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1 **A complex and dynamic redox network regulating oxygen reduction at photosystem I**

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15 Running head: Regulation of oxygen reduction at photosystem I

16 **Abstract**

17 Thiol-dependent redox regulations of enzyme activities play a central role in regulating
18 photosynthesis. Beside the regulation of metabolic pathways, alternative electron transport has been
19 shown to be subjected to thiol-dependent regulation. We investigated the regulation of O₂ reduction
20 at photosystem I. The level of O₂ reduction in leaves and isolated thylakoid membranes depends on
21 the photoperiod in which plants are grown. We used a set of Arabidopsis mutant plants affected in
22 the stromal, membrane and lumenal thiol network to study the redox protein partners involved in
23 regulating O₂ reduction. Light-dependent O₂ reduction was determined in leaves and in thylakoids of
24 plants grown in short day and long day conditions using a spin-trapping EPR assay. In wild type
25 samples from short day, ROS generation was twice the amount of that in samples from long day,
26 while this difference was abolished in several redoxin mutants. An in vitro reconstitution assays
27 showed that thioredoxin m, NADPH-dependent reductase C (NTRC) and NADPH are required for high
28 O₂ reduction levels in long day thylakoids. Using isolated photosystem I, we also show that reduction
29 of a PSI protein is responsible for the increase in O₂ reduction. Furthermore, differences in the
30 membrane localization of thioredoxins m and 2-Cys peroxiredoxin were demonstrated between
31 thylakoids of short day and long day plants. Finally, we propose a model of redox regulation of O₂
32 reduction according to the reduction power of the stroma and the capabilities of the different thiol-
33 containing proteins to form a network of redox interactions.

34

35

36 Introduction

37 Thiol-dependent redox regulations of enzyme activities play a central role in regulating
38 photosynthesis. It has been well established that key enzymes of the Calvin-Benson-Basham cycle are
39 redox-regulated via the thioredoxin (Trx) system (Buchanan, 2016). In the chloroplast Trxs are
40 reduced in the light by ferredoxin in a reaction catalyzed by the Ferredoxin Thioredoxin Reductase
41 FTR. NTRC, a protein with both a NADPH-thioredoxin reductase and a Trx domain, has also been
42 found in chloroplasts (Serrato et al., 2004). NTRC has been shown to be involved in controlling the
43 level of H₂O₂ via its interaction with 2-cys peroxiredoxin (2-Cys-PRX), it participates in redox
44 regulation of a key enzyme in starch biosynthesis (Michalska et al., 2009; Lepistö et al., 2013), of
45 enzymes of the chlorophyll biosynthesis pathway (Richter et al., 2013; Perez-Ruiz et al., 2014) and in
46 regulating the activity of the chloroplast ATP-synthase (Naranjo et al., 2016; Carrillo et al., 2016;
47 Nikkanen et al., 2016; for a recent review see Cejudo et al., 2019). A severe growth inhibition
48 phenotype has been reported for a mutant lacking NTRC and Trx f1 (Thormählen et al., 2015) and in
49 the triple mutant *ntrc-trxf1f2* and the double mutant *ntrc-trxx* (Ojeda et al., 2017a). Both, *ntrc-*
50 *trxf1f2* and *ntrc-trxx* mutants showed a high mortality at the seedling stage (Ojeda et al., 2017b)
51 indicating that NTRC is important for chloroplast redox regulation, for controlling the redox state of
52 several thioredoxins and showing that both redox regulation systems, FTR and the NTRC, are linked
53 via 2-Cys-Prxs (Perez-Ruiz et al., 2017). Interaction between NTRC and Trx m1, Trx m3 and Trx γ 1
54 have been shown by bifluorescence complementation assays in vivo (Nikkanen et al., 2016). Trx m4
55 has been suggested to regulate negatively cyclic electron flow around photosystem I (PSI) (Courteille
56 et al., 2013). Recently, evidence has been provided that m-type thioredoxins form a complex with
57 PGRL1, a protein that is supposed to participate together with PGR5 in cyclic electron flow (Okegawa
58 and Motohasi, 2020; Wolf et al., 2020). Trx m1, Trx m2 and Trx m4 have also been reported to be
59 implicated in the biogenesis of PSII (Wang et al., 2013), showing a broad implication of m-type
60 thioredoxins in the photosynthetic light reactions. Compared to the other isoforms, Trx m3 seems
61 not to be relevant for controlling these processes. A mutant of Trx m3, the less abundant protein of
62 the m-type thioredoxins in chloroplast stroma (Okegawa and Motohasi, 2015), shows unaltered
63 chloroplast performance (Benítez-Alfonso et al., 2009). Tobacco plants overexpressing Trx m were
64 shown to be impaired in photosynthesis but being more resistant to oxidative stress conditions (Rey
65 et al., 2013). Furthermore, Trx m1, Trx m2 and NTRC have been shown to be indispensable for
66 acclimation of photosynthesis under fluctuating light conditions (Thormählen et al., 2017). Taken
67 together, these reports provide experimental evidence for an important role played by NTRC and Trx
68 m isoforms for the rapid acclimation of plants to changes in the light regime, for controlling
69 alternative photosynthetic electron flow and for coping with oxidative stress. Another important
70 protein in the thiol-based chloroplast redox regulatory network is 2-Cys peroxiredoxin (2-Cys PRX),
71 the most abundant peroxiredoxin in the chloroplast (Muthuramalingam et al., 2009). 2-Cys PRXs are
72 reduced by NTRC and by Trxs and detoxify H₂O₂. Most importantly, they also act as a system to
73 reoxidize reduced thioredoxins (Telman et al., 2020).

74 In the thylakoid lumen, candidates for controlling the redox state of protein disulphide bridges are
75 the Lumen Thiol Oxidoreductase 1 (LTO1) and the atypical cytochrome *c*_{6A} (Marceida et al., 2006).
76 The LTO1 protein is a transmembrane protein carrying a C-terminal thioredoxin-like domain typical of
77 oxidoreductases belonging to the protein disulphide isomerase family. The *lto1* mutant had been
78 shown previously to be affected in the assembly of active PSII while PSI electron transport was
79 unaltered upon excitation with far-red light (Karamoko et al., 2011). Since the cysteine residues of

80 LTO1 are at the lumen side of the thylakoid membrane, the redox state of the stroma has to be
81 transmitted to these cysteines. Possible candidates are the transmembrane proteins CCDA and HCF
82 164 (Karamoko et al., 2013; Kang and Wang, 2016; Motohasi and Hisabori, 2010).

83 There is a strong link between the level of reactive oxygen species (ROS) and the thiol system.
84 Superoxide anion radicals ($O_2^{\cdot-}$) are mainly generated by the photosynthetic electron transport at
85 photosystem I (PSI) by the classical “Mehler reaction” or pseudocyclic electron flow. It has been
86 reported that leaves of *A. thaliana* and *N. tabacum* plants grown under short day conditions (SD, 8 h
87 light, 16 h dark) have the double amount of superoxide compared with plants grown under long day
88 conditions (LD, 16 h light, 8 h dark) (Michelet and Krieger-Liszkay, 2012). This extra electron
89 transport in SD plants is used to generate a higher proton gradient and more ATP than found in
90 thylakoids from LD plants. In the presence of an uncoupler, the difference in ROS generation was
91 abolished between the two different thylakoid preparations. Addition of NADPH but not of NADH
92 increased the level of ROS generation in LD thylakoids to the same amount as observed in SD
93 thylakoids. Addition of NADPH to SD thylakoids had no significant effect. In thylakoids from plants
94 lacking NTRC the ROS production was like in SD wild type (wt) thylakoids and the difference between
95 SD and LD thylakoids was abolished (Lepistö et al., 2013). These results point to a redox regulation of
96 ROS generation at the level of PSI.

97 It remains an open question whether NTRC interacts with a protein of PSI at the thylakoid
98 membrane, whether a Trx is involved in the redox regulation of ROS generation at PSI and how the
99 redox state of the stroma is transmitted to the thylakoid lumen. In this study we aimed to establish
100 the interaction between different players of the chloroplast thioredoxin network and their ability to
101 alter the capacity of O_2 reduction at the PSI acceptor side. We measured light-dependent ROS
102 generation on leaves and isolated thylakoids of *A. thaliana* grown under SD or LD conditions in wt, in
103 single mutants: *ntrc*, *trxm4*, *ccda*, *lto1*, *cyt c_{6A}*, in double mutants: *trxm1trxm2* and *2cpab*, and in
104 plants overexpressing NTRC (oeNTRC). The sub-chloroplast localization of the NTRC and Trx m was
105 studied using immunoblots. In vitro reconstitution experiments were performed using thylakoids and
106 purified recombinant Trx m and/or NTRC in order to directly test their effect on light-induced ROS
107 generation.

108

109 **Materials and Methods**

110 *Plant Material*

111 *A. thaliana* wt (Col-0) and mutants were grown for 6 weeks in soil either under short day conditions
112 (8 h continuous white light - 160 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, 21°C/16 h dark, 18°C) or long day conditions (16
113 h continuous white light - 160 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, 21°C /8 h dark, 18°C). All mutants and over-
114 expressing plants used were already described in previous studies: the *trxm4* T-DNA mutant
115 (Courteille et al., 2007) and the *trxm1m2* mutant (Thormählen et al., 2017); the T-DNA insertion
116 mutant of NTRC (*ntrc*) (Lepistö et al., 2009) and transgenic plants overexpressing wild type NTRC
117 protein in *ntrc* background (Toivola et al., 2013); the double T-DNA mutant lacking the two 2-Cys
118 Prxs, A and B (*2cpab*) (Ojeda et al., 2018); the T-DNA mutants lacking CCDA isoforms (Page et al.,
119 2004); the T-DNA mutant lacking Cytochrome c_{6A} (Pesaresi et al., 2009).

120 *Extraction of proteins from leaves*

121 *Arabidopsis* shoots were grinded in liquid nitrogen before homogenization in lysis buffer. The lysis
122 buffer contained 100 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulphate (SDS), 20 mM EDTA and
123 protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MI, USA).

124

125 *Extraction of thylakoids from A. thaliana*

126 Young fully expanded leaves were grinded in 0.33 M sorbitol, 50 mM KCl, 10 mM EDTA, 1 mM MgCl_2 ,
127 25 mM Mes pH 6.1. After centrifugation, the pellet was washed twice with 0.33 M sorbitol, 60
128 mM KCl, 2 mM EDTA, 1 mM MgCl_2 , 25 mM HEPES pH 6.7. After centrifugation, the pellet was
129 resuspended in 0.3 M sucrose, 50 mM KCl, 1 mM MgCl_2 , 20 mM HEPES pH 7.6 (measurement buffer).
130 This procedure was repeated once, and the pellet was resuspended to a final concentration of about
131 1 mg of chlorophyll per ml of thylakoids. All centrifugations were performed at 3,000xg for 3 min at
132 4°C.

133 *Isolation of Photosystem I*

134 Photosystem I was isolated as described in Krieger-Liszkay et al. (2020).

135 *Room-Temperature Spin-Trapping EPR Measurements*

136 Spin-trapping assays with 4-pyridyl-1-oxide-*N-tert*-butylnitrone (4-POBN) (Sigma-Aldrich) were
137 carried out using leaf disks or freshly isolated thylakoid membranes at a concentration of 10 μg of Chl
138 ml^{-1} . Leaf disks were vacuum-infiltrated with the buffer containing the spin trap reagents prior to the
139 illumination and then floating on the same buffer during the illumination. Samples were illuminated
140 for a given time with white light (200 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ in case of leaf disks and 500 $\mu\text{mol quanta}$
141 $\text{m}^{-2}\text{s}^{-1}$ in case of thylakoids) in the presence of 50 mM 4-POBN, 4% ethanol, 50 μM Fe-EDTA, and
142 buffer (25 mM HEPES, pH 7.5, 5 mM MgCl_2 , 0.3 M sorbitol). When indicated, 200 μM NADPH, 0.3 μM
143 Trxm4 and 0.3 μM NTRC were added to the assay before starting the illumination.

144 EPR spectra were recorded at room temperature in a standard quartz flat cell using an ESP-300 X-
145 band (9.73 GHz) spectrometer (Bruker, Rheinstetten, Germany). The following parameters were
146 used: microwave frequency 9.73 GHz, modulation frequency 100 kHz, modulation amplitude: 1G,
147 microwave power: 6.3 milliwatt in 4-POBN assays, receiver gain: 2×10^4 , time constant: 40.96 ms;
148 number of scans: 4.

149

150 *O₂ consumption*

151

152 Measurements of O₂-consumption were performed in a Liquid-Phase Oxygen Electrode Chamber
153 (Hansatech Instruments, Norfolk, England) using isolated PSI ($10 \mu\text{g Chl ml}^{-1}$) in Tricine 20 mM
154 pH 8.0, in the presence of 5 mM MgCl₂, 30 mM NaCl and 5 mM ascorbate, 30 μM 2,6-
155 dichlorophenolindophenol (DCPIP) as exogenous electron donors to P700*.

156

157 *SDS-PAGE and Western Blotting*

158 SDS-PAGE was performed using 8% or 4-20 % polyacrylamide gels. Proteins were blotted onto a
159 nitrocellulose or a PVDF membrane. Labelling of the membranes with polyclonal antibodies,
160 produced in the lab (anti-Trxm; anti-NTRC; anti-2-Cys PRX) or commercially available (PsaF and β -
161 subunit of ATP synthase; Agrisera, Vännäs, Sweden), was carried out at room temperature in 50 mM
162 Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20 and 5% non-fat milk powder. After washing, bound
163 antibodies were revealed with a peroxidase-linked secondary anti-rabbit antibody (Agrisera, Vännäs,
164 Sweden) and visualized by enhanced chemiluminescence.

165 *Redox state of PsaF*

166 Total leaf protein samples were prepared as described above with AMS or mPEG-maleimide (2 mM)
167 added to the extraction buffer. 50 μg protein samples were electrophoresed (4-20 % SDS-PAGE) and
168 PsaF was immuno-detected (PVDF membrane / Chemiluminescence) and signals corresponding to
169 reduced (alkylated) and oxidized forms were quantified using ImageLab software (BioRad).

170 *NTRC Trx reduction assays*

171

172 TRX reduction tests by NTRC were performed using DTNB as already described in Bohrer et al. (2012).

173

174 Results

175 Leaves and thylakoid membranes isolated from *A. thaliana* wild-type (wt) plants grown in
176 short day (SD) conditions generate in the light about twice the amount of ROS compared to those
177 from plants grown in long day (LD) conditions (Fig. 1) as has been previously shown (Michelet and
178 Krieger-Liszkay, 2012). ROS production was measured using an indirect spin trapping assay. In this
179 assay hydroxyl radicals are detected which derive from superoxide anion radicals and hydrogen
180 peroxide in a Haber-Weiss reaction catalyzed by Fe(II) (Michelet and Krieger-Liszkay, 2012). A
181 comparison between ROS production in leaf disks and isolated thylakoids shows that the same
182 differences in ROS production are found in both types of samples (Fig. 1B, C). This demonstrates that
183 in the light the majority of superoxide/hydrogen peroxide is generated by the photosynthetic
184 electron transport chain. Mutants affected in Trx m isoforms or in NTRC lost the difference between
185 SD and LD. The single mutant *trxm4* and the double mutant *trxm1m2* generated similar amounts of
186 ROS like LD plants, while *ntrc* generated high amounts of ROS like SD plants, independently of the
187 photoperiod during their growth. Overexpression of NTRC increased the difference between SD and
188 LD compared to wt. In a similar manner the mutant devoid of 2-Cys PRX A and B showed an overall
189 increase in the ROS production, however, the difference between SD and LD was maintained in
190 *2cpab* (Fig. 1).

191 Furthermore, we found that 2-Cys PRX was slightly more abundant in leaf protein samples
192 from plants grown in LD than in SD when prepared in presence of SDS but not in absence of
193 detergent (Fig. 2A and B), suggesting that 2-Cys PRX is mainly stromal and the difference between SD
194 and LD samples would be attributable to a differential association to thylakoid membranes. Indeed,
195 thylakoids from LD grown plants showed a marked higher amount of 2-Cys PRX (recovered from
196 thylakoid membranes in the presence of SDS) compared to SD, confirming that the difference of
197 global (stromal plus thylakoid-associated) abundance in leaf extracts between photoperiods was
198 attributable to the fraction of membrane bound protein. In LD, the protein was present mainly in its
199 dimeric form, most probably corresponding to the oxidized form as evidenced by the shift of the
200 signal from an apparent mass of about 40 kDa to about 20 kDa reproduced by reduction with DTT
201 giving the monomeric form. In PSI preparations, we could not immune-detect 2-Cys PRX (Fig. 2C)
202 suggesting that the protein was not directly associated to PSI complexes, or lost upon sample
203 preparation due to a loosen association to the photosystem. This latter possibility is strongly
204 suggested by the detection of a faint signal in the supernatant of LD thylakoids resuspended in buffer
205 devoid of detergent.

206 To see whether the amount of Trx m and/or NTRC is altered in plants grown in the two
207 different light regimes, immunoblots were performed using leaf extracts and isolated thylakoids. As
208 shown in Fig. 3, no difference in the total amount of Trx m4 and NTRC was found in leaf extracts.
209 However, the attachment of Trx m differed in SD and LD thylakoids. Trx m4 was found in the
210 thylakoid fraction in SD thylakoids but not in LD thylakoids. A similar membrane localization like for
211 Trx m4 was observed for Trx m2 (Sup. Fig. 1).

212 Attachment of Trx m seems to be required for allowing electron transport to oxygen at PSI.
213 To test whether the lack of membrane-associated Trx was indeed responsible for the lower ROS
214 generation in LD thylakoids, we reconstituted LD thylakoids with purified Trx m4 and/or NTRC
215 proteins in the presence of NADPH (Fig. 4). Addition of NADPH alone stimulated slightly the ROS
216 production in LD thylakoids. A further increase in signal size was observed when Trxm4 or NTRC were
217 added together with NADPH. However, these differences were statistically not significant. When
218 Trxm4 was added together with NADPH and NTRC, LD thylakoids generated three-fold more ROS

219 than without any protein addition. In the absence of NADPH, addition of TRxm4 and NTRC had no
220 effect. The different additives had only a small effect on SD thylakoids. This result points to a redox
221 regulation of O₂ reduction at the level of PSI. To investigate whether there is a direct effect of thiol
222 reduction on the O₂ reduction capacity of PSI, we incubated isolated PSI with the reducing agent
223 TCEP and followed light-dependent O₂ consumption using an O₂ electrode. As shown in Fig. 5, O₂
224 consumption was two times higher in the presence of TCEP. These data show that it is redox
225 regulation of PSI itself that is crucial for the level of O₂ reduction at the acceptor side of PSI.

226 The question arises which subunit of PSI may be redox-regulated. Several subunits of PSI
227 contain cysteines, however most of them are ligands of the iron-sulphur clusters F_x, F_A and F_B and can
228 therefore be excluded as candidates undergoing reversible regulatory redox modifications. In
229 addition, there are two proteins, PsaN and PsaF that may be candidates for disulfide bridge
230 formation. It has been shown previously that the PSI subunit PsaN contains four cysteine residues
231 that can form two disulfide bridges (Motohashi and Hisabori, 2006). PsaF contains at its luminal site 2
232 cysteine residues that are close enough to form a disulfide bridge. PsaF is a transmembrane protein
233 that forms the docking site for plastocyanin at the donor side of PSI and at the acceptor side its C-
234 terminus is in direct neighborhood with PsaE. PsaE forms together with PsaC and PsaD the docking
235 site for ferredoxin. O₂ reduction is supposed to take place at this site. Since PsaF is the most likely
236 candidate for redox regulation, and we did not observe any significant quantitative difference of this
237 protein when comparing SD and LD thylakoid and PSI protein preparations (Fig. 2), we performed
238 redox western assays to explore the influence of light regime on its redox state. Thylakoid
239 membranes were treated with 4'-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) that
240 reacts with thiol groups (alkylation reaction), we separated the proteins by SDS-PAGE and analysed
241 the apparent mass of PsaF by immunodetection. Sup. Fig. 2 shows that two bands were detected
242 when the thylakoids had been chemically reduced with tris(2-carboxyethyl) phosphine prior to the
243 AMS treatment. This indicates that PsaF exists in an oxidized form with cysteines potentially forming
244 a disulfide bridge and, upon addition of a reductant, in a reduced form with AMS accessible thiols.
245 However, without exogenous reductant, in isolated thylakoids from both SD or LD plants only the
246 oxidized form was found, probably due to spontaneous oxidation during membrane sample
247 preparation. Redox Westerns were performed on the mutants used for detection of the ROS levels in
248 Fig. 1. In leaf extracts prepared in presence of SDS and m-PEG-mal (Fig. 6, Sup. Fig. 3) PsaF was
249 immuno-detected as two well distinguishable redox variants that could be quantified by
250 densitometry. We found that relative abundance of PsaF oxidized and reduced forms strongly varied
251 between light and dark samples. In LD, PsaF was reduced equally in all genotypes with a reduction
252 percentage ranging from 24.6 to 32.4 % in the dark, and from 1.5 to 2.9 % in the light. But, we did not
253 observe significant differences in the redox state of PsaF in the two light regimes or between the
254 different mutant lines, with the exception of *ntrc* where PsaF was significantly more reduced in SD
255 (39.7 % in the dark and 8.4 % in the light) than in LD (24.6 % in the dark and 2.4 % in the light), and in
256 comparison to wt (28.4 % in the dark and 2.2 % in the light) and the other mutants analyzed.
257 However, there was no correlation with the ROS levels found in *ntrc*. We concluded that the
258 reduction of PsaF may be not stable enough to catch it during the alkylation treatment procedure.

259 The redox state of the stroma has to be transmitted to the thylakoid lumen to be able to act
260 on thiol/disulphide groups of PsaF. The transmembrane proteins CCDA and HCF164 are likely
261 candidates for the transmission of the redox state from the stromal site to the luminal site of the
262 thylakoid membrane (Motohashi and Hisabori, 2010). LTO1 (Karamoko et al., 2011) and the atypical

263 cytochrome c_{6A} (Marceida et al., 2006) may act as candidates for redox modifications inside the
264 lumen. Fig. 7 shows that the mutants of CCDA (*ccda3* and *ccda4*) and of LTO1 have indeed lost the
265 difference in superoxide production between SD and LD, while loss of cyt c_{6A} has no effect.

266 Discussion

267

268 Redox regulation of linear photosynthetic electron transport and alternative pathways has
269 been reported previously (Johnson, 2003). Courteille and coworkers (2013) showed that cyclic
270 electron transport involving the NDH complex was altered in Trx m4 mutants, Nikkanen et al. (2018)
271 reported the involvement of NTRC in the control of NDH complex-dependent and Naranjo et al.
272 (2021) in PGR5-dependent cyclic flow. Both alternative electron transport pathways, cyclic and
273 pseudocyclic flow, are in competition and down-regulation of cyclic flow is therefore supposed to
274 stimulate pseudocyclic flow. We have shown previously (Michelet and Krieger-Liszkay, 2012) that O_2
275 reduction at PSI is higher in SD thylakoids than in LD thylakoids and that this difference is abolished in
276 the presence of uncouplers. This observation points to a pH- or redox-regulated process taking place
277 at the luminal side of the thylakoid membrane and controlling O_2 reduction at PSI. Indeed, O_2
278 reduction of isolated PSI is stimulated by the thiol-reducing agent TCEP (Fig. 5). At low light
279 intensities, such as those used for the measurements shown in Fig. 5, O_2 is reduced by the terminal
280 electron acceptors, the 4Fe4S clusters F_A and F_B , while at higher light intensities it is reduced by the
281 acceptor A_1 , a phylloquinone (Kozuleva et al., 2021). PsaN and PsaF are the only constitutive protein
282 subunits of PSI containing cysteine residues that can form a disulphide bridge. The most likely
283 candidate for the redox modification affecting O_2 reduction is PsaF for the following reasons: 1. PsaF
284 was found in PSI x-ray structures from pea either in the reduced state (Mazor et al., 2015) or with a
285 disulfide bond (Qin et al., 2015), and 2. PsaF has been identified by a proteomics approach as a
286 redox-affected protein (Ströher and Dietz, 2008). PsaF is a transmembrane protein, and we
287 hypothesize here that, upon a modification of the redox state of cysteines, a long-range structural
288 change affects the neighboring subunit PsaE and thereby the ferredoxin docking site at the PSI
289 acceptor side. Accordingly, O_2 reduction is favored when the disulfide bridge in PsaF is reduced while
290 Fd reduction becomes less efficient. The subunit PsaE seems to be crucial for O_2 reduction at PSI in
291 higher plants (Krieger-Liszkay et al., 2020). Unfortunately, we were not able to detect significant
292 differences in the reduction level of PsaF in the two light regimes and in the mutant lines analyzed
293 where distinct O_2 reduction levels were clearly found (Fig. 1). We cannot rule out at present that
294 PsaN may also be involved in the redox-dependent regulation of O_2 reduction at PSI. Different to
295 PsaF, PsaN is a peripheral luminal protein belonging to the light harvesting complex of PSI with no
296 connection to the acceptor side of PSI. However, in the structure published by Pan et al. (2018) for
297 maize PSI, PsaN forms close contacts with the N-terminal extension of PsaF. Redox modification of
298 PsaN may impose a structural change on PsaF that then, as described above, may exert a structural
299 alteration of the PSI subunits forming the Fd docking site.

300 We also addressed the question how the redox state of PsaF and/or PsaN is controlled in the
301 lumen. *Lto1* mutant has lost the difference between SD and LD (Fig. 7), implying a role of this protein
302 in redox regulation of PSI. The difference between SD and LD is also lost in the mutants *ccda3* and
303 *ccda4* (Fig. 7), demonstrating the importance for CCDA in transmitting the stromal redox state to the
304 lumen in vivo. Mutants of CCDA and LTO1 show high ROS levels independent of the growth
305 photoperiod. We hypothesize that LTO1 keeps PsaF oxidized under LD conditions, while CCDA keeps
306 LTO1 oxidized, leading to low O_2 reduction in LD. In SD conditions, where the redox state of CCDA

307 and LTO1 is highly reduced, the situation changes. Under these circumstances, reduction of PsaF
308 (or/and PsaN) seems feasible (Fig. 8).

309 Trx m has been shown to be the electron donor to CCDA *in vitro* (Motohashi and Hisabori,
310 2010). We propose here that Trx m association to the thylakoid membrane is required for an efficient
311 electron donation to CCDA and onwards to redox regulated proteins in the thylakoid lumen,
312 reduction of PsaF (and/or PsaN) and increase in O₂ reduction (Fig. 7). Trx m association to the
313 membrane in SD conditions (Fig. 3 and Sup. Fig. 1) may be facilitated by the higher proton motive
314 force generated in thylakoids from SD plants (Michelet and Krieger-Liszkay, 2012). Such a mechanism
315 has been shown to play a role for the attachment of the plastid terminal oxidase to the thylakoid
316 membrane (Bolte et al., 2020).

317 The question arises how to integrate 2-Cys-PRX and NTRC into this model. 2-Cys-PRXs are
318 mainly reduced by NTRC and act as an oxidizing system towards reduced Trxs (Nikkanen et al., 2016;
319 Perez-Ruiz et al., 2017; Telman et al., 2020). We suggest that membrane association of 2-Cys-PRX in
320 LD is important to keep CCDA oxidized. As shown in Fig. 2, 2-Cys PRX is associated to the membrane
321 only in LD conditions and is found mostly in its dimeric form. Besides controlling the redox state of
322 Trxs, 2-Cys-PRX is responsible for H₂O₂ detoxification in the stroma (König et al., 2002). H₂O₂
323 detoxification may be more efficient if 2-Cys PRX is attached to the membrane close to the site of
324 superoxide production and conversion into H₂O₂ by membrane-associated SOD. In the absence of
325 NTRC, 2-Cys PRX is not able to fulfill this role, resulting in higher ROS levels independent of the
326 photoperiod (Fig. 1). In the absence of 2-Cys PRX, as expected, the ROS levels are increased, but the
327 difference between SD and LD is maintained (Fig. 1B). According to a previous report (Bohrer et al.,
328 2012) and our measurements of DTNB reduction (Sup. Fig. 4), NTRC is not able to reduce Trxs m in a
329 direct manner when tested at physiologically relevant concentrations.

330 In conclusion, our hypothesis for the redox regulation of pseudocyclic electron flow is based
331 on three mechanisms: 1. The availability of electron acceptors other than O₂ for photosynthetic
332 electron transport, 2. Redox regulation of the different players according to the redox state of the
333 stroma and the capabilities of the different players to form a network of redox-driven interactions
334 and 3. The reversible membrane attachment of Trx m and 2-Cys-PRX that may depend on changes in
335 pH and ion concentration controlled by the proton motive force. Attachment of Trx m4 seems to be
336 necessary to achieve a high reduction state of CCDA and PsaF in SD resulting in high O₂-reduction
337 levels at PSI, while detachment of Trx m and attachment of 2-Cys-PRX in LD seems to favor oxidation
338 of CCDA, and PsaF that is oxidized by LTO1, resulting in low levels of O₂-reduction. However, the
339 connection of redox regulation of PsaF *in vivo* with the photoperiod remains to be demonstrated.

340 Reduction of O₂ in pseudocyclic electron flow is in competition with cyclic electron flow. Both
341 pathways lead to the formation of a proton gradient without generating NADPH. According to
342 Takahashi et al (2013), the redox state of the chloroplast controls the formation of supercomplexes
343 composed of PSI, Cyt *b₆f*, LHCs, PGRL1, FNR. Such supercomplexes are thought to be required for
344 cyclic electron flow (Iwai et al., 2010). The formation of a complex between reduced Trx m and
345 PGRL1 may inhibit cyclic electron flow by preventing the supercomplex formation required for cyclic
346 flow. This suggestion is supported by the recent reports on the interaction between Trx m and PGRL1
347 (Wolf et al., 2020; Okegawa and Motohashi, 2020). As shown recently, Trx x and Trx y play an
348 important role in the acceptor-side regulation of PSI and protection of PSI against photoinhibition
349 under fluctuating light conditions (Okegawa et al., 2023). Future work on the extent of cyclic flow and
350 superoxide production in mutants of the different components of the Trx system will show if both,
351 pseudocyclic and cyclic flow are controlled by the same proteins but in the opposite way.

352

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359

360 **Author Contribution**

361 E.I-B. and A.K-L. designed the project. U.H., B.N., G.S., C.E., H.V., E.I-B. and A.K-L. performed the
362 experiments and analysed the data. P.S. and E.R. participated in discussions. A.K-L. wrote the initial
363 version of the manuscript that was read and revised by all authors.

364

365 **Data availability**

366 Data will be made available on demand.

367

368

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517

518 Figure Legends

519

520 **Figure 1**

521 **Light-induced hydroxyl radical formation in SD and LD leaf disks and washed thylakoid membranes**
522 **in wild type and redoxin mutants.** Generation of hydroxyl radicals originating from $O_2^{\bullet-}/H_2O_2$ was
523 detected by spin trapping with 4-POBN. A: Typical EPR spectra of the 4-POBN/ α -hydroxyethyl adduct
524 are shown. After infiltration with assay medium containing 4-POBN/ethanol/FeEDTA, leaf disks were
525 incubated in the same medium for 30 min in light ($200 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) before detection of the
526 radicals in the medium. Difference in EPR signal size for leaf samples (B) and thylakoid membranes
527 (C). Thylakoid membranes ($20 \mu\text{g ml}^{-1}$) were illuminated for 2 min ($500 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) in the
528 presence of the spin trapping assay before detection of the radical. Grey bars: SD, white bars: LD. All
529 EPR signals were normalized to the signal of SD thylakoids without addition (100%). Mean values are
530 shown ($n \geq 6$, biological replicates; *, $P < 0.05$ (comparison between SD and LD growth conditions for
531 each genotype) according to Tukey test.

532 **Figure 2**

533 **Association of 2-Cys PRX with thylakoid membranes depends on the photoperiod.** Immuno-
534 detection of 2-Cys PRX in (A) soluble leaf extracts ($50 \mu\text{g protein per well}$), (B) thylakoid membranes
535 ($5 \mu\text{g chl per well}$) or (C) PSI complexes ($0.5 \mu\text{g chl per well}$) prepared from plants grown either in
536 short day (SD) or long day (LD) conditions. Immuno-detection of PsaF and coomassie staining (input)
537 were taken as membrane-associated protein extraction and gel loading controls, respectively. SDS
538 (2%) and DTT (10 mM) treatments of membrane samples were performed for 10 min at RT. After
539 centrifugation the supernatant was collected and supplemented with non-reducing loading blue
540 prior heat treatment and gel loading.

541 **Figure 3**

542 **Membrane association of Trx m and 2-Cys PRX.** Immuno-detection of NTRC and Trx m4 in leaf
543 extracts and thylakoid membranes prepared from plants grown either in short day (SD) or long day
544 (LD) conditions. Samples corresponding to $5 \mu\text{g chlorophyll}$ were loaded in each well. The results
545 shown are representative of three biologically independent experiments.

546 **Figure 4**

547 **Reconstitution of ROS generation in LD thylakoids by Trx m, NTRC and NADPH.** Light-induced
548 hydroxyl radical formation in SD and LD thylakoids is shown by indirect spin trapping with 4-POBN.
549 Thylakoid membranes ($20 \mu\text{g ml}^{-1}$) were illuminated for 2 min ($500 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) before
550 detection of the radical. When indicated, $200 \mu\text{M}$ NADPH, $0.3 \mu\text{M}$ Trx m4 and $0.3 \mu\text{M}$ NTRC were
551 added to the assay before starting the illumination. All EPR signals were normalized to the signal of
552 SD thylakoids without addition (100%). Grey bars: SD, white bars: LD. Mean values are shown ($n=3$; *,
553 $P < 0.05$ (comparison with LD no protein added) according to Tukey test.

554 **Figure 5**

555 **Dependence of O_2 consumption by isolated PSI on the redox state.** O_2 consumption was measured
556 with an O_2 electrode using DCPIP/ascorbate as electron donor. Samples were illuminated with green
557 actinic light. When indicated, isolated PSI had been incubated with 1 mM TCEP for 15 min prior to the
558 measurement.

559 **Figure 6**

560 **Redox state of PsaF in dark- and light-adapted plants.** Total leaf protein extracts from WT (Col-0)
561 and redoxins (*ntrc*, *trxm4*, *trxm1m2* and *2cpab*) mutant plants grown in short day (SD) or long day
562 (LD) conditions were prepared in presence of the thiol alkylating agent mPEG-maleimide. After SDS-
563 PAGE, proteins ($50 \mu\text{g}$ per well) were electro-transferred onto a PVDF membrane for PsaF immuno-
564 detection (chemi-luminescence). See Sup. Fig. 2 and 3 for details about the quantification method
565 used and an example of redox western signals. (A) and (B) PsaF redox state in leaves in the dark and
566 in the light, respectively. Values correspond to the mean of 5-6 experiments. Error bars correspond
567 to standard deviation. * and # designate significant differences between mutant and wt genotypes,
568 and between SD and LD (for each genotype), respectively; according to Student's t-test, $P < 0.05$.

569 **Figure 7**

570 **Light-induced hydroxyl radical formation in SD and LD leaf disks of mutants affected in LTO1, CCDA**
571 **(*ccda3* and *ccda4*) and Cyt c_{6A} .** Generation of hydroxyl radicals originating from $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ was
572 detected in leaves by spin trapping with 4-POBN. Grey bars: SD, white bars: LD. All EPR signals were
573 normalized to the signal of SD thylakoids without addition (100%). Mean values are shown ($n=8$, 2-3
574 biological replicates; *, $P < 0.05$ (comparison between growth conditions for each genotype)
575 according to Tukey test.

576 **Figure 8**

577 **Model of redox regulation of O_2 reduction at photosystem I.** Reduced cysteine residues in PsaF
578 favor higher O_2 reduction activity. The redox state of PsaF is modified by two thiol modulating redox
579 systems. The first one is required for reduction of the disulfide bond, the second one for their
580 oxidation. Trx m is a central player for controlling the reduction state of PsaF. Trx m is reduced by
581 FTR. Under SD conditions, Trx m associates to the thylakoid membrane and reduces CCDA which
582 reduces via HCF164 finally PsaF. In LD conditions, reduced PsaF is oxidized by LTO1, which itself is
583 oxidized by CCDA (blue arrows. 2-Cys-PRX attaches to the membrane, allowing the oxidation of
584 CCDA. In addition, it can oxidize Trx m (blue arrows).

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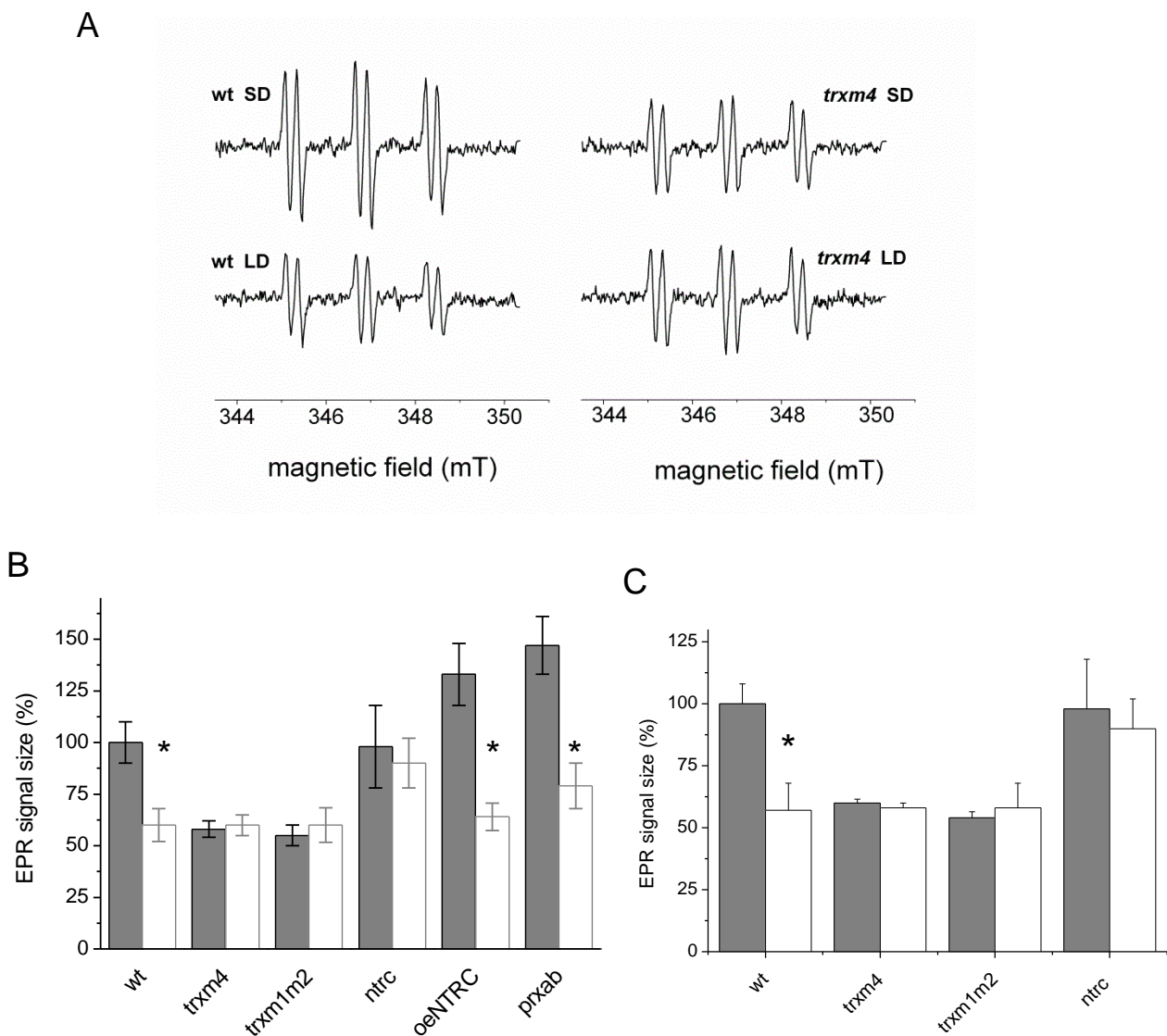


Figure 1

Light-induced hydroxyl radical formation in SD and LD leaf disks and washed thylakoid membranes in wild type and redoxin mutants. Generation of hydroxyl radicals originating from $O_2^{\bullet-}/H_2O_2$ was detected by spin trapping with 4-POBN. A: Typical EPR spectra of the 4-POBN/ α -hydroxyethyl adduct are shown. After infiltration with assay medium containing 4-POBN/ethanol/FeEDTA, leaf disks were incubated in the same medium for 30 min in light ($200 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) before detection of the radicals in the medium. Difference in EPR signal size for leaf samples (B) and thylakoid membranes (C). Thylakoid membranes ($20 \mu\text{g ml}^{-1}$) were illuminated for 2 min ($500 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) in the presence of the spin trapping assay before detection of the radical. Grey bars: SD, white bars: LD. All EPR signals were normalized to the signal of SD thylakoids without addition (100%). Mean values are shown ($n \geq 6$, biological replicates; *, $P < 0.05$ (comparison between SD and LD growth conditions for each genotype) according to Tukey test.

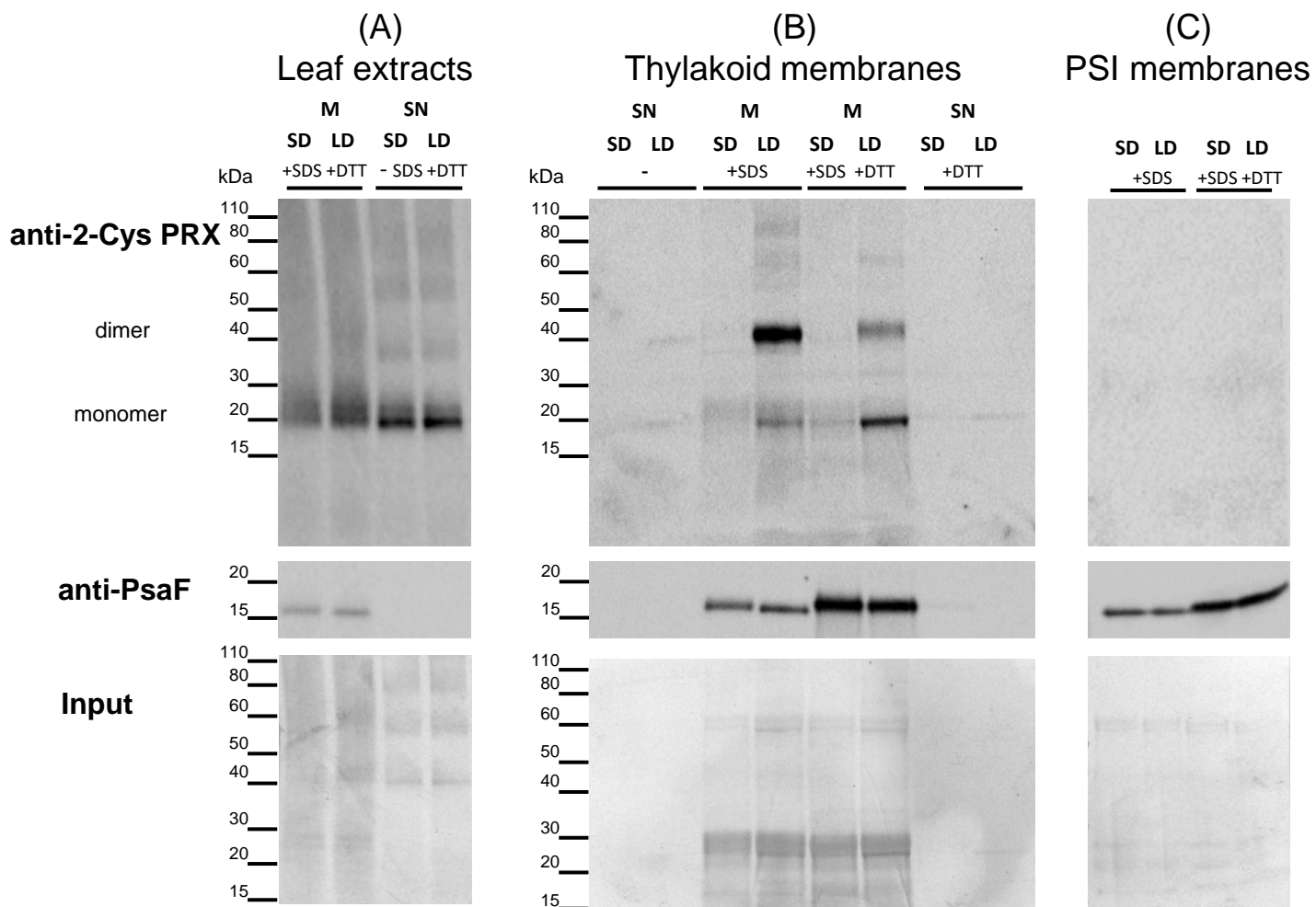


Figure 2

Association of 2-Cys PRX with thylakoid membranes depends on the photoperiod

Immuno-detection of 2-Cys PRX in (A) leaf extracts (50 μg protein per well), (B) thylakoid membranes (5 μg chl per well) or (C) PSI complexes (0.5 μg chl per well) prepared from plants grown either in short day (SD) or long day (LD) conditions. Immuno-detection of PsaF and coomassie staining (input) were taken as membrane-associated protein extraction and gel loading controls, respectively. SDS (2%) and DTT (10 mM) treatments of membrane samples (M) were performed for 10 min at RT. After centrifugation the supernatant (SN) was collected and supplemented with non-reducing gel loading blue prior heat treatment and gel loading.

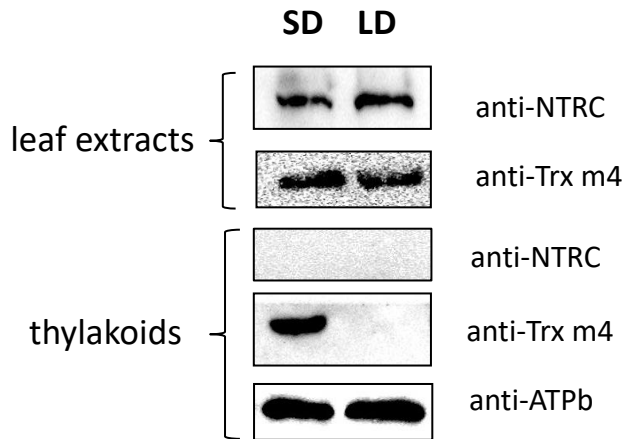


Figure 3

Membrane association of Trx m4 to thylakoid membranes in SD conditions.

Immuno-detection of NTRC and Trx m4 in leaf extracts and thylakoid membranes prepared from plants grown either in short day (SD) or long day (LD) conditions.

Samples corresponding to 5 μ g chlorophyll were loaded in each well.

The results shown are representative of three biologically independent experiments.

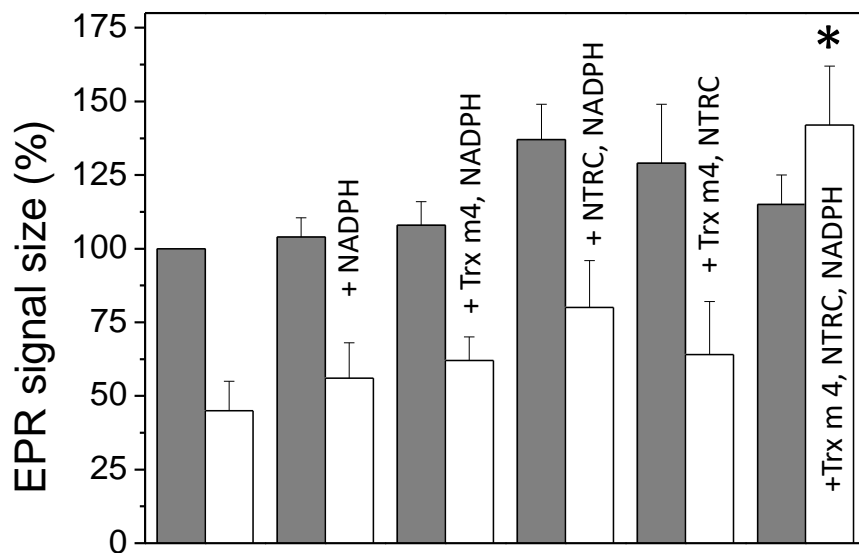


Figure 4

Reconstitution of ROS generation in LD thylakoids by Trxm, NTRC and NADPH.

Light-induced hydroxyl radical formation in SD and LD thylakoids is shown by indirect spin trapping with 4-POBN. Thylakoid membranes ($20 \mu\text{g ml}^{-1}$) were illuminated for 2 min ($500 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$) before detection of the radical. When indicated, $200 \mu\text{M}$ NADPH, $0.3 \mu\text{M}$ Trx m4 and $0.3 \mu\text{M}$ NTRC were added to the assay before starting the illumination. All EPR signals were normalized to the signal of SD thylakoids without addition (100%). Grey bars: SD, white bars: LD. Mean values are shown ($n=3$; *, $P < 0.05$ (comparison with LD no protein added) according to Tukey test.

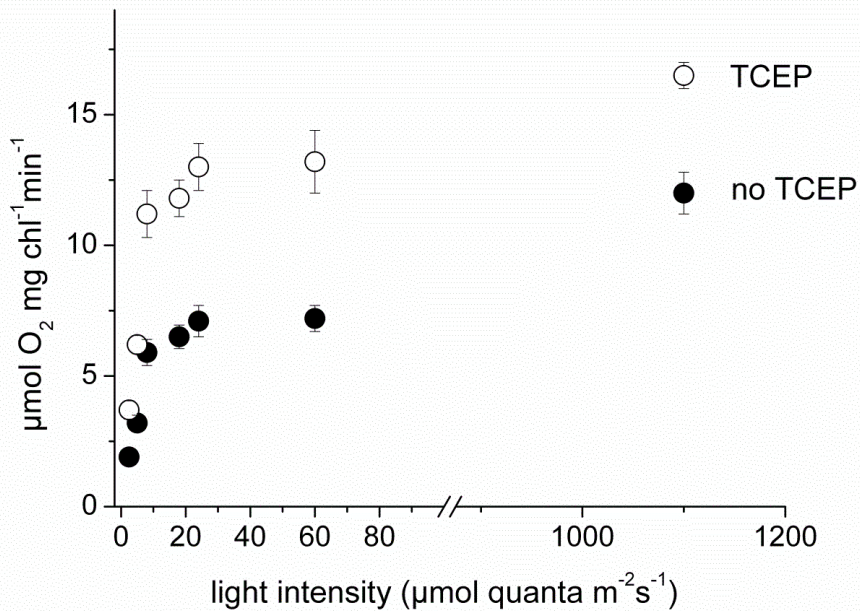


Figure 5

O_2 consumption by isolated PSI as a function of the light intensity.

O_2 consumption was measured with an O_2 electrode using DCPIP/ascorbate as electron donor. Samples were illuminated with green actinic light. When indicated, isolated PSI had been incubated with 1 mM TCEP for 15 min prior to the measurement.

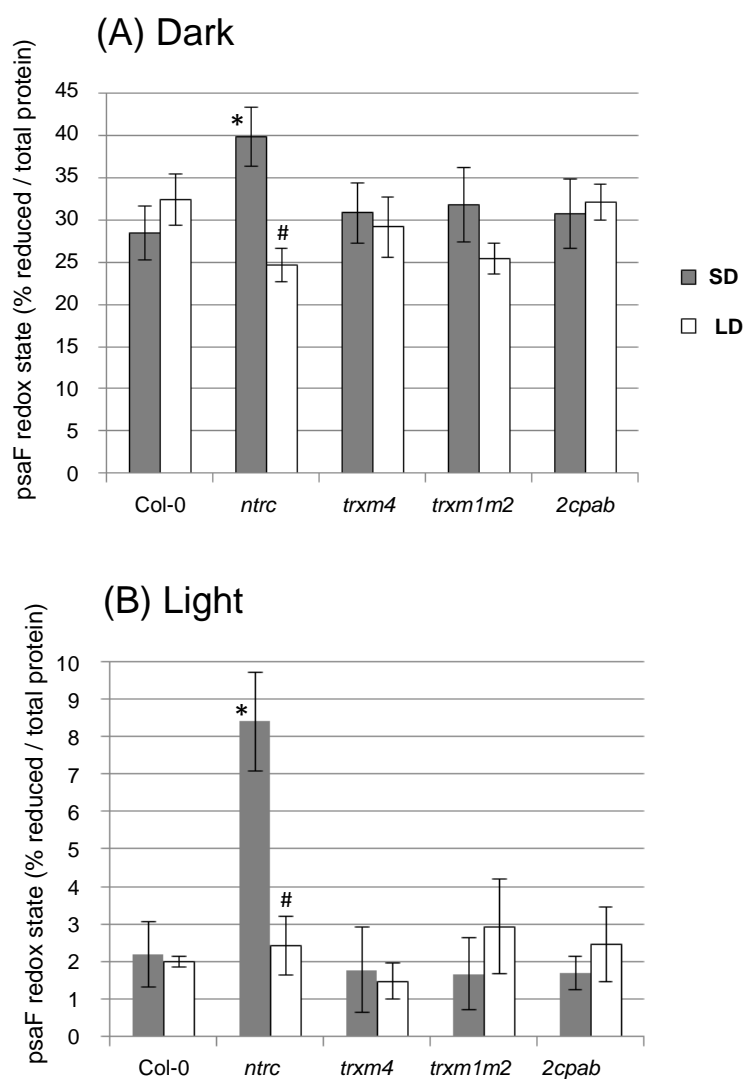


Figure 6

Redox state of PsaF in dark- and light-adapted plants.

Total leaf protein extracts from wt (Col-0) and redoxins (*ntrc*, *trxm4*, *trxm1m2* and *2cpab*) mutant plants grown in SD or LD conditions were prepared in presence of the thiol alkylating agent mPEG-maleimide. After SDS-PAGE, proteins (50 μ g per well) were electro-transferred onto a PVDF membrane for PsaF immuno-detection (chemi-luminescence). See Sup. Fig. 3 for details about the quantification method used and an example of redox western signals. (A) and (B) PsaF redox state in leaves in the dark and in the light, respectively. Values correspond to the mean of 5-6 experiments. Error bars correspond to standard deviation. * and # designate significant differences between mutant and wt genotypes, and between SD and LD (for each genotype), respectively; according to Student's *t*-test, $P < 0.05$.

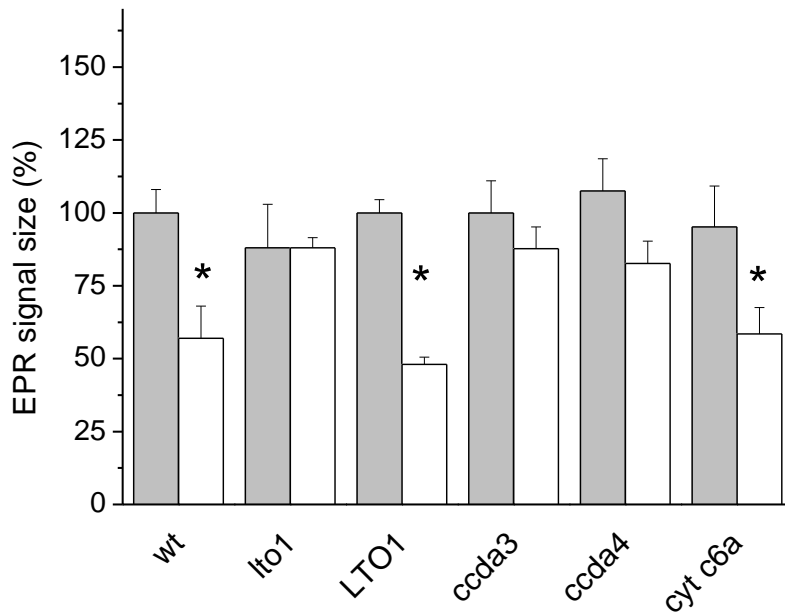


Figure 7

Light-induced hydroxyl radical formation in SD and LD leaf disks of mutants affected in LTO1, 2-Cys-PRX (*prxab*), CcdA (*ccda3* and *ccda4*) and Cyt c_{6A} .

Generation of hydroxyl radicals originating from $O_2^{\cdot-}/H_2O_2$ was detected in leaves by spin trapping with 4-POBN. Grey bars: SD, white bars: LD. All EPR signals were normalized to the signal of SD thylakoids without addition (100%). Mean values are shown (n=8, 2-3 biological replicates; *, $P < 0.05$ (comparison between growth conditions for each genotype) according to Tukey test.

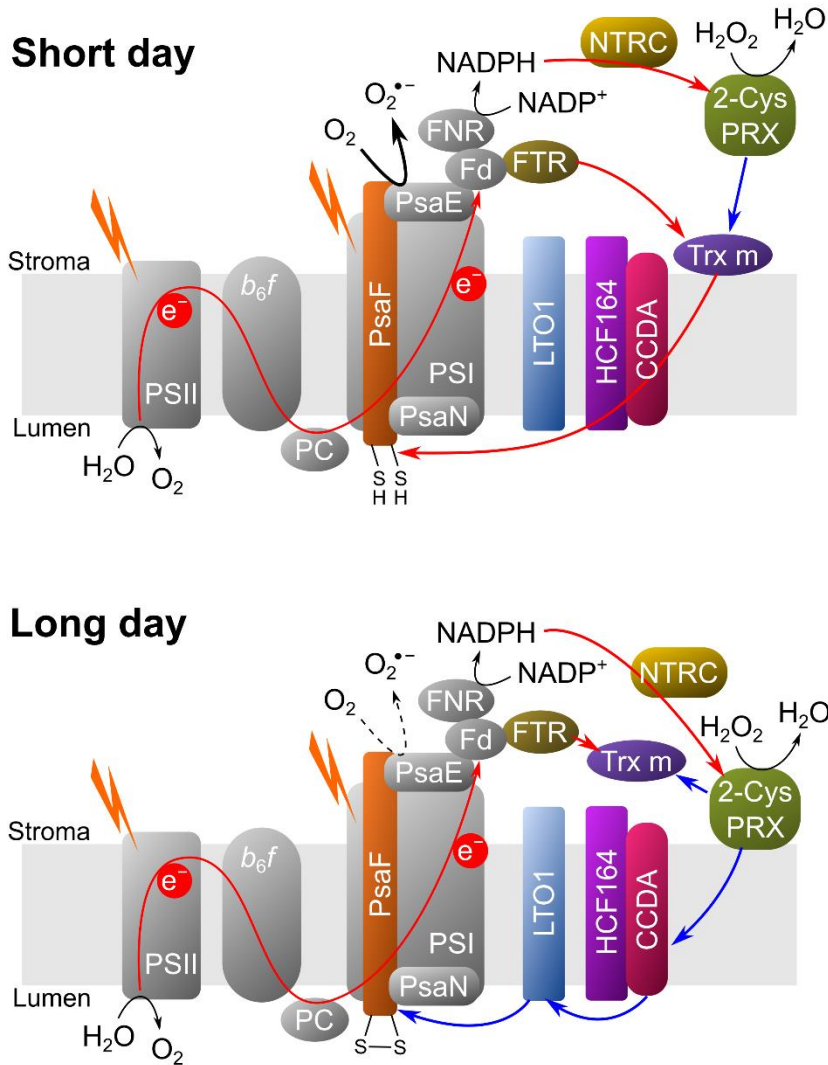


Figure 8

Model of redox regulation of O₂ reduction at photosystem I. Reduced cysteine residues in PsaF favor higher O₂ reduction activity. The redox state of PsaF is modified by two thiol modulating redox systems. The first one is required for reduction of the disulfide bond, the second one for their oxidation. Trx m is a central player for controlling the reduction state of PsaF. Trx m is reduced by FTR. Under SD conditions, Trx m associates to the thylakoid membrane and reduces CCDA which reduces via HCF164 finally PsaF. In LD conditions, reduced PsaF is oxidized by LTO1, which itself is oxidized by CCDA (blue arrows). 2-Cys-PRX attaches to the membrane, allowing the oxidation of CCDA. In addition, it can oxidize Trx m (blue arrows).

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