

Light-dependent reversal of dark-chilling induced changes in chloroplast structure and arrangement of chlorophyll–protein complexes in bean thylakoid membranes[☆]

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Abstract

Changes in chloroplast structure and rearrangement of chlorophyll–protein (CP) complexes were investigated in detached leaves of bean (*Phaseolus vulgaris* L. cv. Eureka), a chilling-sensitive plant, during 5-day dark-chilling at 1 °C and subsequent 3-h photoactivation under white light (200 μmol photons m⁻² s⁻¹) at 22 °C. Although, no change in chlorophyll (Chl) content and Chl *a/b* ratio in all samples was observed, overall fluorescence intensity of fluorescence emission and excitation spectra of thylakoid membranes isolated from dark-chilled leaves decreased to about 50%, and remained after photoactivation at 70% of that of the control sample. Concomitantly, the ratio between fluorescence intensities of PSI and PSII (F736/F681) at 120 K increased 1.5-fold upon chilling, and was fully reversed after photoactivation. Moreover, chilling stress seems to induce a decrease of the relative contribution of LHCII fluorescence to the thylakoid emission spectra at 120 K, and an increase of that from LHCI and PSI, correlated with a decrease of stability of LHCI–PSI and LHCII trimers, shown by mild-denaturing electrophoresis. These effects were reversed to a large extent after photoactivation, with the exception of LHCII, which remained partly in the aggregated form. In view of these data, it is likely that dark-chilling stress induces partial disassembly of CP complexes, not completely restorable upon photoactivation. These data are further supported by confocal laser scanning fluorescence microscopy, which showed that regular grana arrangement observed in chloroplasts isolated from control leaves was destroyed by dark-chilling stress, and was partially reconstructed after photoactivation. In line with this, Chl *a* fluorescence spectra of leaf discs demonstrated that dark-chilling caused a decrease of the quantum yield PSII photochemistry (F_v/F_m) by almost 40% in 5 days. Complete restoration of the photochemical activity of PSII required 9 h post-chilling photoactivation, while only 3 h were needed to reconstruct thylakoid membrane organization and chloroplast structure. The latter demonstrated that the long-term dark-chilled bean leaves started to suffer from photoinhibition after transfer to moderate irradiance and temperature conditions, delaying the recovery of PSII photochemistry, independently of photo-induced reconstruction of PSII complexes.

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Keywords: Bean; Chloroplast and thylakoid structure; Chlorophyll *a* fluorescence; Confocal laser scanning microscopy; Dark-chilling stress; LHCI–PSI; LHCII–PSII

Abbreviations: CS, Chilling-sensitive; Chl, chlorophyll; CLSM, confocal laser scanning microscopy; CP, chlorophyll–protein; Em, emission; Ex, excitation; FFA, free fatty acids; PAM, modulated fluorometer; PAR, photosynthetic active radiation

[☆] Dedicated to Prof. Zbigniew Kaniuga on the 25th anniversary of his initiation of studies on chilling-induced stress in plants.

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1. Introduction

Chloroplasts of higher plants contain internal thylakoid membranes differentiated into stacked (appressed) and unstacked (non-appressed) regions [1,2]. Cylindrical granum stacks of appressed membrane are surrounded by non-appressed helically organized stroma thylakoids. In this three-dimensional membrane system grana stacks consist of 10–20 layers and are interconnected by unstacked lamellae [3]. The

granum and stroma thylakoids contain different photosynthetic complexes [2,4]. In stacked membranes the main chlorophyll *a/b* light-harvesting complexes (LHCII) and minor light-harvesting complexes (Lhcb4, Lhcb5, Lhcb6) form the LHCII–PSII supercomplexes with the dimer of photosystem II (PSII) [5,6]. Moreover, the mobile trimeric form of LHCII also occurs [7,8]. The LHCII–PSII and mobile LHCII trimers build up the macrodomain structure, which is less stable than that of supercomplexes [7–9]. In intact grana, these complexes have been shown to form densely packed aggregates [10,11]. In unstacked lamellae, the photosystem I (PSI), associated with external antenna and composed of four polypeptides (Lhca1–4), forms the LHCI–PSI supercomplexes [12,13].

The degree of thylakoid membrane stacking and lateral segregation of photosynthetic complexes are dynamic phenomena dependent on environmental conditions [3,15]. Light-induced non-photochemical quenching of excitation energy (NPQ) [16] is associated with rearrangement of LHCII complexes [17,18] and formation of large-aggregates within membranes [11]. Regulation of energy distribution between PSII and PSI (state-transition) is related to dislocation of the mobile LHCII, associated with PSII in granum, to PSI in stroma lamellae [15,19]. Association of LHCII with PSI requires the direct interaction between proteins (molecular recognition) [19], as has been indicated in experiments with *Arabidopsis* mutants [20,21]. Thus, under variable light conditions, the utilization of the excitation energy is regulated by structural changes of the thylakoid membranes [11]. Many other environmental stress factors also induce structural changes within the thylakoid membranes. Long-term CO₂ deficit may cause the aggregation and physical disconnection of LHCII from photosystems independently of the yield of PSII photochemistry [22,23]. Dehydration of a desiccation-tolerant plant induced reversible thylakoids destacking associated with EPR-detected conformational transition in membrane proteins [24]. The disassembly of both PSI–LHCI and LHCII trimers was also observed during dark-induced leaves senescence [25]. Moreover, the dissociation of LHCI from LHCI–PSI is one of the effects of reactive oxygen species during photoinhibition [26].

Low-temperature (0–12 °C) treatment of chilling-sensitive (CS) plants affects the chloroplast ultrastructure, resulting in swelling and distorting of thylakoids [27–29]. It is known that chilling treatment in both the light and the dark may affect different sites within the chloroplast, and thus may account for the differences in the effect and magnitude between chilling in the light and in the dark [cf. 30, and references cited therein]. Besides decreased activities of stromal bisphosphatases [31] and Rubisco activase [32], chilling in the light may cause photoinhibition of both PSI and PSII, of which PSII has long been considered the primary target for excessive light. For CS species like common bean and cucumber (*Cucumis sativus* L.), however, selective photoinhibition of the iron–sulfur center of PSI was reported upon exposure to weak [30,33] or high-irradiance levels [34] at chilling temperatures. Analysis of other CS species like pumpkin (*Cucurbita maxima* L.), tobacco (*Nicotiana tabacum* L.) and maize (*Zea mays* L.) indicated that

the preferential inhibition of PSI observed for cucumber cannot be considered as a general phenomenon among CS plants [34,35], and strongly depends on the developmental history of the plant, i.e., the experienced PSII excitation pressure during leaf development [36]. It is reasonable to assume that the recovery of PSII after photoinhibition is much faster than that of PSI, because the reaction center of PSI is heterodimeric and is not a rapid-turnover protein like the D1 protein in PSII. Kudoh and Sonoike [37], therefore, stated that the prolonged inhibition of photosynthesis and bleaching of leaf pigments, after returning chilled leaves to ambient growth temperatures, can be explained as a consequence of irreversible photoinhibition of PSI.

On the other hand, when CS plants are chilled for a longer time (>48 h) in the dark, the limitations of photosynthesis are associated with destabilization of PSII extrinsic proteins and release of manganese from PSII [38–41]. In addition, during chilling of detached leaves, chloroplasts of CS plants accumulate free fatty acids (FFA) due to a high activity of galactolipase [42]. Illumination of tomato (*Lycopersicon esculentum* Mill.) leaves following a 3-day dark-chilling treatment, for 2 h with low light (80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at room temperature, results in almost complete restoration of the O₂ evolution activity, and simultaneously to a decrease of the amount of FFA to the control level [40]. As restoration of both O₂ evolution and Mn content are accompanied by a simultaneous decrease of the FFA level, it is likely that accumulated FFA are involved in the structural rearrangement of thylakoid membranes, resulting in PSII inactivation [40,42]. More recently, the contribution of thylakoid endogenous reductants to disintegration of Mn-cluster during dark-chilling of cucumber leaves has been suggested [41].

In experiments with the temperate soybean (*Glycine max* [L.] Merr.) genotypes, in which the chilling episode was only applied during the night, and the plants were able to recover during the warm photoperiod, it was found that dark chilling-induced inhibition of photosynthesis was predominantly associated with species-specific metabolic restrictions rather than with direct effects on electron transport reactions [43]. Interestingly, a comparative study on the effects of a one-night chilling episode on photosynthetic carbon metabolism between a temperate and a tropical soybean genotype revealed large intra-specific differences in the physiological and biochemical factors involved [44]. From this study, it appeared that dark-chilling imposed a multitude of metabolic limitations in the tropical genotype, in which also impairment of electron transport was involved, whereas stomatal limitation dominated the inhibition of photosynthesis in the temperate one.

Long-term chilling of CS leaves in the dark causes a reversible grana disintegration and thylakoid dilatation [28]. However, there are no data concerning changes in the structure of chlorophyll–protein (CP) complexes under these conditions. The aim of the present study was to characterize chloroplast structure and arrangement of CP complexes in thylakoids isolated from dark-chilled and subsequently photoactivated bean (*Phaseolus vulgaris* L.) leaves by means of fluorescence confocal laser scanning microscopy (CLSM), fluorescence spectroscopy and mild-denaturing electrophoresis, whereas the functionality of PSII was assayed by measuring the maximum

capacity of PSII photochemistry in leaf discs using a PAM fluorometer.

2. Materials and methods

2.1. Plant materials, growth, chilling and photoactivation procedures

Bean (*Phaseolus vulgaris* L. cv. Eureka) plants were grown into perlite-containing pots of 3 L in a climated room (22 °C/20 °C day/night temperature), a photosynthetic active radiation (PAR) of 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during a 16-h photoperiod and at relative humidity of 70–80%. Plants were fertilized with full Knop's nutrient solution. Fully expanded first leaves of 14-day-old plants were used for the experiments. For the control samples the leaves were harvested 30 min after beginning of the photoperiod. For the chilling treatment, the leaves were detached from the plants as for control samples and placed in thermos flasks on damp paper above an ice layer in a cold-room for 5 days in the dark. The temperature and relative humidity inside the thermoses were 1 °C and 100%, respectively. The cold-stored leaves were placed in a closed transparent plastic dish on a layer of water and the adaxial side was also sprinkled with water. For photoactivation, the leaves were transferred to a climate room at 22 °C with a PAR of 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during 3 h.

2.2. Preparation of chloroplasts and thylakoids

For thylakoid membranes preparations, bean leaves were homogenized in (buffer A) 20 mM Tricine–NaOH (pH 7.5) containing 330 mM sorbitol, 15 mM NaCl, 4 mM MgCl_2 and 40 mM ascorbate. The homogenate was filtered and centrifuged at $2000\times g$ for 5 min to obtain chloroplasts pellet. Thylakoids were prepared from chloroplasts by osmotic shock in (buffer B) 20 mM Tricine–NaOH (pH 7.0) containing 15 mM NaCl and 4 mM MgCl_2 . After centrifugation at $6000\times g$ for 10 min the thylakoids were resuspended in (buffer C) 20 mM HEPES–NaOH (pH 7.0) containing 330 mM sorbitol, 15 mM NaCl and 4 mM MgCl_2 . Centrifugation at $6000\times g$ for 10 min was repeated and the pellet resuspended in buffer C and kept on ice in the dark. Thylakoids were always freshly prepared before each experiments and used within 2 h for electrophoresis or fluorescence spectroscopy in order to analyze the chill-induced changes that occur particularly inside the photosynthetic membranes.

Intact chloroplasts were obtained by gentle homogenization of leaves in a semi-frozen buffer, which was identical to the grinding medium applied for preparation of thylakoids (buffer A). The homogenates were filtered and centrifuged at $2000\times g$ for 3 min, and the pellet gently resuspended in buffer C, and used immediately for investigation of chloroplast structure by confocal scanning fluorescence microscopy.

Concentration of Chl *a* and *b*, and Chl *a/b* ratio were quantified spectrophotometrically after extraction with 80% acetone in water [45]. Absorption in the visible range was monitored with a Beckman DU-68 spectrophotometer.

2.3. Determination of Chl fluorescence of thylakoid membranes

Steady-state fluorescence emission (Ex 435, 470 and 490 nm) spectra at 25 °C (298 K) were determined with the Shimadzu RF-5301PC spectrofluorimeter with 3- and 10-nm spectral resolution for excitation and emission, respectively. The thylakoid suspension (6 $\mu\text{g Chl ml}^{-1}$) was prepared in buffer C (pH 7.5) saturated with argon and placed in a sealed quartz cuvette (10-mm optical path length). Each sample was magnetically stirred to prevent settling, and each spectrum was recorded 3 times. The first scan was carried out to eliminate light-dependent changes in fluorescence intensities, which did not exceeded 2%. The second and the third scans were used to obtain an average fluorescence spectrum of the sample.

Steady-state fluorescence spectra at 120 K were monitored with a Spex FluoroMax spectrofluorimeter with 2-nm spectral resolution for excitation and emission. Samples were thermostated using a home-made liquid nitrogen cryostat (Institute of Physics, Polish Academy of Sciences, Warsaw). Temper-

ature was measured directly in the glassy solution by a diode thermometer, with an accuracy of 0.5 K. Thylakoid samples (3 $\mu\text{g Chl ml}^{-1}$) were prepared in 2 ml of 20 mM HEPES–NaOH buffer (pH 7.5) containing 15 mM NaCl, 4 mM MgCl_2 and 80% (v/v) glycerol in 10×10 mm polymethacrylate cuvettes (Sigma, USA). Fluorescence emission (Ex 435, 470, 490 nm) and fluorescence excitation (Em 680 and 740 nm) spectra were recorded in the range of 620–850 nm, and 350–670 and 350–730 nm, respectively. The total optical density of the thylakoid samples did not exceeded 0.1 to minimize inner-filter effects. Low-temperature measurements were performed at 120 K because temperature stability below 120 K was not sufficient, especially near boiling temperature of liquid nitrogen. In addition, further decrease of temperature led essentially to the same relations between fluorescence intensities of the dark-chilled, photoactivated and control thylakoid samples.

Background spectra containing background emission (<5%) and small peaks of Raman scatters were eliminated by subtracting the signal for buffer and/or buffer containing all other components except thylakoid membranes. All emission spectra were corrected for inner-filter effect [46] due to overlap with absorption spectra in the range of 600–800 nm. To compensate for the decrease in fluorescence due to increase of absorption of the thylakoid membranes, the measured fluorescence was multiplied by a correction factor $G(\Delta A(\text{Em})) = \text{antilog}_{10}(-\Delta A(\text{Em})/2)$, where $\Delta A(\text{Em})$ is an increase of absorption at emission wavelengths (Em), when the sample was either chilled or photoactivated. Correction did not exceed 10%, and had no effect on the relative changes of fluorescence intensities (Table 1) due to dark-chilling or photoactivation.

In order to determine the effects of chilling and subsequent photoactivation on the relative contribution of specific CP complexes to the overall fluorescence pattern, fluorescence spectra of thylakoids at both 298 and 120 K were normalized to the same area (100) under the spectrum [47]. The latter is equivalent to the normalization at 688 and 720 nm, respectively, where isoemissive points are observed in the fluorescence-difference spectra (Figs. 1B and 2B).

2.4. Mild-denaturing “green” electrophoresis

CP complexes were analyzed by mild-denaturing polyacrylamide gel electrophoresis following the method of Maroc et al. [48] with some

Table 1

Relative changes (in % of control) of the steady-state fluorescence intensities at the selected maxima of fluorescence emission (A) and excitation (B) bands (λ_{max}) of thylakoids isolated from bean leaves chilled in the dark (5 days, 1 °C) and chilled and subsequently photoactivated (3 h, 22 °C)

A				
Samples	Fluorescence emission spectra (λ_{ex} 470 nm)			
	298 K		120 K	
	680 nm	681 nm	736 nm	
Dark-chilled	49 ± 5 ^a	41 ± 5 ^c	66 ± 3 ^c	
Dark-chilled and photoactivated	71 ± 10 ^b	58 ± 9 ^d	79 ± 11 ^d	
B				
Samples	Fluorescence excitation spectra ($\lambda_{\text{em}}/\lambda_{\text{max}}$, nm)			
	298 K		120 K	
	680/470	740/648	680/473	740/648
Dark-chilled	47 ± 5 ^a	60 ± 4 ^a	41 ± 5 ^c	69 ± 3 ^c
Dark-chilled and photoactivated	70 ± 12 ^b	85 ± 12 ^b	72 ± 11 ^d	91 ± 14 ^d

The data are the means ± S.D. for 7 and 3 separate experiments with respect to fluorescence at 298 K (25 °C) and 120 K, respectively. Thylakoids samples for each experiment were isolated from the same plant cultivation (cf. Sect. 2.3). Statistical significance (P) was calculated for the marked data series and estimated to be no more than: ^a $P < 0.001$, ^c $P < 0.02$ versus corresponding control values and ^b $P < 0.005$, ^d $P < 0.05$ versus corresponding values for thylakoids from chilled leaves, respectively.

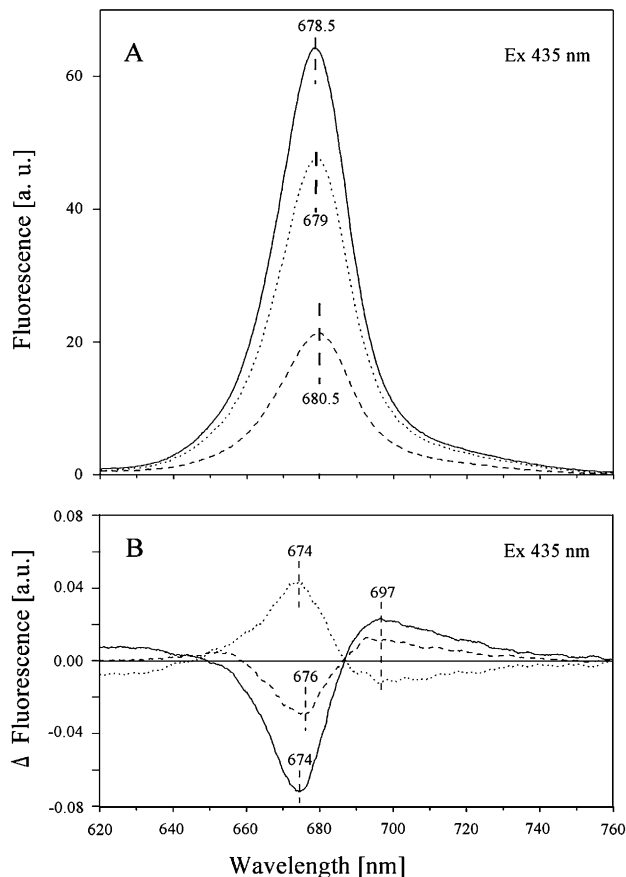


Fig. 1. Effect of dark chilling (5 days, 1 °C) and subsequent photoactivation (3 h, 22 °C) under moderate light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) on steady-state fluorescence emission spectra (Ex 435 nm) at 25 °C (298 K) of (A) thylakoids isolated from control (solid line), dark-chilled (dashed line) and subsequently photoactivated leaves (dotted line). The presented spectra were fully corrected for the inner-filter effects as well as for background fluorescence spectra (Section 2.3), and these presented here are representative for seven separate experiments with variations below 10%. (B) The difference spectra chilling-minus-control (solid lines), photoactivated-minus-control (dashed line) and photoactivated-minus-chilled (dotted line), calculated by subtraction of the spectra normalized to the same area of 100 under the spectrum.

modifications. Electrophoresis was carried out on 4% stacking and 9–17% separating polyacrylamide gels that contained 0.1% (w/v) lithium dodecyl sulfate (LDS). Thylakoids ($0.5 \text{ mg Chl ml}^{-1}$) were solubilized in 20 mM Mes–NaOH (pH 6.6) buffer containing 100 mM sucrose, 15 mM NaCl, 5 mM MgCl_2 , 1.1% n-octyl- β D-glucopyranoside, 0.22% LDS and 62.5 mM dithiothreitol. The electrophoresis was run in 12.5 mM Tris–glycine buffer (pH 8.8) contained 0.005% LDS. Relative intensities of CP bands were quantified by scans analyzed with Quantity One software (Bio Rad).

2.5. Fluorescence confocal laser scanning microscopy (CLSM) and image analysis

Isolated intact chloroplasts ($30 \mu\text{g Chl ml}^{-1}$) were resuspended in buffer C (pH 7.5) containing 6% (v/v) glycerol and $30 \mu\text{M DCMU}$. Suspension was placed on a poly-L-lysine layer ($1 \mu\text{g ml}^{-1}$) and fixed on a microscopic glass. Samples were imaged using a Zeiss LSM 510 confocal laser scanning fluorescence microscope equipped with a PlanApo 63 \times , NA 1.4 objective lens. Excitation was performed at 543 nm output from a helium-neon laser (no neutral density filter). Fluorescence emission was collected through a 560 nm long pass filter, while confocal aperture was set at 106 μm . Z-series (40–47) of 1024×1024 pixels and 8 bit images were collected, and only those

were taken for computer-aided analysis, which were not affected by fluorescence quenching due to the light-exposure in microscope, i.e., exhibited similar fluorescence intensities of the first and the last cross-section slices. Data stacks were deconvolved using AutoDeblur software (AutoQuant).

2.6. Chl *a* fluorescence measurements in leaf discs

Chl *a* fluorescence of control, chilled and photoactivated leaves were measured on the adaxial side of leaf discs (11 mm diameter) using a modulated fluorometer (PAM 2000, H. Walz GmbH, Effeltrich, Germany). Measurements were performed as described by Venema et al. [49] at a temperature of 20 °C in humidified air under ambient O_2 and CO_2 concentrations. The maximum Chl *a* fluorescence level of dark-adapted leaves (F_m) was determined at a saturating flash of white light during 1 s with an irradiance of $4000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The initial Chl *a* fluorescence level of dark-adapted leaves (F_0) was sensitized with red light of about $0.1 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ at a modulation of 1.6 kHz. The quantum yield of PSII photochemistry (F_v/F_m) was calculated from the ratio of variable ($F_v = F_m - F_0$) to maximum Chl *a* fluorescence. Prior to the measurements, leaf discs were dark-adapted for 30 min. Photoactivation was followed during 18 h.

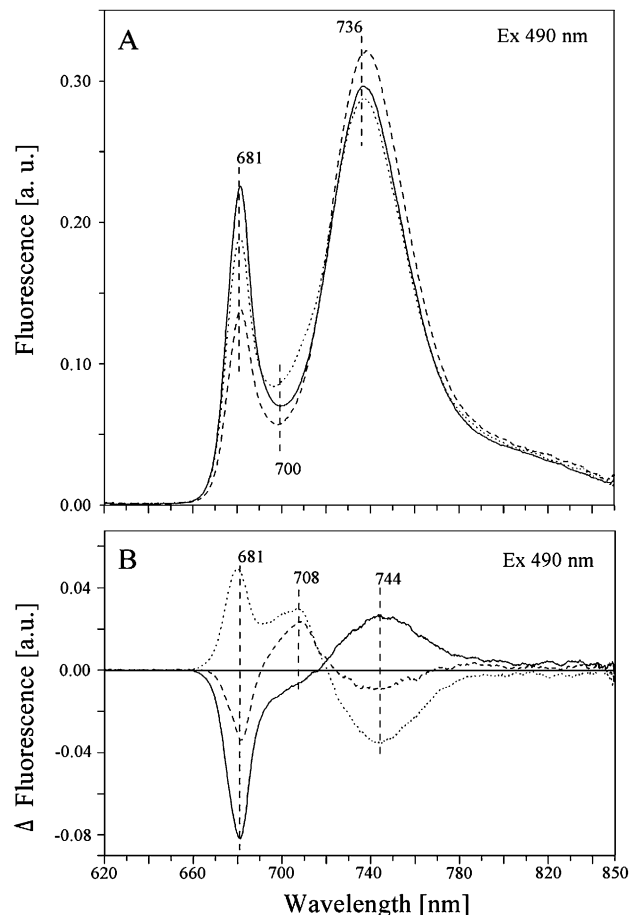


Fig. 2. Effect of dark-chilling and subsequent photoactivation on fluorescence emission spectra (Ex 490 nm) of isolated thylakoids at 120 K. (A) Fluorescence emission spectra of thylakoids isolated from control (solid line), dark-chilled (dashed line) and subsequently photoactivated leaves (dotted line). (B) Fluorescence emission-difference spectra chilled-minus-control (solid), photoactivated-minus-control (dashed) and photoactivated-minus-chilled (dotted). The spectra (A) were normalized to the area of 100 under the spectrum, and the arithmetic differences (B) between them were calculated. The presented spectra are representative for three separate experiments.

3. Results

3.1. Relative fluorescence yields in thylakoid membranes isolated from control, dark-chilled and subsequently photoactivated leaves

Decrease in both Chl content and Chl *a/b* ratio under stress conditions has been suggested to indicate a degradation of Chl molecules along with chlorophyll–protein (CP) complexes [23,25,37]. However, after dark-chilling of bean leaves for 5 days the Chl content on a FW basis was not changed significantly and amounted $98.0 \pm 3.9\%$ of the control level. In subsequently photoactivated leaves the Chl content slightly decreased to $91.6 \pm 5.1\%$. In thylakoid membranes isolated from control, dark-chilled and photoactivated leaves Chl *a/b* ratios were 3.02 ± 0.09 , 3.10 ± 0.15 and 2.91 ± 0.20 ($n=7$), respectively, suggesting that dark-chilling and subsequent photoactivation at moderate light ($200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) did not significantly affect the CP complexes in thylakoids. On the contrary, the fluorescence values estimated at appropriate maxima of fluorescence emission (λ_{ex} 470 nm) and excitation (λ_{em} 680 and 740 nm) spectra changed significantly with respect to control ones. In thylakoids isolated from chilled leaves, the fluorescence intensity value related to PSII (detected at 680 nm) decreased to about 45%, while the PSI associated fluorescence (measured at 736 or 740 nm) diminished to about 65% of that measured for thylakoids isolated from untreated leaves. After photoactivation the fluorescence intensity was recovered to about 68% and 85% of the control level for PSII and PSI fluorescence, respectively (Table 1). Similar results were also obtained for fluorescence emission spectra (λ_{ex} 435 and 490 nm) (data not shown). In addition, the PSI/PSII fluorescence ratio (F736/F681), estimated at 120 K, increased by 1.5 times upon chilling treatment and returned to nearly the control value following photoactivation (Table 2). These observations suggest that the dark-chilling-induced decrease and subsequent photoactivation-mediated recovery in fluorescence are mostly associated with rearrangements of CP complexes inside thylakoid membranes. Although the fluorescence of both photosystems decreased, PSI seems to be less sensitive to dark-chilling stress than PSII.

Table 2

Ratio of fluorescence emissions, F736/F681, at 120 K of thylakoids isolated from control, chilled in the dark (5 days, 1 °C) and chilled and subsequently photoactivated (3 h, 22 °C) bean leaves

Samples	Ratio of fluorescence emission, F736/F681		
	λ_{ex} 435	λ_{ex} 470	λ_{ex} 490
Control	1.6 ± 0.03	1.4 ± 0.04	1.3 ± 0.02
Dark-chilled	$2.8 \pm 0.14^{\text{e}}$	$2.2 \pm 0.11^{\text{e}}$	$2.2 \pm 0.07^{\text{e}}$
Dark-chilled and photoactivated	$2.2 \pm 0.30^{\text{f}}$	$1.7 \pm 0.11^{\text{f}}$	$1.6 \pm 0.13^{\text{f}}$

The data are the means \pm S.D. for 3 separate experiments. Statistical significance (*P*) was calculated for marked data series and estimated to be no more than: $^{\text{e}}P < 0.01$, versus corresponding control values and $^{\text{f}}P < 0.05$ versus corresponding values for thylakoids from chilled leaves, respectively.

3.2. The relative contribution of CP complexes to fluorescence spectra of thylakoids

The maximum of the fluorescence emission spectrum of bean thylakoid membranes in buffer C (pH 7.5) at 25 °C (298 K) are located at 678 nm (Fig. 1A), typical for PSII complexes and their antennae, and consistent with the fact that this band is probably composed of six bands ascribed to these complexes [50]. Although at room temperature, the contribution of PSI and LHCI to the steady-state fluorescence is very low, it cannot be ruled out [51]. Dark-chilling of the bean leaves led to partial quenching of the fluorescence at 678 nm, and shifted the maximum for emission to 680.5 nm. In contrast, large recovery of the parent spectra was achieved by photoactivation of leaves at 22 °C. It follows that fluorescence emission spectra should make possible characterization of the effect of chilling on the chlorophyll–protein complexes in bean leaves.

The difference spectrum at 298 K for normalized emission of thylakoid membranes isolated from dark-chilled leaves, relative to that of thylakoids isolated from control leaves, with Ex 435 nm (Fig. 1B, solid line), and 470 and 490 nm (data not shown), exhibited in each case a negative band at 674 nm and positive band at 697 nm. Since emission spectra used here were fully corrected for the inner-filter effects as well as for background fluorescence spectra (Section 2.3), these bands demonstrate a change of the relative proportion between fluorescence emission bands due to the dark-chilling. The latter is also reflected in the emission-difference spectrum of thylakoids from photoactivated leaves, albeit at significantly diminished intensity of both bands in the difference spectrum due to significant reactivation (Fig. 1B, dashed line). In our opinion, the effect of dark-chilling may be considered as relative decrease of LHCII–PSII as well as an increase of LHCI–PSI fluorescence [50,51]. Moreover, three other possibilities of changes in thylakoid fluorescence upon chilling should be considered, i.e., changes in light scattering [52], the degree of stacking [8] and/or rearrangement of LHCII complexes [18].

The steady-state fluorescence spectra of all three types of thylakoid membranes performed at 120 K exhibited two maxima at 683 and 736 nm (Fig. 2A). Although the fluorescence emission spectra at 120 K may be slightly different to that measured at 77 K [53], it is commonly accepted that thylakoid spectra are highly heterogeneous and consist of six main emission bands corresponding to (i) trimers and monomers of LHCII (~680 nm), (ii) reaction centers and inner antennae of PSII (685 and 695 nm), (iii) aggregated trimers of LHCII (~700 nm) and (iv) core complex and LHC of PSI (720 and 735–740 nm) [13,14,47,53]. In order to visualize relative contribution of individual CP complexes to the fluorescence emission spectrum of the thylakoid membranes at 120 K, fluorescence-emission difference spectra (Fig. 2B) were determined similarly to that at 298 K (cf. Fig. 1B). Difference spectrum of thylakoid membranes isolated from dark-chilled leaves (Fig. 2B, solid line) exhibited a negative band at 681 nm with a shoulder around 695 nm, both attributed to antenna complexes associated with PSII [47], while the

positive wide band with maximum around 744 nm is probably related to both PSI and LHCI complexes [13,14,54]. Intensity of these bands dramatically decreased upon photoactivation (Fig. 2B, dashed line) suggesting significant reconstruction of the parent relationship between CP complexes following photoactivation.

It is worth noting that photoactivation led to increase of the relative emission from the LHCII and PSII, as shown by the positive bands with maxima at 681 and 708 nm, visible in emission-difference spectrum (Ex 490 nm) of thylakoids from photoactivated leaves, relative to that of thylakoids from chilled leaves (Fig. 2B, dotted lines). Since the aggregation of LHCII causes the appearance of the emission band at around 700 nm [23,53,56], it seems possible that photoactivation of leaves led to relative increases of aggregated form of LHCII. In addition progressive decline in the fluorescence intensity at 680 nm accompanied by fluorescence increase at 699 nm occurs upon LHCII aggregation in vitro [56]. Therefore, the fluorescence ratio F_{699}/F_{680} (calculated from non-normalized spectra) equal to 0.34, 0.47 and 0.49 for thylakoids isolated

from control, chilled and subsequently photoactivated leaves, respectively, might be taken as a marker of the extent of LHCII aggregation [23], which is increased in dark-chilled leaves and is not diminished following photoactivation. This indicates that photoactivation does not completely reverse dark-chilling induced changes in CP complexes, in line with a foregoing observation (cf. second paragraph in this Section, and Fig. 1B).

3.3. The relative efficiency of light harvesting in thylakoids isolated from dark-chilled and photoactivated leaves

Normalized excitation spectra at 120 K (Em 680 and 740 nm) reveal differences between thylakoids isolated from control and chilled leaves (Fig. 3A, B) and show the relative energy transfer from absorbing pigments to emitting Chl species reflecting a state of CP complexes [55]. The fluorescence excitation-difference spectra (Em 680 and 740 nm) of thylakoids from chilled leaves, relative to those isolated from control leaves (Fig. 3C, D, solid lines), showed a negative

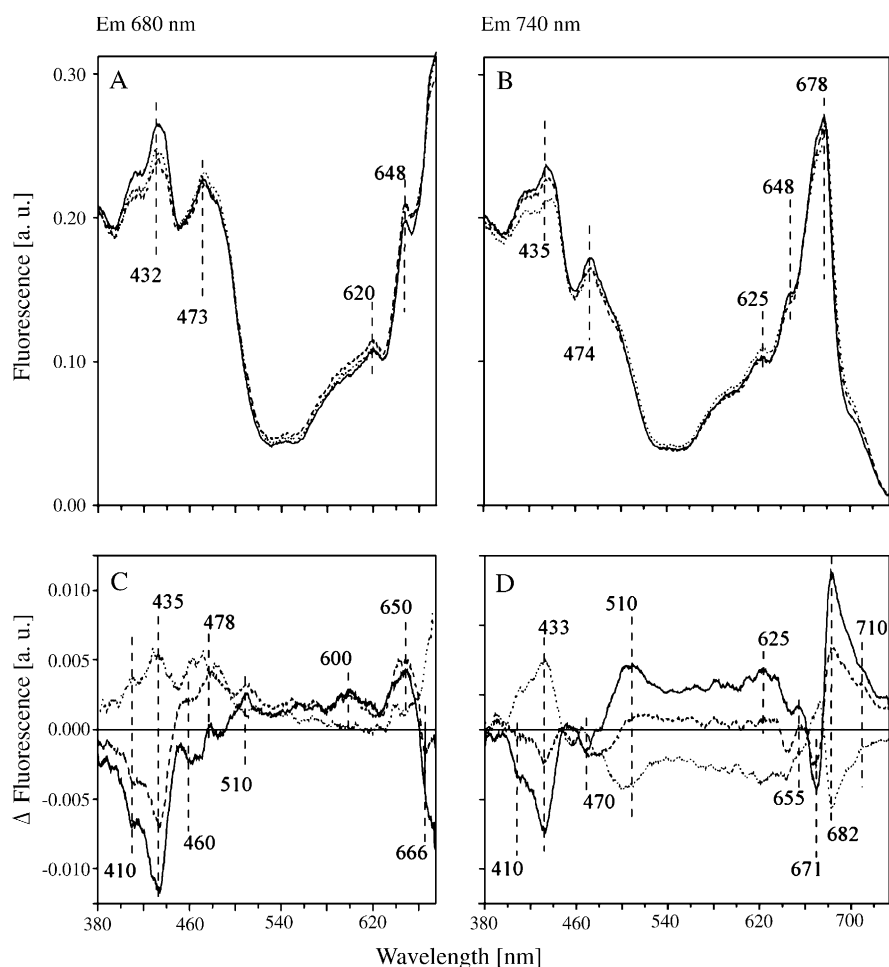


Fig. 3. Effect of dark-chilling and subsequent photoactivation on fluorescence excitation spectra of thylakoids at 120 K, determined for Em 680 nm (A, C) and Em 740 nm (B, D). A and B: spectra of thylakoids isolated from control (solid line), dark-chilled (dashed line) and subsequently photoactivated (dotted line) leaves. C and D: fluorescence excitation-difference spectra of chilled-minus-control (solid line), photoactivated-minus-control (dashed line) and photoactivated-minus-chilled (dotted line). The excitation spectra were measured for the same sample as that used for the fluorescence emission measurements (Fig. 2). The spectra (A, B) were normalized to the area of 100 under the spectrum and the arithmetic differences (C, D) between them were calculated. The presented spectra are representative for three separate experiments.

band at about 433 nm with shoulders between 410–420 and 460–470 nm, as well as positive band at 510 nm, better pronounced in the case of excitation-difference spectrum for Em 740 nm (Fig. 3D, solid line). In addition, the positive bands around 650 nm (broad) and at 682 nm (sharp) were observed (Fig. 3C and D). These difference spectra resemble those reported for the aggregated form of isolated LHCII [55], suggesting that LHCII aggregation might occur during chilling. However, the excitation spectra of thylakoids can be disturbed by light scattering and sieve effects [52]. The excitation-difference spectra (Em 680 and 740 nm) of thylakoids from photoactivated leaves, relative to that of thylakoids isolated from control leaves, showed decrease in intensity of bands at 435 and 682 nm (Fig. 3C, D, dashed lines) in comparison with dark-chilling induced difference spectra (Fig. 3C, D, solid lines). This suggests a partial restoration of the energy transfer from antennae complexes to Chl species emitting at both 680 and 740 nm.

3.4. Dark-chilling induced destabilization of aggregated forms of CP complexes revealed by mild-denaturing electrophoresis

Thylakoid membranes isolated from control, dark-chilled and photoactivated leaves were subjected to analysis by native PAGE to separate the CP complexes (Fig. 4A). The green bands were assigned to CP complexes according to Maroc et al. [48]. CPIa¹, CPIa² were related to undissociated LHCI–PSI complexes while CPI to the reaction center of PSI. The reaction center of PSII and its antennae corresponded to CP47 and CP43 bands, respectively. The CPII¹ and CPII² bands corresponded to oligomeric forms of antenna complexes, mainly to trimeric forms of LHCII, while the monomeric

forms of CP complexes were assigned to CP¹, CP² (LHCII monomers) and CP³.

The electrophoretic patterns obtained for thylakoids isolated from both control and chilled leaves differed in the relative intensity of bands corresponding to undissociated LHCI–PSI (CPIa) and LHCII trimeric complexes (CPII). Mild-denaturing electrophoresis did not completely preserve the CP supercomplexes structure, especially the LHCII–PSII organization [57]. Hence, the presented results illustrate the stability of distinct CP complexes rather than its native organization in thylakoid membranes. The quantitative analysis of gels revealed that the decrease in CPIa bands intensities was associated with a simultaneous increase in intensity of CPI band (Fig. 4B), indicating that the action of detergents (octyl glucoside and lithium dodecyl sulfate) on LHCI–PSI complexes was enhanced in thylakoids isolated from chilled leaves, in comparison with that exhibited in thylakoids isolated from untreated leaves. Furthermore, a decrease in CPII² band intensity might suggest a diminished stability of LHCII trimers. On the contrary, the banding pattern of thylakoid membranes isolated from photoactivated leaves was similar to that for the control sample, suggesting restoration of membrane properties.

3.5. Intact chloroplast structure analysis by confocal laser scanning microscopy (CLSM)

The observation of isolated, intact chloroplasts was performed by CLSM (Fig. 5). Fluorescence images of chloroplasts were obtained from various focal depths creating cross-section optical slices combined into deconvolved images [58,59]. Since PSI fluorescence at ambient temperature contributes for less than 5% to the total Chl fluorescence, the observed

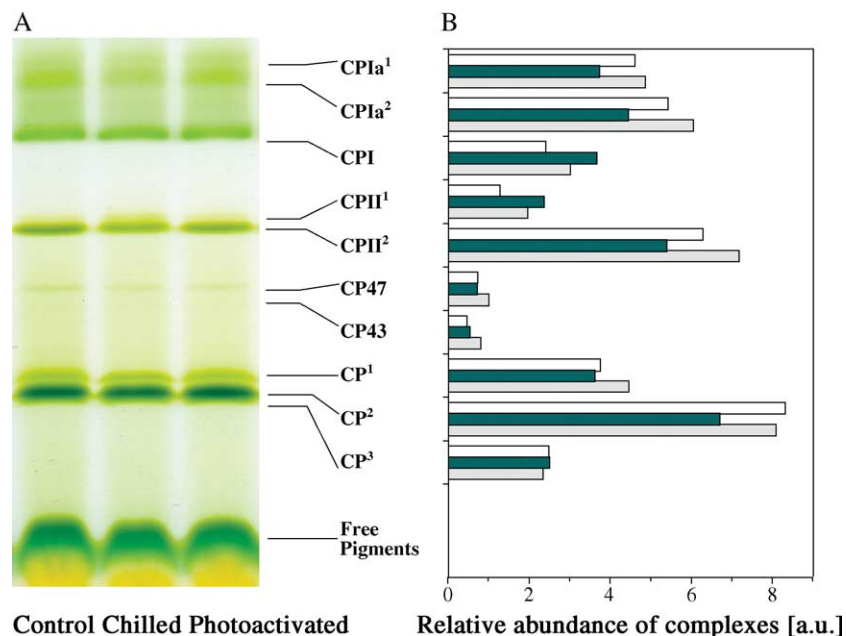


Fig. 4. Analysis of CP complexes in thylakoids with mild-denaturing green gel electrophoresis. (A) Native PAGE of thylakoids isolated from control, dark-chilled and subsequent photoactivated leaves. Each lane is loaded with solubilized thylakoids containing 25 µg Chl. (B) Relative intensities of CP bands. Empty, black and gray bars symbolize the values for control, chilled and photoactivated samples, respectively. The electrophoretic patterns are representative for at least eight separate experiments.

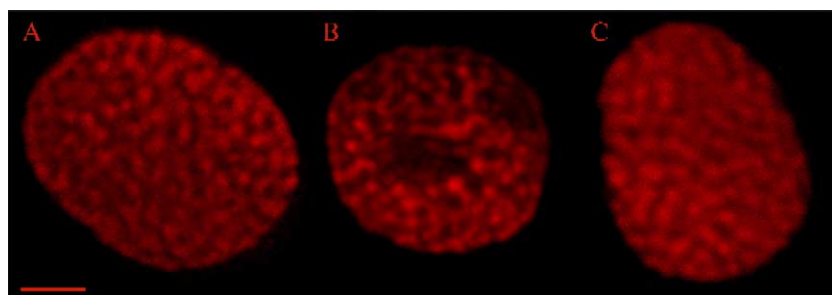


Fig. 5. Deconvolved images of intact chloroplasts analyzed by CLSM. The presented chloroplasts were isolated from control (A), dark-chilled (5 days, 1 °C) (B) and chilled and subsequently photoactivated leaves (3 h, 22 °C). Scale bar is equal to 2 μ m.

fluorescence pattern is mainly attributable to PSII and their antennae [59,60]. In view of the lateral heterogeneity of photosystems [2,4], grana stacks were characterized by bright-fluorescence regular shapes, while the membrane connecting grana regions were not visible (Fig. 5) [59,60].

In chloroplasts isolated from control leaves grana stacks in the range of 0.4–0.5 μ m in diameter were uniformly distributed within chloroplasts (Fig. 5A). Regular grana arrangement was disrupted in chloroplasts isolated from dark-chilling leaves (Fig. 5B), resulting in appearance of non-fluorescent gaps in the range of 1 μ m in diameter. The structure of chloroplasts was much more rough and irregular (Fig. 5B) compared with the fine and regular structure of chloroplasts obtained from control leaves (cf. Fig. 5A). Moreover, chloroplast images revealed a

large heterogeneity and consisted of areas where fluorescence intensity was quenched or enhanced (Fig. 5B). Grana rearrangement of chloroplasts isolated from photoactivated leaves resembled those of control chloroplasts, while the regularity of grana distribution was not completely restored (Fig. 5C). Moreover, the intensity of grana fluorescence was lower and the contrast between granal and intergranal areas was smaller in comparison with that obtained from control leaves (Fig. 5C).

3.6. Effect of dark-chilling and subsequent photoactivation on PSII photochemistry in intact leaves

The effect of dark-chilling on PSII photochemistry and the possibility of subsequent recovery was tested by measuring the initial (F_0) and maximum fluorescence (F_m) values in dark adapted leaf discs. The F_v/F_m ratio, i.e., the maximum quantum yield of PSII, declined by 38% from 0.82 to 0.51 within 5 days of chilling (Fig. 6A). This decrease was fully related to a decrease of F_m and not to any increase of F_0 (Fig. 6B). During subsequent photoactivation of chilled leaves up to 4 h at moderate light and temperature, F_v/F_m decreased further to 27% of the control value. Under these conditions, both F_0 and F_m fell to very low, but almost similar, levels (Fig. 6B). The restoration of F_v/F_m to nearly the control level was observed only after 10 h of photoactivation (Fig. 6A). However, the value of F_v/F_m did not recover completely within 18 h, while F_0 increased significantly, concomitantly with the strong increase in F_m after 7 h of photoactivation (Fig. 6B). These Chl *a* fluorescence measurements revealed a slower recovery of PSII photochemistry in comparison with restoration of thylakoid fluorescence (cf. Tables 1 and 2) and chloroplasts structure (cf. Fig. 5).

4. Discussion

The effect of both long-term dark-chilling and subsequent post-chilling photoactivation on thylakoid membranes organization, chloroplast structure and PSII function was studied in view of data received by application of four complementary methods: steady-state fluorescence spectroscopy, mild-denaturing electrophoresis, CSLM and PAM-fluorometry. The decrease of overall Chl *a* fluorescence caused by 5-day (120 h) dark-chilling (Table 1) is in agreement with a diminished

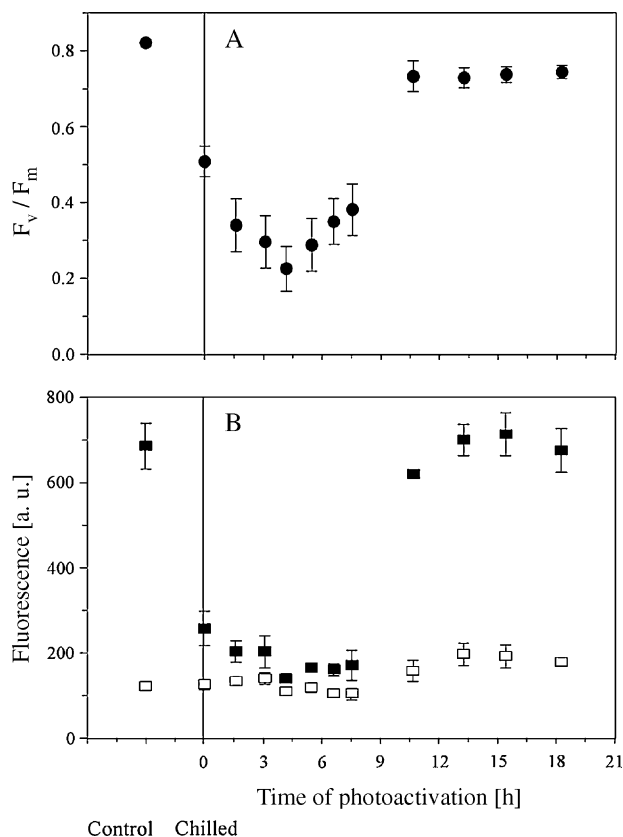


Fig. 6. Effect of dark-chilling and subsequent photoactivation on F_v/F_m (A) as well as F_0 and F_m levels (B) of leaf discs. The data are means \pm S.D. for 3 different plants. F_v/F_m —solid circles, F_0 —empty squares, F_m —solid squares.

stability of LHCI–PSI and LHCII trimers revealed by mild-denaturing electrophoresis (Fig. 4), representing the fingerprint of protein–protein interactions within thylakoid membranes [57]. Since the low-temperature treatment resulted neither in a significant decrease of the Chl content in leaves nor in a change of the Chl *a/b* ratio in thylakoids (cf. Section 3.1), the disturbance of oligomeric structure of CP complexes seems to be the reason for the decline in overall Chl *a* fluorescence. Moreover, changes in the relative contribution of LHCI and PSI versus LHCII and PSII to the fluorescence spectrum of thylakoids isolated from chilled leaves (Tab. 2, Fig. 2) suggest a rearrangement of these complexes, probably most evident with respect to aggregations of LHCII (Figs. 2 and 3, and the last paragraph of Section 3.2). Thus, these data indicate that the storage of detached bean leaves at 1 °C in the dark causes the disassembly and certain rearrangements of CP complexes in thylakoid membranes. Most of these changes are restored to the initial state following post-chilling illumination of leaves at moderate temperature and irradiances (Table 1, Figs. 1–4), indicating the reversibility of the destructive action of long-term (5-day) dark-chilling on CP complexes. However, the picture of low-temperature fluorescence spectra (Figs. 2 and 3) suggests that a part of LHCII remains in an aggregated form.

The ratio of surface area of granum and stroma membranes is presumed to be 1:1 and can vary as a result of environmental conditions [3]. This is usually associated with changes in the macrodomain structure of thylakoids [8,9]. For example, both the dislocation of LHCII towards stroma thylakoids under state transition [61] and the formation of LHCII–PSI complexes in an *Arabidopsis* mutant [21] resulted in changes in grana size. However, little is known about a possible direct impact of thylakoid membranes reorganization on the structure of intact chloroplasts [59]. To our knowledge, this is the first case, where the dark-chilling-induced disturbance of the structure of bean chloroplasts revealed by CLSM (Fig. 5B) may be a consequence of rearrangements of thylakoid supercomplexes (Figs. 1–4). Furthermore, an incomplete reconstitution of the chloroplast structure (Fig. 5C) following the post-chilling photoactivation, may be associated, among other, with aggregation of LHCII (Figs. 2 and 3). Hence, changes in chloroplast structure under long-term dark chilling seem to derive from a reorganizations of CP complexes inside thylakoid membranes.

Dark-chilling induced inhibition of photosynthetic capacity in CS plants, which is related to reduction of electron transport and metabolic restrictions, are species-specific ([43], and references cited therein). Although the quantum yield of PSII photochemistry (F_v/F_m) of cucumber leaves was found to be quite insensitive to short-term (0–48 h) dark chilling [30] and to a number of consecutive nights of chilling [62], our data with bean showed a pronounced decrease of 38% in F_v/F_m after 5 days at 1 °C in darkness (Fig. 6A). This decrease could fully be ascribed to a decrease of the maximum fluorescence level (F_m) (Fig. 6B). The decrease of F_m in the dark is considered to be the overall result of a large-scale inactivation of reaction centers (RCs), simultaneously with a smaller increase in the trapping flux of still active RCs [62]. The mechanism of dark-chilling induced inactivation of RC in the

dark seems to be related to specific chilling-induced events as dissociation of Mn-cluster and extrinsic proteins (PsbP and PsbQ) from PSII [38–41] and hydrolysis of galactolipids [42]. The disintegration of the Mn-cluster in dark-chilled cucumber leaves could be ascribed to its over-reduction. However, the mechanism how and why, remains to be elucidated [41].

The present study illustrates that in bean leaves 3 h of photoactivation was enough for partial reconstruction of thylakoid membranes organization (Figs. 1–3) and chloroplast structure (Fig. 5), while it was too short to recover PSII photochemistry (Fig. 6). Within the first 4 h of the post-chilling recovery period, F_v/F_m decreased further from 38 to 73% of the initial value due to both a decrease of F_m and an increase of F_0 . These changes indicate that after a long-term chilling treatment in the dark, bean leaves become susceptible to photoinhibition of PSII when transferred to moderate light and temperature conditions [35,63]. By comparison, restoration of O_2 evolution after 3 days of dark chilling in tomato was almost complete after 2 h of photoactivation at a low irradiance level, although the membrane conformation was not completely reversed during this time [40]. Experiments with cucumber, under nearly similar conditions as that used by Garstka and Kaniuga [40], illustrated that the extent of light-induced reactivation decreased, when the leaves were dark-chilled for a longer period [41]. In view of these results, we conclude that both the long-term chilling treatment (5 days) and application of moderate level of irradiance during reactivation rendered bean leaves susceptible to photoinhibition, independently of structural reconstruction of PSII complexes that occurred during photoactivation after dark-chilling. The fact that F_v/F_m did not recover completely to the control level, even not after 18 h of photoactivation, could be ascribed to a significant increase of F_0 . Since F_0 originates from Chl *a* antennae associated with PSII RC, the increase in F_0 is indicative for decreased energy transfer from this complex to PSII cores and/or less efficient energy processing in the cores [64]. It appears that there is more than one mechanism that leads to a change in F_0 level under light, and their action probably depend on growth and treatment conditions as well as on the species [65]. The following mechanistic explanations have been proposed (i) the formation of a PSII RC with the quinone acceptor stabilized in the protonated reduced form (Q_AH) [66] or (ii) the (partial) disconnection of PSII RC from the antenna complexes [23]. The irreversible character of the increase in F_0 during long-term photoactivation indicated the occurrence of the latter mechanism in thylakoids of dark-chilled bean leaves during photoactivation. Moreover, the rearrangements of LHCII–PSII and a partial aggregation of LHCII, as observed in thylakoids under these conditions (Figs. 2 and 3, Section 3.2) might cause the increase of the initial Chl *a* fluorescence intensity (F_0) at open centers.

It has been suggested recently, that the arrangement of supercomplexes within thylakoid membranes is the result of protein–protein and lipid-mediated interactions [15,19,67]. The thylakoid proteins have boundary lipids of structural importance [67,68]. Particularly, the interaction of LHCII with monogalactosyldiacylglycerol (MGDG), the main lipid of thylakoid

membranes, is probably indispensable for formation of ordered lamellae, the framework of thylakoids [69]. As the dark-chilling effect is associated with accumulation of FFA and a partial depletion of galactolipids [40,42], thus it may cause disordering of lipid–protein interactions. However, direct interactions of FFA with thylakoid proteins [70,71] cannot be excluded. Further studies are required to elucidate this possibility.

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