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The Effect of Hydrogen Peroxide on the Redistribution of Antenna Complexes Between Photosystems in Higher Plants

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Abstract—One of the adaptive mechanisms used by photosynthetic organisms in response to changing light conditions is redistribution of antenna complexes between the photosystems, a process known as state transitions (ST). This mechanism allows to regulate the amount of light energy absorbed by the photosystems. Numerous studies have reported inhibition of ST at the elevated light intensity; however, the mechanism underlying this process is still debated. We evaluated the effect of H_2O_2 at various concentrations on the ST process in functionally active thylakoids isolated from *Arabidopsis thaliana* leaves and investigated which stage of this process is affected by H_2O_2. To assess the extent of ST, we measured low-temperature chlorophyll *a* fluorescence spectra (650-780 nm) and calculated the F745/F685 ratio, whose changes can serve as an indicator of ST progression. H_2O_2 inhibited ST under the low-intensity light conditions and, furthermore, led to a decrease in the accumulation of phosphorylated Lhcb1 and Lhcb2 proteins involved in ST. This suggests that the observed ST inhibition resulted from the suppression of STN7 kinase activity. Importantly, H_2O_2 in the tested concentrations did not affect the electron transport rate, indicating that the inhibition of STN7 kinase activity was not associated with suppression of the photosynthetic electron transport chain (PETC) activity. The treatment with H_2O_2 did not reduce the level of phosphorylated D1 protein (a product of phosphorylation by the thylakoid STN8 kinase). Taken together, these results demonstrate for the first time the mechanism by which H_2O_2 inhibits STN7 kinase activity and, consequently, the process of ST.

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Keywords: photosynthesis, phosphorylation, state transitions, light-harvesting antenna, hydrogen peroxide

INTRODUCTION

Environmental changes trigger adaptive mechanisms, thus enabling plants to regulate the amount of light quanta absorbed by the photosynthetic apparatus (PA). The efficiency of light energy absorption is determined by the functional size of the light-harvesting antenna of each photosystem, which can dynamically adjust in response to varying environmental conditions. The light-harvesting antenna complexes (LHC) of the photosystems are pigment–protein complexes. In higher plants, they consist of Lhcb1-6 proteins in photosystem II (PSII) and Lhca1-4 proteins in photosystem I (PSI) capable of non-covalent binding of chlorophylls (Chls) and carotenoids [1-3].

One of the key adaptive responses to changes in the light conditions which allows simultaneous regulation of the functional antenna size in both PSI and PSII, is a process known as state transitions (ST). This process is considered to be a mechanism by which plants adjust to changes in the spectral composition of light. Under illumination predominantly exciting PSII, the thylakoid membrane-associated enzyme STN7 kinase becomes activated and phosphorylates the outer light-harvesting complex II (LHCII) proteins, resulting in the electrostatic repulsion of the antenna complexes

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from PSII, followed by their association with PSI, and formation of state 2. Subsequent shading or illumination with light that predominantly excites PSI leads to the dephosphorylation of these proteins and return of LHCII to PSII, and formation of state 1. Dephosphorylation is catalyzed by the PPH1/TAP38 phosphatase, also associated with the thylakoid membrane [4, 5]. Later studies confirmed that ST can proceed under low-intensity white light also (LL).

Substantial progress has been made in understanding the molecular mechanism of ST, including identification of key components involved in this process. Loosely bound LHCII trimeric complexes (L-trimers) migrate between the photosystems, while strongly (S-) and moderately (M-) bound trimers remain associated with PSII [6, 7]. The L-trimers consist of Lhcb1 and Lhcb2 proteins, both of which undergo phosphorylation. However, the roles of these proteins differ: phosphorylated Lhcb2 (Lhcb2-P) is crucial for the LHCII association with PSI [8, 9], whereas phosphorylated Lhcb1 (Lhcb1-P) is involved in the disassembly of grana stacking [10, 11].

Exposure to high-intensity light (HL) leads to a reduced phosphorylation of LHCII proteins, and, consequently, suppression of LHCII migration to PSI, meaning that the ST is inhibited under these conditions [12-14]. In our previous work, we demonstrated that the light intensity at which ST become inhibited varies between plant species [15]. For example, in the model plant *Arabidopsis thaliana*, inhibition of ST occurs at the light intensities above 300 μmol quanta·m^{-2}·s^{-1}, whereas in barley, this level is above 800 μmol quanta·m^{-2}·s^{-1}.

It has been suggested that HL inhibits STN7 kinase activity, resulting in blocking plants in state 1, in which all proteins of LHCII complexes remain bound to PSII. However, the exact mechanism of STN7 inhibition under HL remains unclear [13, 14, 16]. STN7 is a serine/threonine protein kinase containing a single transmembrane domain, with its N-terminus exposed to the thylakoid lumen and its catalytic domain and C-terminus located on the stromal side. Both termini contain two conserved Cys residues [17, 18]. Notably, one Cys residue at the N-terminus is directed towards the thylakoid membrane, while the other is exposed to the lumen. It was shown that STN7 interacts with the cytochrome *b6f* complex via the Rieske protein [17], and the site of STN7 interaction is located between the residues 51 and 97 in the PetC protein of this complex [19]. The binding of reduced plastoquinone (plastoquinol) to the binding site in the cytochrome *b6f* complex activates STN7 kinase [20, 21]: the conformational changes in the binding pocket lead to the repositioning and exposure of the N-terminal Cys residue to the lumen. Under these conditions, the two Cys residues are brought into proximity in the lumen, enabling formation of an intramolecular disulfide bond and then of an intermolecular disulfide bond, resulting in covalently linked the STN7 dimer, which ultimately leads to the kinase activation [19].

The available data on whether STN7 kinase is active in its oxidized or reduced state remain contradictory. Wu et al. [22] demonstrated that the conserved luminal cysteine residues of STN7 are critical for maintaining the oxidized state of this kinase, which is necessary for its enzymatic activity. In contrast, Singh et al. [23] reported that the STN7 kinase remains active in the presence of a reducing agent, indicating that the reduced state of cysteines is required for the catalytic function. However, it remains unclear whether stromal or luminal cysteine residues are responsible for regulating the STN7 activity. Using site-directed mutagenesis, Shapiguzov et al. [19] showed that substitution of the stromal cysteines in STN7 kinase did not impair the ST process, whereas substitution of its luminal cysteines resulted in inhibition of ST.

Several studies have suggested that thioredoxins play a key role in the inhibition of STN7 kinase under HL conditions by reducing stromal Cys residues [24, 25]. For example, in plants overexpressing thioredoxin m, phosphorylation of Lhcb1 and Lhcb2 proteins was suppressed even under LL [26]. So far, the involvement of thioredoxins in the STN7 regulation still has to be proven. It was also proposed that superoxide anion radical, which is potentially produced during plastoquinol oxidation at the quinol oxidation site of the cytochrome *b6f* complex, may influence the activity of *Chlamydomonas reinhardtii* STT7 kinase, an equivalent of STN7 from higher plants [23]. Coordinated oxidation of plastoquinol at this site is expected to reduce the likelihood of superoxide anion radical generation in the cytochrome *b6f* complex.

Hydrogen peroxide is known to oxidize Cys residues in various enzymes [27, 28], including kinases [29, 30]. In a number of studies, including those conducted by our group, it has been proposed that hydrogen peroxide produced in chloroplasts under HL conditions may affect the STN7 kinase activity [23, 31-33]. Roach et al. [34] demonstrated the effect of hydrogen peroxide on the STT7 activity in *C. reinhardtii*. However, this hypothesis has yet to be experimentally verified in the context of hydrogen peroxide effect on the STN7 activity in higher plants. Therefore, the aim of this study was to investigate the effect of hydrogen peroxide on the ST process and activity of STN7 kinase in the isolated thylakoids of *A. thaliana*.

MATERIALS AND METHODS

Plant growth conditions. Wild-type *A. thaliana* plants (Columbia ecotype) were grown in soil under

controlled conditions in a climate chamber at a light intensity of 80-100 μmol quanta·m^{-2}·s^{-1} with a 10 h light/14 h dark photoperiod at 21°C. Thylakoids were isolated from 6-8-week-old plants.

Thylakoid isolation. Intact, photosynthetically active thylakoids were isolated as described by Casazza et al. [35] with modifications: bovine serum albumin (BSA) was included only in the homogenization medium and the first washing buffer. Thylakoids were extracted from *A. thaliana* leaves after the dark period. The leaves were detached and incubated in ice-cold water in the dark for 30 min. Homogenization was performed in a buffer containing 20 mM Tricine (pH 8.4), 5 mM $MgCl_2$, 5 mM EDTA, 10 mM NaCl, 0.4 M sorbitol, 0.5% BSA, and 0.1% sodium ascorbate. The homogenate was filtered through two layers of nylon mesh and centrifuged at 3500*g* for 3 min. The supernatant was discarded, and the pellet was resuspended in a washing buffer containing 0.4 M sorbitol, 0.5% BSA, 5 mM $MgCl_2$, 5 mM EDTA, 10 mM NaCl, and 50 mM Hepes (pH 7.6), followed by the second centrifugation. The washing procedure was repeated using the same buffer without BSA. The pellet was then resuspended in a shock buffer without sorbitol (50 mM Hepes, pH 7.6; 5 mM $MgCl_2$; 5 mM EDTA; and 10 mM NaCl), followed by centrifugation. The final pellet was resuspended in a small volume (0.5-1 ml) of buffer (50 mM Hepes, pH 7.6; 400 mM sorbitol; 5 mM $MgCl_2$; 5 mM EDTA; 10 mM NaCl). During the experimental procedures, thylakoids were kept on ice and in the dark; only freshly isolated thylakoids were used.

Chlorophyll content determination. The content of chlorophyll (Chl) was determined in 96% ethanol extracts as described in [36].

Quantification of Lhcb1-P, Lhcb2-P, and D1-P proteins. The reaction mixture contained thylakoids (Chl concentration, 50 μg/ml), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM Hepes (pH 7.6). Where indicated, hydrogen peroxide (25 or 50 μM) or catalase (300 U/ml) were added. To induce state 2 conditions in the thylakoid PA and promote phosphorylation of Lhcb1 and Lhcb2 proteins, the thylakoid suspension was illuminated for 5 min with a low-intensity red light (LED, λ = 640 nm, 60 μmol quanta·m^{-2}·s^{-1}, LL conditions). For the HL experiments, illumination was performed using red light (LED, λ = 640 nm) at 800 μmol quanta·m^{-2}·s^{-1}. All incubations were carried out with constant stirring. In experiments with the dark-adapted thylakoids, two treatments were used: the "dark" variant, when samples were frozen immediately, and the "dark + ATP" variant, when samples were frozen after 5-min incubation with ATP in the dark. After the incubation, 5 mM NaF was added to inhibit the activity of phosphatases and to preserve protein phosphorylation levels. The resulting samples were used for Western blot analysis.

Protein electrophoresis was conducted under denaturing conditions using 15% polyacrylamide gel in a Mini-PROTEAN cell (Bio-Rad, USA). The samples of thylakoid membranes were dissolved in the loading buffer (pH 6.8) containing 200 mM Tris-HCl, 8% SDS, 0.4% bromophenol blue, and 400 mM DTT, heated at 97°C for 5 min, and centrifuged at 13,000*g* for 5 min in a MiniSpin centrifuge (Eppendorf, Germany). The resulting supernatants were loaded on the gel (1.0 μg of Chl per lane). Precision Plus Protein Kaleidoscope (10-250 kDa; Bio-Rad) were used as molecular weight markers. After electrophoresis, the proteins were transferred to PVDF membranes (Bio-Rad) using a Mini Trans-Blot Cell wet blotting system (Bio-Rad). Primary antibodies were rabbit polyclonal antibodies against Lhcb1-P and Lhcb2-P proteins and D1-P (phosphorylated D1 protein). Secondary antibodies were goat anti-rabbit IgG conjugated with alkaline phosphatase (Agrisera, Sweden). Visualization of the reaction was performed using a substrate kit for alkaline phosphatase conjugates (Bio-Rad). Immunodetection results were scanned and analyzed using ImageJ and OriginPro software.

Measurements of Chl *a* fluorescence spectra at 77 K. The thylakoid membranes were incubated as described previously (see section "Quantification of Lhcb1-P, Lhcb2-P, and D1-P proteins") prior to fluorescence measurements.

Immediately after the dark adaptation (state 1) or light treatment (state 2), the thylakoid suspensions were frozen in liquid nitrogen, and low-temperature Chl *a* fluorescence spectra were recorded. For the dark-adapted thylakoids, two treatment variants were applied: in the "dark" variant, the samples were frozen immediately, while in the "dark + ATP" variant, the samples were incubated for 5 min with ATP in the dark prior to freezing. Where indicated, 10 mM NaF was added before illumination to inhibit the phosphatase activity. The excitation wavelength was 440 nm, and fluorescence spectra were recorded in the range of 650-800 nm using an Ocean QePRO spectrofluorometer (Ocean Optics, USA). The fluorescence maximum at 745 nm (F745) indicated excitation energy transfer to PSI, while the peak at 685 nm (F685) corresponded to PSII [37]. A decrease in F685 and a concomitant increase in F745 indicated a transition from state 1 to state 2. The F745/F685 ratio (PSI/PSII chlorophyll *a* fluorescence peaks ratio) was used as a relative indicator of the ST extent.

Measurement of oxygen uptake rate. The rate of oxygen consumption was measured in a temperature-controlled glass chamber at 21°C using a Clark-type pO_2 electrode. Illumination was provided by a red LED light source (λ = 640 nm) at the light intensity of 60 μmol quanta·m^{-2}·s^{-1}. The incubation medium contained thylakoids (Chl concentration, 20 μg/ml),

100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM Hepes (pH 7.6). Where indicated, hydrogen peroxide (25 or 50 μM) was added to the thylakoid suspension.

Statistical analysis was performed with the OriginPro software. Analysis of variance (ANOVA) was conducted, followed by comparison of mean values using the Holm–Bonferroni method. The differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of hydrogen peroxide on the low-temperature Chl *a* fluorescence spectra. A classical method for assessing the PA transition to state 2 is recording of low-temperature (77 K) Chl *a* fluorescence spectra. This approach enables simultaneous monitoring of Chl fluorescence changes in both photosystems. Upon illumination, an increase in the fluorescence maximum at 745 nm (F745) accompanied by a decrease in the maximum at 685 nm (F685) relative to the dark-adapted spectrum, indicates migration of the LHCII from PSII to PSI, i.e., proceeding of ST. To quantify the extent of ST, we used the F745/F685 ratio.

As shown in Table 1, illumination of thylakoid suspensions for 5 min with the red LL (60 μmol quanta·m^{-2}·s^{-1}) resulted in a statistically significant increase in the F745/F685 ratio from 3.4 ± 0.2 (in the "dark + ATP" condition) to 4.1 ± 0.1, indicating induction of state 2. However, when samples were illuminated in the presence of hydrogen peroxide at concentrations of 25 μM or 50 μM, no significant increase in the F745/F685 ratio compare to dark was observed, suggesting inhibition of the ST process by hydrogen peroxide at used concentrations.

To detect the extent of PA transition to state 2 without possible reverse transition to state 1 under our experimental conditions, NaF (phosphatase inhibitor) was used. Inhibition of TAP38/PPH1 phosphatase prevents Lhcb 1 and Lhcb 2 proteins dephosphorylation and return of the antenna complexes containing these proteins to PSII, i.e., prevents transition back to state 1. However, under our conditions, addition of NaF during illumination did not lead to changes in the F745/F685 ratio, indicating that transition to state 2 had proceeded to its maximum. The F745/F685 ratio after 5-min LL illumination of the thylakoids in the presence of 50 μM H_2O_2 and NaF did not differ from that observed in the absence of NaF. These results confirm that H_2O_2 inhibited PA transition to state 2 and did not affect its reverse transition to state 1 under the given conditions.

We also observed that incubation of *A. thaliana* thylakoids in the dark in the presence of ATP with stirring resulted in a significant increase in the F745/F685 ratio, which may reflect either the occurrence of ST in the dark or the activation of other processes altering the F745/F685 ratio.

The effect of hydrogen peroxide on the accumulation of phosphorylated Lhcb1, Lhcb2, and D1 proteins. The first step in the PA transition to state 2 is activation of STN7 kinase and phosphorylation of Lhcb1 and Lhcb2 proteins. Considering the effect of H_2O_2 on the F745/F685 ratio, we investigated how hydrogen peroxide affected accumulation of these phosphorylated proteins in *A. thaliana* thylakoid membranes under LL illumination (Fig. 1). Incubation of thylakoids in the dark with stirring in the presence of ATP led to a significant increase in the Lhcb1-P and Lhcb2-P levels, which was consistent with the results of the low-temperature recordings of Chl *a* fluorescence spectra (Table 1). The accumulation was more pronounced for Lhcb2-P (Fig. 1). These findings suggest either spontaneous phosphorylation of Lhcb1 and Lhcb2 in the dark in the presence of ATP in the medium or the possibility of STN7 activation in the dark resulting in Lhcb1 and Lhcb2 phosphorylation.

Illumination resulted in a significant light-dependent accumulation of Lhcb1-P and Lhcb2-P in isolated thylakoids compared to the "dark + ATP" variant. Incubation of the thylakoid suspension under

Table 1. Effect of hydrogen peroxide on the F745/F685 ratio

Thylakoid incubation conditions	F745/F685
Dark	2.0 ± 0.2 c
Dark + ATP	3.4 ± 0.2 b
LL	4.1 ± 0.1 a
LL + NaF	4.1 ± 0.1 a
LL + 25 μM H_2O_2	3.6 ± 0.2 ab
LL + 50 μM H_2O_2	3.6 ± 0.1 b
LL + 50 μM H_2O_2 + NaF	3.7 ± 0.1 b

Note. The incubation medium contained: thylakoids (Chl concentration, 50 μg/mL), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM HEPES (pH 7.6); where indicated, hydrogen peroxide was added to the thylakoid suspension at the concentrations of 25 and 50 μM. To initiate the transition to state 2, the suspension was illuminated for 5 min (LED, λ = 640 nm; light intensity, 60 μmol quanta·m^{-2}·s^{-1}), which corresponded to the LL variant. The data are presented as mean ± standard error of mean (SEM); statistically significant differences were determined using ANOVA followed by comparison of mean values using the Holm–Bonferroni method ($p < 0.05$) and are indicated by different letters (a, b, and c), values sharing the same letter are not significantly different ($p \leq 0.05$).

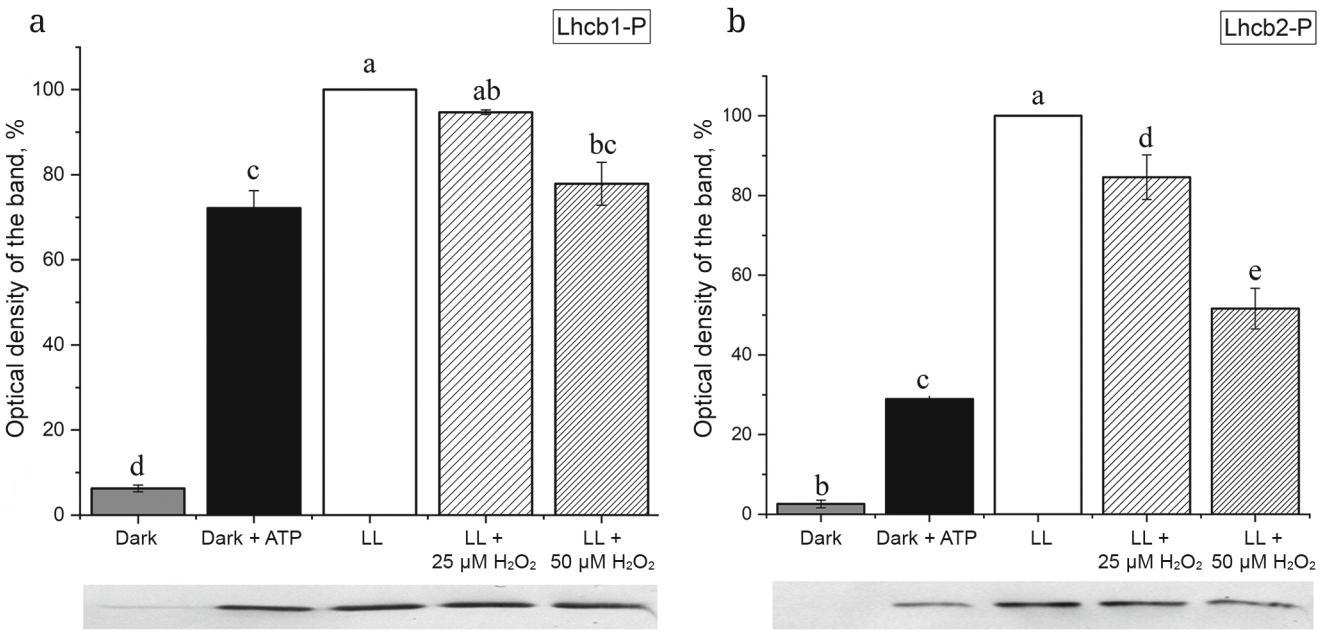

Fig. 1. The effect of hydrogen peroxide on the accumulation of Lhcb1-P (a) and Lhcb2-P (b). The incubation medium contained: thylakoids (Chl concentration, 50 μg/mL), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM HEPES (pH 7.6). Where indicated, hydrogen peroxide (25 or 50 μM) was added to the thylakoid suspension. To initiate the transition to state 2, the suspension was illuminated for 5 min (LED, λ = 640 nm; light intensity, 60 μmol quanta·m^{-2}·s^{-1}). The figure shows representative Western blots and densitometric analysis of the corresponding bands obtained in at least three independent experiments. For each individual membrane, the optical density of the band in the LL variant was taken as 100%. The data are presented as mean ± SEM; statistically significant differences were determined using ANOVA followed by comparison of mean values using the Holm–Bonferroni method ($p < 0.05$) and are indicated by different letters (a, b, c, d, e), bars sharing the same letter are not significantly different ($p < 0.05$).

illumination in the presence of 25 or 50 μM H_2O_2 led to a significant suppression of Lhcb1-P and Lhcb2-P accumulation vs. samples illuminated in the absence hydrogen peroxide; this effect was more pronounced at the higher H_2O_2 concentration (50 μM) (Fig. 1). At this concentration, H_2O_2 fully suppressed the light-dependent accumulation of Lhcb1-P that reached the levels similar to those observed in the "dark + ATP" variant with stirring. The changes in the Lhcb2-P content were less pronounced in the "dark + ATP" with stirring comparing to those registered in the dark in the absence of ATP and stirring. We also observed more significant changes in the light-dependent accumulation of Lhcb2-P than in the Lhcb1-P, as well as a higher inhibitory effect of H_2O_2 addition. The observed differences between the accumulation of Lhcb1-P and Lhcb2-P may be related to their distinct functional roles in the ST process (see "Discussion" section).

The amounts of Lhcb1-P and Lhcb2-P detected in the illuminated samples reflected the balance between the two opposite processes: phosphorylation by STN7 kinase and dephosphorylation by TAP38/PPH1 phosphatase. The suppression of light-dependent accumulation of phosphorylated proteins could result from either decreased kinase activity or increased phosphatase activity. To exclude the influence of H_2O_2 on the activity of TAP38/PPH1 phosphatase, we assessed the light-dependent accumulation of phosphorylated Lhcb1-P and Lhcb2-P in the presence of NaF also. The inhibitory effect of H_2O_2 on the light-induced accumulation of phosphorylated proteins was not affected by NaF (data not shown). Based on these results, we concluded that hydrogen peroxide at the tested concentrations specifically reduces the activity of STN7 kinase.

During illumination of isolated thylakoids, electrons derived from water photolysis in PSII are transferred from components of the photosynthetic electron transport chain (PETC), primarily those belonging to PSI, to molecular oxygen as the only available acceptor, since no alternative electron acceptors were used in our experiments. Electron transfer to oxygen produces superoxide anion radicals, which then generate hydrogen peroxide either through spontaneous dismutation of superoxide anion radicals or via reduction of superoxide anion radical by plastoquinol (see "Discussion" section). An increase in the light intensity leads to the activation of H_2O_2 production in thylakoids, as more electrons enter the PETC and reduce molecular oxygen [38]. Therefore, as an alternative approach to evaluating the effect of hydrogen peroxide on the ST proceeding, we employed the HL conditions that ensured an elevated rate of H_2O_2 generation in the thylakoids.

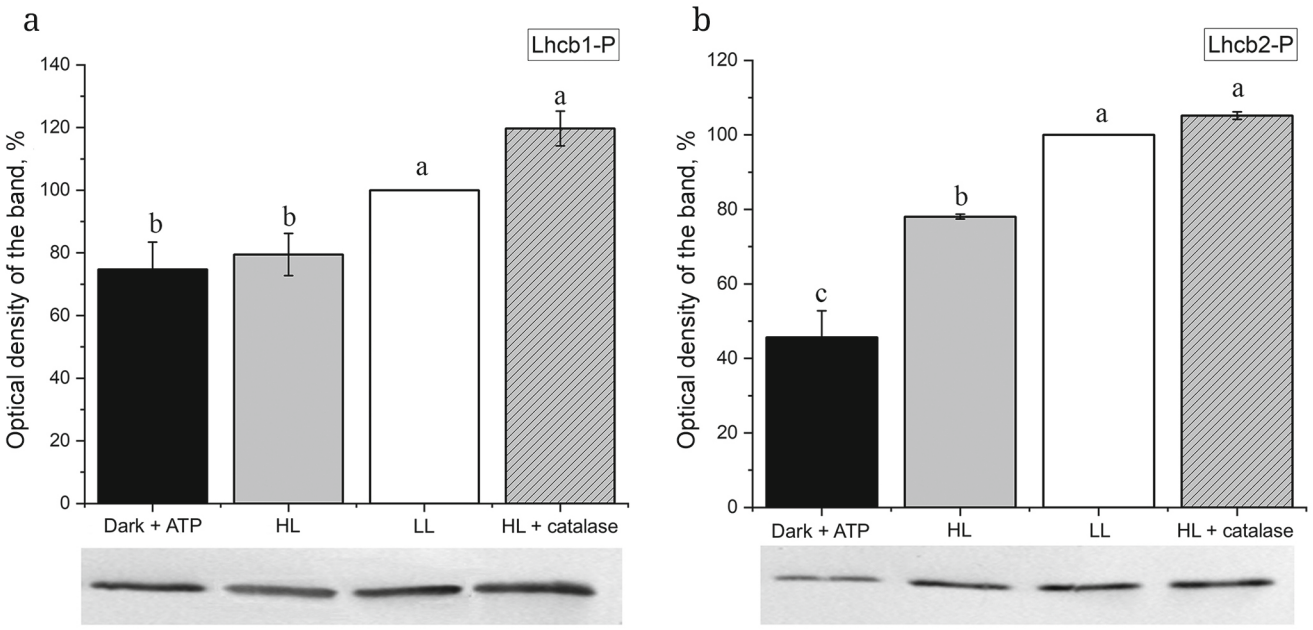

Fig. 2. The effect of catalase on the light-dependent accumulation of Lhcb1-P (a) and Lhcb2-P (b) under HL. The incubation medium contained thylakoids (Chl concentration, 50 μg/mL), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM HEPES (pH 7.6). Where indicated, catalase (300 U/mL) was added to the thylakoid suspension prior to illumination. The "dark + ATP" variant corresponds to thylakoids incubated for 5 min in the above-mentioned medium with stirring. The samples were illuminated for 5 min (LED, λ = 640 nm); LL, 60 μmol quanta·m^{-2}·s^{-1}; HL, 800 μmol quanta·m^{-2}·s^{-1}. The figure shows representative Western blots and densitometric analysis of the corresponding bands obtained in at least three independent experiments. For each membrane, the optical density of the LL band was taken as 100%. The data are presented as mean ± SEM. Statistically significant differences were determined using ANOVA followed by comparison of mean values using the Holm–Bonferroni method ($p < 0.05$) and are indicated by different letters (a, b, c), bars sharing the same letter are not significantly different ($p < 0.05$).

Experiments were conducted in the presence or absence of catalase, an enzyme that decomposes hydrogen peroxide into water and molecular oxygen. It is impossible to use low-temperature Chl *a* fluorescence spectra upon HL illumination to assess the induction or inhibition of ST, since we have previously demonstrated that the HL illumination leads to a strong increase in the F745/F685 ratio unrelated to the ST [15]. Therefore, we evaluated the effect of HL only on the light-dependent accumulation of Lhcb1-P and Lhcb2-P proteins.

Illumination of the thylakoid suspensions in HL (800 μmol quanta·m^{-2}·s^{-1}) failed to induce a marked increase in the Lhcb1-P levels, but caused a statistically significant accumulation of Lhcb2-P compared to that under the "dark + ATP" conditions (Fig. 2). However, the light-dependent accumulation of Lhcb2-P upon HL illumination was significantly lower than under the LL conditions (Fig. 2). The oxygen uptake rate under HL was ~11 μmol O_2/mg Chl per hour, i.e., nearly two times higher than under LL conditions. The addition of catalase under HL resulted in a significant increase in the light-dependent accumulation levels of both Lhcb1-P and Lhcb2-P comparable to those observed under LL (Fig. 2), suggesting that the inhibition of Lhcb1-P and Lhcb2-P accumulation under HL is a peroxide-dependent process. The inhibitory effect of HL was less pronounced than that observed in the presence of exogenous H_2O_2. It can be assumed that under the used illumination conditions, the amount of generated H_2O_2 was lower than the amount of exogenously added H_2O_2, but the hydrogen peroxide produced under HL was generated directly by PETC components, i.e., in close proximity to STN7 kinase.

Several kinases have been identified in *A. thaliana* thylakoid membranes, including STN8 (STN7 kinase paralog), which phosphorylates the PSII core protein D1 [39, 40]. STN7 and STN8 are the main contributors to the thylakoid membrane phosphorylation and are essential for the rapid response to changes in the redox status of the chloroplast PETC. It is known that the maximum accumulation of phosphorylated D1 protein (D1-P) occurs under HL, i.e., under conditions when H_2O_2 generation in chloroplasts is upregulated. It can be assumed that the activity of STN8 *in vivo* is unlikely to be inhibited by hydrogen peroxide, which supports the hypothesis that H_2O_2 specifically affects the STN7 kinase and does not influence the activity of STN8. To examine this suggestion, we assessed the effect of H_2O_2 at the concentrations used in this study on the STN8 activity by analyzing D1-P accumulation in the thylakoid membranes (Fig. 3).

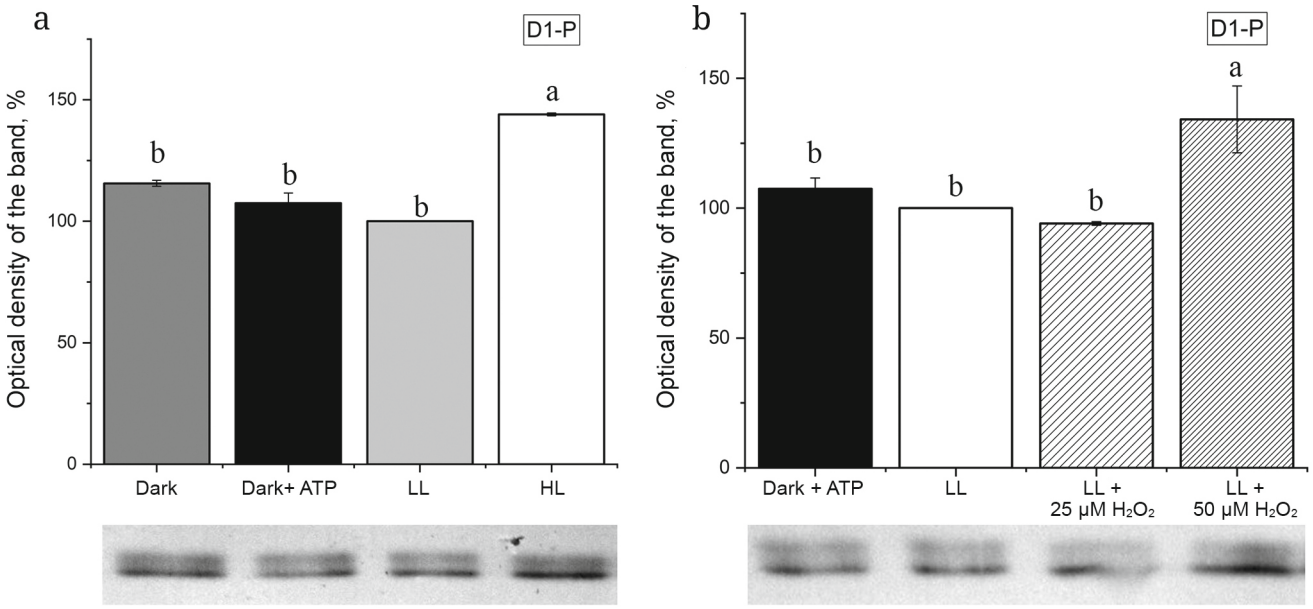

Fig. 3. The effect of varying light intensities (a) and hydrogen peroxide addition (b) on the D1-P protein accumulation. The incubation medium contained thylakoids (Chl concentration, 50 μg/mL), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM HEPES (pH 7.6). Where indicated, hydrogen peroxide (25 or 50 μM) was added to the thylakoid suspension prior to illumination. Illumination was performed for 5 min (LED, λ = 640 nm); LL, 60 μmol quanta·m^{-2}·s^{-1}; HL, 800 μmol quanta·m^{-2}·s^{-1}. Dark, dark-adapted thylakoids frozen without prior incubation; Dark + ATP, thylakoids incubated for 5 min in the above described medium with stirring. The figure shows representative Western blots membranes and densitometric analysis of the corresponding bands obtained in at least three independent experiments. For each membrane, the optical density of the LL band was taken as 100%. The data are presented as means ± SEM. Statistically significant differences were determined using ANOVA followed by comparison of mean values using the Holm–Bonferroni method ($p < 0.05$) and are indicated by different letters (a, b).

Dark-adapted thylakoids (dark in Fig. 3) contained significant amounts of D1-P, in contrast to Lhcb1-P and Lhcb2-P proteins. The amounts of D1-P in the "dark" samples with or without ATP did not differ. Illumination of the thylakoid suspension with LL caused no statistically significant increase in the D1-P accumulation; a noticeable elevation in the D1-P content was detected only after exposure to HL (Fig. 3a). No inhibition of D1-P accumulation was observed after addition of H_2O_2 (Fig. 3b). Interestingly, illumination in the presence of 50 μM H_2O_2 led even to a considerable increase in the D1-P accumulation compared to illumination in the absence of hydrogen peroxide. The level of D1-P after illumination with LL in the presence of 50 μM H_2O_2 was comparable to its level observed after exposure to HL (Fig. 3). Hence, no inhibition of STN8 kinase by hydrogen peroxide was detected at the concentrations used in our study.

Assessment of H_2O_2 impact on the electron transport rate (ETR) in isolated thylakoids. The above-described effects of H_2O_2 may be related not only to its direct action on the STN7 kinase but also to the indirect influence of H_2O_2 on the ETR. To examine whether hydrogen peroxide at the applied concentrations affected ETR in the thylakoid membranes, we measured the oxygen uptake rate in a suspension of isolated thylakoids under the same conditions as those used for evaluating ST and accumulation of phosphorylated proteins.

H_2O_2 at the concentrations used in the study did not decrease the rate of oxygen uptake, i.e., had no negative effect on the ETR in the PETC of isolated thylakoids (Table 2). Therefore, the observed H_2O_2-induced inhibition of ST was not associated with the effect of hydrogen peroxide on the ETR.

Table 2. Effect of hydrogen peroxide addition on the oxygen uptake rate

Variant of thylakoid incubation	Oxygen uptake rate, μmol O_2 per mg Chl per hour
LL	6.9 ± 0.4
LL + 25 μM H_2O_2	7.5 ± 0.6
LL + 50 μM H_2O_2	7.1 ± 0.5

Note. The incubation medium contained thylakoids (Chl concentration, 20 μg/mL), 100 mM sucrose, 2 mM ATP, 1 μM gramicidin D, and 50 mM HEPES (pH 7.6). Where indicated, hydrogen peroxide (25 or 50 μM) was added to the thylakoid suspension prior illumination. The light source was a red LED (λ = 640 nm); light intensity, 60 μmol quanta·m^{-2}·s^{-1}). The data are presented as mean ± SEM.

DISCUSSION

Redistribution of light-harvesting complexes is considered to serve as a mechanism of adaptation to changes in spectral composition of light. However, a recent study by Hommel et al. [41] demonstrated that light that predominantly excites one of the photosystems, as well as the low-intensity white light, which excites both photosystems, exert a similar influence on ST and LHCII protein phosphorylation. Furthermore, it was recently shown that the optimal electron flow ratio through PSII and PSI (~1.02) at LL is achieved by PA transition into state 2 [42]. These findings highlight the significance of this mechanism not only in response to changes in the light spectral composition, but also upon exposure to light after a dark period.

Previous studies conducted in whole leaves have reported accumulation of Lhcb1-P and Lhcb2-P proteins, as well as the possibility of state 2 formation in the dark under stress conditions, such as iron deficiency or heat stress [43, 44]. In this study, we found that incubation of thylakoid membranes isolated from *A. thaliana* plants grown under normal conditions, in the presence of ATP with stirring in the dark resulted in the accumulation of Lhcb1-P and Lhcb2-P proteins (Fig. 1), and partial PA transition to state 2 (Table 1), which could be attributed to spontaneous phosphorylation of Lhcb1 and Lhcb2 or activation of STN7 kinase in the dark in the presence of ATP by light-independent manner. These findings must be taken into account when assessing the extent of ST in isolated thylakoids. We further evaluated the effect of hydrogen peroxide specifically on the light-dependent accumulation of Lhcb1-P and Lhcb2-P, since it is assumed that the *in vivo* influence of H_2O_2 is mainly relevant upon illumination, when hydrogen peroxide is generated by components of the PETC.

Inhibition of ST under HL conditions has been repeatedly demonstrated, although its underlying mechanisms are still debated (see section “Introduction”). In this study, two independent approaches – recording of low-temperature fluorescence spectra and analysis of Lhcb1-P and Lhcb2-P proteins accumulation – were used to demonstrate a role of hydrogen peroxide in the inhibition of ST. Illumination of thylakoid suspensions in the presence of 25 and 50 μM H_2O_2 did not provide a significant increase in the F745/F685 ratio compared to the dark conditions (Table 1), indicating inhibition of the thylakoid PA transition to state 2 upon illumination in the presence of H_2O_2. The transition from state 1 to state 2 can be divided into two stages: (i) activation of STN7 kinase and phosphorylation of Lhcb1 and Lhcb2 proteins and (ii) LHCII migration from PSII to PSI. The observed inhibition of state 2 transition may occur at either of these stages. To determine whether hydrogen peroxide affects the activity of STN7 kinase namely, we assessed the light-dependent accumulation of Lhcb1-P and Lhcb2-P in the absence or presence of exogenous H_2O_2. Illumination of thylakoid suspension in the presence of 25 and 50 μM H_2O_2 led to a significantly lower light-dependent accumulation of Lhcb1-P and Lhcb2-P compared to that under control conditions (Fig. 1).

The inability of exogenous H_2O_2 to fully block the light-dependent accumulation of Lhcb1-P and Lhcb2-P may result from the insufficient H_2O_2 concentrations used in the experiment. However, higher H_2O_2 concentrations inhibited electron transport in the PETC (data not shown), which made interpretation of the obtained results ambiguous. Another possible explanation for the partial inhibition of STN7 kinase is a limited accessibility of its Cys residues to H_2O_2, which is especially relevant if the inhibition mechanism involves Cys residues exposed to the lumen [19]. Furthermore, it is possible that inhibition of STN7 kinase proceeds via multiple mechanisms. Considering that STN7 is thought to be activated through plastoquinol binding to the quinol-oxidizing site in the cytochrome *b6f* complex [20, 45], it can be assumed that the electron transfer rate and, in particular, the rate of plastoquinol oxidation at this site, also directly regulate the kinase activity. In addition, STN7 inhibition may be induced by its dephosphorylation. In *A. thaliana*, STN7 kinase contains four phosphorylated residues (Ser and Thr) near the C-terminus [46], some of which undergo autophosphorylation. Rapid inhibition under HL is associated with a partial dephosphorylation of STN7. Exposure to excessive light intensity can caused complete STN7 dephosphorylation, which has been shown to correlate with its degradation [47, 48]. Inhibition of STN7 kinase by H_2O_2 may be regarded as a necessary step, if the kinase has already been activated at the cytochrome complex level but not yet dephosphorylated for subsequent proteolysis.

Interesting results have been obtained by comparing accumulation of phosphorylated Lhcb1 and Lhcb2 proteins. We found a significant phosphorylation of Lhcb1 in the dark in the presence of ATP, which was considerably greater than phosphorylation of Lhcb2. In contrast, Lhcb2-P was characterized by a clearly manifested light-dependent accumulation and, correspondingly, a more pronounced inhibitory effect of hydrogen peroxide on this accumulation. Previous studies have shown that only ~30% of total Lhcb1 undergoes phosphorylation, which might potentially explain its near-maximum phosphorylation levels in the darkness (Fig. 1), whereas the extent of Lhcb2 phosphorylation can reach 70% [9]. These findings may also reflect the functional differences between Lhcb1 and Lhcb2 in ST, as Lhcb2-P has been shown to play a key role in LHCII docking to PSI [10, 49].

Moreover, it was recently demonstrated that replacement of native Lhcb1 with a protein with eliminated phosphorylation site (Thr → Val) had no effect on ST, whereas the same substitution in Lhcb2 abolished ST entirely [50]. A more pronounced inhibition of Lhcb2-P accumulation observed in this study was consistent with the earlier reported essential role of Lhcb2 in ST.

Previously, it was shown that increasing illumination leads to the increase of hydrogen peroxide production in isolated thylakoid membranes, both under conditions when oxygen is the only electron acceptor and in the presence of methyl viologen (an artificial electron acceptor which is more efficient than molecular oxygen) [31, 51, 52]. Illumination of isolated thylakoids with HL (800 μmol quanta·m^{-2}·s^{-1}) induced a complete inhibition of the light-dependent accumulation of Lhcb1-P and partial suppression of Lhcb2-P accumulation compared to the LL conditions (Fig. 2). Apparently, illumination for 5 min was insufficient for the accumulated H_2O_2 to fully inhibit the light-dependent accumulation of Lhcb2-P (Fig. 2). Another possible explanation for the incomplete inhibition of Lhcb2-P accumulation may be that the complete inhibition of STN7 requires additionally the thioredoxin activity in the stroma, since it was suggested that thioredoxins participate in STN7 inhibition [24, 25].

To verify the role of H_2O_2 in the inhibition of Lhcb1-P and Lhcb2-P accumulation in isolated thylakoids under HL, we illuminated these preparations in the presence of catalase, an enzyme that decomposes H_2O_2. The addition of catalase significantly enhanced Lhcb1-P and Lhcb2-P accumulation upon HL illumination compared to the control (in the absence of catalase). Removal of H_2O_2 molecules prevented STN7 inhibition upon HL illumination of isolated thylakoids, while addition of H_2O_2 in the above-described experiments in LL mimicked the increase in the H_2O_2 levels as in the case of HL inhibiting STN7 kinase. It cannot be excluded that under the LL conditions, we could nor observe the maximum accumulation of Lhcb1-P and Lhcb2-P and complete transition from state 1 to state 2, considering that in this case, H_2O_2 was also generated by PETC components, albeit at a lower rate than under HL.

We also demonstrated that the H_2O_2-mediated inhibition of STN7 kinase was specific. The genome of *A. thaliana* contains a STN7 paralog – STN8 kinase, which is responsible for phosphorylating PSII core proteins, in particular, D1 protein [39, 40]. Under HL conditions, accumulation of D1-P protein is upregulated, which facilitates partial grana unstacking and promotes faster turnover of core proteins [39, 40]. The addition of H_2O_2 to the isolated thylakoid suspensions did not inhibit the STN8 activity (in contrast to STN7), i.e., did not reduce D1-P accumulation (Fig. 3), which confirmed differential regulation of these kinases and highlighted the specificity of H_2O_2 action on STN7. Interestingly, addition of 50 μM H_2O_2 during illumination significantly increased D1-P accumulation (Fig. 3) up to levels comparable to those observed under HL. This is consistent with recent findings of the H_2O_2-mediated inhibition of PBCP phosphatase [53], which dephosphorylates D1-P. It was proposed that the inhibition of PBCP by hydrogen peroxide under HL conditions may account for the observed increase in the phosphorylation levels of PSII core proteins, which results from the inhibition of phosphatase rather than upregulation of the STN8 activity [53].

It has been previously shown that formation of H_2O_2 in chloroplasts occurs not only in the stroma (the so-called stromal H_2O_2) as a result of superoxide anion radical dismutation, but also takes place in the vicinity of thylakoid membrane (membrane-associated H_2O_2) due to the reaction of superoxide anion radical with plastoquinol present in the membrane lipid phase [38, 51]. Increased illumination enhances the overall H_2O_2 production rate in chloroplasts, however, mainly due to the elevated formation of membrane-associated H_2O_2 rather than stromal H_2O_2 [52]. Consequently, the proportion of H_2O_2 generated near the PETC components, including the transmembrane STN7 kinase, increases with an increase in light intensity. Hence, it can be assumed that it is the membrane-associated H_2O_2 that plays a key role in the inhibition of STN7 activity *in vivo*. This suggestion offers a new interpretation of the ambiguous role of plastoquinone pool in ST: although reduced plastoquinone molecules are required for the STN7 activation, illumination with HL (when plastoquinone is mostly reduced) results in the ST inhibition and decreased phosphorylation levels of LHCII proteins [25, 54]. Therefore, the regulatory role of plastoquinone pool redox state in the control of STN7 activity should be considered as a complex system that involves not only plastoquinone reduction level but also H_2O_2 production.

CONCLUSION

In this work, we demonstrated suppression of the PA transition to state 2, specifically, inhibition of STN7 kinase, upon illumination of isolated thylakoid suspensions with LL in the presence of hydrogen peroxide. H_2O_2 removal caused an increase in the light-dependent accumulation of STN7 kinase products (Lhcb1-P and Lhcb2-P) upon HL illumination of isolated thylakoid suspensions. Inhibition of STN7 kinase upon increased illumination may be an important mechanism regulating PA functioning. The involvement of STN7 kinase in the long-term

regulation of the PSII antenna size under increased illumination was shown in [54], which may include changes in its activity and redox or phosphorylation states. Inhibition of STN7 kinase during abrupt changes in light conditions could represent a critical step in triggering the retrograde signaling for the regulation of PSII antenna size by suppressing the biosynthesis of Lhcb proteins. Along with MAP kinases, STN7 acts as a key participant in the reversible phosphorylation in chloroplasts and influences not only the activity of these organelles, but the functioning of the entire cell. Inhibition of STN7 kinase may thus serve as a crucial signal for the adaptive remodeling of reversible protein phosphorylation in plant cells. Several studies have reported STN7 inhibition in response to other abiotic stress factors, such as low temperature [55, 56], NaCl addition, or treatment with H_2O_2, in cultured *A. thaliana* cells [57]. All these abiotic stresses also induce accumulation of reactive oxygen species, e.g., hydrogen peroxide, suggesting that the H_2O_2-mediated mechanism of STN7 inhibition described here may represent a universal response to various stress factors.

Abbreviations. Chl, chlorophyll; HL, high-intensity light; Lhcb1-P and Lhcb2-P, phosphorylated Lhcb1 and Lhcb2 proteins; LHCII, light-harvesting complex II; LL, low-intensity light; PA, photosynthetic apparatus; PSI, photosystem I; PSII, photosystem II; PETC, photosynthetic electron transport chain; ST, state transition.

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Contributions. N.V.B., D.V.V., and M.M.B.-M. conducted the experiments and discussed the results; D.V.V. prepared the draft of the manuscript; N.V.B. and M.M.B.-M. edited the manuscript.

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