Evidence That Cytochrome \textit{b}_{559} Mediates the Oxidation of Reduced Plastoquinone in the Dark*

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The function of cytochrome \textit{b}_{559} in photosystem II (PSII) was investigated using a mutant created in tobacco in which the conserved phenylalanine at position 26 in the \textit{β}-subunit (PsbF) was changed to serine (Bock, R., Kössel, H., and Maliga, P. (1994) \textit{EMBO J.} 13, 4623−4628). The mutant grew photoautotrophically, but the amount of PSII was reduced and the ultrastructure of the chloroplast was dramatically altered. Very few grana stacks were formed in the mutant. Although isolated PSII-enriched membrane fragments showed low chlorophyll fluorescence and thermoluminescence; OEC, oxygen evolving complex.

Photosystem II (PSII) is the membrane protein complex in thylakoid membranes that catalyzes the light-induced oxidation of water and the reduction of plastoquinone. The scaffold of the PSII reaction center is formed by two protein subunits, D1 and D2, where the cofactors for photosynthetic electron transport are located, and by cytochrome \textit{b}_{559}. Cytochrome \textit{b}_{559} is located in close proximity to D1/D2 and is found in the smallest experimentally obtainable PSII complex capable of carrying out light-induced charge separation (1). This cytochrome, encoded by the \textit{psbE} and \textit{psbF} genes, has a heterodimeric structure consisting of one \textit{α}- and one \textit{β}-subunit, both of which form single transmembrane \textit{α}-helices (2). The heme group is ligated by two histidine residues, one located in the \textit{α}- and one in the \textit{β}-subunit, as revealed by x-ray crystallography (3). The heme is bis(histidine) 6-fold-coordinated, as was demonstrated spectroscopically (4, 5). The electron paramagnetic resonance (EPR) spectrum of the oxidized heme iron is anisotropic, with \( g_z \sim 3.00, g_y \sim 2.20, \) and \( g_x \sim 1.50, \) indicative of a low-spin species. These values are consistent with two ligating histidine planes being perpendicular (or nearly perpendicular) to each other (5). Likewise, the orientation of the heme ring was determined to be perpendicular to the membrane plane (6).

Different potential forms for cytochrome \textit{b}_{559} are known: the high potential form, which is found in active oxygen-evolving PSII; the intermediate form; and the low potential form, which is found in PSII with an impaired oxygen-evolving complex (for review, see Ref. 7). The different redox forms of cytochrome \textit{b}_{559} are characterized by small changes of the \( g \)-values in their EPR spectra, indicating very subtle changes in the heme coordination (such as a small change in the relative orientation of the histidine planes). There is an ongoing debate whether one or two cytochrome \textit{b}_{559} units are present per PSII, although most recent publications (for reviews, see Refs. 2 and 7) and the x-ray structure suggest that there is only one (3).

Cytochrome \textit{b}_{559} is an indispensable constituent of PSII, but its function remains unclear. It is a redox-active protein, and both photooxidation and photoreduction of its heme iron have been observed. Because these reactions are very slow and characterized by low quantum yields, it is generally assumed that the cytochrome takes no active part in the primary electron transfer reaction. A direct electron donation from cytochrome \textit{b}_{559} to \textit{P680} by bypassing the physiological donor, the \textit{Mn}-cluster, is observed only under prolonged illumination at 77 K (8, 9). It has recently been shown that electron donation from the cytochrome occurs via a redox-active carotenoid (10) rather than via a redox-active chlorophyll as previously thought.

Several functions have been assigned to cytochrome \textit{b}_{559} in the literature, ranging from its putative participation in the mechanism of water oxidation (11), a function in the assembly of PSII (12−14), to a role in photoprotection (for review, see Ref. 7). Morais \textit{et al.} (13) investigated cytochrome \textit{b}_{559} mutants of \textit{Chlamydomonas reinhardii} in which the heme-ligating histidine of the \textit{α}-subunit was replaced by different amino acids. In contrast to earlier reports of cytochrome \textit{b}_{559} knockout mutants (e.g. 15) or mutations of the heme axial ligands (16) that did not assemble PSII, these mutants assembled few but functional
oxygen-evolving PSII centers, although the cytochrome $b_{559}$ did not contain a heme group. This finding seems to exclude a function of cytochrome $b_{559}$ in photosynthetic water splitting, and it was proposed that cytochrome $b_{559}$ plays a protective role under photoinhibitory illumination. Recently, it has also been suggested that cytochrome $b_{559}$ may function as a plastoquinol oxidase (17, 18).

In higher plants, the cytochrome $b_{559}$ α- and β-subunits are encoded by the psbE and psbF genes in the plastid genome and are cotranscribed as part of a tetracistrionic transcription unit (psbE operon). Here we describe the characterization of a cytochrome $b_{559}$ mutant created in tobacco (Nicotiana tabacum) in which the conserved phenylalanine at position 26 in the β-subunit of cytochrome $b_{559}$ was changed to serine (19). In tobacco, the codon for phenylalanine is present at the DNA level, whereas in spinach the codon for phenylalanine is created post-transcriptionally from a genetically encoded UCU (serine) codon by C-to-U RNA editing (20).

EXPERIMENTAL PROCEDURES

Plant Material—Tobacco (Nicotiana tabacum cv. Petit Havanna) plants were grown in soil at a light intensity of 100 μmol quanta m$^{-2}$s$^{-1}$.

Generation of Antibodies against the α-Subunit of Cytochrome $b_{559}$—Based on published sequences in the data bases, two primers were designed to amplify the gene coding for the α-subunit of cytochrome $b_{559}$ from Narcissus pseudonarcissus L. Total RNA was isolated from daffodil flowers using RNA-Easy kit (Qiagen, Hilden, Germany), and a 280-bp cDNA fragment encoding the entire α-subunit of cytochrome $b_{559}$ was amplified by RT-PCR. The fragment was then cloned into PCR-Blunt II-TOPO vector (Invitrogen), yielding the plasmid pCYT, and the insert was sequenced. The deduced amino acid sequence was highly homologous to previously determined sequences of the cytochrome $b_{559}$ α-subunit gene from other plants. To express the cytochrome $b_{559}$ α-subunit as a GST fusion in Escherichia coli cells, a SmallEcoR I fragment containing the coding region was excised from pCYT and cloned into pGEX-2T (Amersham Biosciences) to yield pGST-cytochrome $b_{559}$. Attempts to purify the fusion protein by affinity chromatography using glutathione-Sepharose failed. Therefore, total E. coli protein extract was separated by SDS-PAGE, and the overexpressed protein was excised from the gel and subsequently purified by electroelution. The purified protein was finally used to immunize mice and a rabbit.

Conventional Electron Microscopy—Specimens were prepared for thin sectioning by fixing small pieces (1 mm$^2$) of tobacco leaves with 1.0% formaldehyde and 0.5% glutaraldehyde in phosphate buffer (pH 7.0). For routine preparations, the samples were dehydrated with ethanol and embedded in epoxy resin (20). Samples for immunolabeling techniques were embedded in LR White (21). Thin sections were performed with the ultramicrotome Ultracut E (Leica, Wetlar, Germany) and prepared on formvar-coated nickel grids and contrasted with uranyl acetate (22) followed by lead citrate (23). Sections to be used for immunolabeling techniques were stained with uranyl acetate only. Ultrathin sections were examined with a Philips CM-10 electron microscope (FEI Company, Eindhoven, The Netherlands) equipped with a Biocam camera (model 792, Gatan, München, Germany). The electron micrographs taken at an acceleration voltage of 60 and 80 kV were processed by a digital image system (Digital Micrograph, Gatan, München, Germany).

Immunolocalization of Cytochrome $b_{559}$—Immunolabeling techniques were carried out on ultrathin sections after embedding and sectioning (24). As primary antibodies we used an anti-α-cytochrome $b_{559}$ antiserum raised against the recombinant protein (25), and 12 nm Colloidal Gold-AffiniPure goat anti-rabbit IgG (H+L) and 12 nm Colloidal Gold-AffiniPure goat anti-mouse IgG were employed as secondary antibodies (Jackson ImmunoResearch Laboratories). In control experiments preimmune serum was used instead of specific primary antibodies.

RESULTs

Chloroplast Ultrastructure and Protein Composition of PSII—Studies of the ultrastructure of wild-type and mutant chloroplasts by electron microscopy (Fig. 1, A and B) showed that the structural organization of the membrane was significantly altered by the single amino acid exchange from phen-
yalanine to serine in the cytochrome b_{559} \(\beta\)-chain at position 26. In the mutant, the structure of thylakoid grana and stroma lamella was disordered, whereas in the wild type the well ordered structure of grana stacks separated by stroma lamella as well as starch granules was nicely seen. The mutant had only small grana stacks, which were less well stacked than in the wild type and showed a much lower degree of structural order. In addition, starch accumulation was much less pronounced in the mutant, and starch granules (when present) were significantly smaller than in the wild type.

Fig. 1E shows a Western blot of thylakoid membrane proteins from wild type and the cytochrome b_{559} mutant stained with antibodies raised against the \(\alpha\)-subunit of cytochrome b_{559}. The alteration in the sequence of the \(\beta\)-subunit of cytochrome b_{559} led to a lower concentration of cytochrome b_{559} relative to the chlorophyll concentration. Western blots conducted with a range of chlorophyll concentrations per sample indicated a reduction of the levels of PsbE to 10–30% of the wild type (data not shown).

Immunolocalization of cytochrome b_{559}, using secondary antibodies coupled to gold particles for detection, showed that the staining of the wild-type chloroplasts was much higher than for the mutant (Fig. 1, C and D). In the wild-type chloroplast a clustering of gold particles can be seen at the grana stacks, whereas in the mutant the less abundant gold particles are more equally distributed. The gold labels, unequivocally identifiable by their intense electron contrast and size (12-nm diameter) were morphometrically evaluated. 92\,39 gold particles were counted per \(\mu\)m\(^2\) for the wild type compared with 20\,13 particles for the mutant, whereas in controls with preimmune serum less than 1 gold particle per \(\mu\)m\(^2\) was counted. The mean values of wild-type and mutant chloroplasts are significantly different at a 99.5% confidence level.

We then investigated the effect of the mutation on other proteins of the photosynthetic machinery. Thylakoids and PSII-enriched membrane fragments (PSII particles) from wild type and the cytochrome b_{559} mutant were compared by Western blot analyses for the abundance of CP47, FNR, OEC 33, and OEC 23. Fig. 2 shows that the amount of the membra-
Fig. 3. Fluorescence emission spectra at 77 K. Thylakoid membranes from the wild type (dashed line) and the cytochrome \( b_{559} \) mutant (solid line) were measured with an excitation wavelength of 440 nm.

Integral PSII protein CP 47 was considerably reduced in mutant thylakoids, whereas there was no significant difference in PSII particles, as expected. All other proteins examined remained largely unchanged in thylakoids. On the other hand, in PSII particles a signal for the 23-kDa protein was not detectable in the mutant, although it was present in thylakoid membranes. This indicates that this luminal extrinsic protein was not attached to PSII. The extrinsic OEC 33 behaved differently, being detectable in PSII particles of both origins. Previous studies have shown that high concentrations of OEC 23 protein are present in the thylakoid lumen (31) and even in etioplasts in the complete absence of PSII (14).

Characterization of the Mutant by 77 K Fluorescence—Fig. 3 shows fluorescence emission spectra at 77 K recorded with thylakoid membranes from the wild type and the mutant. The maxima at 684, 690, and 730 nm are generally attributed to emissions originating predominantly from light-harvesting complex and antenna pigments of PSII (684 and 690 nm) and PSI (730 nm) (32). In the mutant, the ratio of emission at 684/730 nm (ratio PSI/PSII) was lower compared with the wild type, indicating that the amount of PSII was decreased relative to PSI. In addition, in the mutant the first peak was slightly shifted by 2 nm to 682 nm. Absorption spectra at room temperature of thylakoid membranes from mature leaves showed no difference between the wild type and the mutant. Likewise, the chlorophyll \( a/b \) ratio was unchanged, indicating that there were no severe structural changes in the antenna system of PSII (data not shown).

To test whether the photosynthetic electron transport chain was inhibited in the mutant, we measured the activity of PSII, PSI, and the entire electron transport chain in the presence of an uncoupler (Table I). PSII activity was reduced by 60% in thylakoid membranes obtained from the mutant, whereas the PSI activity remained unchanged compared with the wild type. The activity of the total electron transport chain was reduced in the mutant by 75%. Similarly, PSII-enriched membrane fragments isolated according to Ref. 27 showed a 75% lower activity than the wild type. This latter result is somewhat unexpected but could either be because of reduced PSII stability during preparation or a certain degree of PSI impurity. Consequently, the loss in PSII activity may be underestimated.

Absorption Spectra of Cytochrome \( b_{559} \)—To test whether the cytochrome \( b_{559} \) of the mutant contained a heme group, we measured difference absorption spectra of PSII-enriched membrane fragments between 535 and 590 nm. Fig. 4 shows the respective reduced minus oxidized optical difference spectra of the wild type and the mutant (Fig. 4, A and B). Ferricyanide was used as oxidant, whereas hydroquinone, ascorbate, and dithionite were subsequently added as reductants to estimate the potential form of cytochrome \( b_{559} \). As shown in Fig. 4, an absorption maximum was observed at 559 nm, indicative for cytochrome \( b_{559} \) in both mutant and wild type. However, the signal size was approximately five times smaller in the mutant.

Fig. 4. Difference absorption spectra from PSII-enriched membrane fragments of wild-type (A) and cytochrome \( b_{559} \) mutant (B). The following spectra of the cytochrome \( b_{559} \) heme are shown: hydroquinone-reduced minus ferricyanide-oxidized (1), ascorbate-reduced minus ferricyanide-oxidized (2), and dithionite-reduced minus ferricyanide-oxidized (3). The samples contained 120 \( \mu \)g of Chl/ml.

Table I: Activity of the photosynthetic electron transport chain in wild-type and mutant thylakoid membranes

| Mutant | Wild type |
|--------|-----------|
| Thylakoid membranes | | |
| PSII activity | 51 | 120 |
| PSI activity | 156 | 141 |
| Total electron transport | 34 | 145 |
| PSII particles | | |
| PSI activity | 85 | 325 |

To test whether the photosynthetic electron transport chain in wild-type and mutant thylakoid membranes was inhibited in the mutant, we measured the activity of PSII, PSI, and the entire electron transport chain in the presence of an uncoupler. PSII activity was measured as \( O_2 \) evolution using \( \mu \)-phenylbenzoquinone as electron acceptor. PSI activity was measured as \( O_2 \) uptake in the presence of DCMU, DCPIP, ascorbate, Na azide, and methylviologen. All activities were determined in the presence of nigericin as uncoupler.

Characterization of the Mutant PSII Particles by EPR Spectroscopy—To further characterize the electron transport in
PSII of the mutant, EPR spectra arising from radical species formed at the donor and acceptor side of PSII upon 200 K illumination were recorded (Table II). Upon illumination at 200 K, a normal multiline signal of the S2 state of the Mn-containing water-oxidizing complex (33) was detected, showing that the ability of the oxygen-evolving complex to advance from the ing water-oxidizing complex (33) was detected, showing that the ability of the oxygen-evolving complex to advance from the

|                | Mutant  | Wild-type |
|----------------|---------|-----------|
| $F_r/F_m$      | 0.35    | 0.76      |
| EPR signals    | +       | +         |
| S2 multiline   | +       | +         |
| Signal II      | very small | very small |
| $V_m$          | +       | +         |
| Fe-Q$_{A^-}$ signal | +       | +         |

To study the PSII activity—

In dark-adapted mutant samples, no oxidized high-potential cytochrome $b_{559}$ was seen by EPR following incubation with 5 mM potassium ferricyanide (an adequate oxidant for both the cytochrome $b_{559}$ and the non-heme iron). Surprisingly, even under prolonged 77 K illumination no signal of oxidized cytochrome $b_{559}$ could be detected, although the amount of Car$^+$/Chl$^-$. This indicates that the electron transfer reactions associated with PSII were not perturbed by the mutation. However, the EPR signal sizes were significantly smaller in PSII particles from the mutant as compared with the wild type when the samples were measured at an equal chlorophyll basis.

Here, one has to keep in mind that, because of the altered PSII-PSI stoichiometry, the enrichment of PSII relative to PSI was low in PSII particles from the mutant.

To investigate the reduction state of the donor and acceptor side of PSII, we performed thermoluminescence measurements on leaf segments of the mutant and the wild type. In thermoluminescence, the emitted light originates from charge recombination of trapped charge pairs (for review, see Ref. 35). The charge pairs involved can be identified by their emission temperature, which strongly depends on the redox potentials of the charge pairs involved. The B-band, which we investigated here, arises from a recombination of the S2 or S3 state of the oxygen-evolving complex with the semiquinone Q$_{m^-}$ (36). This recombination yields a band at $\sim$30 °C. The intensity of the B-band oscillated with a period of four, which reflects the cycle of the S-states of the Mn cluster. In general, in a dark-adapted sample 75% of the centers are in the S1 state and Q$_B$ is mainly oxidized. In this case, the highest intensity of the B-band (maximum emission at 33 °C) is observed after the first flash, as can be seen clearly with wild-type leaves (Fig. 6, open circles). In contrast, almost no thermoluminescence signal was detected in the mutant after the first flash, whereas after the second flash a large signal (B-band, maximum emission temperature at 35 °C) was observed (Fig. 6, filled circles). This B-band oscillated with a periodicity of four, as expected, showing the highest peak after two and six flashes, respectively. The signal on the fifth flash is again very small. This indicates a normal function of the water-splitting complex and the forward electron transfer in PSII but a shift in the oscillation pattern of the B-band by one flash.
DISCUSSION

We have characterized a tobacco mutant carrying a single point mutation in the plastid $psbF$ gene, resulting in a phenylalanine to serine change at position 26 in the $\beta$-subunit of cytochrome $b_{559}$. This mutation is interesting because it does not directly influence the heme-ligating amino acid residues (one His in the $\alpha$-subunit and one His in the $\beta$-subunit) but affects a highly hydrophobic stretch in the transmembrane helix in the $\beta$-subunit. The mutation leads to a severe phenotype, and we have shown here that it results in a reduced amount of the $\alpha$-subunit of the cytochrome (Fig. 1E), of CP47, and of the extrinsic 23-kDa protein associated with PSII (Fig. 2). Because the cytochrome $b_{559}$ is known to be essential for the PSII assembly process (see below), it is not surprising that the mutant also displayed a significant reduction in the amount of PSII centers, which in turn causes ultrastructural alterations in the thylakoid membrane (Fig. 1). In addition, the reduction state of the electron transport chain was altered in the mutant as demonstrated by chlorophyll fluorescence and thermoluminescence measurements (Figs. 5 and 6).

It has been reported previously that cytochrome $b_{559}$ is essential for the assembly of stable PSII reaction centers (12, 14). Null mutants for the $\alpha$-subunit of cytochrome $b_{559}$ (12) or for both $\alpha$- and $\beta$-subunits (15) are unable to assemble PSII. Mutants in which one of the heme-ligating histidines was exchanged by a different amino acid and in which the cytochrome no longer contained a heme group display inefficient PSII assembly or even a complete failure in this respect (13, 16). In the mutant investigated here, little PSII is present (about 30% as seen by Western blots and immunolabeling), indicating that a mutation not directly affecting the heme-ligating histidine evidently also leads to perturbations of the PSII assembly process.

The low amount of PSII exerts a dramatic effect on the ultrastructure of the thylakoid membranes as can be seen in the electron microscopy images (Fig. 1). Whereas thylakoid membranes of the wild type showed the typical grana stack, grana formation and stacking were strongly reduced in the mutant (Fig. 1). A number of previous studies have suggested that grana stack formation is correlated with the amount of PSII present in the thylakoid membrane (e.g. Ref. 37). It has been proposed that the close appression of grana membranes arises through recognition patterns and contact surfaces formed by LHCII light-harvesting complex proteins that are associated with PSII (38). It thus appears plausible that the low amount of PSII in the mutant and the concomitantly reduced number of recognition sites for grana stacking are responsible for the perturbed chloroplast ultrastructure.

Although PSII activity in thylakoid membranes of the PsbF mutant was reduced, a certain quantity of active PSII was found (Table I) that showed the characteristic EPR spectra (Table II). However, no EPR signal for cytochrome $b_{559}$ was seen. This finding cannot be explained with a mutation-induced loss of the heme group because difference absorption spectra showed a maximum at 559 nm, indicating heme incorporation (Fig. 4). Mutants of *Chlamydomonas reinhardtii*, which carried mutations in the $\alpha$-subunit of cytochrome $b_{559}$, did not contain a heme group and showed no such absorption maximum at 559 nm (13). The lack of detectable EPR signals for cytochrome $b_{559}$ in PSII particle preparations from the mutant could be explained by a broadening of the spectra because of a structural change, such as an increase of the anisotropy caused by H-bonds to the imidazole group of the heme-ligating histidines. The difference absorption spectra also showed a broadening of the $\alpha$-band of the cytochrome $b_{559}$ spectrum.

Sequence inspection of the $\alpha$- and $\beta$-subunits of cytochrome $b_{559}$ reveals that the site of mutation is located in a region with extremely hydrophobic amino acid residues (Phe-Phe-Leu in the $\beta$-subunit and Leu-Phe-Ile in the $\alpha$-subunit). The recently determined crystallographic data for PSII (3) suggest that this region provides the contact site of the two helices. The change from phenylalanine to serine in the mutant could have two effects. The introduction of a small polar amino acid residue might alter the contact site between the $\alpha$- and $\beta$-subunits, leading to a structural change and increased flexibility in the orientation of the two helices. Alternatively, the introduction of a much smaller side group might lead to a closer contact at this site, resulting in an opening of the helices toward the stroma.

Our determination of the distribution of high and low potential forms of cytochrome $b_{559}$ (Fig. 4) showed a lower amount of high potential cytochrome $b_{559}$ in the mutant. However, the fluorescence (Fig. 5) and thermoluminescence signals (Fig. 6) were altered in leaves of the mutant. Recordings of fluorescence curves showed that the dark level of fluorescence ($F_d$) was very high in the mutant, indicating a largely reduced plastohydroquinone pool. In the light, photochemical and non-photochemical quenching occurred, and the fluorescence declined to a level comparable with that seen in wild-type leaves. Upon switching of the actinic light, an increase of the dark-level fluorescence was observed in the mutant, indicating that the same reduction level of the PQ pool was reached as was observed during the dark adaptation prior to the measurement. In thermoluminescence analysis, a normal oscillation pattern of the B-band ($S_2\rightarrow Q_B$ recombination) was observed. Most interestingly, however, the oscillation pattern was shifted by one flash. This shift can be explained either by the presence of $Q_B^{-}$ in the dark within the majority of the reaction centers or by a change in the stability of the $S$-states of the Mn cluster. Normally, in a dark-adapted sample, 75% of the Mn cluster of the PSII centers are in the $S_1$ state and 25% in the $S_0$ state. If the acceptor side of PSII is oxidized, a single flash results in the formation of $S_0\rightarrow Q_B^{-}$, yielding the highest thermoluminescence emission. If the $S_1$ state were less stable in the mutant, the Mn cluster could be in the $S_0$ state in the dark. This in turn would lead to a shift of the oscillation pattern of the B-band by one flash, resulting in the maximum TL emission on the second flash. However, a lower S-state is excluded by our EPR data (Table II); 200 K illumination, which is equivalent to one flash, was sufficient to give a multiline signal ($S_0$ state) in the PSII preparation from mutant leaves, indicating that the Mn cluster was predominately in the $S_1$ state in the dark. The high fluorescence state in the dark and the shifted oscillation of the B-band are therefore indicative of a reduced acceptor side in the dark. If $S_0\rightarrow Q_B^{-}$ and $S_1\rightarrow Q_B^{-}$ are present in the dark, the flash-dependence of the thermoluminescence emission as shown in Fig. 6 is readily explained.

Far-red illumination (intensity: 9 $\mu$mol quanta m$^{-2}$ s$^{-1}$) did not decrease the high dark level of fluorescence. Therefore, it can be excluded that the alteration of the PSII:PSI stoichiometry and increased activity of cyclic electron flow around PSI were responsible for the high reduction state of the plastohydroquinone pool in the mutant.

We therefore conclude that at least one physiological role of cytochrome $b_{559}$ is to keep the acceptor side of PSII oxidized under conditions when forward electron flow does not occur and the plastohydroquinone pool is reduced non-photochemically (e.g. in the dark, in the presence of a high proton gradient or a large concentration of reduction equivalents). It has been proposed recently that cytochrome $b_{559}$ in its low potential form could function as a plastohydroquinone oxidase (17, 18). It has also previously been suggested that photoreduction of the oxidized high
Oxidation of Reduced Plastoquinone by Cytochrome b₅₅₉

potential of cytochrome b₅₅₉ occurs via electron donation from PQH₂ (39). Oxidation of PQH₂ by cytochrome b₅₅₉ would result in delivery of an electron to oxygen and formation of superoxide. Detoxification of superoxide could occur via the superoxide dismutase activity of cytochrome b₅₅₉ as proposed by Ananyev et al. (40). It may also be possible that the electron transfer leading to oxidation of PQH₂ via cytochrome b₅₅₉ involves an alternative oxidase (41) that ultimately reduces oxygen.

The high reduction level of the plastoquinone pool in the mutant may either be a consequence of the thylakoid membrane’s global structural changes caused by a lower level of cytochrome b₅₅₉ or be because of a change of the redox properties of cytochrome b₅₅₉ perturbing the putative plastoquinol oxidase function. The possibility of alterations in the redox properties is supported by the lack of an EPR signal and by the broadening of the α-band of the absorption spectrum.

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