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Kinetic properties and physiological role of the plastoquinone terminal oxidase (PTOX) in a vascular plant

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ABSTRACT

The physiological role of the plastid terminal oxidase (PTOX) involved in plastoquinol oxidation in chloroplasts has been investigated *in vivo* in tomato leaves. Enzyme activity was assessed by non-invasive methods based on the analysis of the kinetics of chlorophyll fluorescence changes. In the dark, the maximum PTOX rate was smaller than 1 electron per second per PSII. This value was further decreased upon light acclimation, and became almost negligible upon inhibition of the photosynthetic performances by reducing the CO_2 availability. In contrast, prolonged exposure to high light resulted in an increase of the overall PTOX activity, which was paralleled by an increased protein accumulation. Under all the conditions tested the enzyme activity always remained about two orders of magnitude lower than that of electron flux through the linear photosynthetic electron pathway. Therefore, PTOX cannot have a role of a safety valve for photogenerated electrons, while it could be involved in acclimation to high light. Moreover, by playing a major role in the control of the stromal redox poise, PTOX is also capable of modulating the balance between linear and cyclic electron flow around PSI during the deactivation phase of carbon assimilation that follows a light to dark transition.

1. Introduction

In the chloroplast, the chlororespiratory pathway ensures the nonphotochemical reduction and oxidation of the plastoquinone pool, in a process by which reducing equivalents (in the form of NADPH, or ferredoxin, [1]) are consumed to reduce the plastoquinone (PQ) pool through the activity of NDH complexes (reviewed in [2–4]). These enzymes can be either multimeric complexes homologues to the respiratory complex 1 (type I NDHs; reviewed in [5]) or monomeric proteins (type 2 NDHs), which are in general found in algae (e.g. [6]). On the oxidising side of the chain, reduced plastoquinones (PQH_2) are consumed to reduce molecular oxygen through the activity of the plastoquinone terminal oxidase (PTOX) a di-iron enzyme that is membrane bound [7,8] and likely localised in the stroma lamellae [9]. Discovered in the 80s in green algae [10], the function of the chlororespiratory chain, and in particular of the PTOX enzyme, is still under debate (see [11] for a recent review). While it is clearly established that PTOX is involved in carotenoid biosynthesis at an early stage of leaf development, pigment biosynthesis is PTOX independent in mature tissues. Indeed, tomato *ghost* or Arabidopsis *immutans* plants (both lacking PTOX activity) do not show any particular carotenoid deficiency when protected from photooxidative damage at an early stage of development [12–14]. Previous work has suggested that PTOX could play a role in the acclimation of photosynthesis to changing environmental conditions including temperature, light and water stress in mature leaves (e.g. [2–4,15,16]). This would reflect the capacity of this enzyme to act as a safety valve for "excess" electrons generated when light absorption overcomes the saturation capacity of the carbon fixation machinery [17]. Moreover, PTOX could also modulate the electron flow rates in the light, by keeping the redox state of the PQ pool oxidised [2,18,19].

To test these hypotheses, we assessed the kinetic parameters of PTOX activity *in vivo*, based on estimates of the redox state of the plastoquinone pool (PQ) derived from chlorophyll fluorescence experiments. Tomato wild type (San Marzano, S.M.) plants were compared to a mutant lacking PTOX (*ghost*), first employing a "classical" PAM-based fluorescence approach, where levels of the minimum fluorescence (Fo) were monitored after exposure to continuous light [20]. This method provides a measurement of the redox state of the

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quinone acceptor of PSII, Q_A. However, this can hardly be translated into measurements of the redox state of the PQ pool due to: *i*. The non-linear relationship between the redox state of Q_A and the fluorescence yield [21] and *ii*. The non-linearity between Q_A and PQ, as expected from the rather large equilibrium constant between the two quinones. Therefore, an alternative approach was used to measure the PQ dynamics more directly. This relies on measurements of the area above the fluorescence induction curves, a parameter that is directly proportional to the number of oxidised electron acceptors present in the chloroplast [22].

Using this approach we were able to compare PTOX activity in dark or light acclimated leaves (both at ambient and at low CO_2 concentrations), or in leaves exposed to high light for several hours. We could clarify the question as to whether PTOX represents or not a safety valve for electron flow [13,23–25] and also examine the influence this enzyme has on the balance between linear and cyclic electron flow.

2. Material and methods

2.1. Plant materials and treatments

Tomato WT (San Marzano S.M.) and PTOX deficient (*ghost*; [26]) seedlings were first grown on soil in a growth chamber under a 16 h day/8 h night regime, in dim light (50 μmol photons m^{-2} s^{-1}) light intensity was chosen to avoid bleaching/variegation of *ghost* leaves) at a temperature not lower than 20 °C. Plantlets were then grown under a light intensity of 100 μmol photons m^{-2} s^{-1} (white light provided by neon tubes) and used to produce the following four different types of plants employed for this work: "Dark" plants were dark-acclimated for at least 5 h prior to measurements; "Light" plants were collected from the growth chamber (after several hours of a "day" period) and dark-incubated for only 15–20 min (to inactivate the Calvin cycle) prior of measurements; "Light-CO_2" plants were placed in an air-tight transparent cabinet containing a CO_2 concentration of 30 ppm (the circulating air stream was depleted of CO_2 through a soda lime filter) and exposed to light (100 μmol photons m^{-2} s^{-1}) for 5 days and then dark-incubated for 15–20 min prior to measurements; "High light" treated leaves were detached from "Light" plants and layered on a Whatman paper (connected to a water reservoir to keep humidity during the treatment). These leaves were covered with a Saran plastic layer to limit evaporation and exposed to 1000 μmol photons m^{-2} s^{-1} for 16 h before measurements. The high light experimental system used a white LEDs source cooled down by air circulation. Therefore, we measured a temperature increase of 1–2 °C maximum at the leaf surface during this treatment. Inhibitors were dissolved in ethanol, diluted to the concentration given in Fig. 2 in a 150 mM aqueous sorbitol solution (to avoid osmotic shock; [27] and infiltrated by a gentle vacuum infiltration using a syringe filled with a solution of the chosen chemical. Controls were treated in the same way and same solution without the chemical.

2.2. Spectroscopic measurements

Spectroscopic measurements were performed on intact leaves with a flash spectrophotometer (JTS 10, Biologic France). P_{700} oxidation kinetics was assessed at 810 nm (after deconvolution of plastocyanin absorption as previously described in [27]. Far-red (FR) illumination was provided by a LED peaking at 735 nm, filtered through three Wratten filters 55 that block wavelengths shorter than 700 nm. When needed, the maximum extent of P_{700}^{+} was estimated by imposing a saturating flash of white light on top of the FR. Actinic light was provided by a red LED peaking at 640 nm. Continuous actinic light was switched off transiently while measuring P_{700} redox changes at the appropriate wavelengths.

2.3. Fluorescence measurements

Data presented in Fig. 1 were measured with a PAM 101/102/103 modulated fluorimeter (Walz, Germany). In these experiments, leaves were exposed to white actinic light (150 μmol photons $m^{-2}s^{-1}$) before measuring the "initial fluorescence" (F_0') level, using the PAM "measuring" beam. When indicated, FR light (720 nm, as provided by the PAM instrument) was applied at the maximum available intensity, to enhance PSI-driven PQH_2 oxidation.

In Figs. 2 and 3, fluorescence was measured with the same apparatus used for absorption spectroscopy. Fluorescence changes were measured upon exposure to red light (80 μmol photons $m^{-2}s^{-1}$) using a blue "measuring" light. Estimation of the size of the PSI and PSII acceptor pools in Fig. 3 was obtained measuring fluorescence induction upon exposures to 1 s of light. After a variable dark time, a second fluorescence induction was measured. A plot of the area above the second fluorescence induction as a function of the dark time between the two consecutive illuminations, yielded a multiphasic profile, which was indicative of the rate of oxidation of the PSII and PSI electron acceptors (see text).

2.4. Chloroplast purification and western blot analysis

Thylakoid membranes were isolated at 4 °C from plants exposed to the different treatments described above. Leaves were briefly blended in ice-cold buffer (20 mM Tricine pH 8.0, 10 mM NaCl, 5 mM $MgCl_2$, 100 mM sucrose containing protease inhibitors), and the resulting suspension was filtered using a syringe equipped with a layer (~1 cm) of fiberglass. Thylakoids were sedimented at 1500×*g* for 3 min in the same buffer without sucrose, and then washed 2 times with the same buffer. They were resuspended in a small volume of the grinding buffer solubilised with a Tris–HCl 50 mM pH 6.8, SDS 2%. The supernatant was recovered after 5 min centrifugation at 18,500×*g* and 7 μg aliquots were loaded onto a 12% acrylamide gel. Two identical gels were run: the first one was used to test protein loading with Coomassie blue staining, while the second one was transferred onto PVDF membrane and blotted using an anti PTOX (1/1000) and an anti NDH-H (1/1500) antibody.

3. Results

3.1. Estimation of the plastoquinone pool redox state using the initial fluorescence (F_o') approach

A widely used approach for addressing the activity of the chlororespiratory electron transfer chain is the "fluorescence rise" approach: upon switching off a continuous illumination, the fluorescence yields decreases to a pseudo "Fo" level and then exhibits a slower increase commonly interpreted as the transient nonphotochemical reduction of the PQ pool [20,28]. We confirmed this observation in tomato plants (Fig. 1A, left panel, black traces). As expected, the rise in fluorescence (hereafter called initial fluorescence or "F_0'") was abolished in leaves submitted to Far Red (FR) illumination (Fig. 1A, grey traces), which preferentially excites PSI and hence leads to the oxidation of the electron donors to PSI, among which are plastoquinols. We observed that the extent of the F_0' increase was slightly larger (Fig. 1A, right panel) in a *ghost* mutant devoid of the PTOX enzyme [26] than in the wild type (S.M.). Moreover, when plants were incubated in darkness for increasing periods of time, the FR-mediated decline of F_0' was persistent for at least 30 min in the mutant while it rapidly disappeared in S.M. (Fig. 1B). This suggests that, after illumination, PQH_2 oxidation by this enzyme was first overcome by injection of electrons into the PQ pool by the other chlororespiratory complex, the NDH. Therefore, a net reduction of the PQ pool is seen. On a longer time scale, PTOX activity becomes prevailing in S.M, leading to the oxidation of the

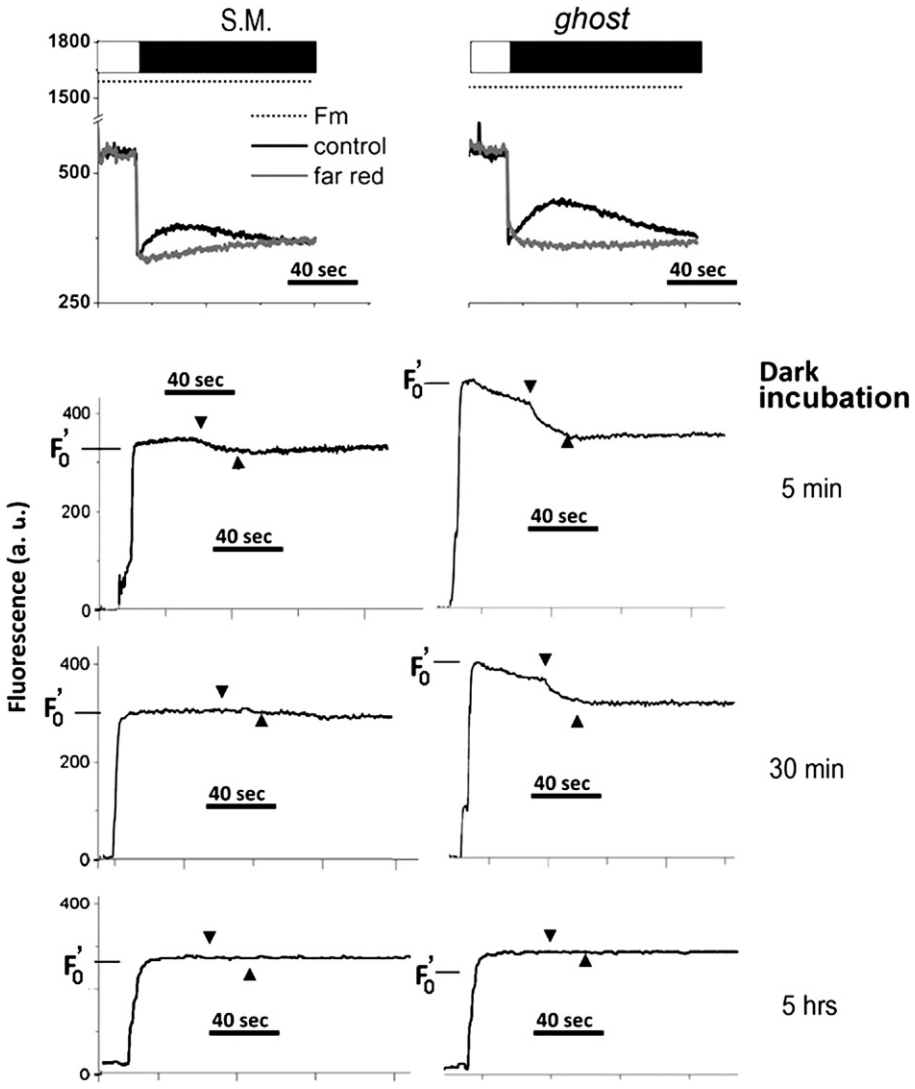

Fig. 1. Chlorophyll fluorescence changes in San Marzano (S.M.) and *ghost* leaves during dark incubation after illumination. Fluorescence was monitored in detached leaves of light-acclimated plants (see description of "light" plants in Material and methods) using a PAM fluorimeter. In panel A, after 10 min light exposure, light was switched off and the transient rise in "dark" fluorescence (monitored by the non-actinic "measuring" light of the fluorimeter) was monitored. In panel B, changes in the initial fluorescence yield (F_0') were recorded after these light-acclimated plants were kept in complete darkness for 5 min, 30 min or 5 h. The steep initial rise to reach F_0' is due to the switching on of the "measuring" light. White boxes above panel A: light on; black boxes: light off. Dots: F_m levels. Arrowheads point to time periods of FR (720 nm) light exposure in order to reoxidise the plastoquinone pool.

pool. In the absence of this activity (*ghost*) PQ oxidation is diminished and therefore PQH_2 has a longer lifetime.

3.2. Estimation of the plastoquinone pool redox state from measurements of the area above the fluorescence induction kinetics

Although the approach employed above provides relevant information on the presence/activity of the chlororespiratory enzymes (e.g. [29]), no quantitative information about the activity of these enzymes can be obtained, because it does not allow quantifying their kinetic properties. This is because changes in the fluorescence yield are expected to occur only when a large fraction of PQ is already reduced, owing to the very large equilibrium constant between the PQ pool (i. e. the substrate of the chlororespiratory enzymes), and Q_A, the primary quinone acceptor of PSII, which modulates the fluorescence yield [21].

To overcome this difficulty, changes in chlororespiratory activity in intact leaves were quantified using an alternative approach first developed in algae [10,30] and more recently in plants [31]. In this approach, quantification relies on measurements of the area above the fluorescence induction curves (see grey area in Fig. 2A), a parameter that is directly proportional to the number of oxidised electron acceptors present in the chloroplast [22]. In dark-acclimated plants, the Calvin cycle is largely inactive and thus constitutes a bottleneck for electron flow. Hence, illumination results in the sequential reduction of all the electron carriers that are in their oxidised state in the dark. This includes the PSI electron acceptors (Ferredoxin, FNR and $NADP^+$), PQ and the primary PSII acceptor Q_A. As a result, a multistep fluorescence increase from the initial level (F_i) to the maximum one (F_m) is seen. Based on this notion, the following protocol was used to quantify the kinetic features of PTOX and of the NDH: plants previously dark acclimated for at least 5 h (hereafter called "dark" plants) were illuminated with 200 μmol photons m^{-2} s^{-1} for 1 s (to fully reduce the PSI and PSII acceptors; solid line Fig. 2A) and then reilluminated a second time after various dark periods. During the dark period, oxidation of some of the primary and of the secondary PSII and PSI electron acceptors takes place, depending on the relative rates of their oxidation processes. This modifies the redox state of some acceptors prior to the second illumination, and therefore alters the shape of the fluorescence kinetics. In particular when the dark period between the two illuminations was short (up to 5 s; open circles

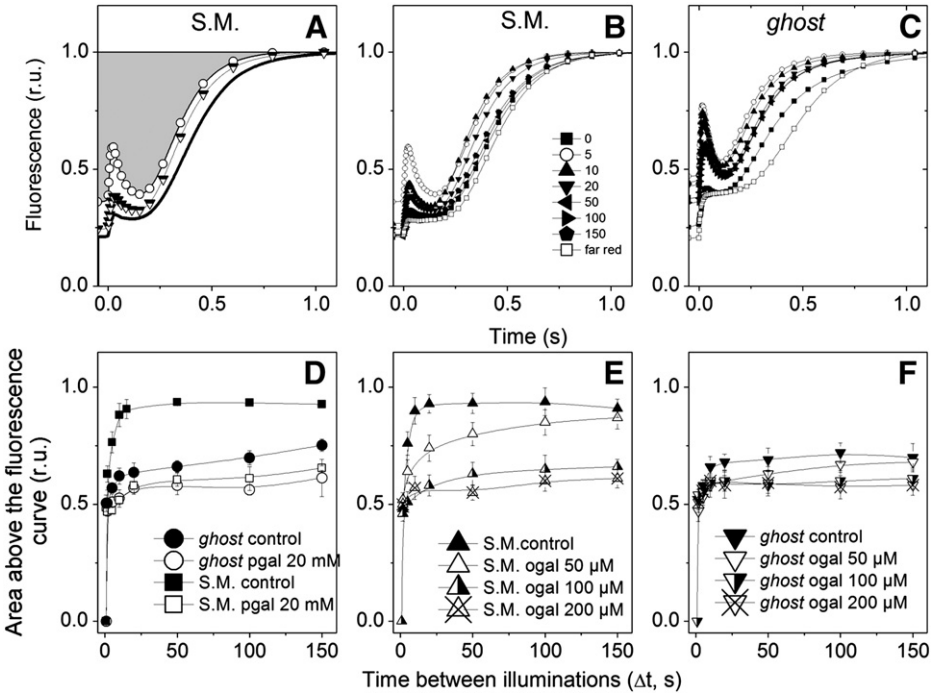

Fig. 2. Fluorescence induction kinetics measured in tomato leaves. Plants were dark-acclimated prior to the experiment as described in Material and methods ("dark" plants). The intensity of the light used to excite fluorescence was 80 µmol photons $m^{-2}s^{-1}$. A. Examples of fluorescence induction curves and bound area above the curve (grey area). Solid line: induction measured during the first light pulse on a dark-acclimated leaf. Open circles: induction during a second light pulse fired 5 s after the first one. Triangle: induction after 20 s between consecutive light pulses. B, C. Fluorescence curves as a function of the duration of dark incubation (from 5 to 150 s) between two consecutive illuminations in wild type S.M. (A) and PTOX-deficient *ghost* (B). D, E, F. Inhibition of PTOX and PTOX-like activities by the PTOX inhibitors propyl-gallate (pgal) and octyl-gallate (ogal). Activities were estimated from the bound area above the fluorescence induction curve as a function of the dark period. The area values have been normalised to the area measured upon exposure to FR light which was set to 1. D: Effect of pgal in SM and *ghost* leaves. E: Effect of ogal in SM leaves. F: Effect of ogal in *ghost* leaves. Note that in both genotypes a dose dependent inhibition is seen. Error bars refer to s.e. of two independent experiments.

in Fig. 2A), a first fast fluorescence rise (t<30 ms) was observed, suggesting that PQ was reduced. This first phase was followed by a slower fluorescence decline (t~50–100 ms) likely reflecting the oxidation of the PQ pool by PSI (*via* the cytochrome b_6f complex). Eventually, an additional much slower rise phase was seen (t>100 ms) resulting from the reduction of all the oxidised electron carriers located

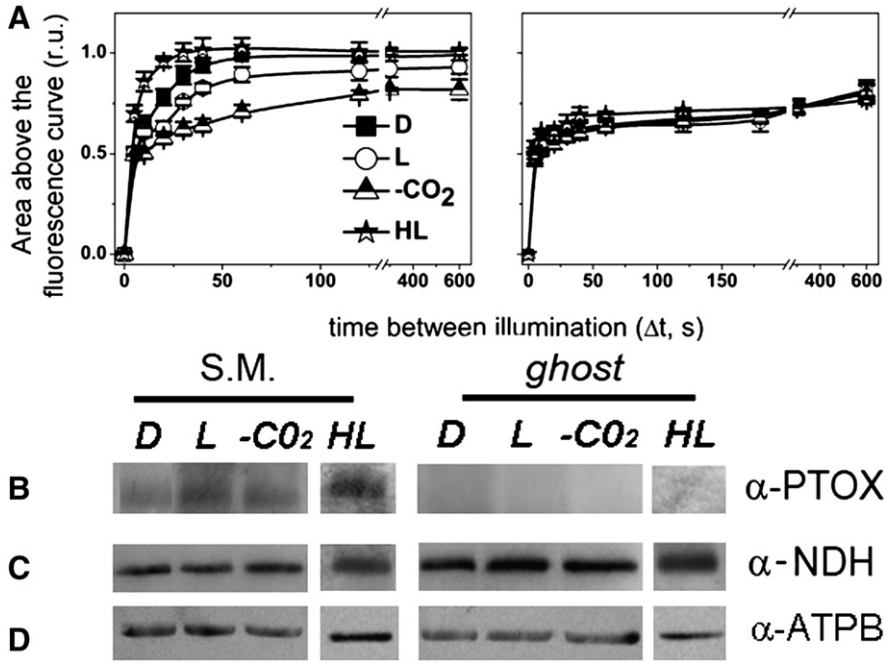

Fig. 3. Effects of acclimation of SM and *ghost* leaves to dark, light, light-low CO_2 and high light conditions on plastoquinone oxidising activities and on PTOX and NDH accumulation. A. Effects on plastoquinone oxidising activities. Area above the fluorescence induction curve as a function of the dark period after 1 s illumination were calculated and normalised as described in Fig. 2. Incubation conditions of "light" (L), "dark" (D) and "low $[CO_2]$" ($-CO_2$) and "high light" (HL) plants are given in Material and methods. Bars refer to s.e. of 6 measurements from three independent experiments. B, C, D. Effects on polypeptide levels. Western blot used an anti PTOX (B) or an anti NDH-H (C) antibody. The ATPB antibody (against the β subunit of the ATP synthase complex) was used as a loading control. These experiments were performed 3 times and a representative immunoblot is shown.

downstream of PSII and ultimately leading to reduction the primary electron acceptors Q_A (which directly modulates the yield of fluorescence emission). After longer dark periods (20 s; triangles in Fig. 2A), both the fast rise and the decline phases disappeared suggesting that the plastoquinone pool had already been oxidised during the dark period between the first and the second illumination. In this case, a plateau was seen, followed by the slow rise phase to Fm.

Fig. 2B and C presents a series of fluorescence induction curves recorded in S.M. (Fig. 2B) and *ghost* (Fig. 2C) leaves, respectively for dark periods ranging from 5 to 150 s. As stated above, the linear relationship between the fluorescence yield and the rate of PSII photoreaction makes the area above the curve (up to the F_m) a linear indicator of the overall amount of oxidised electron acceptors located downstream of PSII (Q_A+ the PQ pool; [10] and PSI [31]. Therefore, a plot of this area as a function of the dark periods between consecutive light pulses (data points from Fig. 2B and C) was employed to quantify the kinetic performances of the processes leading to the oxidation of the PSII and PSI electron acceptors. The observed profiles (black symbols in Fig. 2D) are multiphasic, consistent with the observation of different phases in the fluorescence induction traces.

The fast phase was completed in a few seconds and was insensitive to propyl-gallate (pgal; open symbols in Fig. 2D) and octyl-gallate (ogal; Fig. 2E and F, open triangles), two known inhibitors of PTOX [32]. This suggests that it reflects the oxidation of PSI acceptors, as proposed earlier [31]. Conversely, the slow phase of increase in the area bound above the fluorescence induction curves was sensitive to both ogal and pgal (Fig. 2D–F), suggesting that it reflects the dark oxidation of the reduced PQ pool by PTOX. The inhibition by ogal was dose-dependent (Fig. 2E), in agreement with previous *in vitro* studies [32]. We note however that the inhibiting concentrations were higher *in vivo* than *in vitro*. The assignment of the fast and slow phase to the oxidation of the PSI and PSII acceptors (respectively) was confirmed by the analysis performed in *ghost* leaves, where a fast phase of similar amplitude as in S.M. was detected while only a small oxidation phase could be observed in the dozens of seconds time scale (Fig. 2D, circles).

3.3. Evaluation of plastoquinone oxidation kinetics in tomato leaves

From the kinetic analysis of the profiles shown in Fig. 2, it is possible to derive quantitative information of the influx and outflux of electron undergone by the PQ pool, i.e. on the activity of the NDH and PTOX enzymes. Quantitative data are calculated by taking the fully oxidised case (100% of oxidisable acceptors are oxidised), obtained after illumination with FR of weak intensity (datapoints from Fig. 2B–C, open squares), as a reference. Then changes in the area above the fluorescence trace were evaluated by dividing the area measured at every datapoint by the FR reference. It can be seen (Fig. 2D) that the fast phase (*i.e.* the oxidation of PSI acceptors) accounts for ~50% of the FR area reference, implying that the size of both the PQ and PSI acceptors pools is roughly equal. In these "dark" S.M. plants, the PQ was almost completely oxidised after a relatively short (<1 min) dark incubation (see Fig. 2B and D). This is indicated by the very small effect of FR illumination on the area above the fluorescence, which was increased by only 10%. In these leaves, the slow phase (reflecting the oxidation of the PQ pool) represents 40% of the overall amplitude (calculated as 90%–50%, where 90% is the total amplitude without FR and 50% is the amplitude of the fast phase). Since 50% is the maximum expected value (see above), we conclude that the $PQ/(PQ+PQH_2)$ relative ratio is 0.80 (calculated as 40%/50%) at equilibrium. Moreover, fitting the data of Fig. 2B indicates that the time constant of the slow oxidation component (τ) is ~30 s. This yields an apparent rate constant of 0.033 s^{-1} (calculated as $1/\tau$). Taking into account the fact that the PQ/PSII stoichiometry is 6 (according to [33]), i.e. that there are 12 electrons per PSII, an apparent oxidation rate of ~0.4 s^{-1} can be evaluated. Because the PQ pool undergoes concomitant reduction and oxidation reactions by the NDH and PTOX respectively, the apparent rate constant evaluated above corresponds to the sum of the individual rates of the two processes (k_{NDH} and k_{PTOX}). Because the $PQ/(PQ+PQH_2)$ ratio at equilibrium (0.8) reflects the ratio between the two rate constants ($k_{PTOX}/(k_{PTOX}+k_{NDH})$), one obtains ~0.3 s^{-1} for k_{PTOX} and ~0.1 s^{-1} for k_{NDH} in "dark" leaves.

In *ghost* leaves, a larger effect of FR was seen confirming that disruption of the PTOX gene by this mutation leads to a significant accumulation of reduced PQ in the dark (compare the t = 0 dark-acclimated and FR treated traces cases in Fig. 2C). However, lack of PTOX activity did not result in the full reduction of the PQ pool. Thus, a plastoquinol oxidation activity remains in *ghost*. In these leaves, the slow phase represents ~25% of the overall amplitude (calculated as 75%–50%, where 75% is the total amplitude without FR and 50% is the amplitude of the fast phase; Fig. 2D, black circles). Thus, the $PQ/(PQ+PQH_2)$ relative ratio is 0.5 (calculated as 25%/50%) at equilibrium, which means the residual PQ oxidising activity is similar to that of the NDH. It is worth noting that this remaining oxidising activity is sensitive to the PTOX inhibitors propyl- and octyl-gallate (Fig. 2D and F), indicating that some residual PTOX (or PTOX-like) activity remains in the mutant. However no residual PTOX-related polypeptide could be detected in chloroplasts isolated from *ghost* by an immunoblotting approach using a specific antibody raised against PTOX (see below), as expected since the single PTOX gene is disrupted by a frame-shift mutation in *ghost* [26]. Therefore, we tend to exclude the presence of "residual" PTOX in ghost leaves, and favor instead the possibility that a PTOX-like enzyme exists in this mutant (see discussion). After long (24 h) dark incubation (not shown), this mutant exhibited a fully oxidised PQ pool, which might be explained by the lack of reduced NADPH to feed the NDH activity or by a decreased in NDH activity.

3.4. Influence of light acclimation on the chlororespiratory activities

An important issue concerning chloroplast respiratory-like enzymes is their possible involvement in the photosynthetic electron transfer and/or regulation processes. This possibility was investigated monitoring the influence of light acclimation on the chlororespiratory activity. In S.M. leaves, we observed a significant decrease in the oxidation rate of PQH_2 upon light acclimation compared to dark acclimation (Fig. 3A, compare "light" plants, open circles, with "dark" ones, squares). As discussed above, the slower initial oxidation rate of PQH_2 in the dark should reflect a decrease in PTOX rate. Using the same approach as above, we indeed estimated k_{PTOX} as 0.2–0.25 s^{-1} in light treated plants, while k_{NDH} was essentially unchanged. In *ghost* leaves, light acclimation had no significant effect on the kinetics of redox changes of the PQ pool in the dark (Fig. 3A, right), confirming that the decrease observed in S.M. was due to a specific inhibition of PTOX. This modification in PTOX activity could not be correlated to changes in the amount of this enzyme (Fig. 3B, C, lanes D and L).

The observation of a light-driven decrease in PTOX activity is in disagreement with the hypothesis that this enzyme could act as a safety valve to prevent over-reduction of the electron flow chain in the light. To further investigate the real capacity of PTOX to act as an electron sink under restricted electron flow, we measured PQ oxidation in plants exposed to limiting CO_2 concentration. S.M. and *ghost* whole plants were incubated for five days under 100 µmol photons $m^{-2}s^{-1}$ in a gas-tight cabinet where $[CO_2]$ was lowered to 30 ppm ("light-CO_2" plants). These conditions are known to promote a stress situation that is counterbalanced by the induction of a number of photoprotective processes, for instance by the re-routing of a substantial fraction of photogenerated electrons towards alternative sinks (reviewed in [34]). Strikingly, in leaves of S.M. plants grown under low $[CO_2]$, the rate of PQH_2 oxidation turned out to be even lower than the one measured in light acclimated plants under ambient $[CO_2]$ (Fig. 3A, triangles), suggesting an additional decrease of PTOX performances. In this case, the PQ pool (as estimated from the ratio of the areas in steady state

vs. FR) was ~50% reduced, suggesting that the PTOX and NDH rates were similar, *i.e.* the same situation as observed in *ghost* (*i.e.* k_{PTOX}~ k_{NDH} ~0.1 s^{-1}). Again, such changes in PTOX activity could not be correlated to macroscopic variations in the amount of this enzyme (Fig. 3B, C). These observations are all the more surprising as exposure of tomato plants to high light intensity (1000 μmol photons $m^{-2}s^{-1}$) for several hours ("high-light" plants) resulted in faster PQ oxidation (Fig. 3A, stars; k_{PTOX} calculated to be ~1 s^{-1}), while *ghost* leaves were mostly unaffected (Fig. 3A, right). In this case, changes in PTOX activity were correlated to a limited but detectable increase in PTOX accumulation, while the accumulation of the NDH enzyme remained unaffected (Fig. 3B, C).

3.5. Measurements of plastoquinone oxido-reduction kinetics from P_{700} turnover

Additional evidences for a role of light in modulating PTOX activity was obtained probing the chlororespiratory performances with a spectroscopic approach. When FR excites PSI, and as such induces the oxidation of P_{700}, the kinetic profile of this oxidation is expected to depend on the redox state of the electron donors to P_{700} (including the PQ pool). S.M. and *ghost* tomato plants were light-acclimated for several hours and then exposed to a first FR illumination in order to set the PQ pool in a defined redox state. The leaf was left in the dark for a definite time (10, 30 or 60 s; Fig. 4A), after which a second FR illumination was provided, aimed at measuring the delay in the oxidation of P_{700}. The rationale for this approach is that after a prolonged illumination, the photosynthetic electron transfer is mainly linear (LEF). Deactivation of linear flow (and activation of cyclic flow) only occurs after a long period of dark activation (e.g. [27]). Therefore, after a dark adaptation of 0 to 60 s, electrons available for P700 reduction are generated by the PSII acceptors and the area above the P_{700} oxidation kinetics should only reflect the reduction level of the PSI donors (including the PQ pool). Measuring this parameter as a function of the dark time between two consecutives FR illuminations provides therefore the rate of re-reduction of the PQ pool. As in the case of the area above the fluorescence induction, a calibration for this method was required. This was done by exposing leaves to a saturating light pulse just before detection, in order to probe the kinetics under conditions of fully-reduced PQ pool. The area measured in these conditions was used as a reference for further calculations.

As shown in Fig. 4A and B, the oxidation kinetics of P_{700} (when normalised on the kinetics measured upon full reduction of the PQ pool) was strikingly similar in S.M. and *ghost*. Plots of the corresponding data as a function of the duration of the dark period between two consecutive FR illuminations to evaluate the kinetics of PQ reduction in the dark (Fig. 4C) were similar in the two plant lines. This suggests that, following prolonged light exposure, PTOX activity has decreased so much in the WT that it has become indistinguishable from that observed in the PTOX-deficient line. The observation that, under

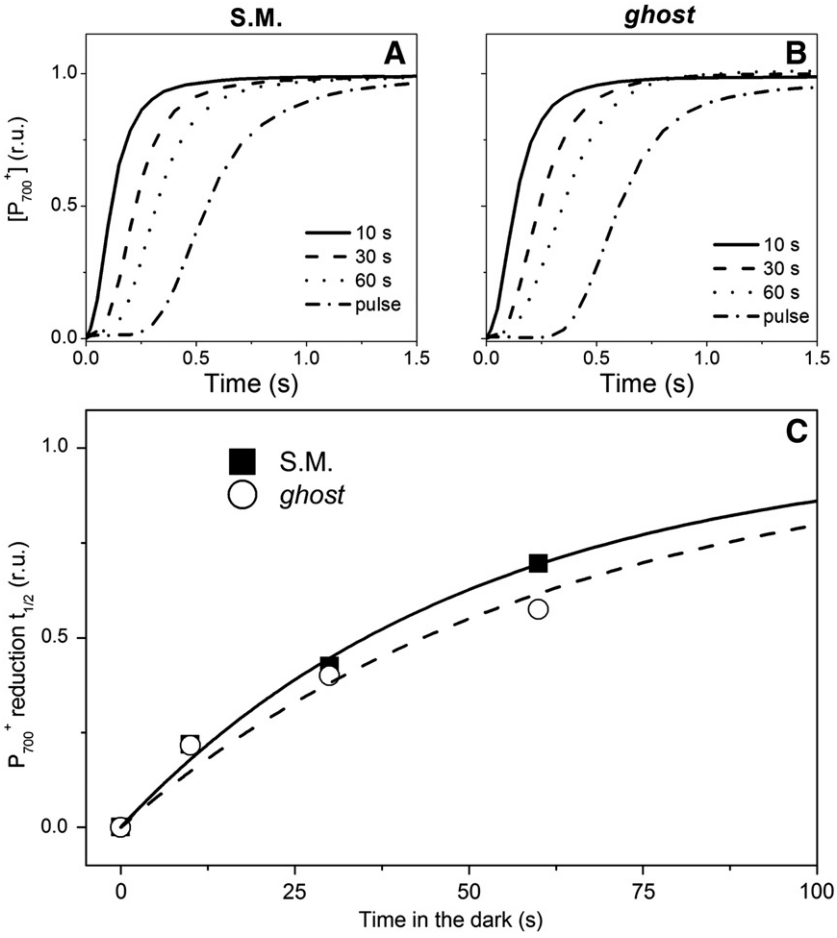

Fig. 4. Probing the rate of PQ reduction in light-acclimated tomato leaves through P_{700} oxidation lag. A,B. P_{700} oxidation by moderate FR light. P_{700} oxidation kinetics was assessed as described in Material and methods. Plants were light-acclimated for several hours, then subjected to a brief FR illumination to induce full PQH_2 oxidation, and thereafter left in the dark during a definite time, before probing the extent of P_{700} oxidation lag. The dash-dot line represents full reduction of the PQ pool, obtained 1 s after a saturating light pulse. C: Plot of the half times of P_{700} oxidation (relative to the fully-reduced PQ pool) as a function of the time left in the dark after initial PQH_2 oxidation. Lines are mono-exponential fits of the experimental data. Squares: S.M. leaves. Circles: *ghost* leaves. Half times were estimated for every P700 kinetics shown in panel A and B and the respective values were plotted as a function of the dark incubation time.

these light-acclimation conditions, the PQ pool progressively gets fully *reduced* after illumination (Fig. 4C) is in contrast with dark-acclimated plants (where the PQ pool gets progressively *oxidised*), and shows that the NDH activity outcompetes the oxidase(s) activity in light-acclimated plants. The reduction kinetics shown in Fig. 4C are compatible with our previous estimates for the NDH performances (~0.15 s^{-1}).

3.6. Possible influence of PTOX on linear (LEF) vs. cyclic (CEF) electron flow

Using the same spectroscopic approach, we tested another possible function of PTOX, *i.e.* the modulation of cyclic electron flow around PSI. A link between chlororespiration and cyclic electron transfer (CEF) has been proposed earlier (e.g. [19,35]). As indicated above, previous work has indicated that while photosynthesis mostly operates in the linear mode after a prolonged illumination, the efficiency of CEF progressively increases upon dark-adaptation of light exposed leaves [27,36]. In dark adapted leaves, the CEF efficiency is maximum [27,36]. During this dark period the PQ pool is under control of the activity of the chlororespiratory enzymes. Measurements of LEF and CEF efficiency were done by measuring the oxidation kinetics of P_{700} upon FR illumination. Indeed, oxidised PSI is re-reduced by electron transfer from PSII very slowly under FR illumination under linear flow conditions. Therefore, P_{700} is rapidly oxidised by FR. On the other hand, electron recycling around PSI by cyclic flow allows re-reduction of P_{700} under FR illumination, thereby slowing down its oxidation [27,36]. Leaves were collected from "dark", "light", "light-CO_2" and "high-light" plants and then exposed to 80 μmol photons $m^{-2}s^{-1}$ for 10 min. This treatment leads to an identical activation state of CO_2 assimilation in all samples, thereby ensuring a maximum LEF efficiency. Leaves were then incubated in the dark for different times before probing the rate of P_{700} oxidation. As shown in Fig. 5, in all the tested conditions (in both S.M. and *ghost* leaves) a fast kinetic and an almost complete oxidation of P_{700} was observed after 3 min of dark incubation. This indicates that at this stage the contribution of CEF was small and identical in all attested conditions. When the duration of the dark incubation was increased, the amount of P_{700} that could be oxidised by FR progressively decreased, suggesting increased electron recycling through CEF. Maximum cyclic flow activity was observed after around 25 min of dark incubation, in agreement with previous findings [36]. Overall, this trend was observed in all the conditions tested, for both S.M. and *ghost* plants. However, differences were observed depending on the previous history of the leaves (*i.e.* on the activity of PTOX, according to the analysis performed in Figs. 2 and 3). In "dark" plants, PTOX deficiency (*ghost*) significantly enhanced the time required to oxidise P_{700} during FR illumination, even after a long dark period (16 or 24 min). This suggests that CEF was recovering faster when PTOX activity is low. In agreement with this hypothesis, a faster recovery of CEF (*i.e.* an enhancement of the time required to oxidise P_{700} during FR exposure) was systematically seen in 'light" and "light-CO_2" leaves (both treatments decrease PTOX activity according to our analysis). Conversely, LEF was more stable in "high light" S.M. leaves (where PTOX activity is higher) as shown by the very fast rate of P_{700} oxidation measured even after a long dark adaptation.

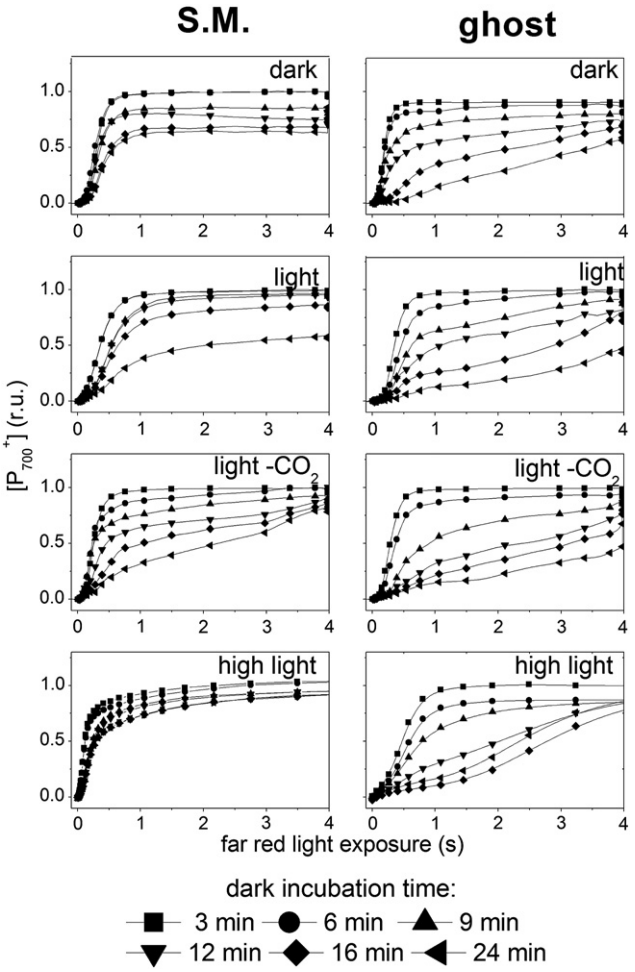

Fig. 5. Changes in the electron flow properties during dark incubation of S.M. and *ghost* leaves acclimated to different conditions. "Light", "Light-CO_2" and "Dark" leaves were obtained as explained in Material and methods, except that light-exposed plants were not dark incubated prior to measurements and all leaves were exposed to 80 μmol photons $m^{-2}s^{-1}$ for 10 min in the spectrophotometer (in order to activate carbon assimilation in the dark-acclimated samples). Light was then turned off for different durations (as indicated by the symbol captions) before exposure to a 4 s FR light pulse (720 nm, intensity 400 μmol $m^{-2}s^{-1}$) with concomitant recording of P_{700} oxidation kinetics as described in Material and methods.

4. Discussion

In this work we evaluated the performances of the chlororespiratory enzymes, using measurements of the area above the fluorescence curves to quantify changes in the redox state of PQ. At variance with the widely employed measurements of the fluorescence rise at the end of illumination, which only provides a qualitative estimate of the redox changes in the PQ pool, this method relies on an observable that is linearly related to the redox state of the plastoquinone pool and therefore allows quantifying the dynamic of the influx and outflux of electrons undergone by this pool (Figs. 2, 3). The validity of this approach is corroborated by testing the sensitivity of the area above the fluorescence curves to known inhibitors of PTOX and by comparing the wild type S.M. and *ghost* mutant (Fig. 2).

Using this approach, we estimated the maximum PTOX activity in tomato plants in different conditions ("dark", "light" "light-CO_2" "high-light" samples), showing that in all cases it is about two orders of magnitude lower than that of its competitor for the oxidation of PQH_2 in linear electron flow, the cytochrome b_6f complex, (the maximum rate of which is ~100 s^{-1} in leaves; [31]). Thus we conclude, in agreement with others [13,23–25,34] that the properties of this enzyme are clearly not compatible with the flux expected for an effective electron sink in all the conditions tested in this work.

Our work also shows the existence of two PTOX activities as revealed by the analysis of *ghost* plants (Figs. 2, 3). In this mutant, where PTOX cannot be detected biochemically (Fig. 3), a PQH_2 oxidation activity is seen, which is sensitive to pgal and ogal (Fig. 2), *i.e.* two typical PTOX inhibitors. This activity is rather slow and therefore cannot oxidise the entire PQ pool, owing to competition with the NDH. We propose that the PTOX-like activity observed in *ghost* plants reflects the presence of small amounts of the mitochondrial alternative oxidase (AOX) protein in the thylakoids, due to dual targeting

of this enzyme to the chloroplast. Recent results have shown that two mitochondrial alternative oxidases (AOX1a and AOX2) can functionally substitute for PTOX when overexpressed in the chloroplast in Arabidopsis chloroplasts [37]. Moreover, the AOX shares with PTOX the sensitivity to the inhibitors used in this study (propyl- and octyl-gallate). On the other hand, it is conceivable that the affinity of this enzyme for PQH_2 is lower than that of PTOX, because ubiquinone rather than plastoquinone is found in mitochondria. The lower abundance and the poor affinity of the AOX for PQ would explain the rather inefficient plastoquinol oxidising activity that is seen in *ghost* plants. This situation observed in tomato plants is somewhat reminiscent of the one observed in the chlorophyte *Chlamydomonas reinhardtii*, where two PTOX activities exist in the chloroplast (respectively called PTOX1 and PTOX2), again with different capacities to oxidise the PQ pool [38]. However, the tomato PTOX activity estimated here is far lower than that calculated in the case of the most active PTOX isoform (PTOX2) of this alga (k_{PTOX2}~4.6 s^{-1}; [38]), and is indeed similar to the one of the less active PTOX gene product, PTOX1 (k_{PTOX1}~0.4 s^{-1}). It is tempting to speculate that the presence of a second, more active, PTOX protein in Chlamydomonas would reflect the necessity to counterbalance non-photochemical reduction of the PQ pool of the monomeric type 2 NDH complex, *i.e.* the enzyme that replaces the multimeric NDH complex normally found in plants and which is far more active than the plant NDH (2 s^{-1} for type 2 NDH, [6]; 0.1 s^{-1} for the tomato NDH, this work). This likely reflects a genetic adaptation, possibly to guarantee a redox homeostasis of the PSII acceptors in the dark. Indeed, evidence in favor of a direct relationship between changes in the two chlororespiratory enzymes has been reported in plants in some extreme environmental conditions [17]. On the other hand, our data, which show independent changes in the relative performances of PTOX and NDH under different environmental conditions, indicate that this model is not always valid, and suggest that PTOX can be regulated independently of the NDH during some acclimation responses. In dark adapted tomato plants, PTOX is more active than NDH, allowing the full oxidation of the PQ pool in the dark, while light exposure - either under CO_2 replete or under CO_2 limited concentration results in a change in the relative performances of the NDH and PTOX, in favour of the former (Fig. 3). Although this observation could reflect an activation of the NDH, as previously proposed [39], our data point to a sustained decrease of the oxidation flux sustained by PTOX, the oxidation rate becoming similar to that of the residual PTOX-like activity observed in *ghost* plants, (where PTOX is absent) under the most extreme conditions. These results argue again against the notion that PTOX acts as a safety valve to counterbalance PQH_2 accumulation under stress conditions. The observed decrease of PTOX rate is not linearly related to changes in its relative abundance in the thylakoids (Fig. 3), suggesting that it is likely caused by the inhibition of its activity. Only in one case, i.e. upon leaf exposure to high light for several hours, we observed an enhanced protein accumulation in the thylakoids, which was paralleled by an increase in the overall plastoquinone oxidising capacity of the leaves *in vivo* (Fig. 3). However, the activity measured in these conditions is still not compatible with the flux expected for an effective electron sink (in agreement with [13,23–25,34]. Thus, the photoprotective role of PTOX (by competing with linear electron flow for electrons) is likely confined to some extremophile plants [17,40] and photosynthetic microorganisms [41–43], in which such a role has been clearly documents.

Overall, our results raise the question of the physiological role of PTOX and the rationale for the observed changes in PTOX rates in dark, in moderate light and under stress conditions (low CO_2, high light). In the dark, this process could serves the purpose of keeping the plastoquinone pool (at least partially) oxidised, to prevent the useless activation of adaptative changes that shape the function and composition of the photosynthetic apparatus in the light, in response to the over-reduction of the plastoquinone pool [44–46], as already proposed [25]. In the light, PTOX cannot actively modulate the redox state of the PQ pool, because its activity is not competing with PQ reduction and oxidation by the components of the linear electron flow. On the other hand, its activity slowly produces reactive oxygen species (ROS; [24]) by reducing molecular oxygen at the expenses of the reducing power stored in the plastoquinone pool. Therefore, diminishing its activity in moderate light could serve the purpose of avoiding the useless, and possibly deleterious, production of ROS. On the other hand, ROS production (in small amounts) could be an advantage in high light, because reactive oxygen species are required to develop the so called "integrated response to high light and pathogen stress" [47]. Enhancing PTOX activity in high light could serve the purpose of amplifying a ROS-triggered signal transduction cascade, the existence of which has been recently shown in algae [48]. Consistent with this hypothesis, studies of PTOX gene expression profile in Arabidopsis (https://genevestigator.com) reveal an accumulation of PTOX transcript upon pathogen exposure of plants, but no such effect upon growth in low CO_2 limited conditions. Unexpectedly, no increase is reported upon short exposure of Arabidopsis to high light. However, significant induction of the PTOX gene has been reported in a study on Arabidopsis plants subjected to high light for 5 h [49], while no significant up-or down-regulation of the PTOX gene has been reported in Arabidopsis plants subjected to other stresses [13], further supporting the notion of a specific PTOX role in plant acclimation to high light.

Finally, our data indicate that despite its overall limited electron flow capacity, PTOX plays a role in modulating the transition from linear to cyclic electron flow during a dark recovery from illumination (Fig. 5). This possible function of PTOX, which was proposed earlier (reviewed in [4]), clearly cannot reflect a direct involvement of this enzyme in electron flow for the kinetic reasons discussed above. Indeed, the PTOX activity in the light is negligible when compared to that of the cytochrome b_6f complex. However, the involvement of PTOX can be conceived considering the current model for the regulation of the relative efficiency of LEF and CEF in plants. In plants, LEF and CEF have been proposed to take place in a freely diffusing system and to be controlled by the redox balance between the PSI donors (including the PQ pool) and its acceptors (discussed in [27,50,51]). According to this model, CEF prevails in dark adapted leaves, where the Calvin cycle is a poor sink for photogenerated electrons, because the electrons transferred to the PSI acceptors cannot be injected into the carbon assimilatory pathway, and are therefore recycled several times around PSI [36,52]. In these conditions, CEF becomes the dominant path of electron flow in the chloroplast. Conversely, the progressive activation of the Calvin cycle, upon light exposure, allows LEF to over-compete CEF by providing an efficient electron sink to the reducing equivalents produced by PSII. In the frame of this hypothesis, the role of PTOX in modulating the relative efficiency of LEF and CEF during a light to dark transitions would reflect its capacity to modulate the redox state of the PQ pool by competing with the NDH. The results in Fig. 5, which show that in all the cases where the activity of PTOX is decreased, the transition from LEF to CEF is faster and more pronounced (and vice versa a very slow transition to CEF is seen when PTOX is enhanced), are in very good agreement with this hypothesis. By modulating CEF efficiency during a light to dark transition, PTOX may play a relevant physiological role, *i.e.* regulation of this electron flow pathway under conditions where this process becomes is predominant. In itself, the notion that the NDH-PTOX chain contributes to setting the stromal redox poise is not unexpected. What is unexpected, at least to us, is that this contribution is major since knocking it down results in a pronounced change in the dynamic of LEF to CEF transition in the dark. By doing so, the activity of the PTOX-NDH chain may prevent overreduction of the PSI electron acceptors, and therefore lower the risk of damage to this complex [53], a phenomenon that is triggered by overreduction of the PSI electron sinks. Consistent with this possibility, photoinhibition of PSI is reduced in Arabidopsis lines overexpressing PTOX [13].

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