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# Plastid Terminal Oxidase (PTOX) protects photosystem I and not photosystem II against photoinhibition in *Arabidopsis thaliana* and *Marchantia polymorpha*

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## Abstract

The plastid terminal oxidase PTOX controls the oxidation level of the plastoquinone pool in the thylakoid membrane and acts as a safety valve upon abiotic stress, but detailed characterization of its role in protecting the photosynthetic apparatus is limited. Here we used PTOX mutants in two model plants *Arabidopsis thaliana* and *Marchantia polymorpha*. In *Arabidopsis*, lack of PTOX leads to a severe defect in pigmentation, a so-called variegated phenotype, when plants are grown at standard light intensities. We created a green *Arabidopsis* PTOX mutant expressing the bacterial carotenoid desaturase CRTI and a double mutant in *Marchantia* lacking both PTOX isoforms, the plant-type and the alga-type PTOX. In both species, lack of PTOX affected the redox state of the plastoquinone pool. Exposure of plants to high light intensity showed in the absence of PTOX higher susceptibility of photosystem I to light-induced damage while photosystem II was more stable compared with the wild type demonstrating that PTOX plays both, a pro-oxidant and an anti-oxidant role *in vivo*. Our results shed new light on the function of PTOX in protection of photosystem I and II.

## Introduction

In nature, plants have to cope with sudden changes in light intensity. Light intensities higher than needed for CO<sub>2</sub> assimilation saturate the photosynthetic electron transport chain and can cause oxidative stress leading to damage to the photosystems. Photosynthetic organisms have a multitude of regulation mechanisms to protect the photosynthetic apparatus in excess light. Among these protection mechanisms there are processes that lead to the dissipation of excess energy as heat,

down regulation of the photosynthetic electron transport and even the degradation and repair of photosystem II (PSII) that is sacrificed protecting photosystem I (PSI) against photodamage (Oguchi et al., 2021; Sonoike, 2011). PSII, different from PSI, has an efficient damage repair cycle so that damage of PSII is not very costly (Li et al., 2018). Alternative electron transport routes like cyclic electron flow and Mehler/Mehler-like reactions are important to protect PSI against photoinhibition (Alboresi et al., 2019). Furthermore, the plastid terminal oxidase PTOX has been shown to be an important sink for electrons upon stress (Stepien and Johnson, 2009; 2018; Streb et al., 2005). PTOX is a non-heme diiron carboxylate enzyme that catalyzes the oxidation of plastoquinol, reducing oxygen to water. Depending on the quinol availability, it can have an anti-oxidant or a pro-oxidant function as has been shown *in vitro* using recombinant PTOX protein (Yu et al., 2014). Upregulation of PTOX expression has been reported for several stress-tolerant plant species acclimated to harsh environments such as drought, high light and high temperature (Ibanez et al., 2010; Quiles, 2006), high salinity (Stepien and Johnson, 2009), low temperature (Ivanov et al., 2012; Streb et al., 2005), and high levels of UV light (Laureau et al., 2013). PTOX is believed to act as a stress-induced safety valve that keeps the acceptor side of PSII oxidized, thereby helping to protect PSII from photodamage (Ivanov et al., 2012; Quiles, 2006; Stepien and Johnson, 2009; Streb et al., 2005). However, overexpression of PTOX1 from *Chlamydomonas reinhardtii* promotes photoinhibition via the production of reactive oxygen species (Ahmad et al., 2020; Heyno et al., 2009) while overexpression of PTOX from *A. thaliana* does not alter the light susceptibility of PSII (Rosso et al., 2006). This may be caused either by a difference in the protein content in the two overexpression systems or by a difference in the regulation between alga- and plant-type PTOX isoforms.

The genome of angiosperms encodes one PTOX while most other photosynthetic organisms possess two isoforms that can either be closely related or belong to a plant-type or an alga-type family (Messant et al., 2021). PTOX mutants called *immutans* (*im*) in *Arabidopsis thaliana* and *ghost* in tomato show, depending on the light intensity, a very strong variegated phenotype (Wetzel et al., 1994). In angiosperms, PTOX is crucial for carotenoid biosynthesis (Carol et al., 1999). Plant carotenoid desaturases use plastoquinone (PQ) as electron acceptor and depend on PTOX for reoxidation of plastoquinol (PQH<sub>2</sub>) to PQ. Single mutants of PTOX in *Chlamydomonas reinhardtii* or in *Marchantia polymorpha* show normal pigmentation (Houille-Vernes et al., 2011; Messant et al., 2021).

The importance of PTOX for protection of the photosynthetic apparatus against photoinhibition remains unclear. Here we use PTOX mutants of *Arabidopsis* and *Marchantia* to study the susceptibility of the photosynthetic apparatus to high light stress. To overcome the strong impact on carotenoid biosynthesis in *im*, we crossed *im* with *Arabidopsis* expressing the bacterial carotene desaturase (CRTI) (Schaub et al., 2005). CRTI introduces conjugation in phytoene by adding four double bonds using oxygen as electron acceptor and, therefore, being independent of PQ. In addition, we generated double mutants of PTOXa and PTOXb in *Marchantia* to show the importance of PTOX in an organism that contains, different to angiosperms, flavodiiron enzymes allowing Mehler-like reaction as additional route of alternative electron flow and keeping the electron transport chain oxidized. Furthermore, we determined the enzymatic activity of both, PTOXa and PTOXb using an *in vitro* approach.

## Results

### **PTOX affects the redox state of the electron transport chain in *A. thaliana* and *M. polymorpha*.**

We crossed *im* with *Arabidopsis* expressing the bacterial carotene desaturase (CRTI) and used chlorophyll fluorescence induction curves and post-illumination fluorescence for screening the F2 generation (Fig. 1). Mutants lacking PTOX in the F2 generation of the *im*CRTI lines show homogeneously green leaves under normal growth light (Fig. 1) and also under fluctuating light (Supplemental Figure S1). There were no significant differences in chlorophyll a/b and chlorophyll/carotenoid ratio between wild type and *im*CRTI lines (Table S1). Two *im*CRTI lines were selected for this study hereafter called *im*CRTI\_1 and *im*CRTI\_2. Upon illumination with low intensity actinic light, in the wild type the fluorescence level reached within the first 10 s a steady state level very close to the minimum fluorescence level  $F_0$ , while both *im*CRTI lines showed a higher fluorescence level in the light (Fig. 1b). This shows that the plastoquinone pool is more reduced in the mutants as expected when PTOX activity is lacking. Moreover, the so-called post-illumination fluorescence rise is higher in *im*CRTI than in Col-0, reflecting a more reduced PQ pool due to the absence of PTOX activity and the loss of chlororespiratory activity.

To investigate whether in *Marchantia* lack of PTOX has the same effect on pigment composition and on the reduction state of the PQ pool as in *Arabidopsis*, we created mutants lacking the two PTOX isoforms PTOXa and PTOXb. *Marchantia*  $\Delta ptoxaptobx_1$  and  $\Delta ptoxaptobx_2$  construction is shown Fig. 2a. Different to *Arabidopsis*, PTOX mutants in *Marchantia* do not show a phenotype independent of the light regime (Fig. 2b). When the growth light intensity was doubled, all genotypes contained less chlorophyll and carotenoids (Fig. S2). No variegated phenotype was observed in the double mutants, showing that carotenoid biosynthesis was not affected in the absence of PTOX (SI Table 1) in contrast to the variegated phenotype reported in the *im* mutant. However, there was a clear difference in the acclimation to the higher growth light intensity. To challenge the photosynthetic apparatus even more, plants were grown in fluctuating light. The double mutants were not more stressed than the other genotypes, only the wild type, Tak-1, was a bit paler than the mutants (Fig. S2).

Both PTOX enzymes, PTOXa and PTOXb, contribute to keep the redox state of the PQ pool oxidized as shown by chlorophyll fluorescence induction upon illumination with actinic light (Fig. 2c). Fluorescence induction was measured by applying a moderate light intensity of red light ( $I=53 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ). At this light intensity, the maximum level of fluorescence was reached faster in the mutants compared with the wild type (Fig. 2c, inset). The single mutants  $\Delta ptoxa$  and  $\Delta ptobx$  showed a fast rise followed by a fluorescence decrease. This transient phase is lacking in the double mutants, and it can be attributed to the remaining PTOX activity in the single mutants. When instead a saturating flash was used like usually done for measuring OJIP fluorescence induction curves, there was no difference, indicating that the size of the PQ pool was the same in all genotypes. The absence of an effect in the mutants in a short saturating flash could be explained by a low activity of PTOX. When the light was switched off, the fluorescence level remained higher in  $\Delta ptoxaptobx_1$  showing that the reoxidation of  $\text{PQH}_2$  in the dark was retarded in the absence of PTOX. The faster fluorescence rise in the first second after onset of actinic light was observed in all mutants, also when only one PTOX isoform was lacking (Fig. 2d).

### Comparison of PTOXa and PTOXb recombinant proteins

We further studied the enzymatic properties of the two PTOX isoforms using recombinant protein and an *in vitro* activity assay. As shown in Figure S3, there are differences in the amino acid sequence of the two proteins, especially in respect to the length at the N-terminal site<sup>16</sup>. In the catalytic site, starting from  $\alpha$ -helix 1, fully conserved residues represent 64% of the total sequences whereas conservative and semi-conservative exchanges are 26% and 6%, respectively. We used purified recombinant PTOXa and PTOXb proteins to determine the maximum catalytic activity of the two

isoforms using saturating concentrations of decylplastoquinol as substrate (Table 1). For PTOXb, the N-terminal extension had been removed since the full-length gene was not expressed in *E. coli*. The enzyme activity of PTOXa was 3 times higher than that of PTOXb.

### **PTOX activity affects the quantum efficiency of PSI**

PTOX competes to a certain extent with linear electron flow from PSII via the PQ pool, the cytochrome *b<sub>6</sub>f* complex and plastocyanin to PSI. Since PTOX oxidizes PQH<sub>2</sub>, it reduces at the same time the number of electrons available for reduction of P700<sup>+</sup>. The number of available electrons determines the level of the so-called donor-side limitation of PSI, Y(ND). When *Marchantia thalli* were exposed to fluctuating light intensities, Y(ND) was high at high light intensities and low at low light intensities independent of the genotype (Fig. 3a). However, the switch from high Y(ND) to low Y(ND) was immediate in the double mutant while this transition was slower in Tak-1, indicating that PTOX oxidizes partly the PQ pool and limits electron donation upon the change from high light to low light. Similar results were obtained for all mutants (Fig. S4). Accordingly, less donor-side limitation (Y(ND)) was observed in the mutants when plants were illuminated with increasing light intensity (Fig. 3b). Each intensity was applied for 3 min before the measurement was taken to ensure that the photosynthetic apparatus was acclimated to the given light intensity. At all light intensities, there was less donor-side limitation in the double mutant than in the single mutant while Tak-1 showed the highest Y(ND). The lower donor side limitation in the mutants resulted in a higher PSI yield (Fig. 3c), while the effective quantum yield of PSII (Y(II)) was the same in all genotypes (Fig. 3d). Acceptor-side limitation (Y(NA)) was very low in all genotypes (Fig. S5) thanks to the presence of flavodiiron proteins (Shimakawa et al., 2017). No differences in non-photochemical quenching (NPQ) were found in the different genotypes (Fig. S5).

### **PTOX protects PSI from photoinhibition and promotes photoinhibition of PSII**

Next, we investigated the protective effect of PTOX against light-induced damage of the two photosystems. Both, *Arabidopsis* leaves and *Marchantia* thalli, were exposed for 3 h to high light (1250  $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ) and photoinhibition of PSI was followed as a loss in the absorption signal at 830 nm, the so-called Pm value, while photoinhibition of PSII was monitored by the loss of the maximum quantum yield of PSII (Fv/Fm). At this light intensity, donor-side limitation of PSI was higher in the wild types than in the mutants (Fig. S6). PSI was more susceptible to photoinhibition in *imCRT1* lines and in  $\Delta\text{ptoxa}\Delta\text{ptoxb}$  mutants (Fig. 4a, c) than in the respective wild types. The opposite was observed for PSII (Fig. 4b, d). PSII was more damaged in respective wild types than in the PTOX lacking mutants. This may be due to the pro-oxidant role of PTOX that has been previously observed in tobacco overexpressing PTOX (Ahmad et al., 2012, 2020; Heyno et al., 2009). In *Marchantia*, PTOX single mutants showed no increase in PSII photoinhibition compared with the double mutants, showing that one PTOX isoform is enough to allow sufficient accumulation of P700<sup>+</sup> that protects against the destruction of the FeS clusters in PSI (Sonoike et al., 1995). On the other hand, one PTOX isoform was also sufficient to protect PSII.

## **Discussion**

The *Arabidopsis imCRT1* lines are homogeneously green and grow well in high light. This makes this mutant an ideal tool to study the effect of PTOX activity on photosynthetic electron transport in angiosperms. Previously the study of PTOX activity in angiosperms was limited to plants grown at low light intensity, a growth condition that permits to avoid the variegated phenotype (Trouillard et al., 2012). Despite in angiosperms no knockout mutants of PTOX have been reported in another

organism of the green lineage. In *Chlamydomonas* a mutant of PTOX2 had been obtained while no mutant of PTOX1 could be isolated (Houille-Vernes et al., 2011). The double mutant in *Marchantia* is the first mutant that lacks all PTOX activity in a plant besides angiosperms.

*In vivo* experiments showed a higher PTOX activity in *C. reinhardtii* (Houille-Vernes et al., 2011) than in the vascular plant tomato (Trouillard et al., 2012) or in *Eutrema salsugine* (Stepien and Johnson, 2009). In *M. polymorpha*, *in vivo*, the deletion of PTOXb has a larger effect on the redox state of the PSII acceptor side than that of PTOXa (Messant et al., 2021). This difference cannot be explained by a difference in the activity of the enzymes but rather by differences in regulation of the enzyme activities. Both purified recombinant PTOXa and PTOXb proteins from *M. polymorpha* showed a higher activity (Table 1) than has been previously reported for rice PTOX (Yu et al. 2014). Here and in the previous study on OsPTOX, the enzymes were expressed as MBP fusion proteins to ensure their solubility. A  $V_{max}$  for MBP-OsPTOX of  $16.6 \pm 0.8 \mu\text{mol O}_2 \text{ mg}^{-1} \text{ min}^{-1}$  has been determined using soluble decyl-PQ at saturating concentration as substrate, while here, using the same enzymatic assay, a 3-fold higher  $V_{max}$  was determined for the truncated MBP-MpPTOXb and a 10-fold higher  $V_{max}$  for MBP-MpPTOXa (Table 1). The difference in activity between the MpPTOX isoforms may be caused by the changes in the amino acid sequence that may affect the substrate accessibility to the catalytic center (Fig. S3). It seems rather unlikely that the N-terminal extension of MpPTOXb affects the catalytic activity in the *in vitro* assay since the helices harboring the catalytic center are unaltered. The N-terminal extension of MpPTOXb may be important for anchoring PTOX permanently to the membrane as has been suggested by Nawrocki et al. (2015). MpPTOXa, the enzyme that is more closely related to angiosperm PTOX isoforms (Messant et al., 2021) may be more loosely attached to the membrane. PTOX in angiosperms has been shown either to migrate within the thylakoid membrane from stroma lamellae to grana stacks (Stepien and Johnson, 2018) or to associate to the thylakoid membrane only in the presence of a high proton motive force (Bolte et al., 2020). Anchoring of the enzyme to the membrane is required for the access to its membrane-localized substrate PQH<sub>2</sub> and PTOXa is supposed to be regulated via relocalization to the membrane under conditions when photosynthetic electron transport is saturated.

In the literature, PTOX is discussed as a safety valve that protects the photosynthetic apparatus and especially PSII upon exposure of plants to oxidative stress conditions such as high light, high salinity, extreme temperatures (Stepien and Johnson, 2009; 2018; Quiles, 2006). However, as shown in Fig. 4 for both, *Arabidopsis* and *Marchantia*, PSII is more susceptible to photoinhibition in the wild types than in the PTOX mutants while the opposite was observed for PSI. Increase in donor-side limitation to PSI and a high level of P700<sup>+</sup> accumulation is known to protect PSI against photoinhibition (Sejima et al., 2014; Cerqueira et al., 2019). We note that the PTOX mutants still can sustain a high level of P700 oxidation, which should be due to other electron sinks such as Calvin-Benson cycle and flavodiiron proteins (Shimakawa et al., 2017). More surprising than the protective effect of PTOX activity on PSI is the higher PSII damage observed in the wild types compared with the mutants. The higher damage of PSII suggests that a larger amount of reactive oxygen species is generated in wild-type plants than in the mutants. It has been shown that overexpression of PTOX1 from *Chlamydomonas reinhardtii* in tobacco promotes oxidative stress, especially the production of superoxide anion radicals (O<sub>2</sub><sup>•-</sup>) (Heyno et al., 2009). In this overexpressor PSII is highly susceptible to photoinhibition (Ahmad et al., 2012; 2020; Heyno et al., 2009). *In vitro*, as a function of the decyl-plastoquinol concentration and pH-value, PTOX has an anti-oxidant or a pro-oxidant role (Yu et al., 2014). At slightly alkaline pH (pH 8.0), PTOX activity favors the production of O<sub>2</sub><sup>•-</sup> when the substrate concentration is high. O<sub>2</sub><sup>•-</sup> production at pH 8.0 is likely caused by the pH-dependent stabilization of the semiquinone PQ<sup>•-</sup> that reduced O<sub>2</sub> to O<sub>2</sub><sup>•-</sup> or by the peroxy intermediate of the Fe-Fe cluster. When the substrate concentration is low, PTOX has an anti-oxidant effect lowering the yield of O<sub>2</sub><sup>•-</sup>

production. This said, PTOX only promotes  $O_2^{\bullet-}$  production when the PQ pool is highly reduced. This is the case at high light intensities largely exceeding the light required for photosynthetic electron transport.

## Conclusion

According to the data presented here, PTOX plays an important role under high light stress, protecting PSI against photoinhibition. Thanks to its PQH<sub>2</sub> oxidizing activity removing thereby electrons from the electron transport chain. In high light, less electrons are available to reduce P700<sup>+</sup>. At the same time, lack of PTOX activity protects PSII against photoinhibition. The negative impact of PTOX on PSII stability in high light is most likely caused by its pro-oxidant role leading to  $O_2^{\bullet-}$  production. Overall, it is beneficial for the plant to sacrifice PSII upon high light stress. Photoinhibition of PSII protects PSI against photoinhibition since it limits electron donation and favours the accumulation of P700<sup>+</sup>. Sonoike has proposed that PSII photoinhibition can be regarded as a protection mechanism for PSI (Sonoike, 2011). PSI photoinhibition is irreversible in the short-term compared with the efficient damage repair cycle of PSII (Li et al., 2018).

## Material and Methods

### Plant material

*Arabidopsis thaliana*. *Arabidopsis thaliana* (Col-0), the PTOX mutant *im*, a transgenic *Arabidopsis* line expressing *CRTI* under 35S promoter control as described in Schaub *et al.*, 2005 here called CRTI, and two lines of crosses *im* x CRTI were grown for 3-4 weeks in soil under a light-dark cycle (16 h-light, 22 °C, 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , white fluorescent lamp/8 h-dark, 20 °C). The lines of the F2 generation which showed no PTOX activity were named *imCRTI\_1* and *imCRTI\_2*. RT-PCR showed that the expression level is low in these lines (Fig. S7)

*Marchantia polymorpha*. A male accession of *M. polymorpha*, Takaragaike (Tak)-1 and all mutants were asexually cultured on one-half-strength Gamborg's B5 agar medium (Gamborg et al., 1968) in the same light conditions as used for *Arabidopsis*. For physiological measurements, 2-3 weeks old thalli were used.

### RT-PCR

For quantitative RT-PCR analysis, plants were subjected to 16 h-light (22 °C, 100  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ , white, fluorescent lamp/8 h-dark, 20 °C) for 2 weeks. Total RNAs were extracted using the Plant Mini RNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions and quantified using a NanoDrop Spectroscopy (Nanodrop 2000, Ozyme, Saint-Cyr- l'École, France). To perform RT reactions, the SuperScript IV Reverse Transcriptase kit (Invitrogen, Carlsbad, USA) was used. qPCR was done using a LightCycler 96 Instrument (Roche, Penzberg, Germany). cDNA was diluted three times with LightCycler 480 SYBR Green I Master (Roche, Penzberg, Germany) reaction medium (1X final concentration) containing 250 nM of each primer (Table S2). mRNA levels were normalized to two reference housekeeping genes (UBIQUITIN CARRIER PROTEIN 21 and YELLOW-LEAF-SPECIFIC GENE). Three technical replicates were performed for each qPCR.

### Genome editing by CRISPR/Cas9 system

The construction of plasmids allowing the transformation of *M. polymorpha* by the CRISPR/Cas9 method was carried out according to the method of Sugano *et al.*, 2014. Six target sequences of interest, three for *PTOXa* and three for *PTOXb*, were inserted into pMpGE\_En03 vectors which were

transferred into pMpGE010 or pMpGE011 vectors by gateway reaction. Each of the  $\Delta ptoxa$  and  $\Delta ptoxb$  single mutants previously characterized (Messant et al., 2021) were transformed with the vectors to mutate the remaining intact PTOX gene. The final transformation of the plants was carried out using the Agar-Trap method (Tsuboyama et al., 2014; 2018) on thalli and gemmae. After sequencing, two double mutants were obtained from the single  $\Delta ptoxb$  mutant transformed with a *PTOXa* gene targeting sequence: CCTATGGAGGCTCTAGG. These different mutants do not differ in the location but in the type of mutation caused by Cas9 (see Fig. S8 for more details). Throughout the manuscript, these two double mutants are called  $\Delta ptoxaptox_{b_1}$  and  $\Delta ptoxaptox_{b_2}$ .

### **Vector constructions and MBP-MpPTOXa and MBP-MpPTOXb $\Delta$ Nter expression, purification from *E. coli* and enzymatic assay**

*PETM-40\_PTOXa construct.* The synthetic optimized gene (Eurofins) of *PTOXa* (Mp3g12150.1) from *Marchantia* was amplified by PCR with the primers [F-PTOXa-Nco1 and R-PTOXa-Xho1]. The amplified fragment was digested with Nco1 and Xho1 and ligated with Nco1/Xho1-digested PETM-40 to produce the vector PETM-40\_PTOXa.

*PETM-40\_133-257-PTOXb (PETM-40\_ΔNter-PTOXb) construct.* First, *PTOXb* gene (Mp7g11430.1) from *Marchantia* was amplified by PCR using the cDNA of Tak-1 as template and the primers [F-PTOXb and R-PTOXb]. The amplified fragments were cloned in PETM-40 vector linearized with Nco1 and Xho1 using the In-Fusion HD Cloning Kit (TaKaRa) to generate the vector PETM-40\_PTOXb. Then, a deletion of a large part of a sequence encoding the N terminal domain (33–257) of the *PTOXb* gene (Mp7g11430.1) were introduced by site-directed mutagenesis using the plasmid PETM-40\_PTOXb as template and synthetic primers F-PET-PTOXb Del and R-PET-PTOXb Del. All plasmids were checked by sequencing. List of primers in SI Table S3. The obtained plasmids were used to transform BL21 (DE3) *E. coli* cells. 20 mL of overnight cultures was inoculated into 1L of LB, grown at 37 °C to an OD 600 nm between 0.6 and 0.8 and induced with IPTG. After induction overnight at 16 °C, the cells were harvested, washed in buffer A (25 mM sodium-phosphate buffer pH 7.6, MgCl<sub>2</sub> 2.5 mM, NaCl 300 mM, glycerol 15 % V/V) and frozen at –80 °C. Purification was carried out on ice. Cells were resuspended in buffer A with DNase, PMSF and lysozyme and sonicated. After centrifugation at 28,000 g for 45 min the supernatant was solubilized for 30 min on ice by slowly adding 6 X CMC n-octyl β-D-glucopyranoside (nOG; 1 X CMC = 25 mM) and then applied to Amylose resin. After washing thoroughly with buffer A containing 2 X CMC nOG; the elution was accomplished with buffer B (50 mM Tris–HCl pH 8.0, MgCl<sub>2</sub> 2.5 mM, glycerol, 10 % V/V, 2 X CMC nOG and 10 mM maltose). Proteins resolved by SDS-PAGE were detected using Coomassie Brilliant Blue. Protein quantification was done by using the Bradford reagent. The activity of recombinant PTOX was measured as described in Yu et al. 2014.

Activity was measured using a coupled enzymatic assay. PQ was reduced to PQH<sub>2</sub> by human DT-diaphorase and PQH<sub>2</sub> oxidized by PTOX. Standard assay conditions containing 10 μg protein per 700 μl assay volume, 100 μM decyl-plastoquinone, 200 μM NADH, 10 μg human DT-diaphorase (Sigma-Aldrich), 50 mM Tris–HCl pH 8.0, 2.5mM MgCl<sub>2</sub> were used. Activities were determined by measuring O<sub>2</sub> consumption with a Clark-type electrode.

### **Chlorophyll fluorescence and P700 measurements**

Chlorophyll *a* fluorescence and P700 absorption was measured using a Dual-PAM-100 fluorometer (Walz, Effeltrich, Germany). Chlorophyll fluorescence curves were measured using a low actinic light



(38  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ). Post illumination fluorescence rise was measured after illumination of *Arabidopsis* leaves for 10 min at 530  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ . PSII and PSI parameters were determined as described in Messant et al. (2021). Pm is the maximal P700 signal upon transformation of P700 from the fully reduced to the fully oxidized state. The following definitions were used according to Klughammer and Schreiber (1994): Effective quantum yield of PSII:  $Y(II)=(F_m'-F)/F_m'$ ; Yield of donor side limitation:  $Y(ND)=1-P700_{red}$ ; PSI quantum yield:  $Y(I)=1-Y(ND)-Y(NA)$ .

### Photoinhibition

*Arabidopsis* leaves and *Marchantia* thalli were exposed to high light (1250  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ) for 3h. Samples were kept for 15 min at room light (8  $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ ) before determining the maximum value for P700 absorption, Pm, and the maximum quantum yield of PSII, Fv/Fm.

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### Author Contribution

M.M, G.S. and A.K-L. designed the project. M.M, U.H.,T.-L.L., A.W, G.S. and A.K-L. performed the experiments and analyzed the data. A.K-L. wrote the initial version of the manuscript that was read and revised by all authors.

### Data availability

Data will be made available on demand.

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**Table 1**

Activity of purified recombinant MBP-MpPTOXa and MBP-MpPtoxb- $\Delta Nter$ .

Sample	Activity ( $\mu\text{mol O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ )
MBP-MpPTOXa	149 +/- 16
MBP-MpPtoxb- $\Delta Nter$	46 +/- 6

The assay contained 10  $\mu\text{g}$  protein per 700  $\mu\text{l}$  final volume, 100  $\mu\text{M}$  decyl-plastoquinone, 200  $\mu\text{M}$  NADH, 10  $\mu\text{g}$  DT-diaphorase, 50 mM Tris-HCl pH 8.0, 2.5 mM  $\text{MgCl}_2$ . Activities were determined by measuring  $\text{O}_2$  consumption at 25°C with a Clark-type electrode.

## Figure Legends

Figure 1. Characterization of *Arabidopsis im* x CRTI lines. **a.** The photo shows 4-week-old plants, wild-type Col-0, PTOX mutant *im*, plant-expressing bacterial desaturase CRTI and *imCRTI\_1* mutant; grown in long day conditions at  $100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ . **b.** Chlorophyll fluorescence induction curves of Col-0 and the *imCRTI\_1* and *imCRTI\_2* lines. Fv/Fm values for Col-0:  $0.853 \pm 0.001$ ; *imCRTI\_1*:  $0.850 \pm 0.012$ ; *imCRTI\_2*:  $0.851 \pm 0.023$ . **c.** Post-illumination fluorescence rise of Col-0 and both *imCRTI* lines after 10 min actinic light ( $530 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ). The flash indicates turning of the actinic light. Representative curves are shown (n=4 for each genotype).

Figure 2. Characterization of *Marchantia polymorpha* PTOX mutant lines. **a.** Design of the PTOX mutants using CRISP/Cas9 technology. *Marchantia* has a "plant-type" and an "alga-type" PTOX (PTOXa and PTOXb respectively). Two membrane-associated (red) and four  $\alpha$ -helices which contain the six iron binding site motifs necessary for the catalytic activity (green) are shown in colored bars. PTOXB possesses a long Nter part. The double mutants were generated by the transformation of  $\Delta\text{ptoxb}$ . All mutants have frameshift mutation in PTOX genes, resulting in shorter polypeptides of PTOX (see details in Materials and Methods and Fig. S3). **b.** Images of the different genotypes grown for three weeks at  $100 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$  and at  $200 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ . **c.** Chlorophyll fluorescence induction curves measured at low actinic light ( $I=53 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ). Arrows indicate the set on/off of the measuring light and of the actinic light. The inset shows a zoom of the fluorescence change upon the onset of the actinic light. Black: Tak-1, blue:  $\Delta\text{ptoxa}$ , pink:  $\Delta\text{ptoxaptox}_1$ . **d.** Fluorescence induction curves upon onset of actinic light for all genotypes starting from the  $F_0$  level corresponding to the zoom in e. Fv/Fm values for Tak-1:  $0.766 \pm 0.001$ ;  $\Delta\text{ptoxa}$ :  $0.774 \pm 0.003$ ;  $\Delta\text{ptoxb}$ :  $0.775 \pm 0.001$ ;  $\Delta\text{ptoxaptox}_1$ :  $0.764 \pm 0.016$ ;  $\Delta\text{ptoxaptox}_2$ :  $0.744 \pm 0.012$ . Representative curves are shown (n=4 for each genotype).

Figure 3. Chlorophyll fluorescence and P700 absorption parameters measured in *Marchantia*. **a.** Changes in PSI donor-side limitation Y(ND) upon illumination with fluctuating light. Light intensity was altered between  $55 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$  (LL) and  $850 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$  (HL). For clarity, only values of a single representative measurement for Tak-1 (black squares) and the double mutant line  $\Delta\text{ptoxaptox}_1$  (pink triangle) are shown. **b.** PSI donor-side limitation Y(ND) as a function of the light intensity. **c.** Quantum yield of PSI, Y(I), as a function of the light intensity. **d.** Effective quantum yield of PSII, Y(II) as a function of the light intensity. B-D: Three replicates from four different independently grown sets of plants, n=12, mean and SD are given.

Figure 4. Photoinhibition of PSI and PSII in *Arabidopsis* (**a, b**) and *Marchantia* (**c, d**). Leaves and thalli were exposed to high light ( $1250 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ ) for 3h. Left, percentage of the loss of maximum absorption change of P700 (Pm) compared to the Pm value prior to the photoinhibition treatment. Fv/Fm values prior to the photoinhibitory treatment for Col-0 were:  $0.813 \pm 0.006$ , *imCRTI\_1*:  $0.814 \pm 0.011$ , *imCRTI\_2*:  $0.809 \pm 0.012$  and for *Marchantia* Tak-1:  $0.766 \pm 0.005$ ,  $\Delta\text{ptoxa}$ :  $0.767 \pm 0.011$ ,  $\Delta\text{ptoxb}$ :  $0.766 \pm 0.008$ ,  $\Delta\text{ptoxaptox}_1$ :  $0.769 \pm 0.06$ ,  $\Delta\text{ptoxaptox}_2$ :  $0.758 \pm 0.016$ . Three replicates from four different independently grown sets of plants, n=12, mean and SD are given. Stars indicate significant differences, compared to the control condition, based on a Mann and Whitney test (\* p<0.05, \*\* p<0.01).