The function of PROTOPORPHYRINOGEN IX OXIDASE in chlorophyll biosynthesis requires oxidised plastoquinone in *Chlamydomonas reinhardtii*

Pawel Brzezowski, Brigitte Ksas, Michel Havaux, Bernhard Grimm, Marie Chazaux, Gilles Peltier, Xenie Johnson & Jean Alric

In the last common enzymatic step of tetrapyrrole biosynthesis, prior to the branching point leading to the biosynthesis of heme and chlorophyll, protoporphyrinogen IX (Protogen) is oxidised to protoporphyrin IX (Proto) by protoporphyrinogen IX oxidase (PPX). The absence of thylakoid-localised plastid terminal oxidase 2 (PTOX2) and cytochrome *b*₆ 若要 complex in the *ptox2 petB* mutant, results in almost complete reduction of the plastoquinone pool (PQ pool) in light. Here we show that the lack of oxidised PQ impairs PPX function, leading to accumulation and subsequently uncontrolled oxidation of Protogen to non-metabolised Proto. Addition of 3(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) prevents the over-reduction of the PQ pool in *ptox2 petB* and decreases Proto accumulation. This observation strongly indicates the need of oxidised PQ as the electron acceptor for the PPX reaction in *Chlamydomonas reinhardtii*. The PPX-PQ pool interaction is proposed to function as a feedback loop between photosynthetic electron transport and chlorophyll biosynthesis.
The tetrapterrole biosynthesis (TBS) pathway leads to biosynthesis of chlorophyll, heme, and siroheme, which are indispensable components of cell metabolism, including energetic processes, such as chloroplast-localised photosynthesis and mitochondrial respiration. In photosynthetic organisms, several enzymatic steps of TBS lead to the biosynthesis of protoporphyrin IX (Proto), which is a common substrate for ferrochelatase (FeCh) and Mg-chelatase (MgCh), two enzymes at the TBS branching point, dedicated to biosynthesis of heme and chlorophyll, respectively. Biosynthesis of Proto is catalysed by protoporphyrinogen IX (Protogen) oxidase (PPX in *C. reinhardtii*, alias PPOX), which removes six electrons and protons from Protogen. However, no information is available on which component accepts the electrons from Proto during oxidation in eukaryotic photosynthetic organisms.

In eukaryotic organisms PPOX belongs to the FAD-containing HemY-type protein family and in plants it is encoded by two nucleus-localised homologous genes, PPOX and PPOX2. PPOX1 is targeted exclusively to plastids, providing Proto for heme and chlorophyll synthesis, while PPOX2 was found in plastid envelope and mitochondria in spinach. However, *N. tabacum* PPOX2 was shown to be solely a mitochondrial protein. In *C. reinhardtii*, PPX is encoded by a single gene and was shown to be targeted exclusively to plastids. Interactions of PPOX with other TBS enzymes, regulatory proteins, or electron acceptors, have not been reported so far.

Photosynthesis relies on a balanced linear electron transfer between photosystem II (PSII), cytochrome bf (cyt bf), and photosystem I (PSI), producing O₂ at the PSI donor side and reducing NADP⁺ at the acceptor side of PSI in the light. The plastoquinone oxidase (PQ) serves as the electron carrier between PSI and cyt bf. In darkness, the linear electron transfer is inactive, but PQ is reduced non-photochemically to plastocyanin in a process called chlororespiration. Plastid terminal oxidase (POTOX), located on the stromal side of the thylakoid membrane, utilises its di-iron centre to oxidise PQH₂ in conjunction with reduction of oxygen to water. Thus, the PQ pool in the *ptox2* mutant is mostly reduced even in the dark. The plastid-localised *PetB* gene encodes cyt bf, a component of the cyt bf complex. The *ptox2 petB* double mutant of *C. reinhardtii* shows a completely photochemically reduced PQ pool in light, due to the electron flow from PSI and a blockage in the linear electron transfer.

Based on the study of *ptox2 petB*, we show that the deficiency in oxidised PQ leads to impairment in TBS, with a pronounced accumulation of Proto, which results from compromised function of PPX. Inhibition of an enzyme usually induces accumulation of the substrate and depletion of the product of the reaction. However, in the case of PPX, it was demonstrated previously that its substrate Proto does not accumulate because it is non-specifically oxidised to Proto, which accumulates as an end-product.

### Results

**DCMU treatment increases light tolerance in *ptox2 petB***

Although two genes encode PTOX in *C. reinhardtii*, PTOX2 was demonstrated to be the major oxidase involved in chlororespiration. To demonstrate the photosynthetic electron transport (PET) capacity in our mutant strains, selected protein accumulation was determined in *ptox2, petB, ptox2 petB*, and the double mutant rescued with the wild-type version of PTOX2, designated *ptox2-R petB*. The *ptox2* mutant is completely devoid of PTOX2, while *petB* lacks cyt b₆. Consequently, *ptox2 petB* is deficient both in PTOX2 and cyt b₆. Because cyt b₆ is an essential subunit of the cyt b₆/f complex, the lack of PetB leads to the absence of cyt b₆/f and it was shown that the synthesis of cyt f, another component of cyt b₆/f, depends on the presence of cyt b₆/subunit IV (PetD) precomplex. Thus, in the present study cyt f was used as an additional control, to confirm the absence of cyt b₆/f (Fig. 1a).

The type II NAD(P)H dehydrogenase (NDA2) is a component involved in chlororespiration in *C. reinhardtii*. As demonstrated by immunoblot, the NDA2 content was similar in all of the mutants examined here (Fig. 1a), which indicates that the chlororespiration process is affected only due to the absence of PTOX2.

The photosynthetic phenotype of the mutants was determined on agar-solidified photoautotrophic medium (tris-phosphate, TP) and compared to growth on heterotrophic medium (tris-acetate-phosphate, TAP). The growth of *ptox2* was similar to WT in all tested conditions (Fig. 1b). However, due to the blockage of electron transfer in PET (Fig. 1c and Supplementary Fig. 1), mutants lacking cyt b₆/f are not able to grow on TP (Fig. 1b). The *ptox2 petB* mutant showed increased light sensitivity on TAP, compared to single *ptox2* or *petB*, or rescued *ptox2-R petB*. The growth of *ptox2 petB* was arrested on TAP at up to 80 µmol photons m⁻² s⁻¹, i.e., two times more than in the absence of DCMU (Fig. 1b). DCMU blocks electron transport at the acceptor side of PSI, observed as a decrease of PSII (Fig. 1c), leading to charge recombination in PSI and generation of O₂⁻. Thus, the increased light tolerance of the double *ptox2 petB* mutant does not reflect a released inhibition of PSI (see control in Fig. 1c) and, generally, it cannot be explained by the direct effect of DCMU treatment on PET.

**Accumulation of Proto in *ptox2 petB* is prevented by DCMU.**

The TBS pathway consists of several highly-regulated steps (Fig. 2). The disturbance of any of these steps usually causes accumulation or deficiency in intermediates and affects the content of the end-products, resulting in altered pigmentation. When grown in TAP-liquid cultures (Fig. 3a), or upon prolonged growth on agar-solidified TAP (not visible on Fig. 1b), the general appearance of *ptox2 petB* was different than *ptox2, petB*, or wild type. The double mutant showed a pale green/yellow phenotype, with a brownish precipitate accumulating in the media (Fig. 3a).

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**The pigment content, including the TBS intermediates and end-products** (Fig. 2) were determined by High Pressure Liquid Chromatography (HPLC) in *ptox2 petB, ptox2, petB, ptox2-R petB*, and wild type. Cultures were grown in TAP either in dark or at 20 µmol m⁻² s⁻¹ light. Additional samples in the same light conditions were treated with DCMU. Proto accumulated in *ptox2 petB* >86-fold compared to *petB*, while it was not detectable in wild type or *ptox2* (Fig. 3b).

Interestingly, treatment with DCMU prevented accumulation of Proto in the media (Supplementary Fig. 2b) and decreased Proto content in the *ptox2 petB* cells to values observed in *petB* or wild type (Fig. 3b).

To test whether Proto accumulation can be also observed in other mutant lines with over-reduced PQ pool, the Proto content was determined in the double mutant devoid of PTOX2 and plastocyanin, *ptox2 pcy*. Over-reduction of the PQ pool in *ptox2*
the absence of cyt b6f and the parameter was used to demonstrate photochemical quenching in cells treated with DCMU (WT, ptox2 petB).

Because non-photosynthetic mutants devoid of cyt b6f do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not synthesize ATP in the light, while certain enzymes of the TBS pathway were shown to require ATP, plastocyanin do not 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the wild-type strain was treated with the PPX inhibitor oxyfluorfen\textsuperscript{42,43}, which resulted in a pale-green/yellowish phenotype (Fig. 3c) and accumulation of Proto already after 24 h in TAP-liquid culture exposed to 20 µmol m\textsuperscript{-2} s\textsuperscript{-1} light (Fig. 3d). Thus, the inhibition of the PPX activity in wild type by oxyfluorfen resulted in a similar Proto accumulation as in \textit{ptox2 petB} without chemical treatment (Fig. 3b).

To get a deeper insight into the effect of the lack of PTOX2 and cyt \textit{b6f} complex, more in-depth analysis of TBS intermediates, end-products, and selected pigments were performed in the double mutant in comparison to wild type treated with oxyfluorfen. It was determined that the entire TBS pathway was deregulated in the wild-type cells treated with oxyfluorfen as well as in \textit{ptox2 petB} (Supplementary Fig. 4). Oxyfluorfen-treated cells accumulated Zn-protoporphyrin (ZnProto), which was not found in the absence of the PPX inhibitor. ZnProto might be formed from Proto, which accepts divalent cations, due to the massive accumulation of this intermediate following oxyfluorfen treatment. It has been observed before that FeCh has a high affinity not only for Fe\textsuperscript{2+}, but also for Co\textsuperscript{2+}, Zn\textsuperscript{2+}, Ni\textsuperscript{2+}, or Cu\textsuperscript{2+}, leading to the formation of the respective metalloporphyrins in vitro, although with FeCh-inhibitory consequences\textsuperscript{44}. Thus, the Zn\textsuperscript{2+} chelation in our experiment in vivo might be due to Fe\textsuperscript{2+} becoming a limiting factor in protoheme biosynthesis (see Fig. 2 for the reference). Indeed, heme levels were lower in \textit{ptox2 petB} compared to other strains grown in the light (Supplementary Fig. 4h), as well as in oxyfluorfen-treated wild type, compared to non-treated cells (Supplementary Fig. 4h). Because it was demonstrated that phytoene desaturase, an enzyme involved in carotenoid biosynthesis, depends on PQ\textsuperscript{45,46}, the content of \textit{β-carotene} was also determined in \textit{ptox2 petB} and wild type treated with oxyfluorfen. Both strains showed a similar decrease in \textit{β-carotene} levels (Supplementary Fig. 4g).

Subsequently, the PPX content was determined in \textit{ptox2 petB}, \textit{ptox2 petB}, and the rescued \textit{ptox2-R petB}. The antibody against PPX immunoreacted with two proteins with an apparent molecular weight of 55 and 59 kDa, as it was previously reported in spinach\textsuperscript{4} and tobacco\textsuperscript{5}. In plants, these protein bands were previously associated with two isoforms, which are either alternatively localised in plastids (PPOX1) or in plastids and mitochondria (PPOX2)\textsuperscript{47,48}. Interestingly, although \textit{C. reinhardtii} possesses only one PPX1 gene, two immune-reacting protein bands were also detected, consistently with previous work of van Lis and coworkers\textsuperscript{3}, but with independently-developed PPX antibody. All strains tested contained similar levels of these two immune-reacting PPX variants, indicating that one protein band potentially corresponds to posttranslationally modified form of PPX, with possible degradation products detected as two additional faint bands, below 55 kDa in \textit{ptox2 petB} (Fig. 3e).

Changes in steady-state levels of tetrapyrrole metabolites can be caused by deregulated or impaired 5-aminolevulinic acid (ALA; Fig. 2) synthesis, the rate limiting step of TBS. ALA synthesis capacity in the dark was similar in \textit{ptox2 petB} compared to \textit{ptox2 petB}, \textit{ptox2-R petB}, and wild type (Supplementary Fig. 5a). Compared to wild type, higher ALA formation was detected in all of the mutants exposed to 20 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} (Supplementary Fig. 5a), which in \textit{ptox2} resulted in higher chlorophyll and heme content compared to wild type (Supplementary Fig. 4e, h). Exposure to 40 µmol photons m\textsuperscript{-2} s\textsuperscript{-1} decreased ALA synthesis in \textit{petB}, \textit{ptox2 petB}, and \textit{ptox2-R petB} (Supplementary Fig. 5b). Thus, altered ALA synthesis rates in the mutants devoid of cyt \textit{b6f} cannot be responsible for Proto accumulation in \textit{ptox2 petB}.

Notably, DCMU treatment increased chlorophyll content in \textit{ptox2 petB} when compared to non-treated \textit{ptox2 petB}, which is indicative that DCMU rescues the phenotype in the double
bands were detected in Supplementary Fig. 6. The HPLC analyses were performed in biological triplicates (n = 3); horizontal bars represent the calculated mean, vertical error bars represent the standard deviation. The source data underlying the graphs is included in the Supplementary Table 1.

**Discussion**

Houille-Vernes and co-workers demonstrated that ptox2 petB shows almost complete reduction of the PQ pool in light9. As shown in the present study, over-reduction of the PQ pool is accompanied by accumulation of Proto, resulting from impaired function of PPX responsible for controlled Proto oxidation. However, because Proto is a substrate of MgCh and FeCh, we examined whether impairment in these enzymatic steps could be responsible for the phenotype in ptox2 petB. Hypothetically, the impairment of MgCh could be twofold. First, the over-reduced PQ pool may directly affect MgCh function. Second, in phosphorylation studies it was proposed that certain TBS subunits and PPX, may be regulated by phosphorylation24,49. Protein phosphorylation was confirmed experimentally for the integral MgCh subunit CHLD of C. reinhardtii and Oryza sativa21, as well as for the regulatory protein GUN4 of A. thaliana, which is phosphorylated in the dark to halt chlorophyll synthesis50. In the light, neither the cyt b6f-deficient mutants petB, ptox2 petB nor the ATPase-deficient fud50 mutant produce ATP in the chloroplast, but Proto accumulation was only observed in the double mutant ptox2 petB (compare Fig. 3b and Supplementary Fig. 3c). We therefore conclude that, although PPX activity may be regulated by phosphorylation24,49, it requires oxidised PQ as an electron acceptor for Proto accumulation. Moreover, addition of DCMU does not increase ATP levels but prevents Proto accumulation in ptox2 petB, because it did not show deficiency in MgProto (Supplementary Fig. 4a). Moreover, addition of DCMU does not increase ATP levels but prevents Proto accumulation in ptox2 petB. In terms of the possible FeCh impairment causing Proto accumulation in ptox2 petB, there is no indication that this enzymatic step requires ATP, and the heme levels are similar with or without DCMU treatment. Finally, Mg2+ or Fe2+ chelation are not redox reactions involving transfer of electrons, and it is unlikely that MgCh or FeCh activity would rely on the PQ pool status, or that they would be directly affected by DCMU. On the other hand, other components of PET upstream from PQ, particularly NDA2 involved in NAD(P)H-dependent PQ reduction, potentially might also affect the PPX activity. However, the NDA2 levels were similar in all of the strains (Fig. 1a), which demonstrated that NDA2 is not responsible for the Proto-accumulating phenotype in ptox2 petB.

Proto is the sole TBS intermediate accumulating in ptox2 petB, while measurements of the ALA synthesis capacity (Supplementary Fig. 5) showed similar trends in all of the mutants lacking cyt b6f. If the ALA synthesis capacity would have shown an increase...
in the double mutant, as opposed to single ptox2 or petB, it might have been indicative of an elevated flow through TBS pathway in ptox2 petB, potentially resulting in accumulation of Proto, due to an impairment or bottleneck in steps downstream from PPX (See Fig. 2 for reference). Because ALA synthesis capacity does not trigger increased metabolic flow through TBS in petB, ptox2 petB, and ptox2-R-petB (Supplementary Fig. 5), Proto accumulation can be solely assigned to an impaired PPX activity. There is a certain variation in ALA synthesis capacity in 20 µmol photons m\(^{-2}\) s\(^{-1}\) light in all of the analyzed mutants (Supplementary Fig. 5a), but a clear pattern emerges from experiment performed in 40 µmol photons m\(^{-2}\) s\(^{-1}\) (Supplementary Fig. 5b). In this light condition, ALA synthesis capacity decreases in all tested non-photosynthetic mutants. Decrease in ALA might be indicative of the oxidative stress caused not only by accumulating Proto in ptox2 petB (Fig. 3b), but also due to the ROS resulting from the blockage of electron transfer in all of the mutants devoid of cyt \(b_{6f}\).

Thus, the double mutant was used to demonstrate dependence of the PPX reaction on the redox state of the PQ pool. It was shown that application of DCMU correlates with reduced Proto level in ptox2 petB and increased tolerance to light (Fig. 1b). It has to be noted, that there is no case in the literature describing a direct effect of DCMU on any enzymatic step of TBS. Moreover, the mode of action of this herbicide has only an indirect character in the study described here. Thus, it can also be concluded that increased light tolerance in DCMU-treated ptox2 petB results from diminished Proto accumulation. Furthermore, DCMU application not only decreased accumulation of Proto in ptox2 petB (Fig. 3b), but also rescues the chlorophyll level in this strain, bringing it back to the contents observed in petB or ptox2-R-petB (compare Supplementary Fig. 4e, f). This is indicative of the restored function of PPX and biosynthesis of Proto in a more controlled fashion, which makes it accessible for MgCh.

Following Möbius and co-workers\(^{51}\), we propose that, similarly to its bacterial counterpart HemG, algal PPX is a thylakoid membrane-bound\(^{3}\) protoporphyrinogen IX/plastoquinone oxidoreductase (Fig. 4). The six electrons and protons extracted from Protoporphyrinogen IX would be transferred to PQ to form 3 PQH\(_2\) molecules. In the dark, O\(_2\) would serve as a terminal electron acceptor via the activity of plastid terminal oxidase PTOX2 and to a lesser extent PTOX1.

By comparison of the oxyfluorfen-treated wild type with non-treated and illuminated ptox2 petB, we conclude that a similar mechanism is responsible for the accumulation of Proto in the double mutant. Moreover, Proto accumulation in ptox2 petB and oxyfluorfen-treated wild type is not the only common denominator. ZnProto might be accumulating in both strains due to the

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**Fig. 4** Schematic representation of a proposed model of the TBS pathway interaction with PET. In normally functioning PET, electrons derived from water are subsequently transferred from photosystem II (PSII) to plastocyanin (PQ) generating its reduced form, plastocyanin (PQH\(_2\)). PQH\(_2\) is then oxidised by transferring the electrons to cyt \(b_{6f}\) and by PTOX2. In the ptox2 petB mutant, the PQ pool is almost completely reduced\(^{9}\), as depicted by the greater PQH\(_2\) accumulation in the double mutant, as opposed to single ptox2 or petB, it might have been indicative of an elevated flow through TBS pathway in ptox2 petB, potentially resulting in accumulation of Proto, due to an impairment or bottleneck in steps downstream from PPX (See Fig. 2 for reference). Because ALA synthesis capacity does not trigger increased metabolic flow through TBS in petB, ptox2 petB, and ptox2-R-petB (Supplementary Fig. 5), Proto accumulation can be solely assigned to an impaired PPX activity. There is a certain variation in ALA synthesis capacity in 20 µmol photons m\(^{-2}\) s\(^{-1}\) light in all of the analyzed mutants (Supplementary Fig. 5a), but a clear pattern emerges from experiment performed in 40 µmol photons m\(^{-2}\) s\(^{-1}\) (Supplementary Fig. 5b). In this light condition, ALA synthesis capacity decreases in all tested non-photosynthetic mutants. Decrease in ALA might be indicative of the oxidative stress caused not only by accumulating Proto in ptox2 petB (Fig. 3b), but also due to the ROS resulting from the blockage of electron transfer in all of the mutants devoid of cyt \(b_{6f}\).

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By comparison of the oxyfluorfen-treated wild type with non-treated and illuminated ptox2 petB, we conclude that a similar mechanism is responsible for the accumulation of Proto in the double mutant. Moreover, Proto accumulation in ptox2 petB and oxyfluorfen-treated wild type is not the only common denominator. ZnProto might be accumulating in both strains due to the
accumulation of Proto per se. Furthermore, alterations in the content of other intermediates showed similar patterns when ptx2 petB and wild type treated with oxygenuxirgen were compared to their respective controls (Supplementary Fig. 4). A complex regulatory network is responsible for transcriptional, translational, and post-translational regulation of TBS. These processes assure balanced metabolic flow through TBS pathway and an adequate supply of TBS end-products at different developmental stages and in response to changing environmental conditions. Metabolic control ensures avoidance of accumulation of tetrapyrrole intermediates, which are capable of generating reactive oxygen species (ROS) and organic radicals. Not surprisingly, the activity of the TBS enzymes also includes redox regulation involving the ferredoxin-thioredoxin (FDX-TRX) system and NTRC. While TRX-FDX derive electrons from PET in the light, NTRC constitutes a NAD(P)H-dependent reductase. Both systems are crucial for TBS regulation. Additionally, a component of the cyclase, YCF54, was shown to act as the scaffolding factor for CHL2 and was recently demonstrated to interact with ferredoxin-NADPH reductase (FRN1), downstream of PSI in A. thaliana. Lack of YCF54 results in accumulation of MgProtoME and decrease of Pchlide, Chlide, and chlorophyll. Thus, it was suggested that FRN1 acts as an electron donor, required for the MgProtoME cyclisation reaction. Here in our work with ptx2 petB, we disclose a different type of regulation, upstream of PSI and cyt b6f, at the level of the PQ pool, more similar to the dynamic PQ pool model proposed for the cyclase regulation in A. thaliana and H. vulgare. PPX-PQ pool interaction in C. reinhardtii is further supported by the presence of a FAD-binding domain in PPX, commonly found in enzymes interacting with plastoquinone, e.g., most of the eukaryotic type II NAD(P)H dehydrogenases or phytoene desaturase. It is very likely that FAD in PPX plays the role of a prosthetic group, mediating transfer of the electrons removed from Proteogen to PQ, and this process is responsible for maintaining functional PPX (Fig. 4).

With increasing precision, biologists are using systems biology to correlate different, well-studied physiological processes in the cell. Not surprisingly, such correlations exist between chlorophyll biosynthesis and chlorophyll (and heme)-dependent photo-synthetic processes, specifically, components of PET. The results presented here provide further evidence for an interaction between the TBS pathway and PET. This regulation of PPX activity simply relies on the availability of oxidised PQ and provides a regulatory control point at the cross-roads between chlorophyll biosynthesis and PET. Thus, in this model, the redox state of the PQ pool acts as a sensor of the electron flow in PET, determining chlorophyll requirements and adjusting its biosynthesis by modulation of PET activity.

Methods

**Chlamydomonas cultures and genetic manipulations.** The wild type (lex4), generation of ptx2 petB, and ptox2 petB were described elsewhere. The ptx2 pcy double mutant was generated by crossing ptx2 (mt9) with pcy (mt7), while fud50 were described. FNR1 acts as an electron donor, required for the MgProtoME cyclisation reaction 88. Thus, it was suggested that FAD in PPX plays the role of a prosthetic group, mediating transfer of the electrons removed from Proteogen to PQ, and this process is responsible for maintaining functional PPX (Fig. 4).

With increasing precision, biologists are using systems biology to correlate different, well-studied physiological processes in the cell. Not surprisingly, such correlations exist between chlorophyll biosynthesis and chlorophyll (and heme)-dependent photo-synthetic processes, specifically, components of PET. The results presented here provide further evidence for an interaction between the TBS pathway and PET. This regulation of PPX activity simply relies on the availability of oxidised PQ and provides a regulatory control point at the cross-roads between chlorophyll biosynthesis and PET. Thus, in this model, the redox state of the PQ pool acts as a sensor of the electron flow in PET, determining chlorophyll requirements and adjusting its biosynthesis by modulation of PET activity.
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