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Experimental evidence suggesting that H_2O_2 is produced within the thylakoid membrane in a reaction between plastoquinol and singlet oxygen

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ABSTRACT

Plastoquinol (PQH_2-9) and plastoquinone (PQ-9) mediate photosynthetic electron transfer. We isolated PQH_2-9 from thylakoid membranes, purified it with HPLC, subjected the purified PQH_2-9 to singlet oxygen ($^1O_2$) and analyzed the products. The main reaction of $^1O_2$ with PQH_2-9 in methanol was found to result in formation of PQ-9 and H_2O_2, and the amount of H_2O_2 produced was essentially the same as the amount of oxidized PQH_2-9. Formation of H_2O_2 in the reaction between $^1O_2$ and PQH_2-9 may be an important source of H_2O_2 within the lipophilic thylakoid membrane.

1. Introduction

Reactive oxygen species (ROS) like singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are ubiquitously formed in plants. Besides causing direct damage to biomolecules, ROS also play important roles in plant signaling [1–3]. ROS are formed in the apoplast, mitochondria and chloroplasts [4–6], and formation of ROS in chloroplasts depends on absorption of light by the photosynthetic apparatus.

$^1O_2$ is mainly formed via interaction of oxygen ($^3O_2$) with the triplet excited state of chlorophyll ($^3Chl^*$), which occurs in the light-harvesting antenna complexes and in the reaction centers of photosystems II and I (PSII and PSI, respectively). In light-harvesting complexes, $^3Chl^*$ can be formed via intersystem crossing from singlet excited chlorophyll ($^1Chl^*$) [7], but transfer of excitation energy to the reaction center shortens the lifetime of $^1Chl^*$, thereby lowering the probability of intersystem crossing. Triplet-triplet energy transfer from $^3Chl^*$ to carotenoids lowers the probability of $^1O_2$ formation in the antenna complexes of both photosystems [8], and the reaction center of PSI does not produce $^1O_2$ [9]. Therefore, the main source of $^1O_2$ appears to be the reaction center of PSII, where the triplet state of the primary donor, $^3P_{680}$, is formed by charge recombination reactions between P_{680}^+ and Q_A^-, the primary quinone acceptor of PSII [8,10]. Furthermore, time-dependent formation of a virtual triplet state of the primary radical pair P_{680}^+ $Pheo^-$ and subsequent recombination may produce $^3P_{680}$ [11], although the short lifetime of the primary pair renders this pathway inefficient.

The rate constants and concentrations of molecules that potentially function as $^1O_2$ quenchers suggest that carotenoids are the main quenchers of $^1O_2$ in the thylakoid membrane. The second-order rate constants of the reaction of $^1O_2$ with thylakoid carotenoids are in the range of 7×10^9 to 1.4×10^{10} M^{-1} s^{-1} [12], much higher than the rate constants of tocopherols with $^1O_2$ [13]. Carotenoids can prevent the formation of $^1O_2$ via direct quenching of $^3Chl^*$. However, the two ß-carotene molecules of the reaction center of PS II are too far away from P_{680} to quench $^3P_{680}$ [14,15], and may therefore mainly function as $^1O_2$ quenchers.

Abbreviations: Chl, chlorophyll; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $HO_2^{\cdot}$, hydroperoxyl radical; MeOH, methanol; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide anion radical; P_{680}, primary electron donor of PSII; Pheo, pheophytin *a*—primary electron acceptor of PSII; PQH·-9 and $PQ^{\cdot-}$-9, plastosemiquinone radical and plastosemiquinone anion radical, respectively; PQH_2-9 and PQ-9, plastoquinol and plastoquinone, respectively; PSI and PSII, photosystems I and II, respectively; ROS, reactive oxygen species; UQH_2-9, ubiquinol-9

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$O_2^{\cdot-}$ and H_2O_2 are produced via one-electron reduction of oxygen at the acceptor side of photosystem I (PSI). This reaction is known as Mehler's reaction, and H_2O_2 is produced by a subsequent dismutation reaction from $O_2^{\cdot-}$. The terminal acceptors F_X, F_A, and F_B likely function as electron donors to oxygen [16]. It has also been suggested that phylloquinone A_1, a secondary electron acceptor in PS I, might donate an electron to oxygen within the thylakoid membrane [17,18]. $O_2^{\cdot-}$ can also be generated on the acceptor side of PS II [19,20]. However, the acceptor side of PSII produces $O_2^{\cdot-}$ at a much lower rate than Mehler's reaction [21].

Mehler's reaction can be fully suppressed with the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transfer from PSII to the plastoquinone pool. However, significant formation of H_2O_2 was shown to occur in isolated thylakoid membranes when the oxidation of PQH_2-9 by the cytochrome b_6/f complex was inhibited with 2,4-dinitrophenylether of iodonitrothymol (DNP-INT) [21]. In this case, H_2O_2 was suggested to be formed from superoxide produced by reduction of oxygen by the plastosemiquinone radical ($PQ^{\cdot-}$-9). The production of H_2O_2 was found to occur mainly within the thylakoid membrane, not via dismutation of $O_2^{\cdot-}$ in the soluble phase [22], and it was suggested that a reaction between $O_2^{\cdot-}$ and PQH_2-9 produces H_2O_2 [21,23]. Thus, PQH_2-9 may convert $O_2^{\cdot-}$ to H_2O_2.

The PQ pool has also been suggested to scavenge $^1O_2$ [24–26]. The suggestion is supported by the high concentration of PQ in the chloroplast together with a modest rate constant of the reaction of $^1O_2$ with PQH_2-9, $0.97 \pm 0.08 \times 10^8$ M^{-1} s^{-1} in acetonitrile [27]. $^1O_2$ can also efficiently react with thylakoid lipids, which leads to lipid peroxidation [28]. Thus, the reaction of PQH_2-9 with $^1O_2$ also slows down the oxidation of lipids by $^1O_2$. The oxidized form (PQ-9) can also quench $^1O_2$ [27]. Earlier work has shown that oxidation of PQH_2-9 by $^1O_2$ produces PQ-9 and hydroxy derivatives of PQ [27]. The results of the present work indicate that the reaction between PQH_2-9 and $^1O_2$ is accompanied with formation of H_2O_2. Oxidation of PQH_2-9 to PQ-9 and H_2O_2 was found to be the main reaction when $^1O_2$ reacts with PQH_2-9, whereas the PQ-hydroperoxides are minor products.

2. Materials and methods

Thylakoid membranes were isolated from pumpkin leaves as described in [21] with some modifications. Leaves were ground in buffer (40 mM HEPES-KOH, pH 7.4, 330 mM sorbitol, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 1 mM ethylenediamine tetraacetic acid, 1% bovine serum albumin) in 20-s cycles for 1 min at 4 °C. The suspension was centrifuged for 5 min at 1500×*g* and the pellet was suspended in osmotic shock buffer (10 mM HEPES, pH 7.4, 5 mM sorbitol and 5 mM $MgCl_2$). The suspension was again centrifuged for 5 min at 1500×*g* and the pellet was suspended in storage buffer containing 10 mM HEPES (pH 7.4), 330 mM sorbitol, 4 mM $MgCl_2$, 10 mM NaCl, and 20% glycerol.

PQH_2-9 was isolated from pumpkin thylakoid membranes. 1 ml of thylakoid suspension at a concentration equivalent to 2 mg of chlorophyll (Chl)/ml was centrifuged at 5000×*g* for 5 min. The supernatant was removed and the pellet was resuspended in 4–5 ml of cold ethyl acetate that had been stored over a dehydrating agent (Molecular Sieves, 4 Å). The extract was filtered with a 0.2 μM PTFE filter and then dried under N_2 stream. The dried sediment was dissolved in 400 μl MeOH and purified with HPLC.

After extraction, PQH_2 was purified using an HPLC-based method [29] with some modifications. Briefly, the HPLC purification was performed with LiChroCART (Germany) C_{18} reversed-phase column (LiChrospher 100, 125 × 4 mm, 5 μM) using an Agilent 1100 series device with absorption and fluorescence detectors (Agilent Technologies, Palo Alto, CA), and using an isocratic solvent system (methanol:hexane, 34:2). The flow rate was 0.75 ml/min, the temperature of the column was maintained at 25 °C, and the injection volume was 20 μl. PQ-9 was detected with absorption at 255 nm, and PQH_2-9 was detected with fluorescence (λ_{ex} = 290 - nm, λ_{em} = 330 nm). Pure PQH_2-9 was collected according to the retention times of the corresponding peaks on the chromatogram using Analyt-FC (Agilent Technologies, Palo Alto, CA) collector. The same HPLC-based method, except that the isocratic solvent system (methanol:hexane, 34:2) was replaced with methanol, was applied to detection of PQ-derived compounds formed in the reaction of $^1O_2$ with PQH_2-9. Reaction of $^1O_2$ with PQH_2-9 was carried out in MeOH containing 10 μM Rose Bengal in a glass cuvette under continuous stirring. Generation of $^1O_2$ was triggered by illuminating the samples with white light. Visible light between 400 and 700 nm was quantified with a Li-Cor quantum sensor (LiCor, Lincoln, NE, USA).

HPLC-MS analyses were carried out by an HPLC/DAD-ESI-MS system consisting of an Agilent HPLC 1200 Series equipped with an absorbance detector (Agilent Technologies, Waldbronn, Germany) and a micrOTOF$_Q$ mass spectrometer (Bruker Daltonics, Bremen, Germany). Chromatographic separations were performed using a LiChroCART C_{18} reversed-phase column (Superspher 100 RP-18, 125 × 4 mm, 5 μM; Merck KGaA, Darmstadt, Germany) with isocratic elution with methanol. The flow rate was 0.7 ml/min and it was reduced to approximately 0.3 ml/min by splitting before the introduction into the ion source. The injection volume was 20 μl. The HPLC system was controlled by Hystar software (version 3.2., Bruker BioSpin). The mass spectrometer was controlled by Bruker Compass micrOTOF control software and operated in the negative ion mode. The capillary voltage was maintained at +4000 V with the end plate offset at −500V. The pressure for nebulizer gas was set at 1.6 bar and the drying gas flow was 8.0 l/min and the drying gas temperature 200 °C. The full scan mass ranged from *m*/*z* 50 up to *m*/*z* 3000. Calibration with 5 mM sodium formate injected via a six-port-valve was used at the end of the HPLC-MS experiment in order to provide high-accuracy mass measurements. The data were handled by Bruker Compass DataAnalysis (version 4.0 SP5).

H_2O_2 was measured with chemiluminescence of the luminol-H_2O_2-horseradish peroxidase system [30]. The chemiluminescence measurements were carried out using an EMI9558B photon multiplier using a set-up earlier designed for thermoluminescence measurements [31]. The reaction medium (330 μl) contained 2.5 mM luminol, 10 mM Tris–HCl buffer at pH 8.4, and 10 U/ml of horseradish peroxidase. The solution was continuously mixed with a magnetic stirrer. The chemiluminescence reaction was triggered by manual injection of 20 μl of sample.

Hydrophobicity analysis was done with the Expasy software [32] using the Kyte-Doolittle approximation [33] and a window size of 19 amino acids.

3. Results

Oxidation of PQH_2-9 to PQ-9 is a two-electron reaction, and therefore oxidation of PQH_2-9 by $^1O_2$ may cause 2-electron reduction of O_2 to H_2O_2. To measure the formation of H_2O_2, PQH_2-9 was directly isolated from pumpkin thylakoid membranes using ethyl acetate as a solvent. The typical HPLC chromatogram of the thylakoid extract is shown in Fig. 1A. The area of the PQH_2-9 peak (indicated as a dashed rectangle in Fig. 1A) was collected to isolate pure PQH_2-9. According to HPLC chromatograms, the purified solution contained mainly PQH_2-9 (Fig. 1B) and small amounts of PQ-9 and a second compound (Fig. 1C), identified with MS analysis as phylloquinone (Fig. 2A). MS analysis also showed that the PQH_2-9 peak of the HPLC chromatogram contained PQ-9-OH and reduced

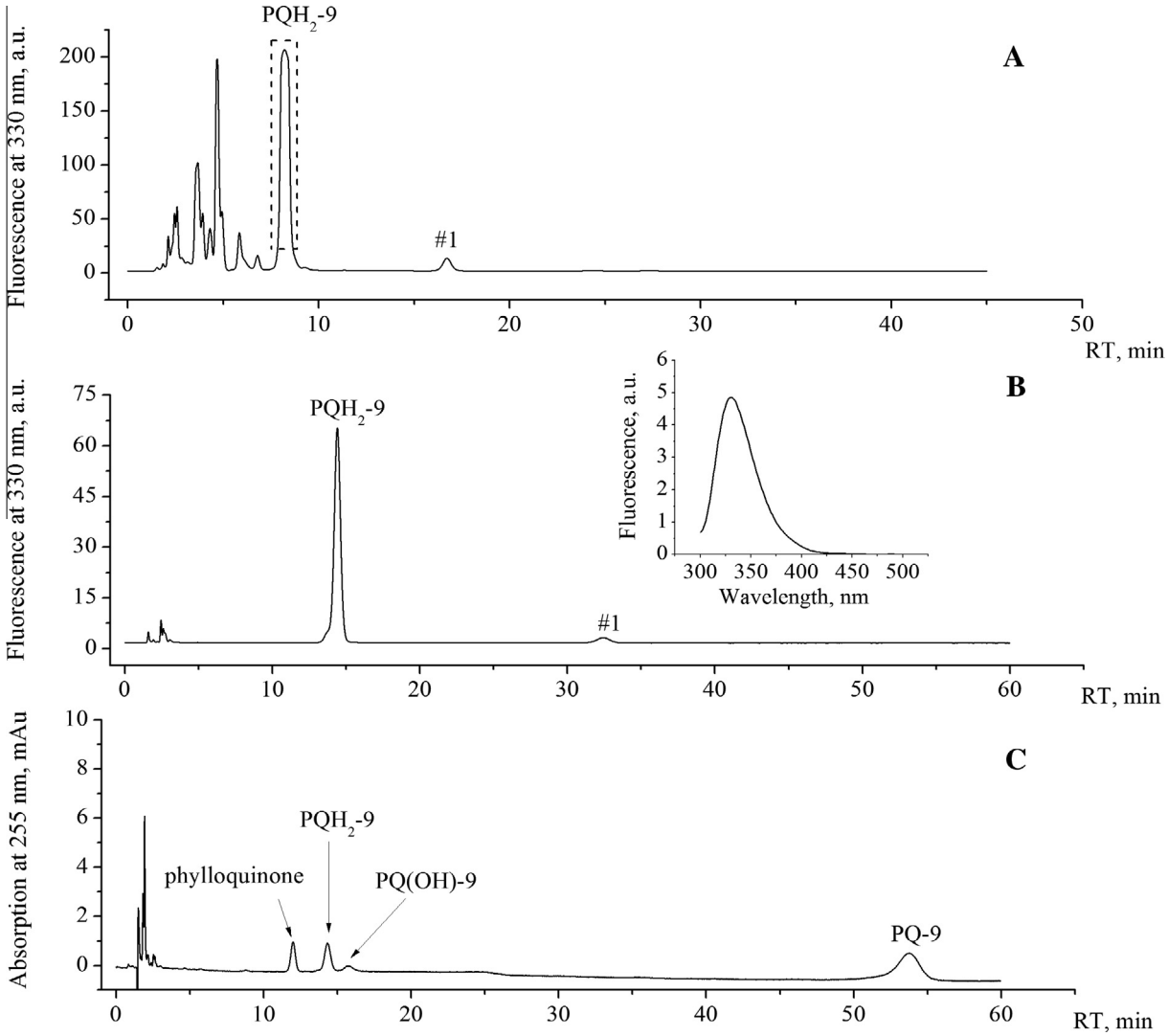

Fig. 1. A chromatogram of unpurified thylakoid extract (A) and chromatograms of purified plastoquinol solution (B and C). PQH_2-9 was detected by fluorescence (A and B) and absorption at 255 nm (C), and PQ-9 by absorption at 255 nm (C). The rectangle in (A) indicates the area of the PQH_2-9 peak which was isolated for purification of PQH_2-9. The emission spectrum of purified PQH_2 is shown as an insert in (B). RT = retention time.

ubiquinone-9 (UQH_2-9) (Fig. 2B). The low absorbance at 255 nm (Fig. 1C) shows that the amount of PQ-9-OH was very low. UQH_2-9 cannot be easily quantified with the HPLC method described here because the peak related to UQH_2-9 overlapped with the PQH_2-9 peak. However, the emission spectrum of the PQH_2-9 solution (insert of Fig. 1B) was identical with the emission spectrum of PQH_2-9 [34], and no detectable fluorescence at 370–490 nm, attributable to of UQH_2-9 (see [34]), could be detected. In addition, a typical chromatogram of both the thylakoid extract and the purified PQH_2-9 solution contained an unknown peak (indicated as peak #1). The ratio between the area of the PQH_2-9 peak and that of peak #1 in both thylakoid extract and purified PQH_2-9 solution was 18–20. We suggest that this peak represents a quinhydrone-type charge-transfer complex of PQ-9/PQH_2-9 [35]. Negative ionization of this complex can lead to destruction of the complex to PQ-9 and PQH^-. In this case, only PQH_2-9, the reduced form of PQ-9, would be detected in the mass spectrum.

Illumination of PQH_2-9 in methanol in the presence of the singlet oxygen sensitizer Rose Bengal (RB; see e.g. [36]) resulted in formation of PQ-9 as the main product (Fig. 3A) and several minor oxidized products associated with oxidation of the isoprenoid side chain (Fig. 3A and B). These findings are in accordance with earlier data [27] except that hydroperoxide groups in the isoprenoid side chain were detected instead of hydroxyl groups. The difference can be explained by the fact that we isolated PQH_2-9 directly from thylakoids whereas earlier experiments have been performed with PQH_2-9 obtained by reducing PQ with $NaBH_4$. The formation of hydroxyl groups in previous works probably resulted from reduction of the hydroperoxides by residual of $NaBH_4$. In the absence of reducing agents, the peroxides are more stable in methanol. Illumination of a methanol solution of PQH_2-9 in the presence of RB did not cause any effect on phylloquinone that is present as a contamination (data not shown). As shown in Fig. 3B, the oxidation of the isoprenoid chain of PQH_2-9 may occur without oxidation of the quinone ring. The products with an oxidized isoprenoid chain but with an intact quinone ring can be detected with fluorescence (λ_{ex} = 290 nm, λ_{em} = 330 nm). The amounts of these products increase with light intensity up to 450 μmol photons m^{-2} s^{-2}. At high light, these products disappear, apparently because the quinone ring, too, becomes oxidized.

NaN_3 is a strong physical quencher of $^1O_2$ and is often used to show involvement of $^1O_2$ in oxidation processes [36]. NaN_3 at 10 mM only partly suppressed the oxidation of 50 μM PQH_2-9 to PQ-9 (Fig. 4) although the second-order rate constant of quenching

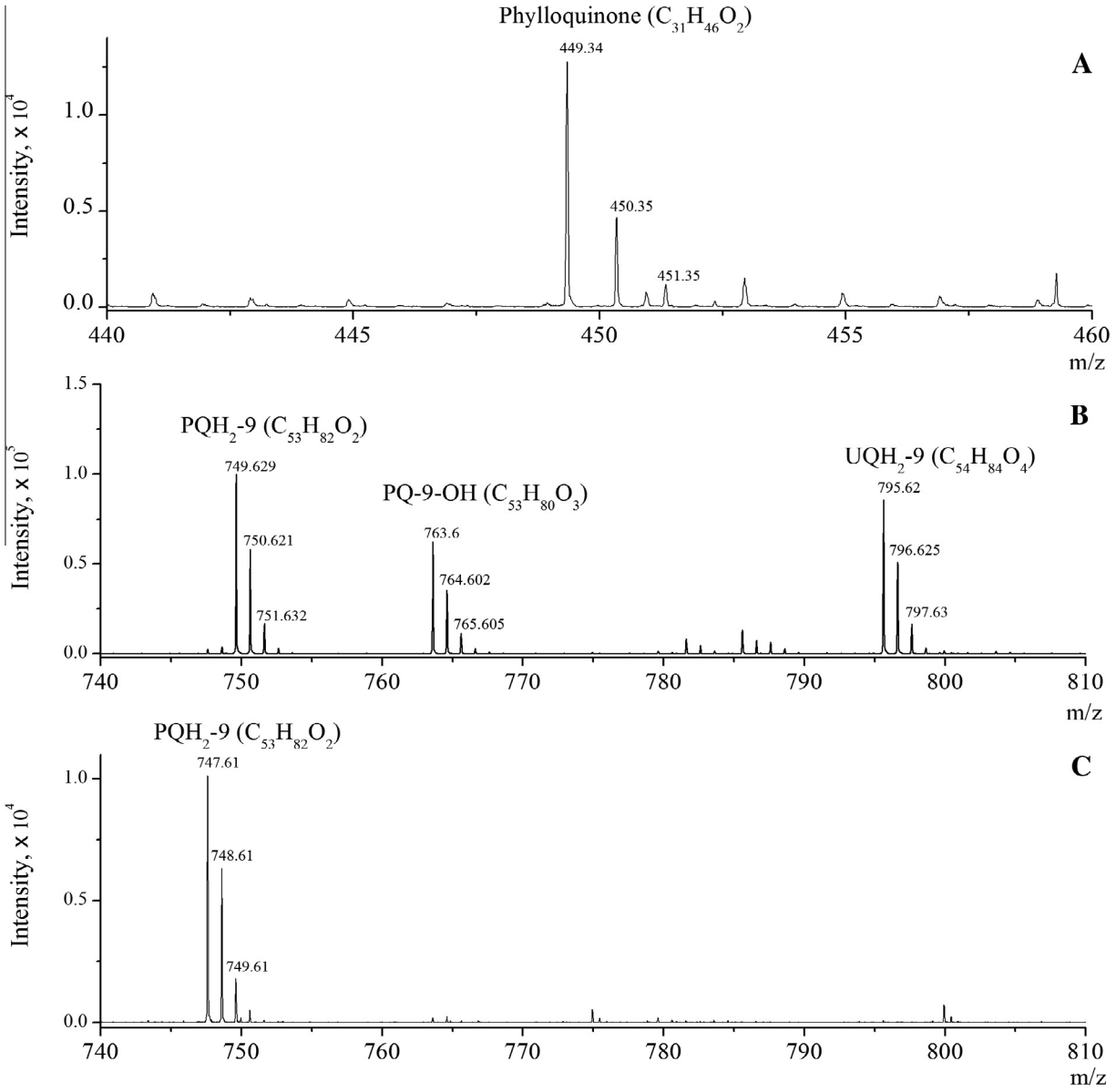

Fig. 2. (A) An average mass spectrum for the peak with retention time 12.1–13.1 min in HPLC (see Fig. 1C); (B) for the peak associated with PQH_2-9 (retention time 13.5–16.5 min, see Fig. 1B); and (C) for the peak associated with PQ-9 (retention time 57–60 min, Fig. 1C).

of $^1O_2$ by NaN_3 in acetonitrile containing 3% of water is 28×10^8 - M^{-1} s^{-1} [37], which is approximately thirty times as high as the rate constant of $^1O_2$ quenching by PQH_2 [27]. The second-order rate constant of $^1O_2$ quenching by NaN_3 in 95% ethanol is 2.3×10^8 - M^{-1} s^{-1} [37]. The effect of NaN_3 on the oxidation of PQH_2-9 by $^1O_2$ may be small because the effective concentration of the N_3^- ion engaged in scavenging of $^1O_2$ in methanol is low due to incomplete dissociation and formation of ion pairs in organic solvents [37]. Incomplete suppression of PQH_2-9 oxidation by NaN_3 can also result from formation of $RB^{3\cdot-}$ and N_3 radical ($N_3^\cdot$) during illumination due to reduction of $^3RB^{2-}$ by N_3^- [38]. The subsequent formation of $O_2^{\cdot-}$ via electron transfer from $RB^{3\cdot-}$to $^3O_2$ can lead to oxidation of PQH_2-9. In addition, $N_3^\cdot$ is able to oxidize PQH_2-9. The yield of $RB^{3\cdot-}$ via reduction of $^3RB^{2-}$ by N_3^- is 0.07 in aqueous solutions [38] but it is possible that $RB^{3\cdot-}$ and $N_3^\cdot$ are significantly produced in methanol at high concentration of NaN_3.

Oxidation of organic molecules by $^1O_2$ often leads to formation of hydroperoxides and endoperoxides [39]. The exposure of PQH_2-9 to $^1O_2$ in methanol mainly caused oxidation of PQH_2-9 to PQ-9, and no significant amounts of stable peroxides or endoperoxides were observed except for the small amount of hydroperoxides of the isoprenoid chain (Fig. 3A and B). Most probably, PQH_2(OOH)-9 containing a peroxide group in the quinone ring is intrinsically unstable and decomposes to PQ-9 and H_2O_2.

We used the luminol-peroxidase system to measure the formation of H_2O_2 via reaction of $^1O_2$ with PQH_2-9. The illumination of PQH_2-9 in methanol the presence of 10 μM RB led to a drastic increase of luminol chemiluminescence (Fig. S1), suggesting that the amount of H_2O_2 exceeded the amount of PQ-9 by several fold (data not shown). This phenomenon was found to result from enhancement of the luminol-H_2O_2-peroxidase chemiluminescence by PQH_2-9 (Fig. 5). Enhancement of the luminol-peroxidase reaction by phenols is well known [40]. Due to the enhancement, the ratio of chemiluminescence intensity to H_2O_2 concentration depends on the concentration of PQH_2-9. For precise measurement of H_2O_2, the samples were divided to two parts after illumination. H_2O_2 in methanol (final concentration 1 μM) was added to one part and an equal volume of methanol was added to the other part. The

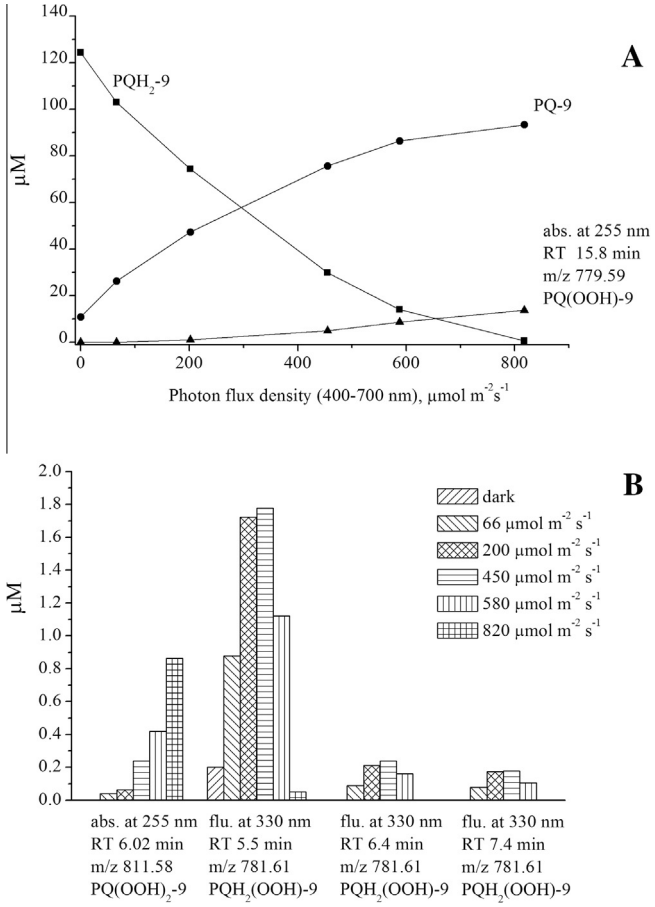

Fig. 3. (A) Light dependent oxidation of PQH_2-9 in the presence of 10 μM RB in methanol. The solution was illuminated for 1 min at each photon flux density. (B) Concentrations of the hydroperoxide derivatives of PQ-9 and PQH_2-9 calculated from the areas of HPLC peaks using absorbance (abs.) or fluorescence (flu.) for quantification.

amount of H_2O_2 was then calculated according to the equation H_2O_2 (μM) = $a/(b-a)$, where a and b are the intensity of the chemiluminescence in the absence and presence of 1 μM H_2O_2, respectively. Using this approach, the amount of H_2O_2 was found to be essentially the same as the amount of oxidized PQH_2-9 (Fig. 6).

The formation of H_2O_2 during of illumination of RB might result from production of $O_2^{\cdot-}$ by electron transfer from $^3RB^{2-}$ to $^3O_2$. $O_2^{\cdot-}$

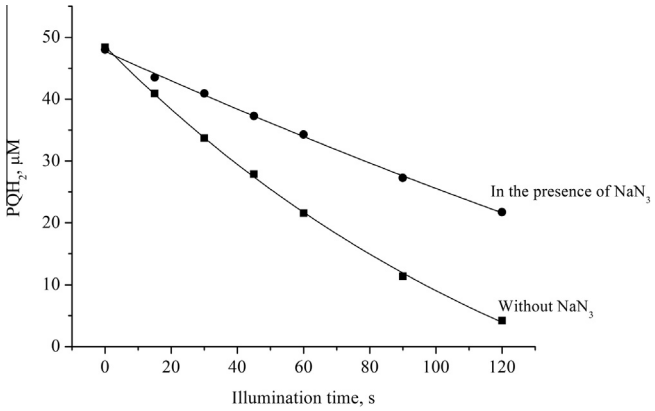

Fig. 4. Effect of 10 mM NaN_3 on oxidation of PQH_2-9 in methanol in the presence of RB under illumination at 300 μmol photons m^{-2} s^{-1}.

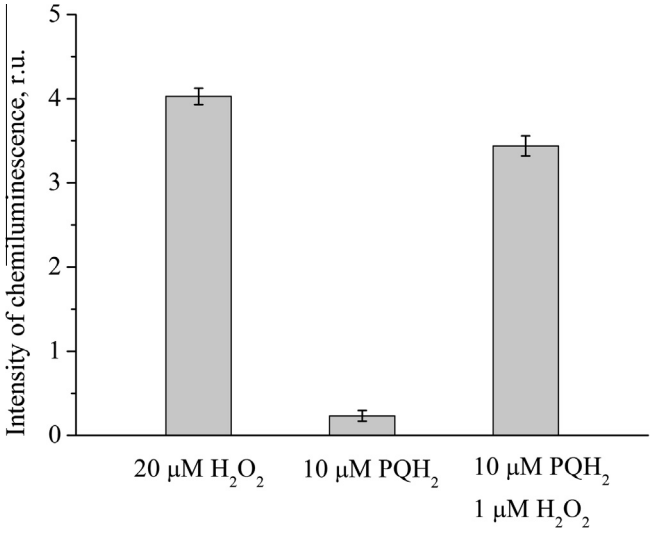

Fig. 5. Chemiluminescence of the luminol-peroxidase system after injection of 20 μl of experimental samples containing 20 μM H_2O_2, 10 μM PQH_2-9, or 1 μM H_2O_2 and 10 μM PQH_2-9, as indicated.

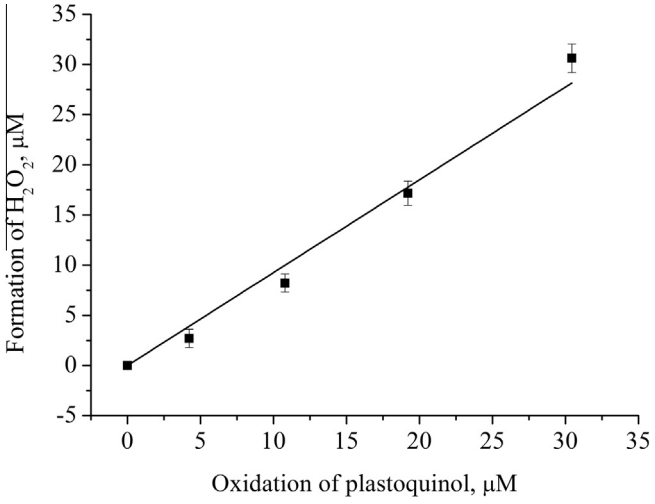

Fig. 6. Dependence of H_2O_2 formation on oxidation of PQH_2-9 by $^1O_2$ in methanol.

can spontaneously dismutate or react with PQH_2-9 to form H_2O_2. However, illumination of RB for 1 min at 1000 μmol photons m^{-2} s^{-1} in methanol produced only 0.25 μM H_2O_2 and 0.75 μM H_2O_2 in the absence and presence of 15 μM PQ-9, respectively (Fig. S2). This H_2O_2 production is very small in comparison to the rates obtained in the presence of PQH_2-9 (Fig. 6). Even at low $^3O_2$ where $^3RB^{2-}$ is not efficiently quenched by energy transfer and therefore electron transfer to $^3O_2$ could occur from $^3RB^{2-}$ or from a reduced substrate produced by electron transfer from $^3RB^{2-}$, the $O_2^{\cdot-}$ yield is less than 1% [41]. Reduction of $^3RB^{2-}$ to $RB^{3\cdot-}$ radical by hydroquinone is an inefficient process, with an $RB^{3\cdot-}$ yield of 0.001 in acetonitrile [38]. A similar yield should be observed for PQH_2-9 in methanol. Thus, the formation of $O_2^{\cdot-}$ via reduction of $^3O_2$ by $RB^{3\cdot-}$ should be low. These data indicate that formation of H_2O_2 mainly proceeds via the singlet oxygen pathway.

4. Discussion

The present data show that reaction of $^1O_2$ with PQH_2-9 produces H_2O_2, thus providing a new mechanism for the formation of H_2O_2 by the PQ-pool of thylakoid membranes. The formation

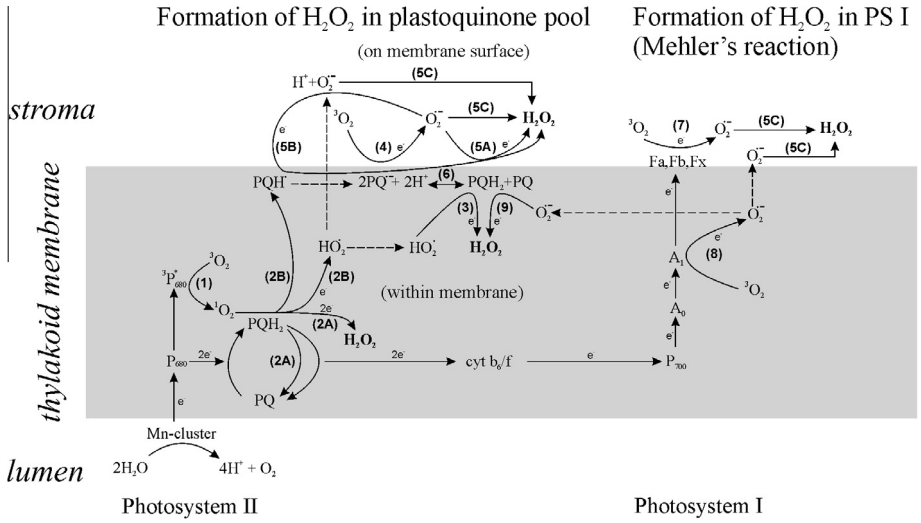

Fig. 7. Mechanisms of H_2O_2 formation in within thylakoid membrane and on membrane surface. Intramembranous formation of H_2O_2 in the PQ-pool is initiated by the formation of $^1O_2$ in PSII (reaction 1). Reaction of $^1O_2$ with PQH_2-9 leads to formation of H_2O_2 and PQ-9 (reaction 2A) or $HO_2^\cdot$ and $PQH^\cdot$-9 (reaction 2B); the $HO_2^\cdot$ formed thereby can form H_2O_2 by oxidizing PQH_2-9 to $PQH^\cdot$-9 (reaction 3). Formation of H_2O_2 on membrane surface starts by reduction of $^3O_2$ to $O_2^{\cdot-}$ by $PQ^{\cdot-}$-9 formed either in reaction 6 or in reaction 2A. H_2O_2 is formed either by dismutation of $O_2^{\cdot-}$ or by reduction of $O_2^{\cdot-}$ by PQH_2-9 (reaction 5A). Formation of H_2O_2 in PS I (Mehler's reaction) begins with reduction of $^3O_2$ to $O_2^{\cdot-}$ by the acceptors F_X, F_A or F_B on membrane surface (reaction 7) or by phylloquinone within the membrane (reaction 8). Reaction 8 might also lead to intramembranous formation of H_2O_2 via reaction of $O_2^{\cdot-}$ with PQH_2-9 (reaction 9).

of H_2O_2 can proceed via oxidation of pure PQH_2-9 to PQ by $^1O_2$ in methanol (Figs. S1 and 6). The same reaction would function as a source of intramembranous H_2O_2 in thylakoids. $^1O_2$ produced in the PSII reaction center can diffuse in the membrane to react with PQH_2-9, as the diffusion length of $^1O_2$ in lipid membrane is in the range of 20–220 nm [42,43]. The reaction between $^1O_2$ and PQH_2-9 is rapid, suggesting that this pathway is an important route for PSII dependent H_2O_2 formation. $^1O_2$ dependent oxidation of the thylakoid PQ-pool has been shown in both *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* during high light stress [26,44]. These data strongly suggest that H_2O_2 is formed via $^1O_2$ dependent oxidation of PQH_2-9 in vivo.

In thylakoid membranes, $^1O_2$ is generated mainly by interaction of $^3O_2$ with $^3P_{680}$ (reaction 1). Reaction of $^1O_2$ with PQH_2-9 can lead to formation of an unstable hydroperoxide adduct of the quinone ring. Decomposition of the hydroperoxide adduct would form H_2O_2 with two alternative mechanisms. Firstly, the hydroperoxide adduct can directly decompose to PQ-9 and H_2O_2 (reaction 2A). Secondly, the hydroperoxide adduct may decompose to form a hydroperoxyl radical ($HO_2^\cdot$) and a protonated plastosemiquinone radical ($PQH^\cdot$-9) (reaction 2B). This indirect mechanism would be similar to that proposed for the oxidation of ascorbate by $^1O_2$ [45]. In the indirect mechanism, H_2O_2 is produced by oxidation of PQH_2-9 to $PQH^\cdot$-9 by $HO_2^\cdot$ (reaction 3).

$$^3P_{680} + {}^3O_2 \rightarrow {}^1P_{680} + {}^1O_2 \quad (1)$$

$$^1O_2 + PQH_2\text{-}9 \rightarrow PQ\text{-}9 + H_2O_2 \quad (2A)$$

$$^1O_2 + PQH_2\text{-}9 \rightarrow PQH^\cdot\text{-}9 + HO_2^\cdot \quad (2B)$$

$$HO_2^\cdot + PQH_2\text{-}9 \rightarrow PQH^\cdot\text{-}9 + H_2O_2 \quad (3)$$

In addition to the reaction between $^1O_2$ and PQH_2-9, PSII also catalyzes formation of H_2O_2 via $O_2^{\cdot-}$ [21]. This type of H_2O_2 formation may occur via reduction of O_2 by $PQ^{\cdot-}$-9 (reaction 4), followed by reduction of $O_2^{\cdot-}$ by PQH_2-9 (reaction 5A), reduction of $O_2^{\cdot-}$ by $PQH^\cdot$-9 (reaction 5B), or by spontaneous dismutation of $O_2^{\cdot-}$ (reaction 5C).

$$PQ^{\cdot-}\text{-}9 + O_2 \leftrightarrows PQ\text{-}9 + O_2^{\cdot-} \quad (4)$$

$$O_2^{\cdot-} + PQH_2\text{-}9 + H^+ \rightarrow H_2O_2 + PQH^\cdot\text{-}9 \quad (5A)$$

$$O_2^{\cdot-} + PQH^\cdot\text{-}9 + H^+ \rightarrow PQ + H_2O_2 \quad (5B)$$

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5C)$$

PQH_2-9 is only very slowly oxidized by O_2 in organic solvents or liposomes [46]. PQH_2-9 can be oxidized via intermediate formation of $PQ^{\cdot-}$-9 [21] which readily reduces O_2 (reaction 4) in aqueous solution, as the redox potentials of the pairs PQ-9/$PQ^{\cdot-}$-9 and O_2/$O_2^{\cdot-}$ are −170 [47] and −160 mV [48], respectively, at pH 7. However, in organic solvent, the respective redox potentials are −400 [49] and −600 mV [50], which renders oxidation of $PQ^{\cdot-}$-9 by O_2 nearly impossible. The physico-chemical reasons presented above suggest that $O_2^{\cdot-}$ is formed on the membrane surface as the result of reduction of $^3O_2$ by $PQ^{\cdot-}$-9 [51]. Furthermore, the subsequent production of H_2O_2 would also occur on the membrane surface because the large solvation energy of $O_2^{\cdot-}$, −1.461 MJ/mol [52], essentially prevents its penetration into the membrane. $HO_2^\cdot$, in turn, would penetrate to the membrane (energy of solvation −29 kJ/mol [52]), but its pKa value is 4.8, and therefore only 0.25% of $O_2^{\cdot-}$ is protonated in the physiological pH of the chloroplast stroma in the light (pH 8 [53]). Therefore, the formation of H_2O_2 by reactions 4-5 would mainly proceed on the membrane surface.

Mechanisms of formation of H_2O_2 on the membrane surface and inside the membrane are connected by the production of semiquinone by reactions 2B and 3. $PQH^\cdot$-9 can also be produced by dismutation (reaction 6A). Deprotonation of $PQH^\cdot$-9 on the surface of membrane (reaction 6B) and subsequent reduction of oxygen by $PQ^{\cdot-}$-9 (reaction 4) would produce $O_2^{\cdot-}$, and H_2O_2 would appear via reactions 5.

$$PQH_2\text{-}9 + PQ\text{-}9 \leftrightarrows 2PQH^\cdot\text{-}9 \quad (6A)$$

$$PQH^\cdot\text{-}9 \leftrightarrows PQ^{\cdot-}\text{-}9 + H^+ \quad (6B)$$

In conclusion, H_2O_2 can be formed in the PQ-pool intramembraneously through oxidation of PQH_2-9 to PQ-9 by $^1O_2$ (reactions 1-3) or on the membrane surface through reduction of O_2 by $PQ^{\cdot-}$-9

(reactions 4-6). Further work is needed to evaluate the importance of these mechanisms. Fig. 7 illustrates the formation of H_2O_2 in PSI (Mehler's reaction) and in the PQ-pool.

PQ-9, the most common prenyllipid in the thylakoid membrane, mediates electron transfer between PSII to PSI and is also involved in cyclic electron transfer around PSI. The redox state of the PQ-pool regulates the expression of both chloroplast and nuclear genes and serves as a major signal in the control of the phosphorylation and dephosphorylation of light-harvesting complex II via the activation of the protein kinase STN7 [54]. It has recently been shown that the STN7 kinase is activated by formation of both intra and intermolecular disulfide bridges [55]. A hydrophobicity analysis of the Arabidopsis STN7 protein showed that cysteins 65 and 70, known to be involved in dimerization [55], are located in a hydrophobic region (Table S1). Thus, H_2O_2 formed in the PQ-pool due to formation of $^1O_2$ in PS II may cause dimerization of STN7. The suggested cooperative signaling that integrates the effects of $^1O_2$ and the redox state of the PQ pool, can function in addition to the independent signaling pathways triggered by $^1O_2$ and H_2O_2 [56].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.011.

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