Thioredoxin m4 Controls Photosynthetic Alternative Electron Pathways in Arabidopsis¹[C][W]

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In addition to the linear electron flow, a cyclic electron flow (CEF) around photosystem I occurs in chloroplasts. In CEF, electrons flow back from the donor site of photosystem I to the plastoquinone pool via two main routes: one that involves the Proton Gradient Regulation5 (PGR5)/PGRL1 complex (PGR) and one that is dependent of the NADH dehydrogenase-like complex. While the importance of CEF in photosynthesis and photoprotection has been clearly established, little is known about its regulation. We worked on the assumption of a redox regulation and surveyed the putative role of chloroplastic thioredoxins (TRX). Using Arabidopsis (Arabidopsis thaliana) mutants lacking different TRX isoforms, we demonstrated in vivo that TRXm4 specifically plays a role in the down-regulation of the NADH dehydrogenase-like complex-dependent plastoquinone reduction pathway. This result was confirmed in tobacco (Nicotiana tabacum) plants overexpressing the TRXm4 orthologous gene. In vitro assays performed with isolated chloroplasts and purified TRXm4 indicated that TRXm4 negatively controls the PGR pathway as well. The physiological significance of this regulation was investigated under steady-state photosynthesis and in the pgr5 mutant background. Lack of TRXm4 reversed the growth phenotype of the pgr5 mutant, but it did not compensate for the impaired photosynthesis and photoinhibition sensitivity. This suggests that the physiological role of TRXm4 occurs in vivo via a mechanism distinct from direct up-regulation of CEF.

In plant thylakoids, photosynthesis involves a linear electron flow (LEF) from water to NADP⁺ via PSII, cytochrome b₆/f, PSI, and soluble carriers. LEF produces NADPH and generates a transthylakoidal electrochemical proton gradient that drives the synthesis of ATP. Besides LEF, cyclic electron flow (CEF) can also occur, involving only PSI (for review, see Johnson, 2011; Kramer and Evans, 2011). These additional reactions include two main distinct pathways involving either the Proton Gradient Regulation5 (PGR5)/PGRL1 complex (Munekage et al., 2002; DalCorso et al., 2008) or the NADH dehydrogenase-like complex (NDH; for review, see Battchikova et al., 2011; Ifuku et al., 2011). The functioning of either CEF pathway, which generates a pH gradient ΔpH without any accumulation of NADPH, is thought to achieve the appropriate ATP/NADPH balance required for the biochemical needs of the plant, especially under certain environmental conditions such as low CO₂ (Golding and Johnson, 2003), heat (Clarke and Johnson, 2001), cold (Clarke and Johnson, 2001), drought (Golding and Johnson, 2003; Kohzuma et al., 2009), high light (Munekage et al., 2004), or dark-to-light transitions (Joliot and Joliot, 2005; Fan et al., 2007). CEF-generated ΔpH is also involved in photoprotection owing to the down-regulation of PSII via nonphotochemical quenching (Munekage et al., 2004; Takahashi et al., 2009). Very recently, the role of the PGR5 protein as a regulator of LEF has been established. It has proved to be essential in the protection of PSI from photodamage (Suorsa et al., 2012).

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The two cyclic pathways are redundant (Munekage et al., 2004), sharing ferredoxin (Fd) as a common stromal electron donor (Yamamoto et al., 2011) and electron carriers from plastoquinone (PQ) to PSI with LEF. Thus, LEF and either of the CEF pathways may be in competition. The molecular events that allow CEF to challenge LEF remain enigmatic, particularly when considering that the conditions that require CEF are also those under which LEF is in excess. Efforts to understand the appropriate functioning of CEF have led to the proposition of several models segregating cyclic and linear pathways at a structural level (for review, see Eberhard et al., 2008; Cardol et al., 2011; Johnson, 2011; Rochaix, 2011). According to the restricted diffusion model, founded on the uneven distribution of the photosynthetic protein complexes in the thylakoids, there is little competition between CEF and LEF, as CEF occurs in stroma lamellae where PSI is concentrated while LEF takes place in the grana stacks.

In line with the supercomplex model, whose relevance was demonstrated in the microalga Chlamydomonas reinhardtii, CEF happens within tightly bound supercomplexes containing PSI, with its own light-harvesting complex (LHCI), the PSII light-harvesting complex (LHCCI), cytochrome b_{6}/f, Fd, Fd NADP reductase (FN), and the integral membrane protein PGRL1 (Iwai et al., 2010). In higher plants, an association between NDH and PSI subunits suggests the formation of such supercomplexes (Peng et al., 2009). The availability of FN, found either free in the stroma or bound to the thylakoids (Zhang et al., 2001), has also been proposed to modulate partitioning between LEF and CEF (Joliot and Joliot, 2006; Joliot and Johnson, 2011). In addition, more dynamic models that illustrate competitive processes involved in the distribution of electrons between the cyclic and linear flows have been proposed. The competition between cytochrome b_{6}/f and FN for electrons from Fd could regulate the segregation between LEF and CEF (Breyton et al., 2006; Yamamoto et al., 2006; Hald et al., 2008). A few years ago, Joliot and Joliot (2006) suggested that the ATP/ADP ratio was one of the parameters that triggered on the transition between LEF and CEF. It was also established that the redox poise of chloroplast stroma contributed to the regulation of the photosynthetic pathway and played an important role in defining the extent of CEF. Breyton et al. (2006) scrutinized this redox regulation and established that the fraction of PSI complexes engaged in CEF could be modulated by changes in the stromal redox state. Overreduction of the NADPH pool involved in the repartition between LEF and CEF (Joliot and Joliot, 2006). The NADPH/NADP\textsuperscript{+} ratio was proposed as a regulator of PGR-dependent CEF in vivo (Okegawa et al., 2008).

All the published data supporting a role for the redox status in the regulation of CEF urged us to investigate a putative role of thioredoxins (TRX) in the regulation of CEF. TRX are ubiquitous disulfide reductases regulating the redox status of target proteins (for review, see Lemaire et al., 2007; Meyer et al., 2009).

In chloroplast, TRX mediate the light regulation of numerous enzymes, among which some belong to the Calvin cycle (for review, see Schürmann and Buchanan, 2008; Montrichard et al., 2009; Lindahl et al., 2011). Global proteomic approaches have revealed that well-known photosynthetic complex subunits may be partners of TRX, such as PsbO in PSI, plastocyanin, Rieske Fe-S protein in cytochrome b_{6}/f, and PsAK and PsAN in PSI (for review, see Montrichard et al., 2009; Lindahl et al., 2011). Furthermore, regarding the regulation of photosynthesis, TRX have also been involved in state transitions (Rintamäki et al., 2000; Buchanan and Balmer, 2005), and their participation in the control of the redox poise of the electron transport chain has also been suggested (Johnson, 2003).

In this work, we have investigated the possible role of TRX in the regulation of CEF. Using Arabidopsis (Arabidopsis thaliana) mutants with altered expression of genes encoding different plastid TRX, we have established in vivo the inhibitor activity of TRXm4 on the NDH-dependent pathway for plastoquinone reduction. This result was confirmed in transplastomic tobacco (Nicotiana tabacum) plants overexpressing the TRXm4 orthologous gene. Moreover, in vitro assays performed with isolated chloroplasts indicated that TRXm4 negatively controls the PGR-dependent electron flow as well.

RESULTS

NDH-Dependent Electron Flow Is Enhanced in the Presence of Thiol Reagents

As a preliminary approach to investigate the putative role of TRX in the regulation of CEF, we examined the effect of N-ethylmaleimide (NEM), a thiol-alkylating agent, on the NDH-dependent alternative pathway. Arabidopsis leaves were infiltrated with NEM, and the NDH-dependent electron flow was probed in vivo using chlorophyll fluorescence (Fig. 1A) and chlorophyll thermoluminescence (Fig. 1B). Control leaves infiltrated with water displayed the transient increase in the baseline level of chlorophyll fluorescence after a light-to-dark transition attributable to PQ and the primary electron-accepting plastoquinone of PSI reduction specifically mediated by the NDH complex (Shikanai, 2007). Accordingly, the postillumination fluorescence transient was not observed in the ndh-1 mutant lacking NDH (Fig. 1A). Infiltration of control leaves with NEM (0.25 or 0.5 mM; Fig. 1B) induced a strong and sustained increase in the chlorophyll fluorescence transient signal. Far-red (FR) light irradiation, which preferentially energizes PSI, rapidly quenched the fluorescence signal to the F0 level, and this effect was reversible, as fluorescence rapidly returned to the previous level when FR was switched off (Fig. 1A). This indicates that the NEM-induced increase in the fluorescence transient is due to a reversible reduction of the PSI acceptors. A similar increase in the postillumination chlorophyll fluorescence transient was obtained using leaves infiltrated with iodoacetamide,
another alkylation reagent (data not shown). In contrast, no transient signal could be detected in leaves of the ndho-1 and pgr5 mutants infiltrated with NEM (Supplemental Fig. S1).

In Arabidopsis, NDH-mediated electron transport in the dark can also be probed by measuring the afterglow (AG) luminescence signal (Havaux et al., 2005; Lintala et al., 2009; Peeva et al., 2012). Following FR illumination at 0°C, an AG thermoluminescence signal peaking at approximately 40°C was detected in control leaves in addition to the B band appearing at approximately 18°C to 20°C (Fig. 1B, WT). This signal reflects a heat-induced electron flow from the stroma to the PQ pool and the PSII centers (Miranda and Ducruet, 1995). In Arabidopsis, and in contrast to other plant species, the AG band is associated mainly with the NDH activity (Havaux et al., 2005; Lintala et al., 2009). This is confirmed by the strong reduction of the AG band in the ndho-1 mutant and its low sensitivity to antimycin A (AA), a specific inhibitor of the PGR-dependent pathway (Fig. 1B, bottom panel). In leaves infiltrated with NEM, the AG thermoluminescence band shifted to lower temperature (from approximately 40°C to approximately 27°C; Fig. 1B, top panel). The shifted AG emission was weakly inhibited in leaves treated with AA (Fig. 1B, top panel, WT+NEM+AA) and could not be detected in ndho-1 leaves, indicating that NDH mediates this phenomenon (Fig. 1B, bottom panel, ndho-1+NEM). This band shift indicates a stimulated electron transfer rate from stroma to the PQ pool: electrons are rapidly transferred to the secondary quinone acceptor at room temperature, leading to charge recombinations within PSII and to an AG thermoluminescence band merged with the B band (Ducruet et al., 2005; Apostol et al., 2006; Peeva et al., 2012). In previous works (Ducruet et al., 2005; Peeva et al., 2012), the downward shift of the peak temperature of the AG band was correlated with an acceleration of CEF, as measured by the rate of rereduction of the oxidized primary electron donor in PSI (P700$^+$) in the dark following a FR light illumination. Leaf absorbance measurements at 820 nm, which

Figure 1. In vivo detection of the NDH-dependent electron flow by chlorophyll fluorescence and chlorophyll thermoluminescence measurements in Arabidopsis leaves infiltrated with NEM. A, Chlorophyll fluorescence of Arabidopsis leaves either infiltrated with water or with NEM at 0.5 and 0.25 mM was monitored with a pulse amplitude-modulated fluorometer. After a 5-min illumination with white light (250 mmol photons m$^{-2}$ s$^{-1}$), light was switched off and the chlorophyll fluorescence transient increase was recorded under low nonactinic light. FR indicates the pulse of FR light (1 s). Measurements were performed 1 h following infiltration. ndho-1, Arabidopsis mutant lacking the NDH complex. B, Chlorophyll thermoluminescence was measured in Arabidopsis leaf discs by increasing leaf temperature from 0°C to 60°C after a 20-s illumination with FR light at 0°C. Heat-induced electron flux from the stroma to PSII acceptors appears as a band (AG) peaking at approximately 40°C. The shoulder appearing at approximately 18°C to 20°C is the B band corresponding to charge recombinations between pre-existing plastoquinone and S2/S3 states. AA indicates 5 mM AA. A.U., Absorbance units; T, temperature; WT, wild type.

Figure 2. Half-times ($t_{1/2}$) of dark rereduction of P700$^+$ in leaves of Arabidopsis plants after a FR light period. WT+H2O or WT+NEM, Leaves of wild-type plants were infiltrated with water or NEM (0.25 mM), respectively. Leaves were irradiated with FR light (more than 715 nm, 120 W m$^{-2}$) and half-time was determined subsequently, after turning off the FR light. Data expressing half-times are means ± SD of four experiments.
reflects changes in the redox state of P$_{700}^+$ were performed on Arabidopsis leaves (Fig. 2). Leaves were first illuminated with FR light, leading to the oxidation of P$_{700}^+$, then rereduction by stromal reductants was recorded in the dark after switching off the FR light. The half-time of the dark rereduction measured in leaves of control (wild type) and NDH mutant (ndho-1) plants was not significantly different (Fig. 2). In contrast, following NEM (0.25 mM) infiltration, the half-time decreased substantially as compared with leaves infiltrated with water, indicating an acceleration of the electron donation from the stroma to PSI via PQ.

Taken together, the chlorophyll fluorescence, P$_{700}^+$ reduction kinetics, and AG data suggested that NDH-mediated electron flow is enhanced in the presence of thiol-alkylating reagents.

NDH Activity Is Increased in an Arabidopsis Mutant Lacking TRXm4

Because TRX are key actors in thiol-mediated redox regulation of chloroplastic enzymes, we examined NDH activity in Arabidopsis mutants lacking chloroplastic TRX. When available, candidate T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Center. Genomic PCR and reverse transcription (RT) analyses were performed to select the knockout mutants (Laugier et al., 2012; Supplemental Figs. S2 and S3). trxnl, trxnm3, trxfl, trxx, and trxy2 mutant lines displayed the typical NDH-dependent transient increase in chlorophyll fluorescence reported in wild-type plants (Fig. 3A). Interestingly, the two trxm4 lines (trxm4-1 and trxm4-2) exhibited a highly stimulated transient (Fig. 3B). PSI excitation using a FR flash rapidly switched off the signal in a reversible way. The stimulated fluorescence transients in trxm4-1 and trxm4-2, which appeared similar to the signal recorded following NEM infiltration (Fig. 1A), indicated that the NDH-dependent electron pathway was up-regulated in plants specifically lacking TRXm4. AG luminescence analysis confirmed the chlorophyll fluorescence transient measurements (Fig. 3C). The downshifted band peaking at approximately 27°C was detected in trxm4-1 (Fig. 3C) and trxm4-2 (data not shown), as observed previously in leaves infiltrated with NEM. Neither the postillumination chlorophyll fluorescence transient nor the shifted AG band was detected in the double mutant ndho-1×trxm4-1 (Fig. 3, B and C).

The NDH-dependent increase in PQ reduction recorded in the trxm4 mutants could be interpreted as the result of an inhibition of the PQ pool reoxidation by the plastid terminal oxidase (PTOX) rather than a stimulation of the NDH activity. However, infiltration of wild-type leaves with n-propyl gallate, a powerful inhibitor of PTOX (Josse et al., 2003), does not induce any change in the chlorophyll fluorescence transient or in the AG signal (data not shown) ascribed to NDH-mediated PQ reduction, suggesting that an inhibitory effect of TRXm4 on the PTOX-dependent oxidation pathway is very unlikely. Thus, we concluded that TRXm4 negatively regulates NDH activity.

Figure 3. Characterization of the NDH-dependent electron flow in Arabidopsis mutants lacking TRXm1, TRXm3, TRXm4, TRXf1, TRXy2, and TRXx. A, Transient chlorophyll fluorescence rise measured under nonactinic light following a 5-min illumination with white light (250 mmol photons m$^{-2}$ s$^{-1}$) in the wild type (WT), trxm1, trxm3, trxf1, trxx, and trxy2. B, Transient chlorophyll fluorescence rise measured as above in the wild type, trxm4-1, trxm4-2, and the double mutant ndho-1×trxm4-1. FR indicates the pulse of FR light (1 s). C, AG chlorophyll luminescence measurements from leaf discs of wild-type, trxm4-1, and ndho-1×trxm4-1 plants after a 20-s FR excitation. A.U., absorbance units; T, temperature. [See online article for color version of this figure.]

The NDH-Dependent Electron Pathway Is Inhibited in Tobacco Plants Overexpressing the TRXm4 Orthologous Gene

The NDH pathway was also measured in transplastomic tobacco plants overexpressing endogenous TRXf and TRXm genes (NiTRXf and NiTRXm, respectively; Sanz-Barrío et al., 2012) from the plastid genome under the control of the strong psbA promoter.
Interestingly, alignment of the NtTRXm complementary DNA sequence with the four Arabidopsis TRXm gene sequences indicated that NtTRXm displayed the highest level of similarity to TRXm4 (data not shown), suggesting that NtTRXm and Arabidopsis TRXm4 are orthologs. While in the tobacco plants overexpressing NtTRXf (o/exTRXf) the postillumination chlorophyll fluorescence increase indicative of NDH activity was similar to the wild type, in the tobacco plants overexpressing NtTRXm (o/exTRXm) it was not detected, indicating that the NDH pathway was inhibited in these plants (Fig. 4). Following infiltration with NEM (0.25 mM; Fig. 4), control tobacco plants (wild type) displayed a highly stimulated chlorophyll fluorescence rise as observed in Arabidopsis (Fig. 1A). In o/exTRXm plants, NEM treatment contributed to the recovery of the NDH-specific chlorophyll fluorescence signal. These results were in agreement with the phenotype of the Arabidopsis mutant trxm4-1, reinforcing our conclusion that, in vivo, the NDH-dependent electron flow is specifically regulated by TRXm4.

**Electron Transport within PSI Is Increased in the Arabidopsis Mutant Lacking TRXm4**

The flow of electron donation from stromal reductants to PSI in the absence of PSII activity was studied in leaves of trxm4 mutants using the kinetics of P700 oxidation in FR light and P700+ rereduction in the dark. In trxm4-1, the rate of P700+ rereduction after FR light was noticeably accelerated compared with that of wild-type and ndho-1 plants (Fig. 5A). The kinetics of P700 photooxidation induced by FR illumination was also monitored in dark-adapted leaves of the wild type and mutants lacking NDH activity or TRXm4 (Fig. 5B, top and bottom panels, respectively). In wild-type leaves, FR light induced a rapid oxidation of P700, reaching the maximum level after about 2 s. There was almost no difference between the kinetic curves of the wild type and ndho-1, as observed previously by Shikanai et al. (1998) and Nandha et al. (2007). Conversely, in trxm4-1, P700 oxidation was much slower, indicating a more important flow of electrons coming from the stroma to the intersystem chain able to maintain PSI in a reduced state. To characterize further the effect of TRXm4 on electron transport, we examined the P700+ level during steady-state photosynthesis in white light (Fig. 6). No difference in the P700 oxidation ratio could be recorded between the wild type, ndho-1, and trxm4 during steady-state photosynthesis at two photon flux densities, 100 and 1,000 μmol photons m−2 s−1.

Taken together, the P700 redox state measurements suggest that TRXm4 inhibits electron transport when PSI is probed with FR light, but this effect appears to have no significant repercussion on PSI photochemistry during steady-state photosynthesis in white light.

**TRXm4 Exerts a Negative Control on Both the PGR- and NDH-Dependent Electron Flows**

Nonphotochemical PQ reduction can be determined in vitro using lysed chloroplasts (Munekage et al., 2002, 2004). PQ reduction activity is monitored as an increase in chlorophyll fluorescence under a weak measuring light (1.0 μmol photons m−2 s−1) upon Fd and NADPH addition (Fig. 7A). In this assay, reduced Fd, the mediator of CEF, is generated by NADPH via the reverse activity of FNR.

In the presence of Arabidopsis TRXm4, which was produced in *Escherichia coli* and purified (Supplemental Fig. S4), PQ reduction activity was partly impaired (Fig. 7B). The inhibition was specific of TRXm4, since the addition of a commercial preparation of reduced TRX from *E. coli* to wild-type chloroplasts induced no change in the PQ reduction activity (Fig. 7B; WT+TRX *E. coli*).
When extensively oxidized by dithiothreitol (DTT) treatment (see “Materials and Methods”), TRXm4 was no longer an inhibitor of PQ reduction (Fig. 7B, ox TRXm4). In this assay, the contributions of the PGR- and NDH-dependent pathways to the total PQ reduction activity can be discriminated using chloroplasts from mutants deficient in either pathway or in the presence of AA, a specific inhibitor of the PGR-dependent pathway. Thus, the effect of TRXm4 on either pathway was investigated. As described previously by Munekage et al. (2002, 2004), in chloroplasts isolated from Arabidopsis mutants lacking either the NDH complex (ndho-1) or PGR5 (pgr5), the PQ reduction activity was decreased, demonstrating the participation of both complexes in CEF around PSI (Fig. 7B). AA, which is a specific inhibitor of the PGR-dependent pathway, mimicked the phenotype of pgr5 (Fig. 7B, WT+AA). When added to an extract of chloroplasts from ndho-1, AA almost fully inhibited PQ reduction (Fig. 7B, ndho-1+AA), indicating that, under our experimental conditions, the full activity recorded in the wild type is the sum of the NDH and PGR activities. PQ reduction activity was measured in isolated chloroplasts following the addition of reduced TRXm4. PQ reduction activity was impaired when TRXm4 was added to chloroplasts of ndho-1 plants lacking the NDH-dependent pathway. Conversely, the addition of TRXm4 had no effect on the reaction with ruptured chloroplasts from the pgr5 mutant. Similarly, incubation of TRXm4 with chloroplasts from wild-type plants premixed with AA did not change the PQ reduction activity. These results show that, contrary to its effect in vivo, TRXm4 lost its capacity to modulate the NDH pathway in vitro, suggesting that the target of TRXm4 may be either lost during the preparation of ruptured chloroplasts or not accessible in the assay.

Interestingly, the in vitro experiments revealed the inhibitory effect of TRXm4 on the PGR-dependent pathway. Whether this effect also occurred in vivo was investigated in tobacco plants overexpressing TRXm4 using the AG thermoluminescence technique (Fig. 8, o/exTRXm). Contrary to Arabidopsis, the AG luminescence signal in tobacco leaves has been shown to rely mainly on the AA-sensitive pathway (Havaux et al., 2005). Figure 8 showed that the amplitude of the thermoluminescence band was drastically reduced in o/exTRXm, indicating that the PGR-dependent pathway was inhibited in these plants. Fd-dependent PQ reduction activity was also probed in vitro using ruptured tobacco chloroplasts (Fig. 9). As reported
previously (Endo et al., 1998; Munekage et al., 2004) and supporting thermoluminescence measurements, we confirmed that the chlorophyll fluorescence increase upon the addition of Fd and NADPH to tobacco chloroplasts was mostly due to PGR activity and, accordingly, was fully impaired by AA. This activity was also fully inhibited in o/exTRXm4 and partly inhibited in the wild type following the addition of TRXm4. Taken together, the experiments performed on Arabidopsis and tobacco chloroplasts show that the NDH activity and also the PGR-dependent electron flow are regulated by TRXm4.

Enhancement of NDH Activity in Plants Lacking the PGR-Dependent Pathway Compensates the Light-Dependent Growth Phenotype But Not Photosynthesis Inhibition or Photoinhibition

NDH appeared essential only in the pgr5 mutant background (Munekage et al., 2004). While single mutants lacking either PGR5 or NDH did not display visible phenotypes when grown under limited light conditions (50 μmol photons m⁻² s⁻¹), the double mutant exhibited severe growth inhibition. Correlated with this phenotype, a drastic inhibition of photosynthesis was recorded in double mutant plants lacking both pathways at low light, which also occurred in the pgr5 single mutant under normal- or high-light conditions. As an attempt to reveal TRXm4 function, trxm4-2 and pgr5 mutants were crossed. Double mutant trxm4-2 × pgr5 plants were grown under normal-light conditions (200 μmol photons m⁻² s⁻¹). Under these conditions, pgr5 plants displayed delayed growth as compared with the wild type (Fig. 10A) but trxm4-2 × pgr5 exhibited an improved growth as compared with pgr5 plants, similar to that of wild-type plants (Fig. 10A). The photosynthetic activity and thermal dissipation, both impaired in pgr5 (Munekage et al., 2004; Figure 10, C and D), appeared similarly impaired in the double mutant trxm4-2 × pgr5 (Fig. 10, B and C), demonstrating that although TRXm4-mediated NDH enhancement (Fig. 10B) did improve the growth of plants lacking the main CEF pathway, the effect was not mediated by improving photosynthesis and/or thermal energy dissipation.

DISCUSSION

TRXm4 Controls the NDH- and PGR-Dependent PQ Reduction Pathways

Supposedly essential for balancing the ATP/NADPH production ratio (Allen, 2003) and photoprotection previously (Endo et al., 1998; Munekage et al., 2004) and supporting thermoluminescence measurements, we confirmed that the chlorophyll fluorescence increase upon the addition of Fd and NADPH to tobacco chloroplasts was mostly due to PGR activity and, accordingly, was fully impaired by AA. This activity was also fully inhibited in o/exTRXm4 and partly inhibited in the wild type following the addition of TRXm4. Taken together, the experiments performed on Arabidopsis and tobacco chloroplasts show that the NDH activity and also the PGR-dependent electron flow are regulated by TRXm4.

Figure 8. AG chlorophyll thermoluminescence measurements in tobacco leaves overexpressing the gene encoding TRXm4. Chlorophyll thermoluminescence was measured in tobacco leaf discs by increasing leaf temperature from 0°C to 60°C after a 20-s illumination with FR light at 0°C. Heat-induced electron flux from the stroma to PSI acceptors appears as an AG band peaking at approximately 40°C. A.U., Absorbance units; WT, wild type.
The role of TRXm4 on CEF required recording P700 oxidation-electron pathway in vivo. Further information on the NDH- and PGR-dependent pathways must be accurately regulated (Kramer et al., 2005; Baker et al., 2007). In this work, using a combination of in vivo and in vitro assays, we explored the role of plastid TRX in the redox control of the photosynthetic alternative electron pathways and established the inhibitory role of TRXm4 on the NDH- and PGR-dependent electron flows.

The regulatory action of TRXm4 on the NDH-dependent pathway was first detected using chlorophyll fluorescence and AG measurements, since these methods enable specific investigation of NDH activity in vivo in Arabidopsis leaves (Havaux et al., 2005; Shikanai, 2007). These experimental approaches revealed that NDH activity was increased when TRXm4 was lacking in Arabidopsis mutants and fully extinguished in tobacco plants overexpressing the gene encoding TRXm4. Chloroplast extracts were incubated with AA (5 mM) or TRXm4 (200 mM). [See online article for color version of this figure.]

Thus, TRXm4 negatively regulates the NDH-mediated electron pathway in vivo. Further information on the role of TRXm4 on CEF required recording P700 oxidation under FR illumination and subsequent P700+ dark reduction kinetics. Indeed, these parameters have been widely used to gain information on the activation of cyclic pathways (Joët et al., 2002; Johnson, 2005; Nandha et al., 2007). In trxm4 leaves, P700 oxidation and reduction kinetics indicated that TRXm4 mutants were able to perform CEF at a higher rate than the wild type (Fig. 5), which could indicate a highly reduced pool of plastocyanin stroma donors in trxm4.

All the above-mentioned methods enable detecting a dark electron flow from the stroma and do not allow exploring the contribution of TRXm4 to CEF regulation in vivo under steady-state photosynthesis. Because it has been established that the operation of CEF under limiting light conditions is unlikely (Heber et al., 1978; Cornic et al., 2000) and PGR-dependent PSI CEF requires adequate light to be fully activated (Shikanai, 2007), a pertinent assessment of the regulating role of TRXm4 needed to be performed under light conditions. Thus, the P700+ level indicative of the electron acceptance capacity from PSI was monitored during steady-state photosynthesis. No difference between the wild type, ndho-1, and trxm4 was recorded under these conditions, suggesting that NDH-dependent flow up-regulation has no effect on photosynthesis. These results are in agreement with the features of trxm4 plants, which displayed no visible phenotype when grown under standard conditions (Supplemental Fig. S3) and no alteration in the light intensity dependence of electron transfer reactions and nonphotochemical quenching (NPQ; Supplemental Fig. S5, A and B). No difference in the NPQ transiently generated in dark-adapted leaves from the wild type, ndho-1, and trxm4-1 exposed to low light could be recorded (Supplemental Fig. SSC), indicating that transient generation of the transthylakoidal pH gradient (Kalituhou et al., 2007) was similar in wild-type and mutant plants. Knocking out genes encoding NDH subunits either in tobacco (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) or in Arabidopsis (Rumeau et al., 2005; Shikanai, 2007) did not affect overall photosynthetic electron transport, and plants did not display any visible phenotype. Thus, it appears logical that up-modulation of NDH activity did not impact noticeably on photosynthetic activity. Only a subtle NPQ change was recorded in trxm4, in agreement with the discrete NPQ increase detected in NDH mutants (Hashimoto et al., 2003; Kamruzzaman Munshi et al., 2005; Rumeau et al., 2005). This is in agreement with previous results that demonstrated that the NDH contribution to photosynthetic electron transport is minor in Arabidopsis, at least when the plants are grown under standard conditions (Shikanai, 2007).

Considering the inhibitory effect of TRXm4 on the PGR-dependent pathway monitored using lysed chloroplasts, it appeared limited, since 75% of chlorophyll fluorescence was recorded when TRXm4 was added (Fig. 7). We ruled out a nonspecific effect of TRXm4, since the inhibition was not detected when E. coli TRX was added to the ruptured chloroplast preparations. However, recorded under very low light (i.e. when the PGR pathway is not fully activated), its physiological significance must be questioned. In planta, the TRXm4-mediated partial inhibition of the PGR-dependent flow probably plays a minor role, since mutants lacking PGR display significant changes in photosynthesis and the role of NDH appeared essential for photosynthesis only (Munekage et al., 2004). Indeed, mutants lacking both the NDH- and PGR-dependent pathways exhibited severe growth phenotypes, and photosynthesis was impaired more severely than in the single mutant pgr5. Therefore, it appeared important to investigate the effect of enhancing NDH activity in the
Double mutants lacking PGR5 and TRXm4 were created. Under standard growth conditions, pgr5×trxm4-2 plants display a favorable phenotype similar to the wild type (WT), indicating that NDH activity enhancement represents an advantage for plant development when the main alternative electron pathway is inhibited. However, the positive effect was not mediated via an improvement of photosynthetic electron flow (Fig. 10B) or NPQ (Fig. 10C), which remained similarly impaired in pgr5×trxm4-2 and pgr5 (Munekage et al., 2004). Thus, although TRXm4 plays an important role in regulating nonphotochemical FQ reduction in chloroplasts, the physiological significance of this regulation still remains to be determined. Possibly, redox regulation of alternative electron flows could be important for some metabolic reactions, as shown previously for carotenoid biosynthesis and the alternative electron transport mediated by the PTOX (Kuntz, 2004).

A Specific Function for TRXm4

Chloroplast TRX are currently subdivided into four types, TRXf, TRXm, TRXy, and TRXx, which are encoded in Arabidopsis by two, four, two, and one
genes, respectively. The biochemical characterization of the four TRXm family members has been extensively performed in vitro (Duek and Wolosiuk, 2001; Issakidis-Bourguet et al., 2001); however, the roles of the different isoforms have not been clearly established yet. Here, we report, to our knowledge for the first time, a specific function for the TRXm4 isoform in vivo. In the past, the characterization of Arabidopsis plants lacking the different TRXm isoforms was attempted. No visible phenotype could be attributed to the TRXm1-, TRXm2-, and TRXm4-deficient plants, at least under standard growth conditions. Only the mutant lacking TRXm3 (named gat1) displayed growth impairment and turned out to be affected in symptlastic trafficking (Benitez-Alfonso et al., 2009). It was suggested that the lack of phenotype of the trxm1, trxm2, and trxm4 mutants could reflect compensation by other TRXm family members and/or functional redundancy. Our results, on the contrary, suggest that TRXm4 could perform a nonredundant and specific function (Fig. 3).

An intriguing question concerns the target(s) of TRXm4 by which NDH- and PGR-mediated electron pathway regulation occurs. In vitro experiments using isolated and ruptured chloroplasts suggest that there are two different targets at least, distinctly mediating NDH and PGR regulation, respectively (Fig. 7). Many studies have been performed in order to identify TRX targets. Most of them were performed in vitro, leading to a list of about 500 candidates in oxygenic photosynthetic organisms, including soluble and membrane proteins (for review, see Montrichard et al., 2009). It must be highlighted that among the numerous targets, neither proteins belonging to the NDH complex nor PGR5 or PGR1 have been found. Specifically, considering TRXm, it was demonstrated that NADP malate dehydrogenase (Collin et al., 2003), Glc-6-P dehydrogenase (Wenderoth et al., 1997), and HCF164 (Motohashi and Hisabori, 2006), which is a TRX-like protein located in the thylakoid membrane, is negatively regulated by PedR. HCF164 is one of the genes encoding the NDHD subunit of the NDH complex in cyanobacteria, is negatively regulated by PedR. In Arabidopsis trxm4 mutants, we could not detect any change in the NDH amount (data not shown), excluding an effect of TRXm4 at the gene expression level. Among the above-proposed targets for TRXm, none appeared as an obvious mediator for NDH or PGR complex regulation.

Recently, a few mutant or transgenic plants exhibiting a stimulated CEF have been described. All of them lack Calvin cycle enzyme activity. Thus, in the Arabidopsis mutant lacking Fru-1,6-bisP aldolase, it was suggested that CEF shifted from the NDH-dependent pathway to the PGR-dependent pathway in response to sink limitation of LEF (Gototh et al., 2010). Jin et al. (2008) obtained an antisense rice (Oryza sativa) line with reduced Rubisco activase activity. In this line, the enhancement of CEF combined with LEF depression was involved in energy dissipation when CO2 availability became restricted. However, a mutant named hcef1 affected in the Fru-1,6-bisphosphatase-encoding gene (Livingston et al., 2010a) and a plant lacking glyceraldehyde-3-phosphate dehydrogenase (Livingston et al., 2010b) were demonstrated to display an enhanced NDH-dependent CEF. Both types of mutants showed substantial increases in CEF linked to an increase in steady-state transthylakoid proton motive force and subsequent activation of the energy-dependent quenching of excess absorbed light energy response. In hcef1, it was proposed that this activation aimed at compensating a need for extra ATP that cannot be fulfilled by alternative mechanisms. Interestingly, it has been demonstrated that the four above-mentioned enzymes of the Calvin cycle are regulated by TRX (Montrichard et al., 2009). Thus, we could suggest that TRXm4 specifically redox regulated any of them. If so, however, an intriguing point remains concerning the lack of a visible phenotype of trxm4. Indeed, plants lacking the Calvin cycle enzyme activities displayed striking phenotypes with inhibited growth (Livingston et al., 2010a). It must be highlighted, however, that in trxm4 leaves, Calvin enzymes are still present and still functioning, and only their redox status may be affected. Consequently, in order to clarify the mechanism underlying the redox regulation of the non-photochemical PQ reduction pathways, the protein target(s) of TRXm4 will have to be identified using, for instance, strategies based on the overexpression of a TRX active-site mutant (Rey et al., 2005).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) plants were grown in soil in a phytotron with an 8-h photoperiod and a photon flux density of 150 μmol m⁻² s⁻¹. The temperature regime was 22°C/16°C (day/night), and the relative humidity was 55%. Arabidopsis T-DNA insertion mutant lines of plastidic TRX were provided by the Salk Institute Genomic Analysis Laboratory (insertion lines trxm3, N526367; trxm4-1, N532538; and trxm4-2, N523810; trxm1 (N653340), trxf (N563799), trxx (N628906), and trxpy (N528065) have been described elsewhere (Laugier et al., 2012). Tobacco (Nicotiana tabacum) plants were grown in soil under controlled conditions in a growth chamber (14-h photoperiod at 25°C followed by a 10-h night at 20°C) and watered with a complete nutrient solution as described previously by Rumeau et al. (2005). Tobacco TRX6 and TRXm coding sequences (GenBank accession nos. HQ338526 and HQ338525, respectively) were amplified by PCR (Sanz-Barrio et al., 2011) and cloned into the pL3 chloroplast transformation vector under the plastid psbA gene promoter (Farran et al., 2008). The resulting expression vectors were bombarded into in vitro-grown tobacco (cv Petit Havana) leaves as described previously (Daniell, 1997). Homoplasmic T1 plants from the o/exTRXf and o/exTRXm transplastomic lines were used for further analysis.

Characterization of the Insertion Mutant Lines

Homozygous T-DNA insertion lines were verified by PCR analysis with the Phire Plant Direct PCR Kit (Finnzymes) using gene-specific primers: for N526367, O5 (5'-ATGCCGTCCGATCATGAC-3') and O6 (5'-TTGGTGAATCATCAATGGT-3'); for N532538 and N523810, O13 (5'-
ACAAAAATCTGCTGTACTCACAGA-3′ and O9 (5′-TACTCGACCAAGAATCCTTTCT-3′). ACTN2 was used as a positive control for each RT-PCR with the same primers as above.

In Vivo Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence was measured at room temperature on detached leaves using a PAM-2000 modulated fluorometer (Walz) as described previously (Rumeau et al., 2005). Briefly, postillumination chlorophyll fluorescence rises were recorded under low nonactinic light following a 5-min actinic illumination (250 µmol photons m⁻² s⁻¹). Chlorophyll fluorescence parameters were measured using a PAM-2000 modulated fluorometer (Walz). A saturating pulse of white light was applied to the leaf, and measurements were recorded during an actinic light illumination (25–2,500 µmol photons m⁻² s⁻¹). The F₀/Fm ratio, which is the ratio of the steady-state chlorophyll fluorescence level and Fm, is the maximal level. NPQ reflects the dissipation of absorbed light energy from PSII as heat. NPQ was calculated as (Fm/Fm') - 1, where Fm' is the maximal fluorescence level in the dark.

Thermoluminescence Measurements

The luminescence emitted by leaf discs (diameter of 12 mm) was measured with a custom-made apparatus as described previously (Ducruet, 2003). The sample was maintained at 0°C for 30 s in the dark, then it was illuminated for 20 s with FR light (more than 715 nm) or after switching off the FR light, respectively. The P700 oxidation ratio was measured as described by Okegawa et al. (2000). The reduction of the primary electron-accepting plastoquinone was measured using the same gene-specific primers as described above for NtS26367, which used O9 (5′-CAATACACTCCTCTCAATGGCGG-3′) and O5 (5′-GTAACCCTATGGTACTCCTG-3′). ACTN2 was used as a positive control for each RT-PCR with the same primers as above.

Redox State of P₄₅₀

Changes in the redox state of the reaction center P₄₅₀ of PSI were monitored via leaf absorbance changes at around 820 nm. A Walz PAM-101 system was coupled to a dual-wavelength P700 unit (ED-P700DW; Walz) was used in the chloroplast preparations. Elution buffer contained 50 mM HEPES (NaOH), pH 7.6, and 20% glycerol.

In Vitro Assay of PQ Reduction by the Cyclic Electron Pathways

PQ reduction activity was measured in ruptured chloroplasts prepared as described by Munekage et al. (2002, 2004). Briefly, leaves of Arabidopsis and tobacco were homogenized in a medium containing 0.4 M sorbitol, 20 mM Tricine/KOH (pH 8.4), 10 mM EDTA, 10 mM NaHCO₃, and 0.15% (w/v) bovine serum albumin. After centrifugation for 90 s at 1,500g, the pellet was gently resuspended in 0.4 M sorbitol, 20 mM Tricine/KOH (pH 7.6), 2.5 mM EDTA (pH 8), 5 mM MgCl₂, 10 mM NaHCO₃, and 0.15% (w/v) bovine serum albumin. Intact chloroplasts were purified by centrifugation on a percoll gradient (mix of 100% Percoll and 2× the suspension medium) above centrifuged at 38,700g (for 55 min). After centrifugation for 10 min at 13,300g, chloroplasts were osmotically lysed in 50 mM HEPES/NaOH (pH 8.0), 7 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 30 mM KCl, and 0.25 mM KH₂PO₄, according to Munekage et al. (2002). The isolation of the primary electron-accepting plastoquinone of PSI and PQ was measured with ruptured chloroplasts diluted in the lysis medium (pH 8.0) at 1.5 mg chlorophyll mL⁻¹ under weak illumination (1.0 µmol photons m⁻² s⁻¹) with electron donors 5 µM ferredoxin and 250 µM NADP⁺. Chlorophyll fluorescence was measured with a PAM-2000 fluorometer (Walz). AA (2 µM), recombinant TRXm4 (200 µM), and commercial TRX E. coli (200 µM Sigma) were added before the measurement.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. In vivo detection of the NDH-dependent electron flow by chlorophyll fluorescence measurements in Arabidopsis leaves infiltrated with NEM.

Supplemental Figure S2. Molecular characterization of the Arabidopsis trxm3 mutant.

Supplemental Figure S3. Molecular characterization of Arabidopsis TRXm4-1 and TRXm4-2.

Supplemental Figure S4. Purification and characterization of recombinant TRXm4.

Supplemental Figure S5. Characterization of the photosynthetic activity of trxm3 mutants.

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Redox Regulation of the NDH and PGR Pathways

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