. After a few minutes of illumination, both F_s and $F_{m'}$ values were identical in WT and PTOX$^+$. At this low irradiance, $F_{m'}$ was close to F_m, showing the absence of non-photochemical quenching. At higher light intensity (50 μmol photons·m^{-2}·s^{-1}), a difference between WT and PTOX$^+$ was also observed, F_s values remained lower in PTOX$^+$ than in WT during the first 3 min of illumination (Fig. 9*B*). When illumination was prolonged, the decrease in F_s was more pronounced in WT, and after 10 min reached a lower level than in PTOX$^+$. It should be noted that variations in F_s values were accompanied by concomitant changes in $F_{m'}$ (Fig. 9*B*). As a consequence, both non-photochemical (qN) and photochemical (qP) quenching parameters were lower in PTOX$^+$ than in WT after 10 min of illumination. This effect on qN and qP was also observed at higher irradi-

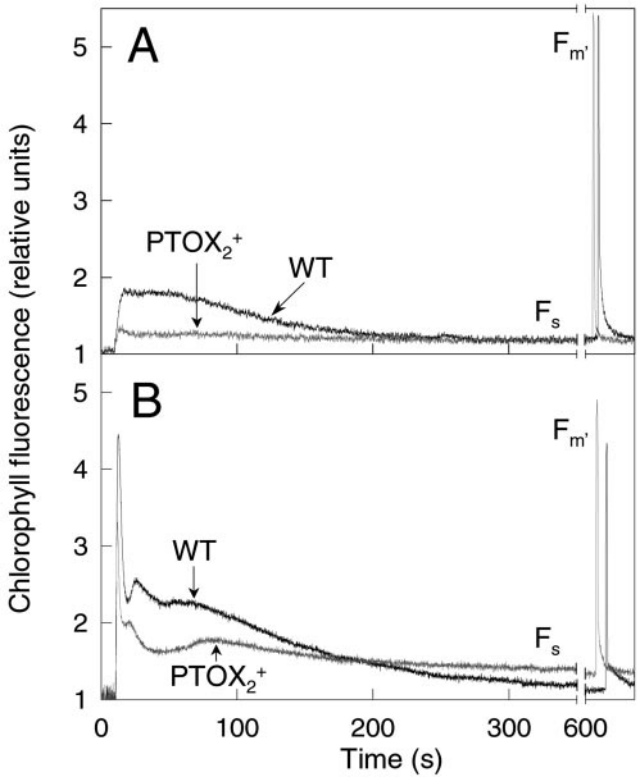

FIG. 9. **Chlorophyll fluorescence induction curves measured during a dark to light transition on attached leaves of WT and $PTOX_2^+$ tobacco plants.** After 10 min a saturating pulse was used to determine $F_{m'}$ and to calculate both qP and qN values. *A*, at an irradiance of 8 μmol photons·m^{-2}·s^{-1}; qP and qN values were identical for WT and $PTOX^+$; *B*, at an irradiance of 50 μmol photons·m^{-2}·s^{-1}. Experimental chlorophyll fluorescence values have been normalized to the F_0 value. qP and qN values (0.956 and 0.212 in WT, respectively) were significantly decreased in $PTOX^+$ (0.914 and 0.111, respectively).

ances (for example at 750 μmol photons·m^{-2}·s^{-1}; Table I). However, at all irradiances, after 1 h of illumination when a steady state was reached, qN and qP of WT and $PTOX^+$ became identical (Table I). Despite these fluctuations in fluorescence quenchings, no significant differences in PS II photochemical yields, measured either at 10 or 60 min, could be evidenced between WT and $PTOX^+$ (Table I). Moreover, measurements of net CO_2 gas exchange at steady state showed no significant difference in quantum yield of CO_2 fixation in air as well as under non-photorespiratory conditions (Table II). In WT and $PTOX^+$, rates of CO_2 fixation measured at saturating irradiance were also similar (Table II).

TABLE I

PS II photochemical yield, chlorophyll fluorescence quenching parameters (qN and qP) measured in WT and $PTOX^+$ attached leaves

Measurements were performed 10 and 60 min after the onset of illumination (750 μmol photons · m^{-2} · s^{-1}). Values are means ± S.D. of 3 experiments.

Chlorophyll fluorescence parameters		WT	$PTOX^+$
$(F_{m'} - F_s)/(F_{m'}$		0.333 ± 0.023	0.339 ± 0.016
After 10 min	qP	0.731 ± 0.011	0.632 ± 0.022
illumination	qN	0.670 ± 0.018	0.593 ± 0.022
$(F_{m'} - F_s)/F_{m'}$		0.350 ± 0.026	0.324 ± 0.032
After 60 min	qP	0.743 ± 0.018	0.759 ± 0.009
illumination	qN	0.599 ± 0.025	0.622 ± 0.033

TABLE II

Quantum yield of CO_2 fixation (Φ_{CO2}) and maximal rate of CO_2 assimilation measured in attached leaves of WT and PTOX tobacco

The maximal rate was measured under a saturating irradiance of 750 μmol photons · m^{-2} · s^{-1}. Quantum yields were measured under photorespiratory conditions (air) and under non-photorespiratory conditions (O_2 1.5% (v/v); CO_2 750 μl · $liter^{-1}$). Number of experiments is indicated in parentheses.

	WT	$PTOX^+$
Φ_{CO2} (air)	0.0481 ± 0.0038 (3)	0.0440 ± 0.0056 (3)
Φ_{CO2} (1.5% O_2; 750 μl · $liter^{-1}$ CO_2)	0.0783 ± 0.0023 (3)	0.0801 ± 0.0021 (3)
Maximal CO_2 assimilation (μmol · m^{-2} · s^{-1})	13.7 ± 0.7 (5)	13.6 ± 1 (5)

DISCUSSION

We have shown in this paper that when expressed in tobacco, At-PTOX is targeted to the chloroplasts and functions as a PQ oxidase. The activity of At-PTOX could be evidenced in intact leaves, following either photochemical or non-photochemical reduction of PQs and also in thylakoids, when PQs were reduced by exogenous NADH. Based on chlorophyll fluorescence and mass spectrometric measurements performed on thylakoids, we propose that At-PTOX drives PQ oxidation using molecular O_2 as a terminal electron acceptor. This agrees with previous conclusions reached from mass spectrometric measurements on *Chlamydomonas* mutants deficient in PS I (24). Because the Ndh complex is involved in the non-photochemical reduction of the PQ pool (8, 9, 34) (see Fig. 3*C*) and At-PTOX is involved in its non-photochemical oxidation, we conclude that a chlororespiratory electron transfer involving the plastid Ndh complex, the PQ pool, and At-PTOX occurs from NAD(P)H to O_2 in chloroplasts of transgenic tobacco expressing At-PTOX. In the dark, the redox status of PQs therefore depends on an equilibrium between its reduction by the Ndh complex and oxidation by PTOX.

In thylakoid membranes, PS I reaction centers and ATPase complexes are essentially located in stroma lamellae, whereas PS II are restricted to grana, cytochrome b_6/f complexes being found in both types of membranes. Like the Ndh complex (11, 38, 39), At-PTOX was found mainly in stroma lamellae, indicating that chlororespiration is restricted to stroma lamellae and is absent in granal thylakoids. Previously, the involvement of a propyl gallate-sensitive PQ oxidase in chlororespiration had been evidenced in *Chlamydomonas* cells (24). It was proposed that an At-PTOX homologue was functional in *Chlamydomonas* thylakoid membranes (24), but the corresponding gene has not yet been identified (16). In higher plants, first evidence for the existence of chlororespiration was based on the effect of respiratory inhibitors such as cyanide (40) or CO (41). Such effects cannot be explained by the inhibition of PTOX, because this protein was reported to be insensitive to these compounds (24, 25). This was confirmed in this study by the insensitivity to cyanide of the slow phase of the chlorophyll fluorescence decay. Therefore, the effects of respiratory inhibitors such as cyanide or CO more likely result from the inhibition of mitochondrial respiration that has been reported to affect the redox state of the PQ pool due to the existence of redox interactions between chloroplasts and mitochondria (16, 42). Such effects may alternatively reflect the existence of an alternative PQ oxidation pathway sensitive to cyanide and CO. In this respect, Fig. 7 indicates the existence in chloroplasts of a propyl gallate-insensitive mechanism for PQ oxidation. Recently, Casano *et al.* (6), studying a reconstituted system containing the Ndh complex and a plastidial hydroquinone peroxidase, proposed the existence of a PQ oxidation pathway using hydrogen peroxide as a terminal acceptor.

In addition to an involvement in dark reactions, we have

shown that At-PTOX may interact with photosynthetic electron transport reactions in illuminated leaves. In WT plants, a transient over-reduction of photosynthetic electron carriers occurs during the induction phase of photosynthesis. This is due to the fact that the photosynthetic carbon reduction cycle is not operative in the dark, because some of the enzymes of the cycle require light-induced activation by reduced thioredoxins (43). In transgenic tobacco plants expressing At-PTOX, the transient over-reduction of photosynthetic electron carriers is greatly decreased, indicating that electrons are diverted to O_2 via PTOX. This suggests that PTOX can potentially prevent over-reduction of PQs in the light. In plant mitochondria, alternative oxidase has been suggested to function as an "energy overflow," its activity being increased when the cytochrome pathway is saturated with electrons (44). Overexpression of alternative oxidase in this organelle has been shown to limit the generation of reactive oxygen species by preventing over-reduction of electron carriers (45). It should be noted, however, that expression of At-PTOX did not result in increased resistance of transgenic lines to photoinhibition (data not shown).

Differences in qN and qP values between WT and $PTOX^+$ were transitorily observed during the 10–30-min period of illumination, whereas the photochemical yield of PS II remained identical in both WT and $PTOX^+$. The fact that both photochemical yield of PS II and rate of CO_2 fixation are identical suggests that at the end of the transitory induction period of photosynthesis, the oxidase function of PTOX does not contribute to drive significant electron flow compared with photosynthetic carbon reduction and oxidation cycles. On the other hand, lower qN and qP in $PTOX^+$ between the initial induction period and steady state suggests that the pH gradient is lower and that PS II acceptors (Q_A) are more reduced compared with the WT. A lower pH gradient could indicate that cyclic electron reactions around PS I are down-regulated in $PTOX^+$. Cyclic electron reactions around PS I have been reported to be controlled by the redox poise of some electron carriers; this effect was possibly mediated by molecular O_2 (46). Overexpression of PTOX, by modifying the redox poise of intersystem electron carriers, may perturb the establishment of cyclic electron transfer reactions. Interestingly enough, a role of chlororespiration in the control of cyclic electron flow around PS I was recently deduced from photoacoustic measurements performed in leaves under low O_2 concentration (12). The fact that chlororespiration and cyclic electron transfer reactions around PS I operate within the same membranes (stroma lamellae, see Ref. 16) further strengthens the hypothesis of a functional link between these two activities.

At steady state, qP and qN values were similar in WT and $PTOX^+$, indicating that both the redox state of Q_A and the pH gradient reached similar levels. This may indicate that at steady state the contribution of cyclic electron flow around PS I is decreased compared with its high activity during the induction phase when terminal electron acceptors are not fully available. Alternatively, this effect might reflect the involvement of regulatory mechanisms that could be turned on under these conditions. For instance, the expression of some nuclear genes, like *cab* genes encoding light harvesting complex apoprotein, has been shown to be controlled by the redox state of PQs (47). The higher reduction of the PQ pool observed in $PTOX^+$ during the induction of photosynthesis may trigger such long term adaptation processes and explain why similar pH gradients and Q_A redox state are finally reached at steady state in both types of plants. Analysis of gene expression in $PTOX^+$ plants should inform us of the possible existence of such adaptive mechanisms.

If the role of At-PTOX in PQ oxidation could be demonstrated in transgenic tobacco, the involvement of a functional PTOX in WT tobacco appears more difficult to establish. A faint band, specific to the native *ptox* transcripts, was amplified in WT tobacco by RT-PCR (Fig. 1*A*). However, by using an antibody raised against At-PTOX, no signal corresponding to native PTOX was detected in insoluble proteins prepared from WT tobacco leaves (Fig. 1). This may be due to the fact that either the antibody raised against the *Arabidopsis* enzyme does not cross-react with the tobacco enzyme or that the native enzyme is present in too small an amount to be detected. The latter hypothesis is the most probable, because this antibody cross-reacts with chromoplast preparations from pepper, another Solanacae species (25), and also with chloroplast preparations from *C. reinhardtii* (24). In this respect, a doublet that may correspond to the native tobacco PTOX was detected in purified stroma lamellae preparations probed with the *Arabidopsis* antibody (data not shown). It should be noticed that the plastid Ndh complex, the other probable component of chlororespiration, has been reported to be present in leaves in very low amounts (4, 39). The slight effect of propyl gallate on the slow phase of the chlorophyll fluorescence decay measured in WT leaves (Fig. 3), may reflect a contribution of the native tobacco PTOX to the oxidation of PQs. In agreement with this interpretation, it has been reported recently (12) that in tobacco leaves the re-reduction rate of the oxidized primary electron donor in PS I (P_{700}^+) is increased by propyl gallate. This effect was interpreted as the re-routing of electrons toward PS I when the putative tobacco plastid terminal oxidase is inhibited. Since PTOX most likely represents a minor component of thylakoid membranes, at least when plants are grown under normal conditions, a regulatory role (for instance in the control of cyclic electron flow) seems more probable than a direct bioenergetic role. However, more work remains to be done to determine clearly the involvement of native PTOX in leaves.

Different lines of evidence suggest that PTOX might become more abundant at particular developmental stages or under particular growth (or stress) conditions. In higher plant chloroplasts, the role of PTOX in carotenoid biosynthesis has been demonstrated from the analysis of *Arabidopsis* and tomato mutants (21, 22, 25). The variegated phenotype of the *Arabidopsis* mutant *immutans* was explained by an involvement of PTOX in phytoene desaturation, an important step in carotenoid biosynthesis occurring during the early stage of the greening process (21, 22). As suggested previously (16, 19), native PTOX might be more abundant in non-green plastid under conditions where the photosynthetic apparatus is not functional. High amounts of PTOX were reported in achlorophyllous membranes prepared from chromoplasts of red pepper fruits, where carotenoid biosynthesis is particularly active (25). Overexpression of At-PTOX did not influence the leaf carotenoid content, thus indicating that the PTOX level is not a limiting factor regulating carotenoid biosynthesis. Interestingly, the *IM* (or *PTOX*) promoter was shown to be active, and *IM* mRNAs were expressed ubiquitously in *Arabidopsis* tissues and organs throughout development, arguing in favor of a more global role for this protein in plastid metabolism (48).

In C_4 plants, subunits of the Ndh complex have been reported to be much more strongly expressed in bundle sheath chloroplasts than in mesophyll chloroplasts (49). In bundle sheath chloroplasts, only low levels of PS II are detected. In these cells, ATP required for CO_2 fixation is generated by cyclic electron transport around PS I. Interestingly, it was recently reported that bundle sheath cells from C_3 leaves have photosynthesis features close to those of C_4 leaves (50). It will be interesting to determine whether *ndh* and *ptox* genes are more strongly expressed in bundle sheath chloroplasts of C_3 plants

than in mesophyll cells and participate in the regulation of cyclic electron transfer reactions around PS I.

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REFERENCES

1. Scherer, S. (1990) *Trends Biochem. Sci.* **15,** 458–462
2. Bennoun, P. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 4352–4356
3. Peltier, G., Ravenel, J., and Verméglio, A. (1987) *Biochim. Biophys. Acta* **893,** 83–90
4. Sazanov, L. A., Burrows, P. A., and Nixon, P. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1319–1324
5. Cuello, J., Quiles, M. J., Albacete, M. E., and Sabater, B. (1995) *Plant Cell Physiol.* **36,** 265–271
6. Casano, L. M., Zapata, J. M., Martin, M., and Sabater, B. (2000) *J. Biol. Chem.* **275,** 942–948
7. Guedeney, G., Corneille, S., Cuiné, S., and Peltier, G. (1996) *FEBS Lett.* **378,** 277–280
8. Burrows, P. A., Sazanov, L. A., Svab, Z., Maliga, P., and Nixon, P. J. (1998) *EMBO J.* **17,** 868–876
9. Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9705–9709
10. Joët, T., Cournac, L., Horvath, E. M., Medgyesy, P., and Peltier, G. (2001) *Plant Physiol.* **125,** 1919–1929
11. Horvath, E. M., Peter, S. O., Joët, T., Rumeau, D., Cournac, L., Horvath, G. V., Kavanagh, T. A., Schafer, C., Peltier, G., and Medgyesy, P. (2000) *Plant Physiol.* **123,** 1337–1349
12. Joët, T., Cournac, L., Peltier, G., and Havaux, M. (2002) *Plant Physiol.* **128,** 760–769
13. Cleland, R. E., and Bendall, D. S. (1992) *Photosynth. Res.* **34,** 409–418
14. Bendall, D. S., and Manasse, R. S. (1995) *Biochim. Biophys. Acta* **1229,** 23–38
15. Corneille, S., Cournac, L., Guedeney, G., Havaux, M., and Peltier, G. (1998) *Biochim. Biophys. Acta* **1363,** 59–69
16. Peltier, G., and Cournac, L. (2002) *Annu. Rev. Plant Biol.* **53,** 523–550
17. Bennoun, P. (1994) *Biochim. Biophys. Acta* **1186,** 59–66
18. Hoefnagel, M. H. N., Atkin, O. K., and Wiskich, J. T. (1998) *Biochim. Biophys. Acta* **1366,** 235–255
19. Nixon, P. J. (2000) *Philos. Trans. R. Soc. Lond-Biol. Sci.* **355,** 1541–1547
20. Cournac, L., Latouche, G., Cerovic, Z., Redding, K., Ravenel, J., and Peltier, G. (2002) *Plant Physiol.*, in press
21. Wu, D. Y., Wright, D. A., Wetzel, C., Voytas, D. F., and Rodermel, S. (1999) *Plant Cell* **11,** 43–55
22. Carol, P., Stevenson, D., Bisanz, C., Breitenbach, J., Sandmann, G., Mache, R., Coupland, G., and Kuntz, M. (1999) *Plant Cell* **11,** 57–68
23. Carol, P., and Kuntz, M. (2001) *Trends Plant Sci.* **6,** 31–36
24. Cournac, L., Redding, K., Ravenel, J., Rumeau, D., Josse, E. M., Kuntz, M., and Peltier, G. (2000) *J. Biol. Chem.* **275,** 17256–17262
25. Josse, E. M., Simkin, A. J., Gaffe, J., Laboure, A. M., Kuntz, M., and Carol, P. (2000) *Plant Physiol.* **123,** 1427–1436
26. Odell, J. T., Nagy, F., and Chua, N. H. (1985) *Nature* **313,** 810–812
27. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., and Fraley, R. T. (1985) *Science* **227,** 1229–1231
28. Genty, B., Briantais, J.-M., and Baker, N. R. (1989) *Biochim. Biophys. Acta* **990,** 87–92
29. Rumeau, D., Cuiné, S., Fina, L., Gault, N., Nicole, M., and Peltier, G. (1996) *Planta* **199,** 79–88
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Lichtenthaler, H. K., Wellburn, A. R. (1983) *Biochem. Soc. Trans.* **11,** 591–592
32. Groom, Q. J., Kramer, D. M., Crofts, A. R., and Ort, D. R. (1993) *Photosynth. Res.* **36,** 205–215
33. Harris, G. C., and Heber, U. (1993) *Plant Physiol.* **101,** 1169–1173
34. Cournac, L., Guedeney, G., Joët, T., Rumeau, D., Latouche, G., Cerovic, Z., Redding, K., Horvath, E., Medgyesy, P., and Peltier, G. (1998) in *Photosynthesis: Mechanism and Effects* (Garab, G., ed) pp. 1877–1882, Kluwer Academic Publishers Group, Dordrecht, The Netherlands
35. Delosme, R. (1967) *Biochim. Biophys. Acta* **143,** 108–128
36. Cournac, L., Josse, E. M., Joët, T., Rumeau, D., Redding, K., Kuntz, M., and Peltier, G. (2000) *Philos. Trans. R. Soc. Lond-Biol. Sci.* **355,** 1447–1453
37. Kautsky, H., Appel, W., and Amann, H. (1960) *Biochem. Z.* **332,** 277–292
38. Berger, S., Ellersiek, U., Westhoff, P., and Steinmuller, K. (1993) *Planta* **190,** 25–31
39. Sazanov, L. A., Burrows, P., and Nixon, P. J. (1996) *Biochem. Soc. Trans.* **24,** 739–743
40. Garab, G., Lajko, F., Mustardy, L., and Marton, L. (1989) *Planta* **179,** 349–358
41. Feild, T. S., Nedbal, L., and Ort, D. R. (1998) *Plant Physiol.* **116,** 1209–1218
42. Bennoun, P. (1998) in *The Molecular Biology of Chloroplasts and Mitochondria in Chlamydomonas* (Rochaix, J.-D., Goldschmidt-Clermont, M., and Merchant, S., eds) pp. 675–683, Kluwer Academic Publishers Group, Dordrecht, The Netherlands
43. Ruelland, E., and Miginiac-Maslow, M. (1999) *Trends Plant Sci.* **4,** 136–141
44. Vanlerberghe, G. C., and McIntosh, L. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 703–734
45. Maxwell, D. P., Wang, Y., and McIntosh, L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 8271–8276
46. Ziem-Hanck, U., and Heber, U. (1980) *Biochim. Biophys. Acta* **591,** 266–274
47. Escoubas, J. M., Lomas, M., LaRoche, J., and Falkowski, P. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10237–10241
48. Aluru, M. R., Bae, H., Wu, D. Y., and Rodermel, S. R. (2001) *Plant Physiol.* **127,** 67–77
49. Kubicki, A., Funk, E., Westhoff, P., and Steinmuller, K. (1996) *Planta* **199,** 276–281
50. Hibberd, J. M., and Quick, W. P. (2002) *Nature* **415,** 451–454
51. Mills, W. R., and Joy, K. W. (1980) *Planta* **148,** 75–83

Conclusion Générale

Dans le but de comprendre le rôle du complexe plastidial Ndh, nous avons obtenu des plants de tabac transplastomiques dont le gène *ndhB*, codant pour une des sous-unités, a été inactivé. L'étude de transformants homoplasmiques nous a permis de montrer que le complexe Ndh, normalement présent dans les thylacoïdes lamellaires des plantes de type sauvage, est absent chez les plantes transplastomiques. Par des mesures de fluorescence de chlorophylle nous avons montré que le complexe Ndh est fonctionnel chez les plantes de type sauvage et qu'il est impliqué dans la réduction non photochimique du pool de PQ. Les plantes transplastomiques dépourvues du complexe Ndh présentent un développement et une croissance similaires aux plantes de type sauvage dans la plupart des conditions environnementales étudiées. Par contre, dans des conditions de déficit hydrique modéré, les plantes mutantes présentent une capacité d'assimilation photosynthétique et une croissance réduites, ces effets étant supprimés en présence d'une concentration elevée en CO_2. En traitant des plants de tabac par de l'acide abscissique, une hormone provoquant la fermeture stomatique, les mêmes retards de croissance ont été observés chez les mutants. Nous avons conclu de ces expériences que les différences observées étaient liées à une teneur interne en CO_2 faible, conditions favorisant la photorespiration. Nous avons fait l'hypothèse que la demande en ATP, accrue en conditions de photorespiration élevée, n'était pas satisfaite chez les plantes mutantes et que le complexe Ndh était impliqué dans la fourniture d'ATP, probablement à travers sa participation au transfert cyclique des électrons autour du PS I.

Pour tester l'hypothèse d'une participation du complexe Ndh au transfert cyclique des électrons autour du PS I, nous avons étudié l'effet de l'antimycine A, composé connu pour inhiber *in vitro* les photophosphorylations cycliques. Nous avons observé que l'activité photosynthétique de disques foliaires de plantes mutantes était significativement plus sensible à l'action inhibitrice de l'antimycine A que celle de plantes de type sauvage. Nous avons proposé l'existence de deux voies de transfert cyclique autour du PS I, l'une insensible à l'antimycine A et impliquant le complexe Ndh et l'autre, sensible à l'antimycine A, impliquant probablement la FQR. En se basant sur l'action inhibitrice de l'antimycine A mesurée dans des conditions où la composition atmosphérique est variable, nous avons conclu que dans des conditions où la demande en ATP est faible (peu de photorespiration), chacune des voies peut indépendamment satisfaire la demande. Par contre, dans des conditions où la

demande en ATP est plus forte (forte photorespiration), le fonctionnement des deux voies est nécessaire pour assurer une croissance optimale.

Afin de mettre en évidence de manière plus directe le transfert cyclique des électrons autour du PS I, nous avons eu recours à la spectrométrie photoacoustique. Dans un premier temps, en accord avec les données publiées antérieurement (Herbert et al., 1990 ; Havaux, 1991; Malkin et al., 1992), seules de faibles activités de stockage photochimique attribuables au transfert cyclique des électrons autour du PS I ont pu être mises en évidence. En se basant sur l'hypothèse que l'activité du transfert cyclique pourrait être contrôlée par l'état rédox de certains transporteurs d'électrons, nous avons effectué des mesures de stockage d'énergie photochimique dans des conditions où le pool de PQ est partiellement réduit. Dans ces conditions, l'activité cyclique est fortement stimulée. L'étude des plantes mutantes nous a permis de distinguer deux voies de transfert cyclique, dont une voie rapide, impliquant le complexe Ndh.

Dans le but de déterminer si PTOX, une oxydase impliquée dans la biosynthèse des caroténoïdes récemment découverte dans les chloroplastes, pourrait jouer un rôle dans les réactions de transfert d'électrons au sein des chloroplastes nous avons produit des plantes de tabac transgéniques surexprimant la protéine d'*Arabidopsis thaliana.* L'étude des plantes transgéniques produites nous a permis de montrer que PTOX est impliquée dans l'oxydation non-photochimique du pool de PQ et utilise l'oxygène moléculaire comme accepteur terminal d'électrons. Nous avons montré qu'à l'obscurité, le niveau rédox du pool de PQ résulte d'un équilibre entre une voie de réduction non photochimique impliquant le complexe Ndh et une voie d'oxydation non photochimique impliquant PTOX. Nous avons conclu de l'ensemble de ces expériences à l'existence, dans les chloroplastes des végétaux supérieurs, d'une chaîne chlororespiratoire de transporteurs d'électrons. Bien que les transferts d'électrons de type chlororespiratoires soient limités, PTOX fonctionne à la lumière comme une "soupape de sécurité" permettant de limiter la réduction transitoire du pool de PQ. La surexpression de PTOX perturbe l'établissement de quenchings de fluorescence de la chlorophylle reliés à l'établissement du gradient de protons. Nous avons émis l'hypothèse que l'activité du transfert cyclique des électrons autour du PSI était affectée chez ces mutants. Plusieurs hypothèses ont été avancées pour expliquer les phénomènes de régulations impliqués dans l'établissement du transfert cyclique. Le PS II, en réduisant les transporteurs d'électrons situés entre les deux photosystèmes pourrait enclencher le transfert cyclique (Heber et Walker, 1992). Il a également été proposé que l'oxygène moléculaire participe à ces mécanismes, le transfert cyclique des électrons autour du PS I mesuré sur des chloroplastes étant stimulé en

conditions de faibles concentrations en oxygène moléculaire (Ziem-Hanck et al., 1980 ; Hormann et al., 1994). Ces phénomènes ont été initialement interprétés par une possible compétition entre transfert cyclique et pseudocyclique (Hormann et al., 1994). D'après l'ensemble de nos expériences, nous proposons que l'activité de la chlororespiration, en permettant un contrôle fin de l'état rédox de certains transporteurs d'électrons, régule l'activité du transfert cyclique autour du PS I (Figure 1). Cette hypothèse est compatible avec la localisation des complexes photosynthétiques au sein des membrane thylacoïdiennes, le complexe Ndh et PTOX étant localisés comme les PS I, les ATPases et une partie des complexes cyt b_6/f dans les lamelles du stroma. Au cours du transfert linéaire d'électrons le couplage entre le PSII et le PSI s'opère grâce à la diffusion latérale des plastocyanines des thylacoïdes granaires vers les thylacoïdes lamellaires. La diffusion des plastocyanines étant beaucoup plus rapide que celle des plastoquinones, ceci aboutit à l'existence *de facto* de deux pools de PQ relativement indépendants. L'état rédox du pool de PQ des lamelles du stroma est donc directement tributaire de l'activité chlororespiratoire. Un déséquilibre transitoire entre les phénomènes de réduction et d'oxydation non photochimique du pool de PQ peut mener à une réduction des transporteurs des lamelles et ainsi stimuler l'activité de transfert cyclique des électrons autour du PS I. Mais l'activité du transfert cyclique des électrons peut être inhibée lorsque la chaîne de transfert d'électrons entre les deux photosystèmes est totalement réduite (Ziem-Hanck et al., 1980). PTOX pourrait jouer le rôle de "soupape de sécurité" évitant ainsi l'engorgement de la chaîne de transfert d'électrons et l'inhibition du transfert cyclique. Ainsi, les flux d'électrons du pool de PQ vers PTOX, mêmes s'ils sont relativement faibles, pourraient réguler des transfert d'électrons quantitativement plus importants du transfert cyclique.

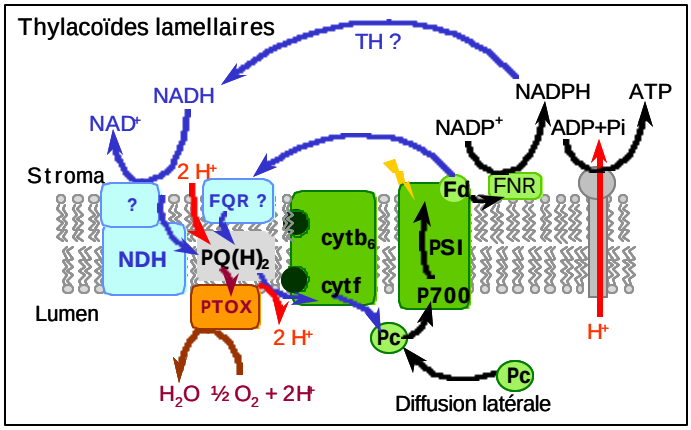

Figure 1. Modèle proposé présentant les interactions entre transfert cyclique des électrons autour du PS I et chlororespiration au sein des thylacoïdes lamellaires des plantes supérieures. Le transfert linéaire des électrons est présenté en noir, le transfert cyclique en bleu, la chlororespiration en marron et les translocations de protons en rouge. La nature moléculaire de la FQR reste inconnue. La présence d'une activité transhydrogénase (TH) reste à établir. L'orientation de PTOX vers le lumen est arbitraire.

A l'issue de ce travail de thèse, diverses questions restent posées. L'existence d'un flux d'électrons de type chlororespiratoire transitant à travers le complexe Ndh, le pool de PQ et l'activité quinol oxydase de PTOX est maintenant démontré. Cependant, le rôle physiologique de cette activité reste à établir. A ce titre, l'étude des plantes transgéniques surexprimant PTOX mériterait d'être approfondie afin de déterminer si le transfert cyclique des électrons est réellement affecté chez ces plantes. L'étude des capacités photosynthétiques sous des conditions où l'activité photorespiratoire varie permettrait d'étudier la réponse des plantes transgéniques à des variations dans la demande en ATP.

Il a été démontré que l'état rédox de la chaîne de transport d'électrons intersystème et plus particulièrement du pool de PQ et du cyt b_6/f permet un contrôle de l'expression de certains gènes photosynthétiques. Les phénomènes impliqués pourraient notamment jouer un rôle dans les processus d'adaptation de l'appareil photosynthétique lors de changements des conditions environnementales (Allen , 1992). La chlororespiration, de par son activité de réduction et d'oxydation du pool de PQ, pourrait être indirectement impliquée dans le contrôle de l'expression de certains gènes photosynthétiques. La régulation différentielle de l'expression de certains gènes pourrait être suivie chez des mutants *ndhB*⁻ ou $PTOX^+$ (et $PTOX^-$) à l'aide de puces à ADN. A ce titre, il pourrait être intéressant d'effectuer des croisements entre les plantes mutantes $ndhB^-$ et $PTOX^+$ qui ont été obtenus sur le même matériel végétal afin d'obtenir des plantes au sein desquelles le niveau redox du pool de PQ tendrait à être très oxydé.

La voie de transfert cyclique des électrons autour du PSI qui est sensible à l'antimycine A implique potentiellement une activité FQR. Cependant, cette enzyme n'est pas caractérisée au niveau moléculaire. D'autre part, des voies alternatives d'oxydation du pool de PQ ont été décrites, impliquant une activité hydroquinone peroxydase récemment détectée au sein des thylacoïdes des végétaux supérieurs (Zapata et al., 1998) et capable de réoxyder le pool de PQ sur des systèmes membranaires reconstitués (Casano et al., 2000). Le clonage des gènes codant pour ces deux enzymes s'avère donc une étape importante de l'étude des processus de réduction et d'oxydation non photochimique du pool de PQ. Dans ce cadre, les mêmes puces à ADN pourrait permettre de détecter des voies alternatives d'oxydation qui seraient réprimées chez le mutant de tabac $PTOX^+$ ou surexprimées chez les mutants Immutans ($PTOX^-$) d'*Arabidopsis thaliana* et des voies alternatives de réduction du pool de PQ qui seraient surexprimées chez les mutants ndhB⁻.

Si nos études ont permis de conclure à l'implication du complexe Ndh dans les réactions de transfert cyclique des électrons autour du PSI, le rendement énergétique de ce mécanisme

demeure inconnu. En effet, l'efficacité du transfert cyclique, c'est-à-dire la quantité d'ATP produite par nombre d'électrons transférés dépend du caractère électrogénique ou non-éléctrogénique du complexe Ndh. L'étude de ces mécanismes de translocation de protons du stroma vers le lumen pourrait s'effectuer *in planta* à l'aide de sondes fluorescentes marquant les variations de pH. Le complexe Ndh et PTOX étant peu abondants, l'étude de l'électrogénicité du complexe Ndh et de la chlororespiration pourrait être réalisée *in vitro* dans des systèmes reconstitués ou dans des systèmes ou le complexe Ndh est abondant et les complexes photosynthétiques absents (au sein des étioplastes, par exemple).

La nature des sous-unités formant le domaine catalytique et la nature du substrat du complexe Ndh restent à déterminer. Il est à noter qu'une démarche de double hybride a été entreprise au cours de cette thèse. En criblant une banque d'ADNc d' *Arabidopsis thaliana* en utilisant des protéines appâts correspondant aux sous-unités solubles du complexe Ndh telles que les peptides NDH-H,I,J et K, nous avions pour objectif de détecter d'éventuelles sous-unités solubles du complexe Ndh codées par le génome nucléaire et impliquées dans l'activité catalytique. Cependant, cette stratégie n'a pas abouti. Les sous-unités solubles du complexe Ndh exprimées en fusion avec la protéine Gal4 sont incapables d'interagir entre elles dans un organisme hétérologue tel que la levure. Une approche *in planta* semble plus prometteuse. Ainsi, un crible de mutants nucléaires pourrait s'effectuer à travers l'analyse de la fluorescence de la chlorophylle sur des jeunes plantules. L'absence de pic transitoire d'augmentation de la fluorescence après une période d'éclairement serait le critère choisi pour obtenir des mutants affectés dans la réduction non photochimique du pool de PQ et permettre de trouver des sous-unités "manquantes" du complexe Ndh.