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Singlet oxygen and non-photochemical quenching contribute to oxidation of the plastoquinone-pool under high light stress in *Arabidopsis*

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ABSTRACT

The redox state of plastoquinone-pool in chloroplasts is crucial for driving many responses to variable environment, from short-term effects to those at the gene expression level. In the present studies, we showed for the first time that the plastoquinone-pool undergoes relatively fast oxidation during high light stress of low light-grown *Arabidopsis* plants. This oxidation was not caused by photoinhibition of photosystem II, but mainly by singlet oxygen generated in photosystem II and non-photochemical quenching in light harvesting complex antenna of the photosystem, as revealed in experiments with a singlet oxygen scavenger and with *Arabidopsis npq4* mutant. The latter mechanism suppresses the influx of electrons to the plastoquinone-pool preventing its excessive reduction. The obtained results are of crucial importance in light of the function of the redox state of the plastoquinone-pool in triggering many high light-stimulated physiological responses of plants.

1. Introduction

Under natural conditions, plants are exposed to frequent changes in light intensity and the photosynthetic apparatus must respond to these changes to harvest solar energy efficiently and to avoid photodamage during elevated irradiance. When incident light intensity is too high to be utilized, it causes over-excitation of the photosynthetic apparatus and several short- and long-term acclimatory mechanisms are elicited from non-photochemical quenching to stimulation of gene expression of nuclear-encoded proteins.

The redox state of the plastoquinone-pool (PQ-pool) in thylakoids is believed to play function of a major redox sensor in chloroplasts which triggers and mediates many cellular responses to variable environment, especially to changes in light quality and intensity. It has been shown that the redox state of the PQ-pool regulates phosphorylation of the light-harvesting complex II (LHCII) via a cytochrome b_6-*f*-dependent kinase [1–3], energy dissipation in photosystem I [4], chloroplastic gene expression of proteins of photosystem II and I complexes (PSII and PSI) [5–9], expression of nuclear-encoded genes for ascorbate peroxidase (APX1 and APX2) [10–13], superoxide dismutase [14,15], carotenoid biosynthesis [16] and others [17]. In most of these cases, the redox state of the PQ-pool was differentially affected by the addition of photosynthetic electron transport inhibitors: DCMU and DBMIB or by treatment of plants with light enriched in 700 and 680 nm wavelength [8,18]. Both of these treatments antagonistically regulate oxidation and reduction of the PQ-pool and can simulate opposite redox states of the PQ-pool under natural conditions.

In several experiments performed recently, cDNA microarray technique was applied to monitor the influence of the redox state of the PQ-pool on expression of thousands of genes [19–21]. However, some of the obtained results were contradictory. Despite these extensive studies, little is known on the mechanism of redox sensing of the PQ-pool and retrograde signal transduction from chloroplast to nucleus. In the recent extensive microarray study [21], 24,000 nuclear *Arabidopsis* genes were screened for the response during medium- and high-light irradiance. Among 663 differentially expressed genes under these conditions, a total of 50 were reverted by DCMU and suggested therefore to be regulated by the redox state of the PQ-pool. It was found that promoter regions of these genes contained short, conserved elements that are probably regulated by shared transcription factors [21].

In plant cells, PQ is synthesized and resides in chloroplasts, mainly in thylakoids and plastoglobuli [22]. In thylakoid membranes, plastoquinone constitutes photochemically redox active chloroplast fraction (PQ-pool), while the fraction found in plastoglobuli is photochemically non-active and is supposed to be the storage site of this prenyllipid necessary for its antioxidant action [22,23]. Both the plastoquinone fractions together make up the total plastoquinone

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DPA, diphenylamine; HL, high light; HPLC, high-performance liquid chromatography; PQH_2, reduced plastoquinone, plastoquinol; PQ, plastoquinone; PS, photosystem; TNM, tetranitromethane

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content of leaves and chloroplasts that can be measured in extracts of leaves using HPLC. Plastoglobuli are especially increased in size and number in older and sun-exposed leaves [22–24]. Both the above mentioned fractions refer to PQ-9 (PQ-A), containing unmodified isoprenoid side-chain. Besides this prenyllipid, also other plastoquinones are known, like PQ-C with a hydroxyl group in the side-chain or PQ-B, a fatty acid ester of PQ-C [25].

It is generally supposed that during high light illumination of plants, the PQ-pool of thylakoids is highly reduced, however, no direct evidence is given in most of the studies. On the contrary, oxidation of reduced plastoquinone in plastoglobuli was observed under high light conditions [26]. Since knowledge on the redox state of the PQ-pool during high light stress is of crucial importance for understanding physiological responses of plants under these conditions, we have performed study on the redox state of the PQ-pool during the stress, taking advantage of the recently developed method [27].

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana plants (wild-type Col-0, *npq4* mutant) were grown under low light conditions (90 μmol photons $m^{-2}\,s^{-1}$, 22 °C, 12 h photoperiod). High light of 2000 μmol photons $m^{-2}\,s^{-1}$ intensity was provided by 400 W halogen lamp with 0.5% $CuSO_4$ solution as a heat filter. For analysis, 5 week-old plants were used.

2.2. Determination of the size and redox state of photochemically active and non-active PQ fractions in leaves

The size of the photochemically active PQ-pool (in thylakoids) and the redox state of the photochemically non-active PQ (in plastoglobuli) of *Arabidopsis* leaves were measured by HPLC as described in Ref. [27] in detail. For the experiments, fully grown leaves of similar size (older leaves) were used. In short, one part of the leaves was illuminated shortly with strong light (2000 μmol photons $m^{-2}\,s^{-1}\times 15$ s) to fully reduce the PQ-pool and immediately homogenized in mortar with cold ethyl acetate. The extract was transferred to an Eppendorf tube, evaporated in a stream of nitrogen and analyzed by HPLC. The other part of leaves was infiltrated with 50 μM DCMU, illuminated shortly (500 μmol photons $m^{-2}\,s^{-1}\times 15$ s) to fully oxidize the PQ-pool and the leaves were then treated as before. The redox state of PQ was compared from both conditions and this allowed to calculate the extent of photochemically active PQ-pool and photochemically non-active PQ fraction in leaves, as well as the redox state of the latter fraction. The redox state of the PQ-pool in *Arabidopsis* leaves during high light stress (2000 μmol photons $m^{-2}\,s^{-1}$) was determined using the above data and those obtained from analysis of the illuminated plants. In the case of DCMU, DPA and TNM, the leaves were infiltrated with the solution of the compounds in tap water and were left for 5 min at low light conditions before the high light illumination.

PQ-C was determined using HPLC as described before [28].

2.3. Fluorescence measurements

Maximum quantum yield of photosystem II (F_v/F_m) was measured using PAM-210 instrument (Walz, Germany) on control or high light-treated leaves that were dark-adapted for 10 min before the measurement. Non-photochemical quenching, $NPQ=(F^0_m-F'_m)/F'_m$ [29], was measured with PAM-101 instrument (Walz, Germany) using saturating pulse of 4500 μmol photons $m^{-2}\,s^{-1}$ intensity and actinic light intensity of 2000 μmol photons $m^{-2}\,s^{-1}$. F^0_m is the maximum fluorescence level after application of the saturating pulse to dark-adapted leaves, while F'_m is the maximum fluorescence after application of the saturating pulse during actinic light illumination of leaves.

2.4. Oxygen evolution measurements

Before the isolation procedure, both control and high light-treated plants were incubated on ice in the dark for 10 min. Then 2 rosettes of each plant were homogenized for 5 s with a blender in 50 ml of HB buffer (0.45 M sorbitol, 20 mM Tricine-KOH, pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$, 5 mM ascorbate, 1 mM $MnCl_2$), filtered through nylon cloth and centrifuged at 1000 $g\times 2$ min. The sedimented chloroplasts were suspended in 1 ml of HB buffer. The oxygen evolution measurements were performed with Clark-type electrode (Hansatech, U.K.) in 50 mM Hepes buffer containing 20 mM NaCl and 5 mM $MgCl_2$ (pH 7.5) at 20 °C. Chlorophyll concentration was 25 μg/ml and 1 mM ferricyanide with 100 μM *p*-benzoquinone was added as electron acceptors. Saturating light intensity of 1500 μmol photons $m^{-2}\,s^{-1}$ was used.

2.5. Fractionation of leaves

Two rosettes of control and high light treated plants were homogenized for 5 s with a blender in 50 ml of HB buffer, filtered through nylon cloth and centrifuged at 1000 $g\times 2$ min (supernatant1). The sedimented chloroplast-enriched fraction was osmotically shocked by the addition of 5 ml of distilled water, following 5 cycles of freeze–thawing in liquid nitrogen to release plastoglobuli from thylakoids. Then, the suspension was centrifuged at 100,000 *g* for 100 min and the supernatant (supernatant2) containing the plastoglobuli fraction was extracted with ethyl acetate. The organic fraction was transferred to an Eppendorf tube, evaporated in a stream of nitrogen and analyzed by HPLC. The sediment containing plastoglobuli-free thylakoids was extracted with methanol and analyzed as before.

2.6. Chlorophyll and xanthophyll pigments determination

Chlorophyll concentration was determined spectrophotometrically in acetone or methanol solutions according to Ref. [30]. Carotenoids of the xanthophyll cycle (violaxanthin, antheraxanthin, zeaxanthin) were determined by HPLC as described in Ref. [31].

3. Results

We have found that *Arabidopsis* WT plants grown under low light conditions showed 50% of foliar plastoquinone in the photochemically active form (PQ-pool) and the other 50% was in photochemically non-active form localized outside thylakoids, probably mainly in plastoglobuli [23] (Table 1). The latter plastoquinone fraction was predominantly in the reduced state. When the plants were exposed to continuous high light illumination, a fast oxidation of the PQ-pool was observed (Fig. 1). Already after 15 min of high light stress, more than 80% of the pool was oxidized. This observation was against the commonly accepted view that the PQ-pool is highly reduced during high light conditions. In order to find the possible sources of fast

Table 1
Size of the PQ-pool, of the photochemically non-active PQ (PQ_{NP}), its redox state and the relative content of total PQ (oxidized + reduced) to chlorophyll in leaves of *Arabidopsis* WT and *npq4* mutant grown under low-light conditions. Data are means ± SE ($n=3$). PQ-pool is the photochemically active (thylakoid) PQ fraction, while PQ_{NP} is photochemically non-active (plastoglobuli) PQ fraction of chloroplasts. Total PQ is PQ-pool and PQ_{NP} in both redox forms.

	PQ-pool (% total)	PQ_{NP} (% total)	Redox state of PQ_{NP} (%reduced)	PQ_{tot}/1000 Chl (mol/mol)
WT	50.0 ± 6.1	50.0 ± 6.1	70.2 ± 7.3	30.6 ± 1.1
npq4	47.7 ± 1.9	52.3 ± 1.9	73.3 ± 3.0	30.0 ± 0.9

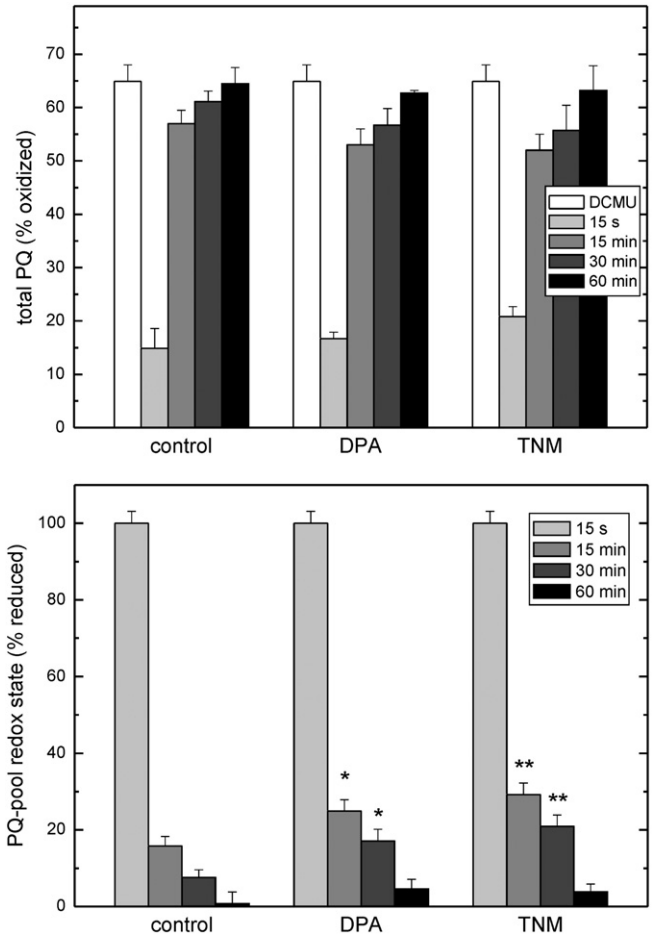

Fig. 1. The effect of high light on: (top) the redox state of total foliar plastoquinone and on the calculated redox state of photochemically active PQ-pool (bottom) in *Arabidopsis* WT control plants, DPA- and TNM-treated plants. The concentration of diphenylamine (DPA), a singlet oxygen scavenger, was 10 μM while that of tetranitromethane (TNM), a superoxide scavenger, was 5 μM. The data are means ± SE ($n = 3$–5). In the lower panel, the asterisks indicate significance of differences *vs.* control (*$P<0.05$, **$P<0.01$).

oxidation of the PQ-pool, we considered oxidation of the PQ-pool by singlet oxygen generated in PSII during high light stress. For this purpose, an analogical experiment was performed in the presence of diphenylamine (DPA), an active and specific singlet oxygen scavenger used previously in several studies to test the contribution of this reactive oxygen form in physiological reactions [28,32]. In the presence of DPA, inhibition of PQ-pool oxidation was observed by about 10%, indicating that singlet oxygen is involved to some extent in PQ-pool oxidation during the stress. When the leaves were illuminated in the presence of tetranitromethane (TNM), an superoxide scavenger, the inhibition of PQ-pool oxidation was even more pronounced (Fig. 1). Since the inhibitory effect of both of the tested compounds could be also due to their inhibition of the photosynthetic electron transport, we measured the effect of these compounds on oxygen evolution in isolated chloroplasts. While DPA showed no effects in this respect, TNM inhibited oxygen evolution by about 20%. Therefore, the effect of TNM on the redox state of the PQ-pool cannot be regarded as specific for superoxide.

One of the possible reasons of oxidation of the PQ-pool under high light illumination could be photoinhibition of PSII. In order to verify this possibility, maximum quantum yield of PSII was measured during the stress. However, the decrease in F_v/F_m values was not pronounced (Table 2). Similarly, oxygen evolution activity of PSII decreased only by about 10% in the course of the stress (Table 2).

Table 2
The effect of high light on total PQ content (oxidized and reduced), maximum quantum yield of photosystem II (F_v/F_m) in leaves of *Arabidopsis* WT and oxygen evolution of *Arabidopsis* WT chloroplasts. For F_v/F_m determination, the whole rosettes were dark-adapted for 10 min before the measurements. Oxygen evolution was measured on osmotically shocked chloroplasts using 1 mM ferricyanide with 100 μM benzoquinone as electron acceptors. Data are means ± SE ($n = 3$). n.d. — not determined.

High light (min)	F_v/F_m	O_2 evolution (μmol/mg Chl/h)	PQ_{tot}/1000 Chl (mol/mol)
0	0.846 ± 0.001	98.9 ± 0.8	30.6 ± 1.1
10	0.786 ± 0.007	n.d.	n.d.
15	n.d.	n.d.	25.9 ± 1.3
20	0.773 ± 0.010	n.d.	n.d.
30	0.783 ± 0.013	88.7 ± 1.9	25.0 ± 0.4
40	0.751 ± 0.022	n.d.	n.d.
50	0.780 ± 0.017	n.d.	n.d.
60	0.767 ± 0.020	89.5 ± 1.6	28.3 ± 1.2

These data indicate that photoinhibition of PSII was negligible, therefore it could be concluded that activity of PSII in PQ-pool reduction was not significantly changed during the high light illumination and cannot account for the observed PQ-pool oxidation.

The factor that could affect accuracy of the determination of PQ-pool measurements during high light stress is a possible oxidation of PQ to other products such as hydroxy-PQ (PQ-C) or any other similar products that were observed *in vitro* studies [33]. However, the analysis of PQ-C during the high light stress showed that its level changed from 0.71 ± 0.08 mol/1000 Chl to 1.22 ± 0.16/1000 Chl after 1 h of the illumination, *i.e.* the increase was 0.5 mol PQ-C/1000 Chl, corresponding to 1.7% of the total PQ amount in leaves. At the same time, the total PQ amount in relation to chlorophyll did not change considerably (Table 2). These data indicate that PQ oxidation to PQ-C could be not the reason for the observed oxidation of the PQ-pool. The total (oxidized and reduced) level of PQ in relation to chlorophyll in *Arabidopsis* leaves is lower than that reported for other plant species [34–36] but the relative PQ/chlorophyll ratio is known to be strongly influenced by growth conditions and age of *Arabidopsis* and other plant species [22,23,27,34,35].

We have also considered another factor influencing reliability of the determination of PQ-pool extent during the high light stress. The calculations are based on the assumption that during the stress the proportion of the PQ-pool and photochemically non-active PQ do not change, as well as does not change the redox state of the photochemically non-active PQ. In order to verify this, we have performed fractionation of *Arabidopsis* leaves of control and high light-treated plants to obtain chloroplast-enriched fraction, plastoglobuli and thylakoid membranes (Table 3). Comparing the PQ to Chl

Table 3
The proportion of total (oxidized + reduced) PQ (PQ_{tot}) to chlorophyll and its redox state in *Arabidopsis* WT leaves and different fractions during isolation of plastoglobuli-free thylakoids in control and high light-treated plants. *In the case of chlorophyll-free plastoglobuli fraction (supernatant2), the proportion of PQ_{tot} in this fraction to that of thylakoids is given. See Materials and methods for further details. The data are means ($n = 2$–3) and SE was ≤10% of the given values. HL — high light.

Fraction	PQ_{tot}/1000 Chl (mol/mol)	Redox state of PQ_{tot} (% oxidized)
Leaves — control	30.1	27.7
Leaves — 1 h HL	29.3	29.8
Filtered homogenate — control	23.4	64.8
Filtered homogenate — 1 h HL	23.5	72.3
Chloroplast fraction — control	19.1	59.6
Chloroplast fraction — 1 h HL	18.8	71.8
Supernatant1 — control	20.7	65.8
Supernatant1 — 1 h HL	19.9	72.6
Thylakoids — control	13.4	100
Thylakoids — 1 h HL	14.2	100
Supernatant2 — control	17.2% of thylakoids level*	96
Supernatant2 — 1 h HL	13.4% of thylakoids level*	100

proportion in the chloroplast fraction and in leaves before and after the stress, there is significant loss of PQ in the chloroplast fraction (Table 3). PQ is supposed to be localized rather exclusively in chloroplasts although some literature data indicate also its presence in some other cell compartments [37]. The above data indicate that during isolation of the chloroplast-enriched fraction, much of PQ present in plastoglobuli is released to the supernatant (supernatant1) (Table 3). The redox state of PQ in this fraction did not change significantly between control and high light treated plants. Plastoglobuli-free thylakoids contain the same PQ to Chl proportion in high light-treated and untreated plants and these levels correspond to approximately half of those found in leaves and this corresponds well with the calculations of the PQ-pool size (Table 1). The supernatant 2 fractions, corresponding to plastoglobuli firmly bound to thylakoids that were released during the freeze–thaw procedure, contained similar proportions of PQ. Both thylakoids and plastoglobuli contained only the oxidized PQ, because of the oxidation of plastoquinol during the relatively long isolation procedure.

In order to examine contribution of non-photochemical quenching to the oxidation of the PQ-pool, we measured the effect of high light stress on the redox state of PQ-pool also in *Arabidopsis npq4* mutant. The obtained data (Fig. 2) show that PQ-pool oxidation in the mutant was slower that in the WT by about 10% in the beginning of the high light stress. The difference was not high but significant. The physiological state of both the mutant and the WT plants were the same as indicated by the same extent of PQ-pool, photochemical

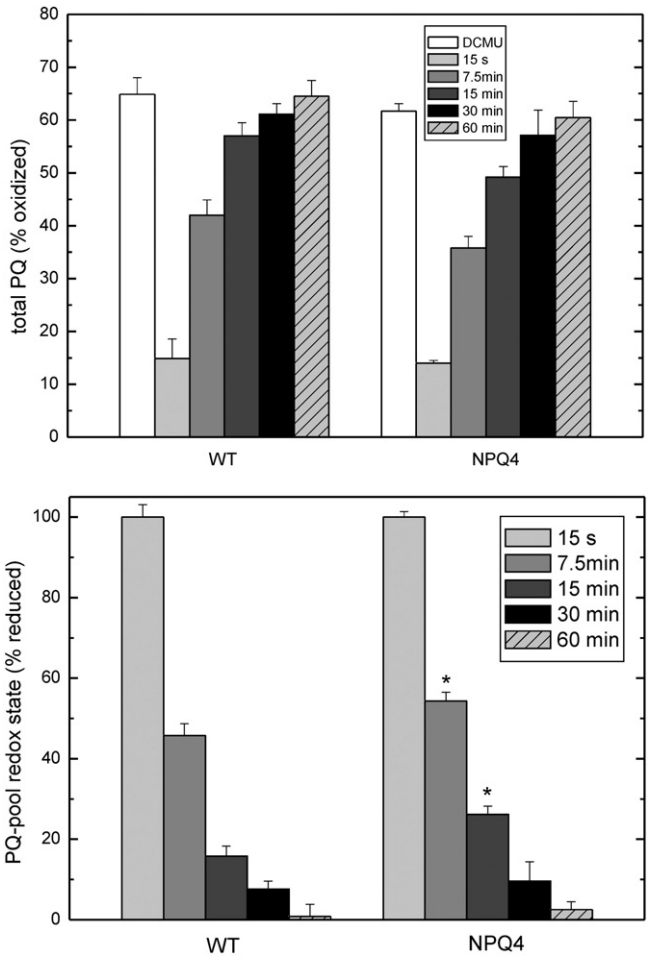

Fig. 2. The effect of high light on: (top) the redox state of total foliar plastoquinone and on the calculated redox state of photochemically active plastoquinone-pool (bottom) in *Arabidopsis* WT plants and *npq4* mutant. The data are means ± SE ($n = 3–5$). In the lower panel, the asterisks indicate significance of differences *vs.* control (*$P<0.05$, **$P<0.01$).

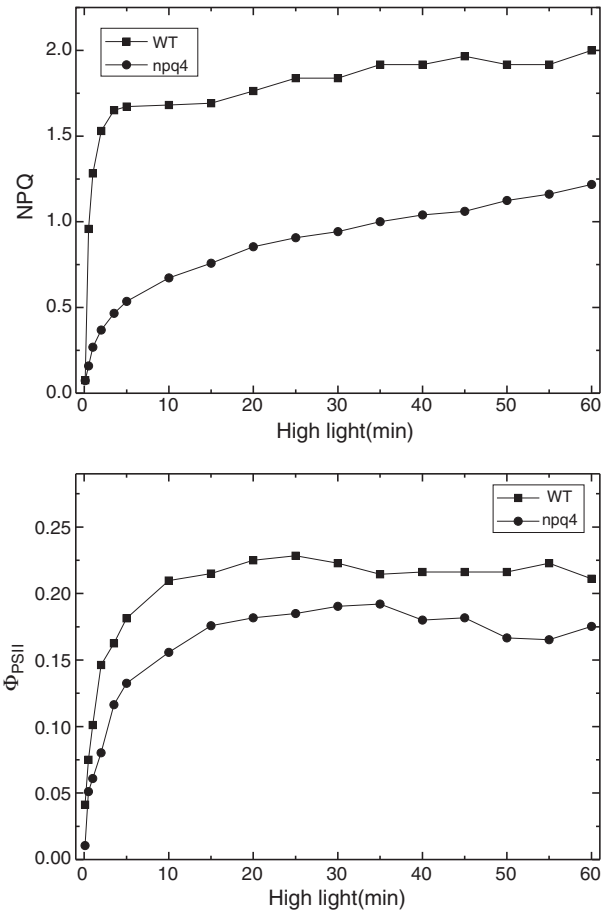

Fig. 3. Non-photochemical quenching (NPQ) and quantum yield of photosystem II (Φ_{PSII}) during illumination of *Arabidopsis* WT and *npq4* mutant. Actinic light of 2000 μmol photons m^{-2} s^{-1} intensity was applied.

non-active PQ, their redox state and the total PQ to Chl proportion (Table 1). The measurements of non-photochemical quenching for both type of plants indicate that in the case of the WT the quenching was more pronounced and reached the linear phase already after 5 min of illumination, while for the mutant, at 15–20 min of high light stress (Fig. 3). The quantum yield of PSII for both plants saturated at the similar illumination time and was only slightly lower for the mutant, as compared to the WT. These data indicate that the linear

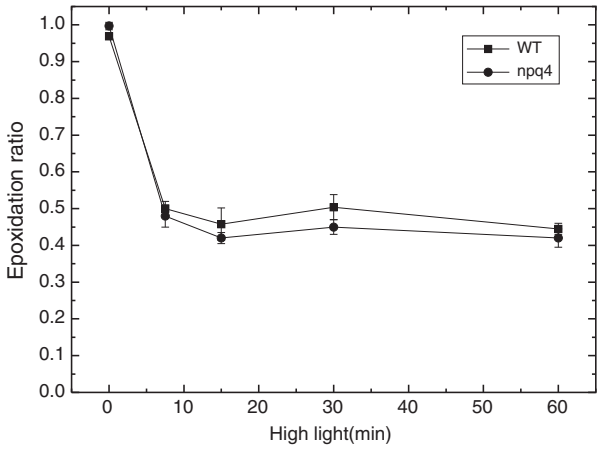

Fig. 4. Epoxidation ratio (EPS = (V + 1/2A)/(V + A + Z)) in leaves of *Arabidopsis* WT and *npq4* mutant during high light illumination. The data are means ± SE ($n = 3–5$). V, violaxanthin, A, antheraxanthin, Z, zeaxanthin.

electron transport rate, which is proportional to Φ_{PSII} [29], is similar in both cases even at the long illumination time. The *npq4* mutant shows partially impaired non-photochemical quenching due to loss in *psb*S protein that is responsible for energy transfer between LHCII complexes to the reaction center of PSII, however the other components of the non-photochemical quenching mechanism, like xanthophyll cycle-based mechanism is functional to the same extent as in the WT (Fig. 4).

4. Discussion

The results of our experiments revealed that PQ-pool is relatively fast oxidized during high light stress of low light-adapted *Arabidopsis* plants, the observation that is against the common view. Nevertheless, oxidation of plastoquinol in plastoglobuli of *Ficus elastica* leaves upon illumination was already reported [26]. It was also suggested in that paper that electrons from plastoquinol are used for deepoxidation of violaxanthin in thylakoid membranes. Moreover, the fact that upon darkening there is re-epoxidation of zeaxanthin to violaxanthin (an oxidation process), that is associated with a reduction of plastoquinone to plastoquinol in plastoglobuli, might be a hint that electrons removed from zeaxanthin flow back to the plastoquinol [26].

Several control experiments performed in the present study showed that the obtained data are reliable and not caused by possible side-effects, such as significant oxidation of the photochemically non-active PQ fraction in plastoglobuli, change in the proportion of the PQ-pool and the photochemically non-active PQ fraction during high light stress. It was also shown that photoinhibition of PSII was insignificant during the stress and could not be responsible for the observed effects. The presented data indicate that one of the reasons of the fast oxidization of the PQ-pool was oxidation of plastoquinol by singlet oxygen generated in enhanced amounts during the stress. Singlet oxygen was shown to be the main reactive oxygen species formed in PSII by deactivation of triplet state chlorophyll of the reaction center [38,39]. It was also found to be the major reactive oxygen species involved in photooxidative damage to plants [40]. It has been shown in many experiments, performed both *in vitro* and *in vivo* that plastoquinol is an effective singlet oxygen scavenger and it is oxidized to plastoquinone in this reaction [28,33,41]. The contribution of superoxide to the oxidation of the PQ-pool seems to be less evident because TNM, the superoxide scavenger, inhibited the electron flow in thylakoids. Although it was suggested that superoxide could oxidize plastoquinol in thylakoids [42] the data obtained *in vitro* indicate that superoxide shows considerably higher reducing activity towards quinone forms of prenyllipids than in the oxidation of the reduced forms [43]. Among other possible pathways of PQ-pool oxidation, plastoquinol oxidase(s) engaged in chlororespiration can be considered, like plastoquinol terminal oxidase (PTOX) [44] or cytochrome b_{559} [45]. However, our control experiments (results not shown) using octyl gallate, a PTOX inhibitor [46], showed no effect on PQ-pool oxidation rate during the high light stress. Our results of the experiments obtained with the *npq4* mutant indicate that non-photochemical quenching shows also significant contribution to the PQ-pool oxidation. The quenching lowers excitation of PSII reaction center, preventing it from over-excitation and photoinhibition. This results in lower PSII-reducing activity and prevents over-reduction of the PQ-pool. It is generally believed that under uninhibited conditions, PQ-pool oxidation by cytochrome b_6f is slower than its reduction by PSII and that during saturating light intensity, PQ-pool is highly reduced. Over-reduction of the PQ-pool could be harmful for the photosynthetic apparatus since it might cause accumulation of triplet states of PSII reaction center and enhanced production of singlet oxygen [38,39]. Thus, keeping PQ-pool in a partially oxidized state is crucial for photoprotection under high light conditions. Oxidation of the PQ-pool correlated well with the increase in quantum yield of PSII (Figs. 2 and 3) and this provides high quantum yield of PSII during high light stress. In light of our results, it is interesting to note that a hypothetical PQ oxidation system during acclimation of tobacco leaves to high light conditions was suggested [47]. In this research, during the acclimation, also other different PQ-pool oxidation systems (photorespiratory carbon oxidation, water–water cycle, heat dissipation processes) were considered in preventing accumulation of electrons in the photosynthetic electron transport chain [47]. Moreover, oxidation of the PQ-pool during high light stress observed in our studies raises the question as to the mechanism of redox-activated signaling pathways mediated by the PQ-pool in chloroplasts under high light stress. Certainly, considering the present results, these concepts must be at least partially revised. On the other hand, it should be also taken into consideration that in our experiments, as in many others with the application of high light stress, the plants grown under low light conditions were exposed to high light intensity that the plants never experienced during their life, *i.e.* the situation probably not frequently found under natural conditions.

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