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 O2, thus avoiding over-reduction of plastoquinones, when photosynthetic CO2 assimilation was not fully induced. We proposed that PTOX, by modulating the redox state of inter-system electron carriers, may participate in the regulation of cyclic electron flow around photosystem I.

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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.

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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 O2 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 500 μmol photon·m⁻²·s⁻¹ supported by halogen lamps (HQL T 400/WDY, 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'-CCCGCGGATCCGGCACGGATGCACAGTTCTCGAG-3' and 5'-CCGGACCTTACTATTACTGATGGTTTGGT-AGGC-3', respectively, containing an XhoI and an SstI 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 expression vector (pKYL7X1). Expression of At-PTOX was driven by a double sequence of the cauliflower mosaic virus 3S-labeled constitutive promoter (26). The recombinant plasmid was introduced into Agrobacterium tumefaciens through electroporation into a phytotube cell wall (GenBANK™ accession number H9262). The recombinant plasmid was introduced by Agrobacterium tumefaciens electroporation into tobacco plants containing 0.3 M sorbitol, 5 mM NaCl, 10 mM MgCl2, 2.5 mM NaHPO4, 50% glucose (20 mM) and glucose oxidase (2 mg ml⁻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 O2 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 MgCl2, 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% (ν/ν) Percoll, respectively. After centrifugation in a swing out rotor at 3500 × g for 15 min, intact chloroplasts were recovered from the 40-90% Percoll interphase, washed with 60 ml of medium A, pelleted at 2000 × g for 3 min, and osmotically lysed by resuspension in 10 mM MgCl2 and 1 mM phenylmethylsulfonyl fluoride for 30 min. Lysed chloroplasts were diluted at a final concentration of 200 μM of chlorophyll·ml⁻¹ in 30 mM Hepes-KOH buffer, pH 7.5, containing 0.3 M sorbitol, 5 mM NaCl, 10 mM MgCl2, 2.5 mM NaHPO4, 50% (ν/ν) glycerol, 1 mM phenylmethylsulfonyl fluoride. Aliquots of the chloroplast preparation were stored at −20 °C. For O2 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 MgCl2. For O2/Fm′ fluorometry, samples were measured at room temperature (25 °C) and leaf vapor pressure deficit was maintained around 1 kPa. Leaves were illuminated using a portable gas exchange system (LI-6400, Li-Cor Inc, Lincoln, NE) and photochemical yield was measured on chloroplast samples using a pulse-amplified photoacoustic detector (MAIHAK, Hamburg, Germany). This mixing system was also used for fluorescence measurements in attached leaves. Photostationary CO2 Fixation Measurements on Attached Leaves—Net CO2 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 O2 and N2 concentrations were provided by mixing pure gases. N2 was measured using a paramagnetic O2 analyzer (MAIHAK, Hamburg, Germany). This mixing system was also used for fluorescence measurements in attached leaves. Quantum yield of CO2 fixation in air and under non-photorespiratory conditions (O2 15% (ν/ν); CO2 3% μlitr−1) was calculated from the slope of the linear portion of the light response curve (5 measurements at irradiances between 40 and 100 μmol photons·m⁻²·s⁻¹ and 10 measurements at 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 MgCl2 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 were resuspended in the supernatant were precipitated with acetone (80% final concentration). Denaturing SDS-PAGE was performed as described by Laemmli (30) using a 10% polyacrylamide gel (w/v). Proteins were loaded onto 0.45-μm nitrocellulose membranes (Schleicher & Schuell) and were incubated with the purified anti-At-PTOX serum (24). Immunoblotting was performed using the chemiluminescence Western blotting kit (Amersham Biosciences).
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RESULTS

Expression of At-PTOX in Tobacco—Transgenic tobacco plants expressing the At-PTOX cDNA sequence under the control of the doubled constitutive 35S promoter of the cauliflower mosaic virus were generated by Agrobacterium-mediated transformation. Two lines, PTOX1- and PTOX2- , showing a particularly strong expression of the transgene were selected among six transformant lines and were further studied (Fig. 1A). Note that although no signal was observed in WT tobacco (Fig. 1A, upper panel), a faint band was detected after re-amplification (Fig. 1A, lower panel). We checked that amplified RT-PCR fragments, including the faint band amplified in WT tobacco (Fig. 1A), 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. 1B). 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 PTOX1- and PTOX2- 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. PTOX1- and PTOX2- 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 F0 Fluorescence Increase—When intact WT leaves were illuminated for a few minutes and then placed in the dark, a transient increase in the F0 chlorophyll fluorescence level occurred (Fig. 3A) (see Refs. 32 and 33). The post-illumination fluorescence transient was absent in PTOX- leaves, and the F0 fluorescence level rapidly decreased after switching off the light (Fig. 3B). As reported previously, the fluorescence increase was absent in Ndh-less mutants (Fig. 3C), 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 redux 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 F0 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 (Fm). 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 (QA) in redux 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. 4B). 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. 4A, 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
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. 5A 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/H11001 leaves 4 s after a pulse illumination was close to that measured in dark-adapted leaves (Fig. 5B). 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. 4A), possibly indicating the contribution of a putative tobacco PTOX in PQ oxidation.

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 $b_{597}$ 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 $b_{597}$
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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 CO₂ 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. 9A), indicating a highly efficient plastoquinol oxidation before the activation of PS I. After a few minutes of illumination, both Fₗ and Fₘₚ values were identical in WT and PTOX⁺. At this low irradiance, Fₘₚ was close to Fₘ, 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, Fₗ values remained lower in PTOX⁺ than in WT during the first 3 min of illumination (Fig. 9B). When illumination was prolonged, the decrease in Fₗ was more pronounced in WT, and after 10 min reached a lower level than in PTOX⁺. It should be noted that variations in Fₗ values were accompanied by concomitant changes in Fₘₚ (Fig. 9B). 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-
steady state was reached, *q*ₙ and *q*ₚ 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 (8).

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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 (O₂ 1.5% (v/v); CO₂ 750 μl · liter⁻¹) CO₂ assimilation measured in attached leaves of WT and PTOX tobacco

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 | PTOX²⁺ |
|------------------------------------|----|-------|
| *(Fₚₐₓ – F₀)/Fₚₐₓ* after 10 min illumination | *q*ₚ | 0.332 ± 0.023 | 0.339 ± 0.016 |
| *q*ₙ | 0.731 ± 0.011 | 0.632 ± 0.022 |
| *(Fₚₐₓ – F₀)/Fₚₐₓ* after 60 min illumination | *q*ₚ | 0.350 ± 0.026 | 0.324 ± 0.032 |
| *q*ₙ | 0.743 ± 0.018 | 0.759 ± 0.009 |

**TABLE II** Quantum yield of CO₂ fixation (Φₐ₁₄) and maximal rate of CO₂ 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 (O₂ 1.5% (v/v); CO₂ 750 μl · liter⁻¹). Number of experiments is indicated in parentheses.

| | WT | PTOX²⁺ |
| | | | |
| Φₐ₁₄ (air) | 0.0481 ± 0.0038 (3) | 0.0440 ± 0.0056 (3) |
| Φₐ₁₄ (1.5% O₂; 750 μl · liter⁻¹ CO₂) | 0.0783 ± 0.0023 (3) | 0.0801 ± 0.0021 (3) |
| Maximal CO₂ 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 O₂ 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. 3C) 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 O₂ 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 *b₅f* 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 in both WT and PTOX$^+$ during 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 (QA) 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 QA 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 QA 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. 1A). 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 Solanaceae 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$_4$ 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$_4$ plants.
than in mesophyll cells and participate in the regulation of cyclic electron transfer reactions around PS I.

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