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ORIGINAL ARTICLE

Contrasting effect of dark-chilling on chloroplast structure and arrangement of chlorophyll–protein complexes in pea and tomato: plants with a different susceptibility to non-freezing temperature

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Abstract The effect of dark-chilling and subsequent photoactivation on chloroplast structure and arrangements of chlorophyll–protein complexes in thylakoid membranes was studied in chilling-tolerant (CT) pea and in chilling-sensitive (CS) tomato. Dark-chilling did not influence chlorophyll content and Chl *a*/*b* ratio in thylakoids of both species. A decline of Chl *a* fluorescence intensity and an increase of the ratio of fluorescence intensities of PSI and PSII at 120 K was observed after dark-chilling in thylakoids isolated from tomato, but not from pea leaves. Chilling of pea leaves induced an increase of the relative contribution of LHCII and PSII fluorescence. A substantial decrease of the LHCII/PSII fluorescence accompanied by an increase of that from LHCI/PSI was observed in thylakoids from chilled tomato leaves; both were attenuated by photoactivation. Chlorophyll fluorescence of bright grana discs in chloroplasts from dark-chilled leaves, detected by confocal laser scanning microscopy, was more condensed in pea but significantly dispersed in tomato, compared with control samples. The chloroplast images from transmission-electron microscopy revealed that dark-chilling induced an increase of the degree of grana stacking only in pea chloroplasts. Analyses of O-J-D-I-P fluorescence induction curves in leaves of CS tomato before and after recovery from chilling indicate changes in electron transport rates at acceptor- and donor side of PS II and an increase in antenna size. In CT pea leaves these effects were absent, except for a small but irreversible effect on PSII activity and antenna size. Thus, the differences in chloroplast structure between CS and CT plants, induced by dark-chilling are a consequence of different thylakoid supercomplexes rearrangements.

Keywords Chloroplast and thylakoid membrane structure · Chilling-sensitive · Chilling tolerant · Chlorophyll–protein complexes · Cation-induced thylakoid stacking · O-J-D-I-P chlorophyll fluorescence · Confocal laser scanning microscopy · Dark-chilling stress · Pea · Tomato

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

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Abbreviations

Chl	Chlorophyll
CLSM	Fluorescence confocal laser scanning microscopy
CP	Chlorophyll–protein
CS	Chilling-sensitive
CT	Chilling-tolerant
Em	Emission wavelength
Ex	Excitation wavelength
FFA	Free fatty acids
PAR	Photosynthetic active radiation
TEM	Transmission electron microscopy

Introduction

Depending on their evolutionary background, tolerance of plants to low temperature varies between species and cultivars. According to the temperature range, plants have been divided into chilling-sensitive (CS) plants, plants susceptible to temperatures below 12°C, and chilling-tolerant (CT) plants, resistant to low but non-freezing temperatures. A third group, freezing-tolerant plants, acquired frost tolerance after a period of exposure to low, non-freezing temperatures in the adaptative cold acclimation process. The latter caused modifications in membrane structure (Los and Murata 2004) and lipid composition (Welti et al. 2002), leading to an enhanced antioxidant defense and to changes in levels of many metabolites (Cook et al. 2004). Recently, transcriptome studies, performed mainly with freezing-tolerant *Arabidopsis*, showed that cold acclimation is associated with an activation of multiple genetic regulatory pathways (Chinnusamy et al. 2006; van Buskirk and Thomashow 2006). In CS and CT plants the genetic response to low temperature has not fully been recognized, even though a number of cold-induced transcriptional factors were identified in CS plants (Kim et al. 2001; Yang et al. 2005 and references therein).

Low-temperature treatments of CS plants like tomato affect the thylakoid electron transport, ribulose-1,5–bisphosphate carboxyleas/oxygenase (Rubisco) and other enzymes involved in the carbon reduction cycle and control of stomatal conductance (Allen and Ort 2001; Venema et al. 2005 and references cited in both). However, plants chilled in the light and in the dark revealed substantial difference both in the scale of inhibition of photosynthesis and the primary mechanisms involved (Allen and Ort 2001). Chilling-induced photoinhibition involves damage to iron–sulfur clusters at the reducing side of photosystem I (PSI; Scheller and Haldrup 2005 and references therein) and to both oxidizing and acceptor side of photosystem II (PSII; Govindachary et al. 2004). In CT plants like barley and *Arabidopsis* (Tjus et al. 1998; Zhang and Scheller 2004), and in CS plants such as pumpkin, tobacco, maize and tomato PSI damage with concomitant damage of PSII was observed (Barth and Krause 1999; Govindachary et al. 2004). The preferential inhibition of PSI in several different species, e.g. in CS cucumber (Kudoh and Sonoike 2002 and references therein) and CT potato (Havaux and Davaud 1994), was also reported. This difference in susceptibility of individual photosystems to chilling-induced photoinhibition depends on the developmental history of plants (Ivanov et al. 1998) and does not allow to identify PSI as a primary target of light-chilling injury (Barth and Krause 1999; Govindachary et al. 2004). However, in contrast to PSII photodamage, during subsequent recovery at non-chilling temperature, the PSI core complex is not repaired but completely degraded, which results in prolonged inhibition of photosynthesis (Kudoh and Sonoike 2002; Zhang and Scheller 2004). This chilling-induced photoinhibition in CS plants is closely related to the downstream of the antioxidant system (Foyer et al. 2002; Zhou et al. 2004), especially superoxide dismutase activity (Choi et al. 2002).

Thus, light-chilling can be regarded as a type of photoinhibition in which oxidative stress plays an important role, whereas the effects of dark-chilling are mainly induced by chilling alone. Yet the mechanisms involved in dark-chilling are not clearly defined. Short-term chilling treatments in the dark stalled the circadian rhythms in chlorophyll *a*/*b* binding protein and Rubisco activase mRNA expression (Jones et al. 1998) and enhanced the abundance of Rubisco small subunit transcripts (van Heerden et al. 2003a). Experiments with soybean grown for several days in a chilling-night/warm photoperiod cycle, showed susceptibility of both fructose-1,6-bisphosphatase and photochemistry to a dark-chilling treatment (van Heerden et al. 2003a, b). However, depending on the soybean genotypes, dark-chilling imposed diverse physiological and biochemical limitations including stomal conductance, metabolic restriction and impairment of electron transport (van Heerden et al. 2003b, 2004). In tomato, the specific activity of Rubisco declines in parallel to photosynthesis rates to 50% of the control values within 14 day of chilling at low non-photoinhibitory light conditions (Brüggemann et al. 1995). This decline in Rubisco activity is linked to the oxidation of thiol groups which result in the formation of disulfide bonds (Brüggemann 1995). It has been suggested that this oxidation is the result of a non-specific oxidation of individual RubisCO molecules in the chloroplast by ROS, which have occasionally not been detoxified (Brüggemann et al. 1995).

The decrease in PSII activity in leaves of CS plants chilled in the dark for 3–5 days was associated with the release of manganese from the oxygen-evolving complex (OEC) of PSII (Kaniuga et al. 1978) and with the destabilization of PSII extrinsic proteins (Shen et al. 1990; Garstka and Kaniuga 1991). Moreover, dark-chilling of detached leaves of CS, but not CT plants, induced the accumulation

of free fatty acids (FFA) in chloroplast membranes, due to a high activity of endogenous galactolipase (Gemel and Kaniuga 1987; Kaniuga 1997). Following 3 days dark-chilling and subsequent 2 h photoactivation of CS tomato leaves at room temperature with low light (80 µmol photons m^{-2} s^{-1}), the O_2 evolution activity was almost completely restored and simultaneously the amount of FFA decreased to the control level (Garstka and Kaniuga 1991). These observations indicated that accumulated FFA are involved in dark-chilling induced inactivation of PSII (Kaniuga 1997). Moreover, a contribution of thylakoid endogenous reductants to disintegration of Mn-cluster during dark-chilling of cucumber leaves has been suggested (Higuchi et al. 2003).

Chloroplasts of higher plants contain thylakoid membranes differentiated into cylindrical granum stacks of appressed (stacked) membranes which are surrounded by non-appressed (unstacked) helically organized stroma thylakoids (Mustárdy and Garab 2003). The PSII and PSI complexes differentially embedded in granum and stroma membranes (Danielsson et al. 2004), are organized into large supercomplexes with specific peripheral antenna complexes, chlorophyll *a*/*b* light-harvesting complexes, LHCI and LHCII. The supercomplex LHCI–PSI, consisting of the monomeric PSI core complex, four LHCI subunits (Lhca1-4; Klimmek et al. 2005) and a temporarily bound LHCII complex (Kouril et al. 2005), is localized in unstacked thylakoid regions (Danielsson et al. 2004). In stacked membranes variable amounts of the trimers of LHCII and minor light-harvesting complexes (Lhcb4, Lhcb5, Lhcb6) form, together with the dimer of the PSII core, the LHCII–PSII supercomplexes (Dekker and Boekema 2005), named photosynthetic unit PSIIα (Kirchhoff et al. 2004b). The monomeric form of PSII, named PSIIβ, does not bind the LHCII trimers and is exclusively located in stroma lamellae (Dekker and Boekema 2005). Moreover, the mobile trimeric form of LHCII also occurs in thylakoid membranes (Dekker and Boekema 2005). LHCII–PSII and trimeric LHCII supercomplexes build up macrodomain structure or megacomplexes which determine the thylakoid structure (Kirchhoff et al. 2004a; Dekker and Boekema 2005). Changes in the degree of thylakoid membrane stacking observed in response to environmental factors, e.g. under variable light conditions (Rozak et al. 2002), are closely related to the rearrangement of chlorophyll–protein (CP) supercomplexes (Kirchhoff et al. 2004a).

Four-day dark-chilling of detached leaves of CS bean and tomato, but not of CT spinach, caused a significant change in chloroplast ultrastructure, e.g. partial dilatations of grana. These perturbations were reversed following illumination of leaves with low light at room temperature (Gemel et al. 1986). However, the mechanism involved in dark-chilling-induced chloroplast injury has still not been elucidated (Kratsch and Wise 2000). Garstka et al. (2005) have reported that in CS bean dark-chilling-induced disturbance of chloroplast structure might be a consequence of rearrangements of CP supercomplexes—LHCI–PSI and LHCII trimers. These effects were reversed to a large extent after post-chilling photoactivation, with the exception of LHCII, which remained partly in the aggregated form (Garstka et al. 2005).

The aim of the present investigation was to compare the chloroplast structure and arrangement of CP complexes in thylakoids isolated from dark-chilled and subsequently photoactivated leaves of CT pea (*Pisum sativum* L.) and CS tomato (*Lycopersicon esculentum* Mill.). Studies were performed with the use of the fluorescence confocal laser scanning (CLSM), transmission electron microscopy (TEM), fluorescence spectroscopy and mild-denaturing electrophoresis as well as fast Chl *a* fluorescence kinetics. Opposite changes in the rearrangement of CP complexes in pea and tomato thylakoids following dark-chilling stress were observed. Moreover, to the best of our knowledge, the increase of the degree of grana stacking in CT pea chloroplasts, as a consequence of the dark-chilling treatment, is reported for the first time.

Materials and methods

Plant materials, growth, chilling and photoactivation procedures

Pea (*Pisum sativum* L. cv. Baron; PlantiCo Zielonki, Babice Stare, Poland) and tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker; Laboratory of Plant Breeding, Wageningen University, The Netherlands) plants were grown in 3–l perlite-containing pots in a climate room (22°C/20°C day/night temperature), at a photosynthetic active radiation (PAR) of 200 µmol photons m^{-2} s^{-1} during a 16 h photoperiod and relative humidity of 60–70%. Plants were fertilized with full Knop's nutrient solution. Fully expanded leaves of 14 and 21 day-old pea and tomato, respectively, were used for experiments. The leaves for the control samples were harvested 30 min after the beginning of the photoperiod due to induce photosynthetic reactions. For the chilling treatment the leaves were detached from the plants and placed in thermos flasks on damp paper above an ice layer in a dark cold-room for 5 days. Inside the thermoses the temperature was 1°C and the relative humidity 100%. The cold-stored leaves were placed in a closed transparent plastic dish on a water layer and their adaxial side was also sprinkled with water. For photoactivation, leaves were transferred to a climate room at 22°C with a PAR of 200 µmol photons m^{-2} s^{-1} for 3 h.

Preparation of chloroplasts and thylakoids

Thylakoid membranes and intact chloroplasts were prepared as described previously (Garstka et al. 2005). Thylakoids were always freshly prepared before each experiments, kept on ice in the dark and used within 2 h for both electrophoresis and fluorescence spectroscopy. For investigation of chloroplast structure by confocal scanning fluorescence microscopy intact chloroplasts were used immediately after isolation procedure. Concentrations of Chl *a* and *b*, were quantified spectrophotometrically after extraction with 80% acetone in water (Hipkins and Baker 1986). From these data the Chl *a*/*b* ratio was calculated.

Determination of Chl fluorescence in isolated thylakoids

Steady-state fluorescence spectra of isolated thylakoids at 25°C (298 K) and 120 K were determined as described previously (Garstka et al. 2005). 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 120 K were normalized to the same area (100) under the spectrum (Andreeva et al. 2003).

Analysis of fast Chl *a* fluorescence kinetics

Chl *a* fluorescence induction curves were recorded in 30 min dark-adapted control, chilled and photoactivated detached pea and tomato leaves with a PEA fluorometer (Plant Efficiency Analyzer, Hansatech Instruments Ltd, King's Lynn, Norfolk, UK) in a darkened room at 20°C under dim green light. Fluorescence data were collected at a sampling rate of 0.01, 10 and 100 ms in the time ranges 0.01–2, 2 to 10^3, and above 10^3 ms, respectively, with 12 bit resolution (Strasser et al. 2004). The fluorescence rise was induced by red light of 3,200 µmol (photons) m^{-2} s^{-1} during 3 s (ensuring full closure of all PSII RCs to obtain a stationary intensity of the maximum fluorescence, F_M) provided by an array of six light-emitting diodes (peak 650 nm) focusing on an exposed crossing area of a fixed sample of 4 mm in diameter. The induction curve is commonly denoted by the so-called O-J-D-I-P rise and accounts for the occurrence of a dip (D) between the J and I level in high intensity light.

The induction curve has been shown to be composed of a photochemical (O-J-D-) phase and two distinguishable photo-electrochemical (D-I-, and I-P-) phases in the 0.01–2, 2–50, and 50–200 ms time range, respectively (Schreiber 2004; Vredenberg et al. 2005, 2006). It has been outlined in detail (Vredenberg 2004) that the initial rise of the photochemical O-J-D phase is theoretically determined by a simple rate equation (Eq. 14 in this reference). The single turnover (STF)-induced variable fluorescence-rFv^{STF} (relative to $F_o = 1$), the light excitation rate *k*L and the rate constant of the donor side quenching release, denoted here as *k*1, are the determinant parameters in this equation. Thus, for example, the experimental $F(t)/F_o$ rise in the dark-adapted control tomato leaf during the initial 0.5 ms of illumination, at the intensity used is quantitatively identifiable with rFv = 1.8 and *k*L and *k*1 equal to 2.9 and 12.3 ms^{-1}, respectively (see Table 2). A change in any of these parameter values after chilling and/or during photoactivation points to a change in photochemical activity (rFv), antenna size (*k*L) and electron transport at the PSII-donor side (*k*1). Moreover a disproportional change in *k*L and *k*1 shows up as a change in the degree of sigmoidicity of the initial rise, quantifiable with the parameter *p*. In common practice *p* is taken as an empirical measure of the so-called PSII connectivity or grouping (Joliot and Joliot 1964; Strasser et al. 2004, but see Vredenberg 2004 for a challenging view on an alternative interpretation of sigmoidicity of the fluorescence induction).

Mild-denaturing "green" electrophoresis

CP complexes were analyzed by mild-denaturing polyacrylamide gel electrophoresis, 4% stacking and 9–17% separating that contained 0.1% (w/v) lithium dodecyl sulfate (LDS), following the method of Maroc et al. (1987) with some modifications (Garstka et al 2005). Thylakoids (0.5 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. Relative intensities of chlorophyll–protein (CP) bands were quantified by scans analyzed with Quantity One software (Bio Rad).

Microscopical methods

For fluorescence laser scanning confocal microscopy (CLSM) isolated intact chloroplasts (30 µg Chl ml^{-1}) were resuspended in 20 mM Hepes–NaOH (pH 7.5) buffer containing 330 mM sorbitol, 15 mM NaCl, 4 mM $MgCl_2$, 6% (v/v) glycerol and 30 µM DCMU. The suspension was placed on a poly-L-lysine layer (1 µg ml^{-1}) and fixed on a microscopic glass. Samples were imaged as described previously (Garstka et al. 2005). Z-series (10–25) of 1,024 × 1,024 pixels and eight bit images were collected. To improve signal-to-noise ratio data stacks were deconvolved and combined in a final image using the Huygens Suite 2.8 software (Scientific Volume Imaging, Hilversum, The Netherlands).

Samples of each experimental stage were taken for electron transmission microscopy (TEM). Pieces of about 1–4 mm^2 were cut from the middle part of the leaf. The material was

fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer at pH 7.4 for 2 h, washed in buffer and post-fixed in 2% OsO_4 at 4°C in 0.05 M cacodylate buffer for about 12 h. The specimens, dehydrated in a graded acetone series, were embedded in the low viscosity Spurr resin (ERL, Sigma-Aldrich) and cut on a Leica UCT ultramicrotome. Sections conventionally stained with uranyl acetate (Chemapol, Praha, Czech Republic) and lead citrate (Serva, Heidelberg, Germany) were examined with a JEM 1200EX electron microscope. The length of grana and stroma lamellae as well as number of membranes in grana was estimated in quadrilateral area of 3 million nm^2 using Digital Micrograph software (Gatan Inc., Pleasanton, CA, USA).

Results

Effect of dark chilling and photoactivation on Chl content and fluorescence in leaves and in isolated thylakoid membranes

It has been demonstrated that a diminished Chl content and Chl *a/b* ratio under stress conditions indicate degradation of Chl molecules along with CP complexes (Kudoh and Sonoike 2002). However, in our experiments, the total Chl content in dark-chilled and subsequently photoactivated leaves of pea and tomato as well as the Chl *a/b* ratio in isolated thylakoid membranes did not alter significantly (Table 1a), indicating that dark-chilling and subsequent photoactivation at moderate light do not damage the CP complexes in chloroplasts of the two species.

Fluorescence intensities at maxima of fluorescence emission (λ_{ex} 470 nm) and excitation (λ_{em} 680 and 740 nm) spectra at 25°C were invariable in thylakoids isolated from control, chilled, and chilled and subsequently photoactivated pea leaves (Table 1b), suggesting that the CP complexes in pea thylakoids are stable under low-temperatures. On the contrary, in thylakoids from dark-chilled tomato leaves fluorescence intensities in both emission and excitation fluorescence spectra were diminished to about 66% of that measured in thylakoids isolated from untreated leaves. However, the fluorescence intensity in thylakoids isolated from tomato leaves was completely recovered after photoactivation (Table 1b). It is commonly accepted that the reversible Chl fluorescence decline is related to reversible changes in organization of CP complexes, e.g. following depletion of Mg^{2+} from thylakoids (Kirchhoff et al. 2000). Thus, in view of the observations presented in Table 1a, b, we assume that the dark-chilling-induced decrease and subsequent photoactivation-mediated recovery of fluorescence in tomato thylakoids may be associated with rearrangements of CP complexes.

Relative contribution of CP complexes to 120 K fluorescence emission spectra of thylakoids

The steady-state fluorescence emission spectra of thylakoid membranes at 120 K, which are only slightly different from those measured at 77 K (Ruban et al. 1995), enable the detection of the relative contribution of individual CP complexes to the Chl fluorescence in thylakoid membranes. The two main bands of thylakoid emission spectrum at about 680 and 740 nm were highly heterogeneous and consisted of at least six emission bands corresponding to (1) trimers and monomers of LHCII (~680 nm), (2) reaction centers (~685 nm), (3) inner antennae of PSII (695 nm), (4) aggregated trimers of LHCII (~700 nm), (5) core complex and (6) LHC of PSI (720 and 735–740 nm; Andreeva et al. 2003; Klimmek et al. 2005). The fluorescence intensities of emission spectra were different in thylakoids isolated from pea and tomato leaves and estimated to about 300 and 180 a.u. (arbitrary units) at 680 nm, respectively. Therefore,

Table 1 Total leaf contents of chlorophyll (Chl *a* + *b*), Chl *a/b* ratio in thylakoids *a* and relative changes (in % of control) in the steady-state fluorescence intensities at selected maxima (λ_{max}) of fluorescence emission (λ_{ex} 470 nm) and excitation (λ_{em} 680 and 740 nm) spectra of thylakoids *b* of control, dark-chilled (5 days, 1°C) and dark-chilled and subsequently photoactivated (3 h, 22°C) CT pea and CS tomato leaves

Leaf treatment	Pea			Tomato		
a	Chl *a* + *b* (mg/g FW)		Chl *a/b* ratio	Chl *a* + *b* (mg/g FW)		Chl *a/b* ratio
Control	3.05 ± 0.15		2.98 ± 0.09	3.03 ± 0.08		3.02 ± 0.17
Dark-chilled	3.03 ± 0.23		2.86 ± 0.06	3.07 ± 0.21		2.87 ± 0.06
Dark-chilled and 3 h photoactivated	3.04 ± 0.27		2.89 ± 0.14	2.72 ± 0.16		2.88 ± 0.05
b	$\lambda_{ex}/\lambda_{max}$	$\lambda_{em}/\lambda_{max}$		$\lambda_{ex}/\lambda_{max}$	$\lambda_{em}/\lambda_{max}$	
	470/680	680/470	740/670	470/680	680/470	740/670
Dark-chilled	106 ± 2	105 ± 4	113 ± 6	63 ± 13	66 ± 15	74 ± 10
Dark-chilled and 3-h photoactivated	102 ± 7	102 ± 8	100 ± 10	106 ± 8	104 ± 6	96 ± 6

The data represent mean values ± SD for five and four separate determinations of Chl content *a*, the steady-state fluorescence intensities *b* at 25°C (298 K), respectively. Similar results as those in panel b were also obtained for fluorescence emission spectra at λ_{ex} 435 and 490 nm

in order to determine the chilling effect on the relative contribution of specific complexes to an overall fluorescence pattern in thylakoids from both species, fluorescence emission and excitation scans were normalized to the same area (100) under the spectrum (Figs. 1, 2).

The steady-state fluorescence emission spectra (Ex 490 nm) at 120 K in thylakoids isolated from control leaves exhibited two maxima at 681.5 and 733 versus 682 and 734 nm in pea and tomato, respectively (Fig. 1a, c). Dark-chilling of pea leaves slightly decreased the Chl fluorescence intensity ratio of the main bands (F733/681.5) from 1.31 ± 0.11 in thylakoids isolated from control leaves to 1.16 ± 0.12 ($n = 3$) in thylakoids isolated from chilled ones. Moreover, one nanometer red shift was observed in the spectrum of thylakoids from dark-chilling pea leaves in comparison with that of the control one (Fig. 1a). Photoactivation of leaves resulted in a partial restoration of the F733/681.5 ratio up to 1.21 ± 0.14, but the maximum at 734 nm did not recover to the control value. On the contrary, the Chl fluorescence intensity ratio of the main bands of tomato thylakoid spectra (734/682 nm), which in control thylakoids was 0.95 ± 0.13 and increased up to 1.68 ± 0.18 in thylakoids isolated from chilled leaves, decreased down to 1.22 ± 0.19 in thylakoids isolated from photoactivated leaves. Moreover, the maximum of the spectrum in thylakoids isolated from chilled leaves shifted to 732 nm, compared to 734 nm in the spectrum of thylakoids isolated from control leaves. The long-wavelength maximum returned to the control value in thylakoids from photoactivated tomato leaves (Fig. 1c).

Dark-chilling of pea leaves led to an increase of the relative emission from LHCII and PSII at about 678 nm accompanied by a simultaneous decrease of emission from the PSI core at 718 nm, as shown by emission-difference spectrum of thylakoids (Fig. 1b, solid line). The positive band at 681 nm related to PSII/LHCII, and the negative band at 718 nm, related to PSI, were also observed in thylakoids isolated from photoactivated pea leaves, indicating that photoactivation did not reverse the dark-chilling-induced changes in CP complexes (Fig. 1d, dashed line). On the contrary, difference spectrum for normalized emission of thylakoid membranes isolated from dark-chilled tomato leaves, relative to that of thylakoids isolated from control leaves (Fig. 1b, solid line), exhibited a negative band at 681.5 nm, related to the fluorescence emission from LHCII and a positive band at 729 nm, probably due to emission from LHCI complexes (Fig. 1d, solid line). Intensities of these bands decreased upon photoactivation (Fig. 1d, dashed line) suggesting a significant photoactivation-induced reconstruction of the parent relationship between CP complexes.

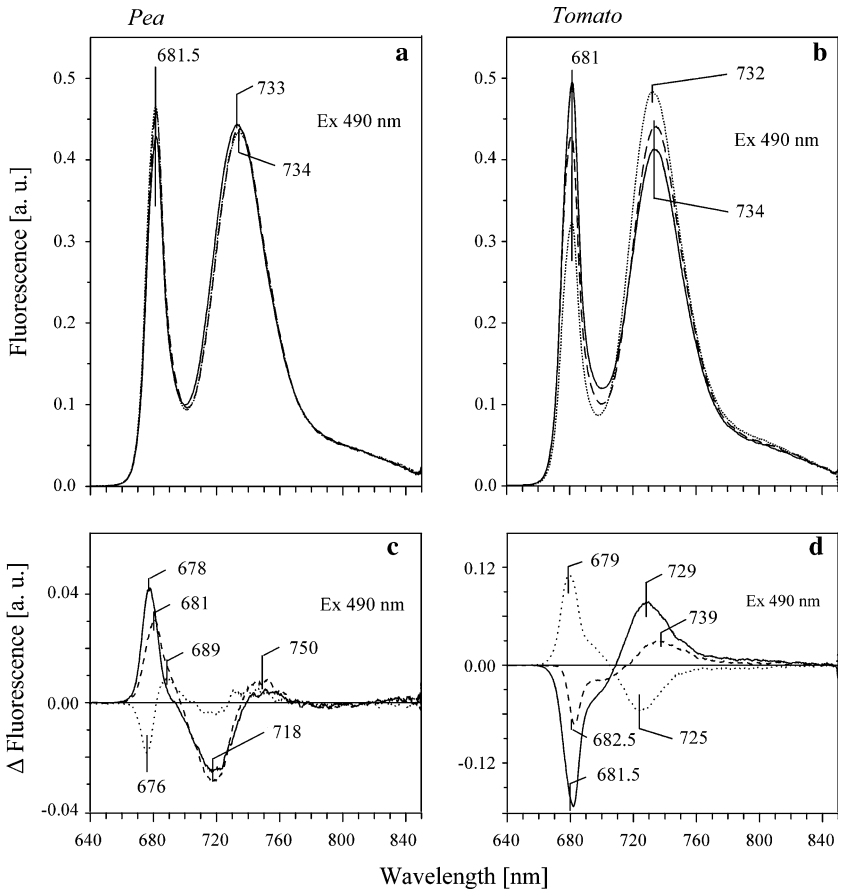

Fig. 1 Effect of dark-chilling and subsequent photoactivation on fluorescence emission spectra (*Ex* 490 nm) of isolated pea (**a**) and tomato (**c**) thylakoids (120 K) isolated from control (*solid line*), dark-chilled (*dotted line*), dark-chilled and subsequently photoactivated (*dashed line*) leaves. **b**, **d** Fluorescence emission-difference spectra of chilled-minus-control (*solid*), photoactivated-minus-control (*dashed*) and photoactivated-minus-chilled (*dotted*) calculated for the emission spectra (**a**, **c**) normalized to the area of 100 under the spectrum. The presented spectra are representative for three independent experiments

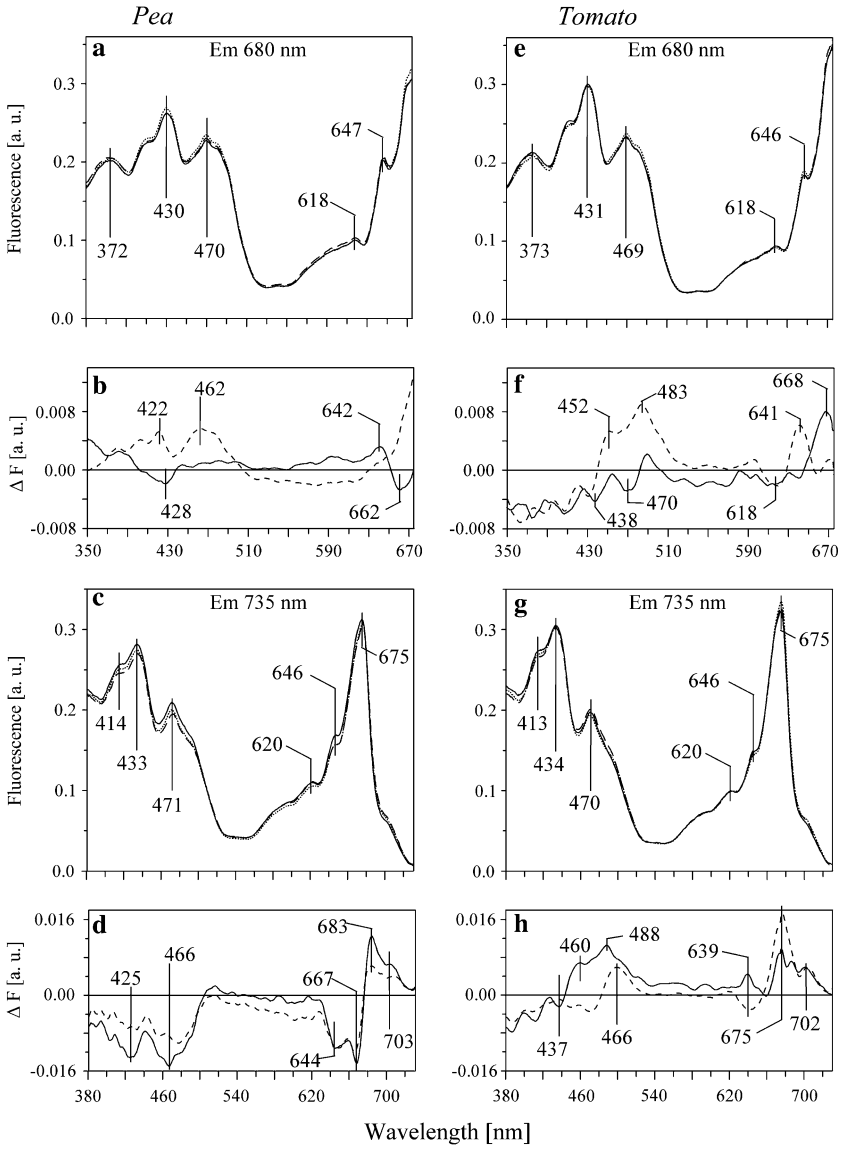

Fig. 2 Effect of dark-chilling and subsequent photoactivation on fluorescence excitation spectra (Em 680 and Em 740 nm) of pea (**a**, **c**) and tomato (**e**, **g**) thylakoids (120 K) isolated from control (*solid line*), dark-chilled (*dotted line*), dark-chilled and subsequently photoactivated (*dashed line*) leaves. **b**, **f**, **d**, **h** Fluorescence excitation-difference spectra of chilled-minus-control (*solid line*), photoactivated-minus-control (*dashed lines*) calculated for the respective excitation spectra (**a**, **e**, **c**, **g**) normalized to the area of 100 under the spectrum. The excitation spectra were measured for the same sample as that in Fig. 1, and data presented here are representative for three independent experiments

Relative efficiency of light harvesting in thylakoids from dark-chilled and subsequently photoactivated leaves

The 120 K steady-state fluorescence excitation spectra (Em 680 and 735 nm) of thylakoids isolated from leaves of both species were used to study the relative energy transfer from absorbing pigments to Chl species emitting at 680 nm (PSII; Fig. 2a, e) and at 735 nm (PSI; Fig. 2c, g). These spectra showed typical excitation bands in the Soret region (400–500 nm) due to light-harvesting by Chl *a* and Chl *b* (430–434 and 470 nm), respectively, and carotenoid pigments, as well as bands in the red wavelengths around 600–700 nm solely due to excitation of Chl *a* (675 nm) and Chl *b* (646 nm; Ruban et al. 1997). Calculated difference excitation spectra (Em 680 and 735 nm) revealed differences between thylakoids isolated from control and chilled or chilled and subsequently photoactivated leaves, reflecting the state of CP complexes under stress conditions (Fig. 2b, d, f, h).

Chilling of pea leaves did not influence the excitation spectrum of PSII (Em 680) significantly (Fig. 2b, solid line). The excitation-difference spectra (Em 680) of thylakoids from chilled tomato leaves showed a noticeable negative band in the Soret region (410–480 nm) and a positive band at 668 nm (Chl *a*; Fig. 2f, solid lines) relative to those isolated from control leaves. However, photoactivation of chilled leaves of both species caused a significant increase of Chl *b*-related bands (at 462 nm in pea vs. 483 and 641 nm in tomato thylakoids; Fig. 2b, f, dashed lines), suggesting an increase of energy transfer efficiency from antennae complexes to PSII.

Fluorescence excitation-difference spectra of PSI (Em 735 nm) of thylakoids isolated from dark-chilled leaves differed significantly between the two species (Fig. 2d, h).

Spectra of thylakoids isolated from chilled pea leaves, showed negative bands in the Soret region at 425 and 466 nm, as well as negative bands at 644 and 667 nm in the red region, relatively to those isolated from control leaves (Fig. 2d, solid lines). In addition, positive bands at 683 nm (sharp) and 703 nm (broad) were observed. The excitation-difference spectra (Em 735 nm) of thylakoids isolated from photoactivated pea leaves, showed a decrease of band intensity at 425, 466 nm in comparison to dark-chilling induced difference spectra (Fig. 2d, dashed line). It suggests only a partial restoration of the energy transfer from antennae complexes to Chl species of PSI in thylakoids isolated from photoactivated pea leaves.

Dark-chilling of tomato leaves led to relative increase of the Chl *b*- and Chl *a*-related peaks, as shown by positive bands with maxima at 460, 488 and 675 nm, visible in the excitation-difference spectrum (Em 735 nm) of thylakoids isolated from chilled leaves, relative to those from control leaves (Fig. 2h, solid line). This observation indicated the increase of relative efficiency of light harvesting by PSI in comparison with efficiency of energy transfer from antennae complexes to PSII during dark-chilling of tomato leaves (cf. Fig. 2f, solid line). The intensity of these bands significantly decreased upon photoactivation (Fig. 2h, dashed line) suggesting a partial restoration of the interaction between CP complexes observed in thylakoids isolated from control leaves.

Effect of dark-chilling and subsequent photoactivation on stability of CP complexes revealed by mild-denaturing electrophoresis

Thylakoid membranes isolated from control, dark-chilled and photoactivated leaves of pea and tomato were analyzed by mild-denaturing electrophoresis to separate the CP complexes (Fig. 3a, b). Electrophoretic patterns obtained for thylakoids isolated from control leaves of both pea and tomato revealed a similar composition of green bands, which were assigned to CP complexes, according to Maroc et al. (1987). Both $CPIa^1$ and $CPIa^2$ bands were related to undissociated LHCI–PSI complexes while the CPI band was assignated to the reaction center of PSI (Fig. 3a, b). The reaction center of PSII and its antennae corresponded to CP47 and CP43 bands, respectively. The $CPII^1$ and $CPII^2$ bands were related to oligomeric forms of antenna complexes, mainly to trimeric forms of LHCII, while the monomeric forms of CP complexes were assigned to CP^1, CP^2 (LHCII monomers) and CP^3 (Fig. 3a, b).

Significant differences in electrophoretic patterns were visible for thylakoids isolated from dark-chilled pea and tomato leaves (Fig. 3a, b). In pea thylakoids no significant changes of relative intensity of bands corresponding to CP complexes were noticed, whereas in tomato thylakoids a

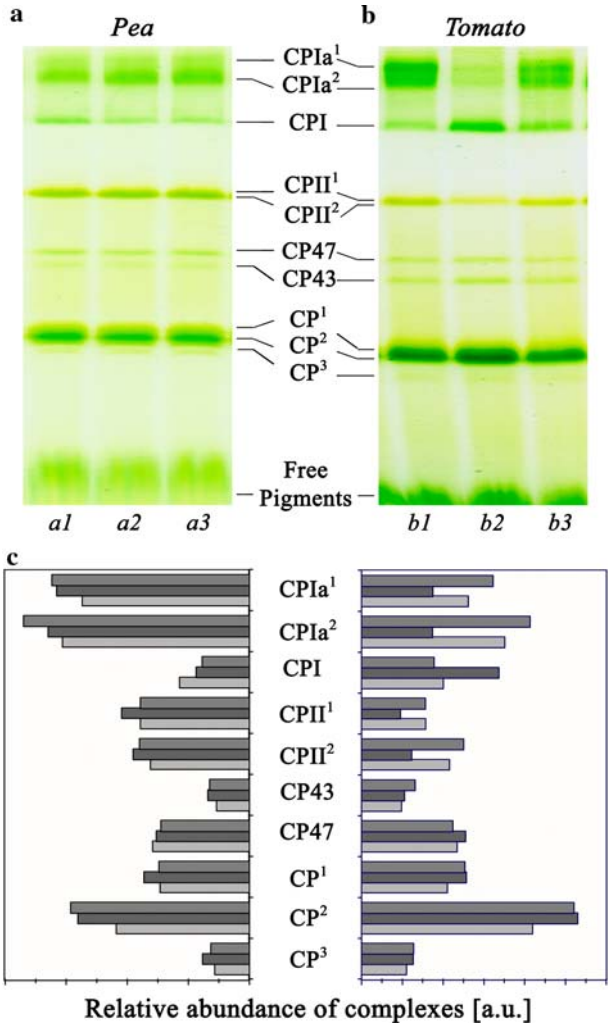

Fig. 3 Mild-denaturing green gel electrophoresis analysis of CP complexes in thylakoids. Native PAGE of thylakoids isolated from control (*a1*, *b1*), dark-chilled *a2*, *b2*), dark-chilled and subsequently photoactivated (*a3*, *b3*) pea (**a**) and tomato (**b**) leaves, respectively. **c** Relative intensities of CP bands quantified by scans analysed with Quantity One software (Bio Rad). *Gray*, *black* and *light-gray* bars correspond to the values for control (*a1*, *b1*), chilled (*a2*, *b2*) and photoactivated (*a3*, b3) samples, respectively. The electrophoretic patterns and their quantifications are representative for at last seven independent experiments

decrease in the intensity of bands related to undissociated LHCI–PSI (CPIa) and LHCII trimeric complexes (CPII) was observed. Mild-denaturing electrophoresis did not preserve the structure of CP supercomplexes completely, especially the LHCII–PSII organization (Allen and Staehelin 1991). Hence, the presented results illustrate the stability of distinct CP complexes, rather than its native organization in thylakoids. Quantitative analysis of mild-denaturing electrophoresis pattern of pea thylakoids isolated from dark-chilled leaves did not reveal significant changes in CP bands intensity (Fig. 3c) as well as in content in free chlorophyll (not shown).

On the contrary, in thylakoids isolated from dark-chilled tomato leaves, a considerable decrease of CPIa band intensities was associated with a simultaneous increase of intensity of CPI band (Fig. 3c), indicating a reduced stability of LHCI–PSI complexes in thylakoids isolated from chilled leaves compared with that observed in thylakoids isolated from control leaves. Furthermore, a decrease of intensity of both CPII bands, corresponding to LHCII trimers, by almost 40% and an increase of free Chl content by about 28% (not shown) in relation to the control sample were observed. It indicates an enhancement of the action of detergents (octyl glucoside and lithium dodecyl sulfate) on CP complexes in thylakoids isolated from chilled leaves. Electrophoretic patterns of thylakoid membranes isolated from photoactivated leaves of the two species (Fig. 3a, b), as well as contents of free Chl (not shown) were similar to those of the control samples, suggesting a restoration of membrane properties.

Distribution of Chl fluorescence inside chloroplasts revealed by confocal laser scanning microscopy (CLSM)

Fluorescence images from CLSM of intact chloroplasts isolated from pea and tomato leaves were obtained from several different focal depths, creating cross-section optical slices. The observed fluorescence is mainly attributed to chlorophyll emission of PSII (Em 680) and their antennae (LHCII), because the PSI fluorescence (Em 735) at ambient temperature is rather weak and the chloroplast suspension contains DCMU, an enhancer of PSII fluorescence (Mehta et al. 1999). In addition, the presence of DCMU and methylviologen (MV), an electron acceptor from PSI, did not noticeably change the CLSM images, despite the fact that MV specifically quenched the fluorescence at room temperature at around 735 nm (data not shown). Therefore, brightly-fluorescing regular shapes were predominantly attributed to grana stacks, consisting of LHCII–PSII supercomplexes (PSIIα) and trimeric LHCII (Danielsson et al. 2004; Dekker and Boekema 2005). However, since PSI fluorescence cannot be ruled out absolutely, the dim fluorescence signal may result from both PSII and PSI localized in intergranal regions (Mehta et al. 1999).

At low Mg^{2+} concentrations isolated chloroplasts do not exhibit the characteristic stacked thylakoids and PSII and PSI are homogeneously distributed within thylakoid membranes, whereas at higher cation concentrations the segregation of the photosystems and restacking of grana are observed (Kirchhoff et al. 2000, 2004 and references therein; Kaftan et al. 2002). Therefore, in order to confirm that the main source of Chl fluorescence within thylakoid regions comes from PSII/LHCII, a control experiment concerning the Mg^{2+}-effect on the distribution of Chl fluorescence in isolated intact pea chloroplasts was performed (Fig. 4). Under the experimental conditions applied, CLSM images showed that 8 mM $MgCl_2$ caused a condensation of Chl fluorescence in brightly fluorescent discs (Fig. 4b) compared with the normally used cation concentration (4 mM $MgCl_2$, 15 mM KCl; Fig. 4a). On the contrary, after depletion of cations from the incubation buffer a wide dispersion of Chl fluorescence within chloroplasts was observed (Fig. 4c).

In chloroplasts isolated from control pea leaves the majority of the bright fluorescence was located in round bodies in the range of 0.5 μm in diameter, uniformly distributed within chloroplasts (Fig. 5a1), corresponding to grana (cf. Fig. 4). Images of chloroplasts isolated from dark-chilled pea leaves revealed a greater heterogeneity (Fig. 5a2). The chloroplast image resembles that of chloroplasts incubated with 8 mM (cf. Fig. 4b), which suggests an increase of PSII fluorescence in stacked regions of thylakoids. Distribution of fluorescence in chloroplasts from photoactivated pea leaves (Fig. 5a3) was more homogenous compared to images from control and dark-chilled pea plants. Thus, incomplete restoration of parent thylakoid membrane structure is suggested.

Similarly to chloroplasts of control pea leaves, the structure of chloroplasts isolated from control tomato leaves was quite regular with brightly fluorescent grana in

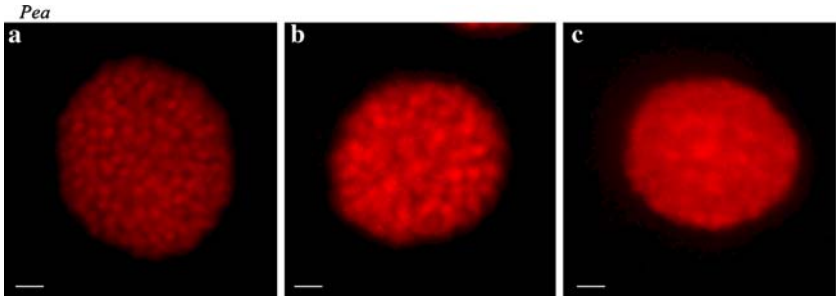

Fig. 4 Effect of different Mg^{2+} concentrations on Chl fluorescence analysed by CLSM in intact chloroplasts isolated from control (without chilling) pea leaves. Chloroplasts were incubated during 10 min. in 20 mM Hepes–NaOH (pH 7.5), 330 mM sorbitol, 6% (v/v) glycerol, 30 μM DCMU buffer comprising: **a** 15 mM NaCl, 4 mM $MgCl_2$ (standard conditions), in the presence of 8 mM $MgCl_2$, **c** without any cations. *Bar* 1 μm. Images are representative for three independent experiments

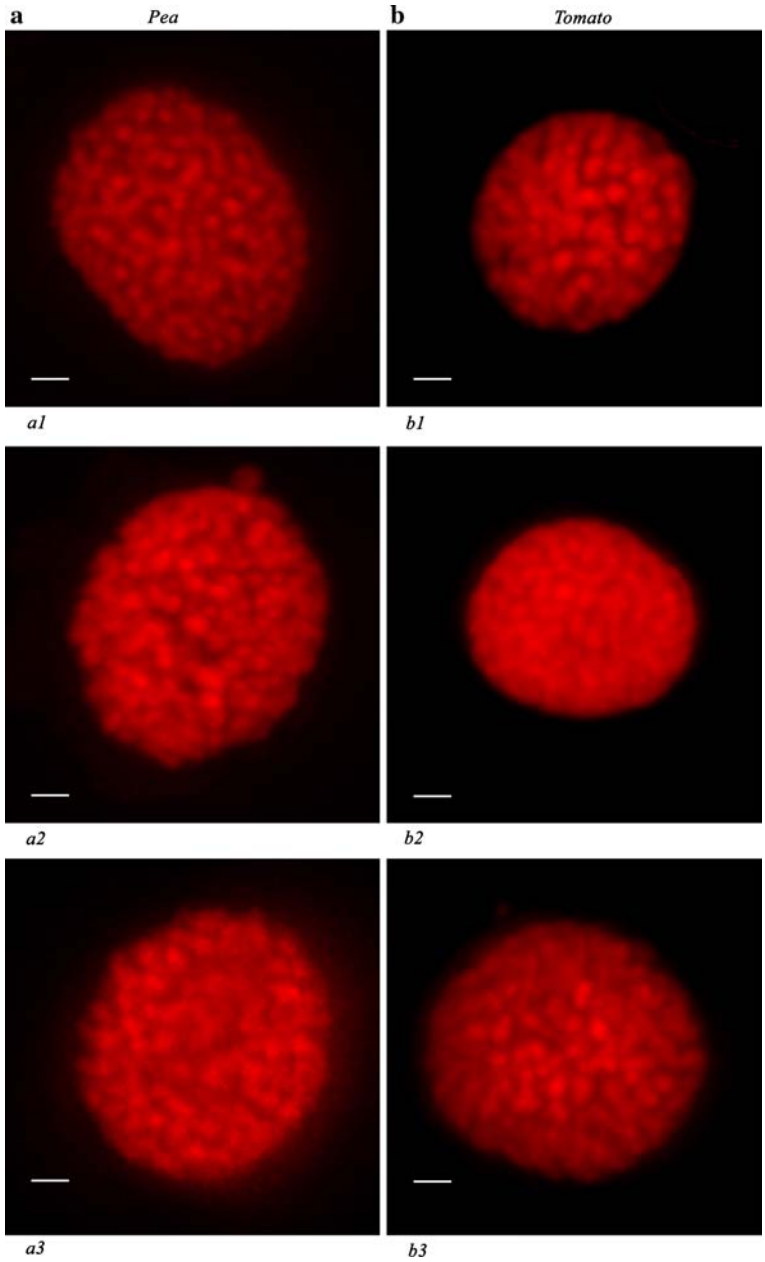

Fig. 5 Images of intact chloroplasts isolated from pea (**a**) and tomato (**b**) leaves analysed by CLSM. Chloroplasts were isolated from control (*a1*, *b1*), dark-chilled (5 days, 1°C; *a2*, *b2*) and chilled and subsequently photoactivated leaves (3 h, 22°C; *a3, b3*). After isolation procedure chloroplasts from all plants were resuspended in 20 mM Hepes–NaOH (pH 7.5), 330 mM sorbitol, 6% (v/v) glycerol, 30 μM DCMU buffer comprising 15 mM NaCl and 4 mM $MgCl_2$. *Bar* 1 μm. Images are representative for six independent experiments

the range of 0.5 μm in diameter (Fig. 5b1). However, in chloroplasts of chilled tomato leaves (Fig. 5b2), in contrast to pea thylakoids (Fig. 5a2), a great fluorescence homogeneity was observed, blurring the clear distinction between grana and stroma as apparent in pea (Fig. 5b2). The picture of a chloroplast from chilled tomato leaves was very similar to that one observed for a cation-depleted pea chloroplast (cf. Fig. 4c), what suggests thylakoid destacking and/or migration of PSII to stroma lamellae. Partial return to fluorescence distribution and therefore to parent arrangement of grana was observed in chloroplasts isolated from photoactivated tomato leaves (Fig. 5b3), although fluorescence distribution was not as regular as in control ones (Fig. 5b1).

Analysis of chloroplast structure by transmission electron microscopy (TEM)

The general view of chloroplasts revealed by TEM is different in pea and tomato leaves. There are more thylakoid membranes per chloroplast area in control, dark-chilled and photoactivated leaves of tomato than in pea (Fig. 6a, b). Although electronograms of control chloroplasts showed a different distribution of thylakoid membranes, more thylakoids in tomato and less in pea (Fig. 6a1, b1), the ratio of the total length of grana thylakoids to the total length of stroma thylakoids was similar in both pea and tomato chloroplasts and equal to 3.8 ± 0.4 and 3.6 ± 0.2, respectively (Fig. 6c, lanes a1, b1).

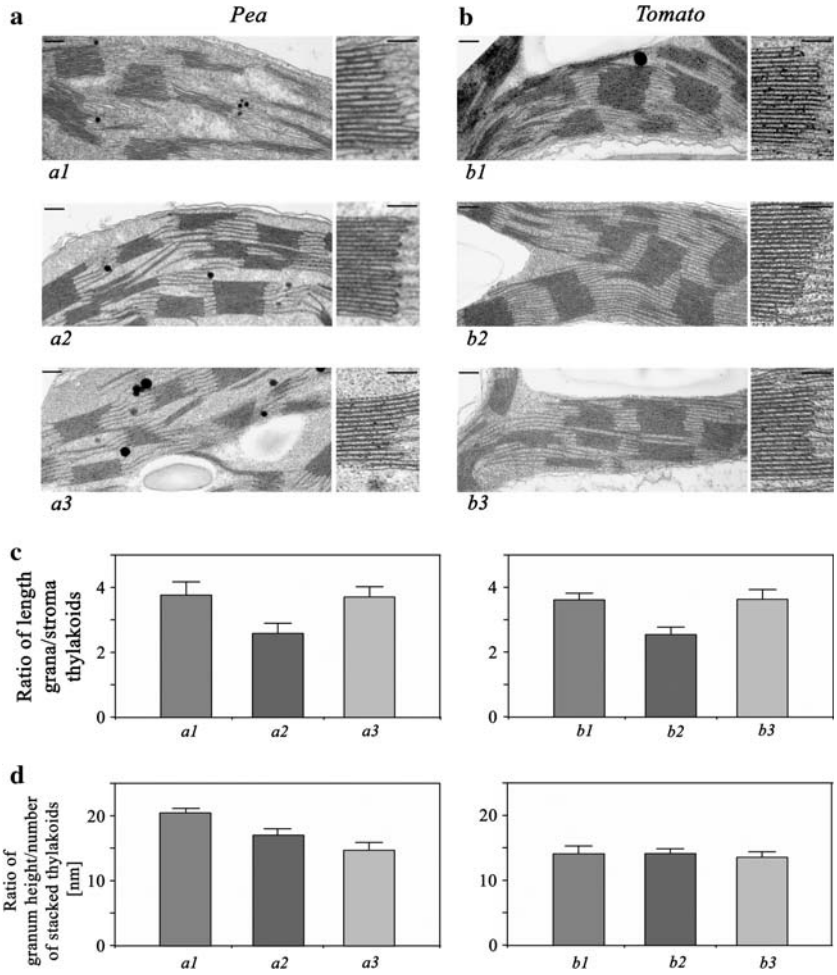

Fig. 6 Effect of dark-chilling and subsequent photoactivation on ultrastructure of chloroplasts in leaves of pea (**a**) and tomato (**b**). Electron micrographs of chloroplasts of control (*a1*, *b1*), dark-chilled (*a2*, *b2*), dark-chilled and subsequently photoactivated leaves (*a3*, *b3*). *Bar* 200 nm, and 100 nm (*insets*). **c** Average value of the ratio of the length of grana thylakoids to the length of stroma thylakoids. **d** Average value of the ratio of height of granum to the number of thylakoids in particular granum. *Gray*, *black* and *light-gray* bars indicate the values for control (lanes *a1*, *b1*), chilled (lanes *a2*, *b2*), and chilled and subsequently photoactivated (lanes *a3*, *b3*) leaves, respectively. The data are mean ± SD of 21 images from three independent experiments

In chloroplasts of control pea leaves, heights of particular grana stacks were large mainly due to relatively big thylakoid lumen (Fig. 6a1, inset), therefore the ratio of height of granum to the number of thylakoids in particular granum, corresponding to vertical distance from one pair of membranes to the next pair, was estimated to 20.5 ± 0.6 nm (Fig. 6d, lane a1). In chloroplasts of chilled pea leaves there were more stroma thylakoids, comparing to control ones (Fig. 6a2) and the ratio of total length of grana thylakoids to the total length of stroma thylakoids decreased to 2.6 ± 0.3 (Fig. 6c, lane a2). On the other hand, average vertical distance between thylakoid membranes in grana, estimated to 17 ± 1 nm, was lower compared with control (Fig. 6a2, inset, Fig. 6d, lane a2) due to either a smaller thylakoid lumen and/or a stronger appression of grana thylakoids. Chloroplasts of photoactivated pea leaves in principle resembled that of the control ones (Fig. 6a3). The ratio of the length of grana/stroma thylakoids returned to the control level (Fig. 6c, lane a3), but vertical distance between thylakoids in the grana (14.7 ± 1.1 nm, Fig. 6d, lane a3) indicated that membrane remained similarly appressed as in chloroplasts of chilled leaves.

In chloroplasts of chilled tomato leaves there were more stromal thylakoids than in control leaves (Fig. 6b1, b2) and the average ratio of length of grana/stroma thylakoids decreased from 3.6 ± 0.2 to 2.5 ± 0.2 (Fig. 6c, lanes b1, b2). Photoactivation of tomato leaves led to the reconstruction of parent proportion of grana/stroma thylakoid length (Fig. 6b3) estimated to 3.6 ± 0.3 (Fig. 6c, lane b3). This dark-chilling-induced and photoactivated-returned phenomenon observed in tomato chloroplasts resembles that in pea. However, in contrast to pea chloroplasts, the ratio of the vertical distance from one pair of membranes to the next pair in granum, estimated to 14.1 ± 1.2 nm in tomato control chloroplasts (Fig. 6d, lane b1), did not change significantly in chloroplasts from dark-chilled and subsequently photoactivated tomato leaves (Fig. 6d, lanes b2, b3).

Analysis of PSII functioning from Chl *a* fluorescence measurements

The effect of dark chilling and subsequent recovery on PSII functioning was tested in vivo by analyzing the kinetics of the Chl *a* O-J-D-I-P fluorescence transient. The parameters

related to photochemical rate, activity and organization of the PSII photosynthetic machinery of dark-adapted pea leaves was not changed much after 5 days of dark chilling but decreased slightly after 3 h and more of subsequent photoactivation (Table 2, Fig. 7). This decline was related to a 10–25% decrease of photochemical activity probably due to slight photoinhibition under prolonged photoactivation (Table 2). Conversely, 5 days of dark chilling in tomato caused a pronounced decrease in the amplitudes and time course of the induction curve (Fig. 7, Table 2). The decrease was related to a substantial decrease of rFv (and F_m) and a small but distinct increase of F_o (Fig. 7). During subsequent 3-h photoactivation of chilled tomato the photochemical activity decreased further down to 12% of the control (Table 2). A partial recovery of photochemical activity occurred concomitantly with a disproportional change in rates of excitation and quenching release during prolonged photoactivation. This indicated an increase in antenna size and degree of sigmoidicity of the initial fluorescence rise. A 24 h photoactivation period caused a 33% recovery of the chilling treatment under the present conditions in tomato.

Discussion

In contrast to CT plants a significant structural injury of chloroplasts has been observed under chilling stress in CS plants (Kratsch and Wise 2000). The underlying reasons of the different structural responses have been studied in the present investigation under both long-term dark-chilling and subsequent post-chilling photoactivation of detached leaves of CT pea and CS tomato. Complementary methods like steady-state fluorescence spectroscopy, mild-denaturing electrophoresis, CSLM, TEM and transient Chl *a* fluorometry have been applied in order to determine the relationship between stress-induced rearrangements of CP complexes and changes in thylakoid structure. The data support our hypothesis that dark-chilling induces different

Table 2 Values of theoretical parameters (rows 3–5) determining the onset kinetics of light-induced variable Chl *a* fluorescence and matching the O-J-D-I-P fluorescence rise in a pea and a tomato leaf in the 0.01–0.2 ms time range before (control), and after 0-, 3-, 9- and 24 h photoactivation period following a 1°C dark-chilling treatment of 5 days

Chl *a* fluorescence parameters										
	Pea					Tomato				
	Control	0 h	3 h	9 h	24 h	Control	0 h	3 h	9 h	24 h
L	4	4	5	5	4	3	10	2	4	5
kl	10	9	9	9	9	12	10	>100	45	26
rF_v^{STF}	2	1.9	1.6	1.6	1.6	1.8	0.6	0.2	0.5	0.6
Photochemical activity	100	89	80	75	77	100	33	12	27	33
Relative antenna size	1	1.1	1.2	1.2	1.1	1	3	0.6	1.4	1.9
Degree sigmoidicity (*p*)	1.5	1.7	2	2	1.7	1	4	0	0.3	0.7

Parameters *k*L and *k*1 refer to rate constants (in ms^{-1}) of light excitation and release of fluorescence quenching, respectively and rF_v^{STF} to the relative variable fluorescence induced by a saturating single turnover excitation. The degree of sigmoidicity *p* has been approximated by estimating for each treatment (column) the closest fit of the theoretical $F_v(t)$ (= $[F(t)-F_o]/rF_v^{STF}$) versus the fraction (S) of RCs with reduced acceptor with the hyperbolic relation $F_v = S/[1 + p\ (1-S)]$. Parameter values are accurate within approx. 5%

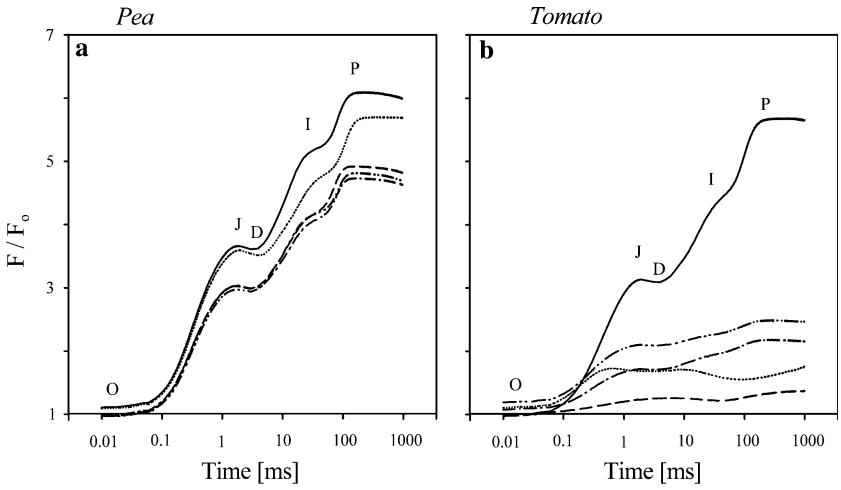

Fig. 7 Typical Chl *a* fluorescence transient (O-J-D-I-P) in control (*solid line*), dark-chilled (*dotted line*), dark-chilled and subsequent 3 h (*dashed line*), 9 h (*dashed-dotted* line), 24-h (*dashed-double dotted line*) photoactivated detached leaves of pea (**a**) and tomato (**b**). Chl *a* fluorescence (*F*) values were normalized to the lowest measured initial fluorescence level ($F_o = 1$)

arrangements of CP complexes in thylakoid membranes of CT pea versus CS tomato chloroplasts.

As demonstrated earlier (Garstka et al. 2005), long-term chilling of the two species does not result in a significant decrease in leaf Chl content and Chl *a*/*b* ratio in thylakoids, indicating no degradation of CP complexes. Therefore, the dark-chilling-induced decline and photoactivation-related restoration of overall Chl *a* fluorescence in tomato might be explained as reversible changes in the oligomeric structure rather than degradation of CP complexes.

An increase in the relative contribution of LHCII and PSII versus decrease in PSI to the fluorescence spectrum of thylakoids isolated from chilled pea leaves (Fig. 1a, b) suggests a rearrangement of these complexes. The PSII complex in grana thylakoids is organized as a dimeric supercomplex binding various numbers of LHCII and building the photosyntetic unit PIIα (Dekker and Boekema 2005). Since the PIIα complexes are excitonically connected (Kirchhoff et al. 2004b), an enhancement of PSII fluorescence may be associated with an increase in connectivity between PSIIα in grana thylakoids. Moreover, as PSI is located in grana margins and stroma lamellae (Danielsson et al. 2004), a decrease in fluorescence emission from PSI (Fig. 1a, b) as well as a decline of energy transfer from the antennae complexes to PSI reaction center (Fig. 2d) indicate rearrangements of LHCI–PSI complexes in this region of thylakoid membranes. The increase of PIIα with the simultaneous decrease of PSI fluorescence might also indicate slight energy spillover from PSI to PSII (Jajoo et al. 1998; Pesaresi et al. 2002).

On the contrary, in thylakoids isolated from chilled tomato leaves a decrease in relative contribution of LHCII and PSII to the fluorescence spectrum versus increase of LHCI band intensity (Fig. 1c, d) suggests the separation and a decrease in connectivity between PSIIα units in grana thylakoids. Like under the ion-dependent unstacking of thylakoids (Jajoo et al. 1998), a decrease in Chl fluorescence might be associated with the migration of PSII and LHCII from grana discs to stroma lamellae and intermixing with other complexes, PSI and ATPase (Kirchhoff et al. 2004b). Thus, a relative enhancement of LHCI-related bands in the fluorescence emission spectrum (Fig. 1d), as well as an increase in the Chl *b*-related peak in the excitation spectrum of PSI (Fig. 2h) indicate significant changes in arrangements of CP complexes in stroma lamellae. Moreover, upon dark-chilling treatment of tomato leaves, the PSI/PSII ratio in thylakoids increases 1.8-fold suggesting that PSI seems to be less sensitive to a low-temperature treatment compared to PSII. The increase of the PSI/PSII ratio (Fig. 1c) with a simultaneous decrease of the Chl *b*-related peak in the excitation spectrum of PSII (Fig. 2f) may indicate the increase of energy transfer from the mobile pool of LHCII to PSI (Jajoo et al. 1998; Pesaresi et al. 2002). In addition, the substantial decrease of overall Chl fluorescence in tomato thylakoids caused by dark-chilling (Table 1b), suggests that under these conditions energy spillover is less important than destabilization of CP complexes.

In contrast to pea (Figs. 1b, 2d), tomato leaf photoactivation induces a reconstruction of control arrangements of CP complexes in its thylakoids (Figs. 1d, 2f, h). The three-times higher changes in the relative contribution of individual CP complexes to fluorescence spectra in tomato as compared to pea thylakoids is remarkable. It seems likely that the storage of detached leaves of CS tomato at 1°C in the dark causes alterations in organization of photosynthetic complexes which are more significant than in CT pea.

Different effects of dark-chilling on properties of pea and tomato thylakoids were found by mild-denaturing electrophoresis (Fig. 3), representing the fingerprint of protein–protein interactions within membranes (Allen and Staehelin 1991). The absence of changes in pea electrophoretic pattern indicates the stabilization of CP complexes under the dark-chilling stress. Slight increase of PSII fluorescence in thylakoids from dark-chilled leaves (Fig. 1a, b) might be related to interaction between multicomplexes; PSIIα connectivity and/or energy spillover (Kirchhoff et al. 2004b). On the contrary, a diminished stability of LHCII and LHCI–PSI complexes in tomato thylakoids might suggest a dark-chilling induced loosening of protein interactions in grana and stroma lamellae. These changes might lead to a decrease in connectivity between photosynthetic units (Kirchhoff et al. 2004b) resulting in a significant decrease in overall Chl fluorescence (Table 1b). Although the effects of dark-chilling on membrane properties of pea and tomato are different, photoactivation led to restoration of control electrophoretic patterns in thylakoids of two species studied (Fig. 3a, b), suggesting a return to parent protein interactions in membranes.

CLSM images of intact pea chloroplasts incubated with 8 mM $MgCl_2$ compared with those incubated without cations confirmed that the main source of bright fluorescence comes from grana stacks (Fig. 4b, c), i.e. from PSIIα and LHCII trimers (Kirchhoff et al. 2004b), while dim fluorescence might come from both PSII and PSI in intergranal regions (Mehta et al. 1999). In dark-chilled pea chloroplasts (Fig. 5a2), similarly to chloroplasts incubated with 8 mM (cf. Fig. 4b), pronounced fluorescence heterogeneity was observed and the density of brightly fluorescent bodies indicated an increase of PSII fluorescence in stacked regions of thylakoids. On the contrary, in chloroplast obtained from dark-chilled tomato leaves fluorescence homogeneity was observed and the dispersion of fluorescence, similar to that observed for cation-depleted pea chloroplasts (cf. Fig. 4b), suggests thylakoid destacking and/or migration of PSII to stroma lamellae (Fig. 5b2; Kirchhoff

et al. 2004b). The fluorescence image of chloroplasts isolated from photoactivated pea leaves (Fig. 5a3) is more homogenous compared to that of dark-chilled plants (Fig. 5a2). However, compared with thylakoids from dark-chilled pea leaves, the fluorescence spectra indicate that an arrangement of CP complexes inside membranes does not change after photoactivation (Figs. 1b, 2d). Thus, as revealed by CLSM, the restoration of fluorescence pattern in tomato chloroplast following leaf photoactivation (Fig. 5b3), is much more evident than in pea chloroplasts and is in agreement with fluorescence data (Figs. 1d, 2f, h), suggesting a restoration of PSII fluorescence in grana thylakoids (Kirchhoff et al. 2004b).

In view of changes in the membrane structure shown by TEM, it seems likely that the dark-chilling of pea leaves induces an increase in the degree of thylakoids stacking (Fig. 6d, lane a2) resulting in the enhancement of relative PSII fluorescence in thylakoids (Figs. 1b, 5a2; Kirchhoff et al. 2004b; Dekker and Boekema 2005). An increase in stroma thylakoid length in chloroplasts of dark-chilled tomato leaves (Fig. 6c, lane b2) seems to be correlated with a dispersion of PSII fluorescence revealed by CLSM (Fig. 5b2) and with a decrease of PSII (Fig. 1d) and with overall Chl fluorescence (Table 1b). According to Albertsson et al. (2004) the relative fraction of stroma thylakoids in different plant species is constant and amount to about 20%. This is consistent with our results concerning control and photoactivated chloroplasts (Fig. 6c, lanes a1, b1, a3, b3), but not chloroplasts from dark-chilled leaves (Fig. 6c, lanes a2, b2) of both species. Although, the ratio of the length of grana to stroma thylakoid from dark-chilled leaves is similar in both species, the low-temperature stress caused the contrasting arrangements of CP complexes (Figs. 1b, d; 5a2, b2). The changes in the length of stroma lamellae seems to be independent of the degree of thylakoid stacking in the two species (Fig. 6c vs. d). These data may be related to those obtained by the use of atomic force microscopy (Kaftan et al. 2002) indicating that due to Mg^{2+}-depletion the unstacking of thylakoids starts at the stromal-exposed edges of the appressed grana domains and leads to their merging with the stroma lamellae. Grana diameter may increase due to the unstacking process, however, the grana height remains relatively constant (Kaftan et al. 2002). Thus, merging of the edges of grana membranes to stroma lamellae and vice versa could proceed independently of vertical interactions between membranes in central parts of grana domains, as observed in dark-chilled pea and tomato leaves (Fig. 6d).

Dark chilling and subsequent photoactivation of CT pea thylakoids resulted in slight irreversible changes in 120 K fluorescence (Figs. 1b, 2d) and increase of stacking (Fig. 6d). The nearby invariability of kinetic fluorescence parameters in vivo, except for photochemical activity and degree of sigmoidicity, suggests that chilling in pea leaves mainly, if not exclusively, affects the antenna size of PSII RCs to some extent (Table 2, Fig. 7a). Apparently, the dark-chilling effect in this CT plant is limited to a structural modification of thylakoids. A quite different effect is observed in CS tomato chloroplasts. In this species in vivo major changes in fluorescence kinetics and yield suggest a substantial decrease of PSII photochemistry and alterations in structural organization under dark-chilling conditions (Fig. 7b, Table 2). This is in qualitative agreement with the morphological observations in isolated thylakoids (cf. Figs. 1d, 5b2). Moreover, a slow and incomplete recovery of PSII photochemistry is observed in association with restoration of thylakoid fluorescence (Fig. 1d) and chloroplast structure (cf. Figs. 5b3, 6b3). The increase of F_o (Fig. 7b) may be related to disconnection of PSII RC from the peripheral antenna complexes (Zhu et al. 2005) and is in agreement with our fluorescence (Fig. 1d) and structural data (Fig. 5b2).

The present study indicates that 3 h of photoactivation of dark-chilled tomato leaves was enough for at least partial reconstruction of the thylakoid membrane organization (Figs. 1, 2, 3) and chloroplast structure (Fig. 5b3, 6b3), while it was too short to recover PSII photochemistry (Fig. 7b). These data are in agreement with those reported for CS bean leaves (Garstka et al. 2005), suggesting that a long-term dark-chilling treatment and application of moderate level of irradiance during first time of photoactivation render leaves of CS plants susceptible to photoinhibition, probably similar to the chilling-induced damages of oxidizing and acceptor side of PSII observed in CS tomato, maize and cucumber leaves (Govindachary et al. 2004). Prolonged photoactivation of tomato leaves leads to 33% recovery of PSII photochemistry and an altered structural organization (Fig. 7b, Table 2). The latter is evident from the increased antenna size and the persistent change in degree of sigmoidicity after 24 h photoactivation. There is a priori no explanation for the incomplete restoration of decreased photochemistry and altered structural organizations upon chilling after a 24 h recovery period in the CS plant (Table 2, Fig. 7b). However, one should keep in mind that the data of Table 2 and Fig. 7 originate from in vivo measurements and are not hampered by invasive treatments like chloroplast isolation and—preparation necessary for the photometric and structural studies (Table 1, Figs. 1, 2, 3, 4, 5, 6). The recovery of chilling effects in the intact leaf (cell) may be retarded due to temporary limitations of some substrates or cofactors that probably are released in connection with the isolation and preparatory procedures of the chloroplast samples.

The fluorescence (Figs. 1, 2) and CLSM data (Fig. 5) suggest that the pattern of dark-chilling-induced changes in chloroplasts of CT pea and CS tomato resembles the pattern

described for cation-dependent stacking versus unstacking phenomena (Fig. 4; Kim et al. 2005). However, it is possible, that changes in chloroplast structure observed under low-temperature treatment and subsequent photoactivation are more complex that those observed under ion-dependent treatments. In addition, dark-chilling-induced dispersion of Chl fluorescence in CS bean chloroplast detected by CLSM (Garstka et al. 2005) is smaller than in tomato chloroplast (Fig. 5b2). The difference may be related to partial aggregation of LHCII, which takes place in bean (Garstka et al. 2005) but not in tomato thylakoids (Fig. 1d). These results demonstrate some differences in response to low-temperature treatment of the two CS plant (tomato and bean).

The level of unsaturated molecular species of phosphatidylglycerol (PG), affecting the phase separation of membrane lipids, is positively correlated with chilling tolerance in many wild plant species and transgenic lines of tobacco and *Arabidopsis* (Nishida and Murata 1996). On the other hand, overall lipid fluidity decreases when the chilling-sensitivity increases (Gang et al. 1990), suggesting a higher probability of low-temperature-induced membrane rigidification in thylakoids of CS than of CT plants (Los and Murata 2004). Recently an involvement of boundary lipids in relatively weak interactions leading to supercomplex associations, in particular among mobile LHCII trimers and LHCII–PSII has been proposed (Kirchhoff et al. 2002). Thus, the opposite response to dark-chilling in thylakoids of CT and CS plants may be correlated with qualitatively/quantitative differences in overall and boundary lipids. Moreover, in thylakoids of CS plants the dark chilling-induced accumulation of FFA and a partial depletion of galactolipids (Gemel and Kaniuga 1987; Garstka and Kaniuga 1991; Kaniuga 1997) may additionally disorder the lipid phase, causing the dissociation of supercomplexes.

Alternations in organization of CP complexes, especially LHCII observed till now include investigations on (1) cold-acclimation of freezing-tolerant evergreen plants and winter cereals (Ensminger et al. 2006) as well as on (2) low-temperature growth of CS plants (Caffarri et al. 2005; Yang et al. 2005), both due to cold-induced changes in expression/accumulation of individual proteins. Our earlier data (Garstka et al. 2005) and present investigations concern the direct, reversible effect of low temperature on lipid–protein and protein–protein interactions affecting organization of thylakoid supercomplexes. The different changes in chloroplast ultrastructure of CT versus CS plants may be a direct consequence of contrasting rearrangements of CP complexes, suggesting a multilateral adaptive process to chilling conditions.

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