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Structural stability and properties of three isoforms of the major light-harvesting chlorophyll a/b complexes of photosystem II

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ABSTRACT

Three isoforms of the major light-harvesting chlorophyll (Chl) a/b complexs of photosystem II (LHCIIb) in the pea, namely, Lhcb1, Lhcb2, and Lhcb3, were obtained by overexpression of apoprotein in *Escherichia coli* and by successfully refolding these isoforms with thylakoid pigments in vitro. The sequences of the protein, pigment stoichiometries, spectroscopic characteristics, thermo- and photostabilities of different isoforms were analysed. Comparison of their spectroscopic properties and structural stabilities revealed that Lhcb3 differed strongly from Lhcb1 and Lhcb2 in both respects. It showed the lowest Qy transition energy, with its reddest absorption about 2 nm red-shifted, and the highest photostability under strong illuminations. Among the three isoforms, Lhcb 2 showed lowest thermal stability regarding energy transfer from Chl b to Chl a in the complexes, which implies that the main function of Lhcb 2 under high temperature stress is not the energy transfer.

1. Introduction

The major light-harvesting chlorophyll (Chl) a/b binding complex of photosystem II (PS) (LHCIIb) is the most abundant pigment–protein complex on earth. It constitutes more than 40% of the photosynthetic membrane protein and about half of the total thylakoid pigments [1]. High-resolution structural analysis of LHCIIb reveals that the complex contains three *trans*-membrane α-helixes, one amphipathic α-helix and one 3_{10}-helix on the lumenal side. It combines, as cofactors, 14 Chls (8 Chl a and 6 Chl b), two luteins (Lut), one neoxanthin (Neo) and one violaxanthin (Vio) per monomer [2,3].

In oxygenic photosynthesis, LHCIIb fulfils a variety of functions in harvesting solar energy, photoprotection, maintaining grana stacking and the lateral segregation of the complexes in a thylakoid membrane, and adjusting the excitation energy distribution between PSII and PSI [1,3,4]. LHCIIb is not only important for harvesting light energy, it is also involved in the response of plants to changes of environmental conditions, particularly under high temperatures and strong illumination [5,6]. The basic functional units of LHCIIb are trimers, consisting of various combinations of three isoforms: Lhcb1, Lhcb2 and Lhcb3. Progress in understanding the functions of different isoforms of LHCIIb has been achieved by observing an antigenic *Arabidopsis thaliana* plant in which the transcription of Lhcb2 genes was silenced (asLhcb2), which, in turn, eliminated both Lhcb1 and 2 [7,8]. A normally minor and monomeric LHCII (Lhcb5) took the position of Lhcb1 and 2, formed trimers and maintained most of the functions of Lhcb1 and Lhcb2. However, the Lhcb5-trimers lack the capacity to be phosphorylated, which results in their reduced adaptation ability under excessive light conditions in asLhcb2 [7,9]. It is Lhcb1 and Lhcb2 that are important in light harvesting, state transition and non-photochemical quenching [7,9].

LHCIIb, as the major energy-capturing apparatus for photosynthesis, is organized into chiral macrodomains in the thylakoid membrane, which facilitates an effective long-range energy migration to photosystem II [10]. Although the Chls are packed densely in LHCIIb, the photostability of Chls regarding photobleaching under strong illumination is much higher than that of free pigment because of the functional architecture of LHCIIb [11]. Under natural conditions, LHCIIb is confronted with continuously changing environments and often faces strong light and high temperature stresses. The long-range chiral structure undergoes flexible light-induced structural rearrangements

Abbreviations: Car, carotenoid; CD, circular dichroism; Chl, chlorophyll; DM, n-dodecyl-β-D-maltoside; LHCIIb, the major light-harvesting Chl a/b complexes of photosystem II; Lhcb1, Lhcb2 and Lhcb3, different subunits of LHCIIb; *Lhcb1, Lhcb2, and Lhcb3*, genes of the respective subunits of LHCIIb; HPLC, high-performance liquid chromatography; PS, photosystem; Lut, lutein; Neo, neoxanthin; Vio, violaxanthin; T_m, apparent dissociation temperature; T_{mf}, apparent dissociation temperature regarding energy transfer from Chl b to Chl a

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which might be related to the adaptation of plants to excessive light stresses [12–14]. High temperature is another external factor influencing the structure of LHCIIb macro-domains, which is correlated with different light intensities [15]. It has been demonstrated that trimer-to-monomer transition under high light intensity and temperature plays important roles in adjusting the chiral structure of LHCIIb [16] or breakdown of the protein [17]. Since LHCIIb is a membrane-inserted pigment–protein complex, the stability of these complexes is dependent on the protein density and lipid composition in the thylakoid membrane [18]. Although there are many observations dealing with the LHCIIb structural stability at the level of macro-organizations [19,20], trimeric structures[21], little is known about the thermal stability or photostability characteristics of different isoforms of LHCIIb, which might be important for understanding the effect of different monomers in performing the functions of LHCIIb.

Due to the difficulties in purifying these highly homologous membrane proteins, all the experiments conducted to investigate LHCIIb thermal stability were focused on the isolated LHCIIb, which was a mixture of Lhcb1, Lhcb2 and Lhcb3 [21–23]. It has been demonstrated that, via detailed spectroscopic analysis (linear dichroism spectra, circular dichroism (CD) spectra, the triplet-minus-singlet spectra) and biochemical analysis (thermolysin digestion and polyacrylamide gel electrophoresis (PAGE)), the properties of recombinant LHCIIb (including orientation of pigments, excitonic interactions between chromophores, organization and folding of the complexes and the energy transfer) are all similar to those of native LHCIIb [24,25]. In view of these observations, recombinant LHCIIb provides a unique opportunity for studying the characteristics of each isoform of LHCIIb in an in vitro environment. Standfuss and Kühlbrandt [26] and Caffarri et al. [27] successfully reconstituted the three isoforms of LHCIIb of *Arabidopsis* and of barley, respectively, in vitro. They analysed the functional roles based on the spectroscopic characteristics and trimer formation properties. In this paper, we have extracted three genes coding Lhcb1, Lhcb2 and Lhcb3 respectively from the pea (*Pisum sativum* L.), and analysed sequence alignments of the three different isoforms in three species (*Arabidopsis*, barley, and pea). Furthermore, we have obtained the three isoforms, Lhcb1–3 by reconstituting apoproteins overexpressed in *E. coli* with pigments extracted from pea, and analysed their spectroscopic properties and structural stabilities under elevated temperature and strong illumination. Based on these findings, the possible physiological functions of the three different isoforms of LHCIIb are discussed in this paper.

2. Materials and methods

2.1. Preparation of LHCIIb

Total RNA from 14-day-old pea (*P. sativum* L.) seedlings were used to synthesise cDNA by using random primers and a RT-PCR kit (Invitrogen, Carlsbad, CA, USA). Different Lhcb genes were amplified from 1 μL of cDNA in a 20 μl PCR reaction containing 0.2 μmol L^{-1} each of the forward and reverse primers for different Lhcb isoforms and 0.5 μL (200 U μL^{-1}) and Taq DNA polymerase (Promega, USA). The forward and reverse primers for amplifying *Lhcb1-3* genes were as follows: 5′-C(A/G)ATGGC(T/C)GCTTCATC(A/C)ATGGC-3′ (*Lhcb1* forward primer), 5′-CC(C/T)CTTCACAAAAACACAATACGAC-3′ (*Lhcb1* reverse primer); 5′-CATGGCCACMTCWGCTATCC-3′ (*Lhcb2* forward primer), 5′-CATTCTCAYTKTCCRGGGAC-3′ (*Lhcb2* reverse primer); and 5′-CATGGC ATTG-(G/A)TG(T/G)CAGCTAC-3′ (*Lhcb3* sense primer), 5′-C(T/A)(G/A)ATGTTTA(C/A)GCACCAGGCAC-3′ (*Lhcb3* reverse primer). The PCR was run 40 cycles with 30 s at 94 °C, 30 s at 60 °C, and 120 s at 72 °C, followed by a final extension step at 72 °C for 10 min. The PCR product was cloned into the pMD-18T vector (Takara, Shiga, Japan) and transformed into *E. coli* strain DH5α. The sequences of the genes were checked by sequencing and aligned with pea LHCII genes in the gene bank, and the homologies were found to be between 95% and 98%. The accession numbers for the three genes in the gene bank are AY845253, AY845254, and AY845255 for *Lhcb1-3*, respectively.

The restriction sites (NdeI and XhoI) were introduced in the 5′ and 3′ termini of the three *Lhcb* genes via PCR with the primers for the three different genes, respectively. The primers were as follows: 5′-ACATATGAGGAAGTCTGCTACCAC-3′ (*Lhcb1* forward primer), 5′-ACTCGAGATTTCCTGGAAGGAAGTTGG-3′ (*Lhcb1* reverse primer), 5′-CATATGCGTCGTACTGTGAAGAG-3′ (*Lhcb2* forward primer), 5′-CTCGAGTTGTCCAGGGTAAAAGTT-3′ (*Lchb2* reverse primer), 5′-CATATGGGAAATGATTTGTGGTATGG-3′ (*Lhcb3* forward primer), 5′-CTCGAGCGCACCAGGCACAAAC-3′ (*Lhcb3* reverse primer). The NdeI–XhoI fragments containing *Lhcb* genes were inserted into the pET-23(a) expression vector (Novagen, USA) and transferred into *E. coli* strain BL21. By introducing restriction site XhoI, an additional sequence CTCGAG was added to the 3′ end of the three genes, which provided additional Leu and Glu residues to the expressed polypeptides. All the genes were checked by sequencing and aligned with the *Lhcb* sequences in gene-bank.

The apoproteins of different Lhcb species were overexpressed and isolated with the method described in [28], and then reconstituted with thylakoid pigments, isolated from pea, with the method developed by Paulsen et al [28]. The reconstituted pigment–protein complexes were loaded onto a sucrose density gradient (10% to 40% sucrose, 0.1% *n*-dodecyl-β-D-maltoside (DM), and 5 mmol L^{-1} phosphate buffer (pH7.5)), and centrifuged at 230,000 ×*g* at 4 °C for 18 h (SW-40 rotor, Beckman, Palo Alto, CA). After the ultracentrifugation, the bands corresponding to monomeric LHCIIb were collected, checked with partially denaturing PAGE as described in [29], and used for further analyses.

2.2. Pigment quantification

The pigment stoichiometries were analysed for the monomeric Lhcbs with a high-performance liquid chromatography (HPLC) (Waters 600, USA). Pigments were extracted from the monomeric Lhcb with 2-butanol according to the method described in [30]. The 2-butanol extraction was applied to a C_{18} reversed-phase column (250 mm×4.6 mm, 5 μm particle size, Non-Encapped Zorbax ODS) (Agilent, USA), and pigments were separated according to the method described in [31] and quantified by comparing integrated peak areas with calibrated ones of known pigment standards.

For quick Chl quantification, the optical density of Chls dissolved in 80% acetone were measured with a Shimadzu UV-VIS 2550 spectrophotometer (Shimadzu, Kyoto, Japan) and the concentrations were calculated according to the method described in Porra et al [32].

2.3. Absorption spectra

Absorption spectra were recorded using a Shimadzu UV-VIS 2550 spectrophotometer at room temperature. The wavelength step was 0.5 nm, the scan rate was 100 nm/min and the optical pathlength was 0.5 cm.

2.4. Circular dichroism spectra and temperature treatment

CD spectra were collected on a Jasco-810 spectropolarimeter (Jasco, Japan). The measurements were carried out using quartz cuvettes with a pathlength of 0.1 cm. The spectra were measured from 400 to 750 nm at the scan rate 100 nm min^{-1}.

Changes of the CD spectra in the visible range (400–750 nm) and CD signal at 492 nm during a temperature gradient were employed to evaluate the thermal stability of the different isoforms from the viewpoint of stability of complex conformation. The change of CD signal at 492 nm, measured at time-intervals of 0.2 min, and change of the CD spectra in the visible range (400–750 nm), measured at data-intervals of 5 °C were monitored on the Jasco-810 spectropolarimeter equipped with a temperature-control unit. Temperature scans were from 20 °C to 90 °C at a rate of 1 °C min^{-1}. Change of the amplitude of the CD signal at 492 nm was then fitted with a sigmoid curve ($Y=a/(1+\exp(-(X-b)/c))$) based on a Marquardt algorithm procedure. The quality of the fits was assessed using residual R^2 criteria set to 0.95 and plots of residuals. The inflection point (b) was taken as the dissociation temperature (Tm). Since the measurement was done at a continuously changing temperature, no attempt was made to reach equilibrium at individual temperatures; hence the dissociation temperatures measured here must be regarded as apparent dissociation temperatures.

2.5. Fluorescence emission spectra and temperature treatment

The fluorescence emission spectra were measured with a Hitachi F-4500 spectrofluorimeter (Hitachi, Japan) at 4 °C. The samples were diluted to 10 μg Chl mL^{-1} with a pre-cooled dilution buffer (5 mmol L^{-1} phosphate dilution buffer (pH 7.5), 0.1% DM, 12.5% sucrose). The fluorescence emission spectra were measured from 600 to 750 nm, with the excitation wavelength set to 480 nm, and slit width set to 5 nm for both excitation and emission.

In order to compare the thermal stabilities of different isoforms of monomeric Lhcbs from the viewpoint of energy transfer in the complexes, changes of fluorescence emission spectra of the complexes upon excitation at 480 nm during a temperature gradient and the decrease of energy transfer from complex-bound Chl b to Chl a upon gradual dissociation of the complexes at 37 °C were observed.

To measure the changes of the fluorescence emission spectra during progressive raising temperature process, the reconstituted monomeric Lhcb1–3 after the ultracentrifugation were heated in a temperature treatment profile from 20 to 70 °C with temperature increment rate at 1 °C min^{-1}. Fluorescence spectra were measured at every 5 °C interval. After the temperature treatment, the samples were diluted and the fluorescence emission spectra were measured with the same method as mentioned above.

The decay of Chl a fluorescence emission upon excitation at 480 nm was monitored continuously in the spectrofluorimeter thermostated to 37 °C according to the method described in Yang et al [27]. Measurements were taken for 15 min at 1-s intervals. The course of fluorescence decay was fitted into two-exponential kinetics ($Y=Y_0+A_1\cdot\exp$

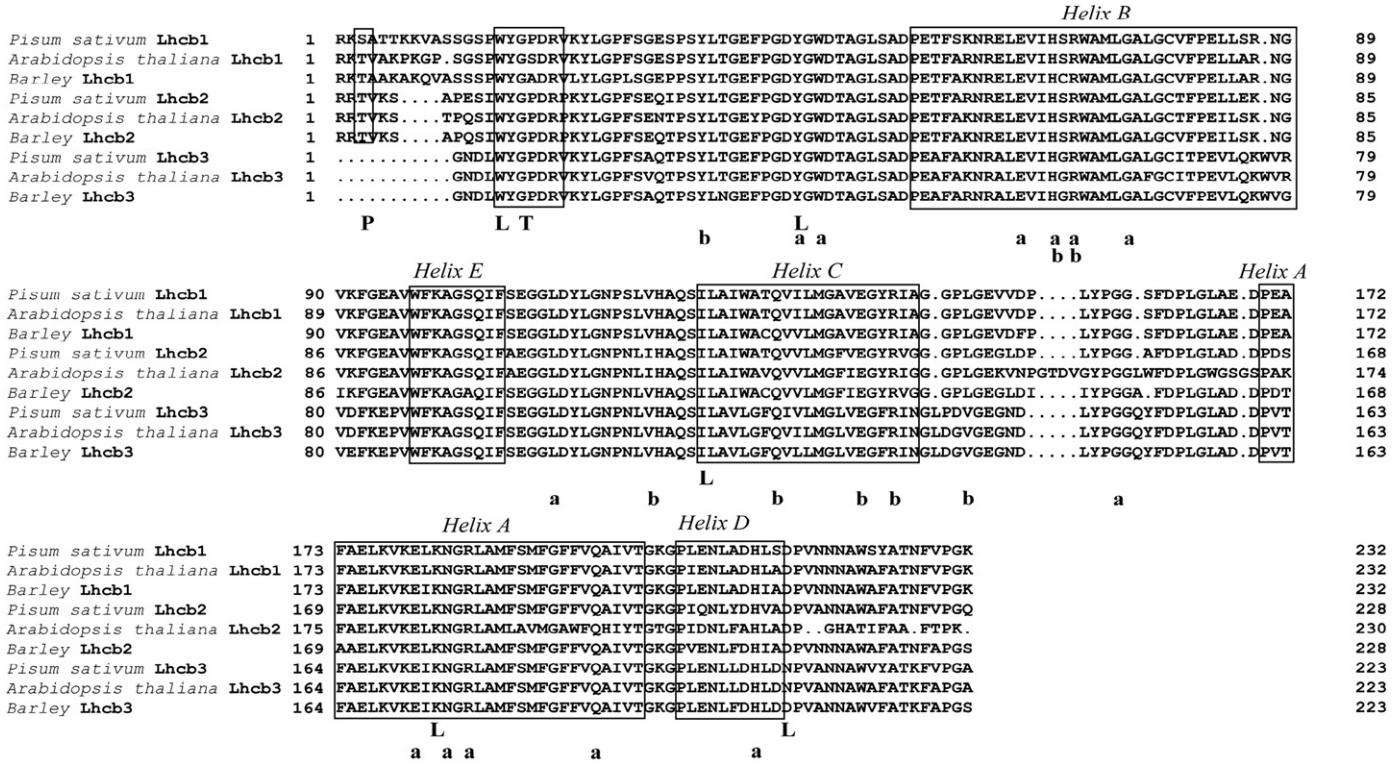

Fig. 1. Alignment of primary sequences of Lhcb1, 2 and 3 from *Pisum sativum* L., *Arabidopsis thaliana* [24] and barley [25]. Different features are indicated: black rectangles, α-helix; P, phosphorylation site; T, trimerisation motifs; L, lipid binding sites; a, Chl a binding sites; b, Chl b binding sites.

$(-k_1 X)+A_2 \cdot \exp(-k_2 \cdot X)$). The lifetime constants (τ_1 and τ_2) of the complexes were calculated on the basis of the rate constant ks ($\tau=(\ln 2)/k$). The quality of the fitting was evaluated using residual R^2 criteria. The R^2 criterion was set to 0.95 for a good fitting.

2.6. Photobleaching

The concentration of the samples was adjusted with a dilution buffer so that the absorption at the maximum of Chl a in the Q_y region was 0.75. The complexes were then illuminated with white light (4000 μmol m^{-2} s^{-1}) under continuous stirring. After each time interval, the cuvette was removed from the light source, and the absorption spectrum was measured with a Shimadzu UV-VIS 2550 spectrophotometer in the range of 600–750 nm [11]. The whole pigment absorption was measured upon integration of the absorption spectra in the Qy region.

3. Results

3.1. Sequence alignment of Lhcb1–3

Fig. 1 shows the sequence alignment of different Lhcbs and a comparison of the same isoforms in the different species [26,27]. The amino acid sequences are highly conservative in different species, with more than 75% similarity for the same isoform. When comparing the sequences of the pea Lhcb with those of *Arabidopsis* [26] and barley [27], the similarities are 90.13% and 89.66% for Lhcb1, 76.50% and 89.91% for Lhcb2 and 95.96% and 93.27% for Lhcb3, respectively. Lhcb3 shows the highest conservation, while the Lhcb2 presents the highest divergence among different species. Sequence alignment of different Lhcb polypeptides of pea reveals that the highest similarity (81.47%) exists between Lhcb1 and Lhcb2. The other pairs – Lhcb1 and Lhcb3, and Lhcb2 and Lhcb3 – are less similar (72.65% and 74.46% similarity respectively). Most of the divergence is located at loop regions (Fig. 1).

The ligands of cofactors in different apoproteins were analysed on the basis of high resolution analysis of the crystal structure of LHCIIb [2,3]. The Chl binding sites in different apoproteins showed minor divergence: The Chlb 608 binding site L148 in Lhcb1 and Lhcb2 had been changed to V148 in Lhcb3; while Chlb 605 binding site V119 in Lhcb3 and Lhcb1 had been changed to I119 in Lhcb2. The binding site for PG and DGDG were all conserved in the three isoforms.

It has been proposed that the Ser and Thr in the N-terminal are the phosphorylation sites [33,34] and the motif WYXXXR in N-terminal [35] and the W222 in C-terminus [36] are necessary for trimerization. The phosphorylation sites are conservative in Lhcb1 and 2, but are missing in Lhcb3 since the latter is short of 14 amino acids in the N-terminal, while these of trimerization are all conservative in all the three isoforms.

3.2. Pigment component of different recombinant isoforms of LHCIIb

Table 1 shows the pigment stoichiometries of reconstituted Lhcb1–3 monomers, calculated on the basis of pigment per 2 Lut as described in [29]. All the LHCIIb isoforms showed similar pigment stoichiometries. Each monomer binds 13.7 to 14.9 Chls and approximately 1 Neo, with Chl a/b ratio equals to 1.11 for Lhcb1, 1.20 and 1.26 for Lhcb2 and Lhcb3, respectively. Nevertheless, our result indicated that each monomer bound only about 0.1 Vio, which was inconsistent with the result shown

Table 1
Pigment stoichiometry of different LHCIIb isoforms

Pigment	Lhcb1	Lhcb2	Lhcb3
Chl a/b	1.11±0.01	1.20±0.01	1.26±0.01
Chl a+b	13.70±0.11	14.26±0.13	14.94±0.10
Chl a	7.20±0.06	7.78±0.06	8.34±0.07
Chl b	6.50±0.07	6.48±0.08	6.60±0.04
Lutein	2.00	2.00	2.00
Neo	0.91±0.01	1.07±0.02	0.91±0.02
Vio	0.10±0.01	0.11±0.01	0.09±0.00

The pigment contents of the complexes from sucrose density gradient were determined with HPLC. The values are normalized, assuming that each LHCIIb monomer binds 2 Luts. The data are given as the averages of 8–10 individual reconstitution and measurements±standard derivation.

in the crystal structure [2], in which each monomer bound 1 Vio. This discrepancy could be attributed to the fact that the Vio in LHCIIb is a loosely bound pigment, which can be removed by very mild detergent. Ruban et al [37], using a particularly mild fractionation method, could find only 0.2 Vio in native LHCIIb monomer. Recombinant LHCIIb shows characteristics similar to those of the native isolated LHCIIb in this aspect [38,39]. In our experiment, different reconstituted isoforms bound similar amount of Vio, which implied that the differences in the measured transition energy and thermo- or photostabilities in different isoforms could not likely to be attributed to the differences in the binding Vio in different isoforms.

3.3. Absorption spectra

The absorption spectra of each isoform of LHCIIb measured at room temperature are presented in Fig. 2A, normalized to the reddest Chl a absorption in the Qy region. The maxima in the Qy region were around 672 nm and 651 nm, and in the Soret region around 436 nm and 472 nm, for all the isoforms.

The fourth deviation of the absorption spectra revealed three components peaking at 666 nm, 672 nm and 680 nm attributed to Chl a absorption in the Qy region. The red-most component (680 nm) was 2-nm red-shifted in Lhcb3. In the Soret region, there were three major differences located at 436 nm, 471 nm and 490 nm, respectively. The absorption maximum of Lhcb3 peaking at 437 nm, and was split into two peaks (436 and 439.5 nm) for both Lhcb 1 and 2; the absorption maximum at 471 nm for Lhcb1 and Lhcb2 was missing in the absorption spectra of Lhcb3 and the absorption maximum around 490 nm for Lhcb1 and Lhcb2 was blue-shifted in Lhcb3. Comparing the absorption spectra among different species [26,27], we can see that the absorption of Lhcb3 in all the species showed two common divergences compared to the other two isoforms: (1) The reddest Chl a absorption in the Qy region was 2 nm red-shifted; and (2) The absorbances at 471 nm and 490 nm in the Soret region were diminished.

3.4. Fluorescence spectra

Fluorescence emission spectrum was used to probe the energy transfer in the recombinant proteins [40]. We have observed that the fluorescence emission spectra were essentially identical (there was one major emission around 680 nm), irrespective of whether Chl a, Chl b or carotenoid (Car) were excited at 440 nm, 480 nm, or 500 nm respectively (Fig. 3 shows Chl b excitation; data not shown for Chl a and Car excitation). These results indicated a nearly complete energy transfer from Chl b to Chl a, and from other chromophores to Chl a in the reconstituted complexes.

More detailed analysis of the fluorescence emission spectra of different isoforms (Fig. 3, inset) showed that in comparison to the emission peak of Lhcb1, that of Lhcb2 was slightly blue-shifted, and of Lhcb3 was about 2 nm red-shifted. The same results were observed in *A. thaliana* [26] and barley [27].

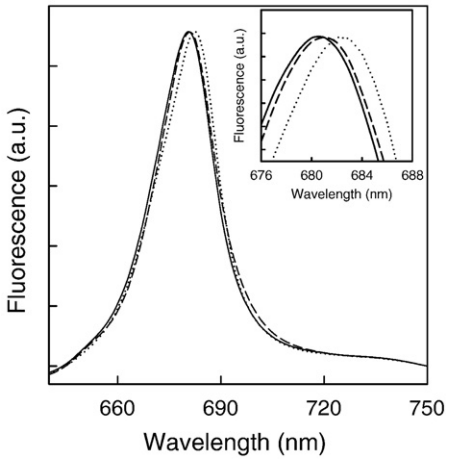

Fig. 3. Fluorescence emission spectra of Lhcb1 (solid line), Lhcb2 (dashed line) and Lhcb3 (dotted line). The samples were excited at 480 nm. Fluorescence spectra were normalized to the maximum.

3.5. Circular dichroism spectra

Circular dichroism (CD) in the visible range reflects the dipole–dipole interactions between chromophores and therefore is a powerful method to study conformation of the pigment–protein complexes. The CD spectra of the three isoforms of LHCIIb (Fig. 4) presented common features resembling those of native monomeric LHCIIb at room temperature [25], with three major negative peaks around (−)680 nm, (−)650 nm, and (−)491nm and a positive peak at (+)669 nm. The three isoforms showed similar features, except that Lhcb3 showed 1 nm red shift in the (−)650 nm peak and reduced amplitude at (+)669 nm in the Qy region.

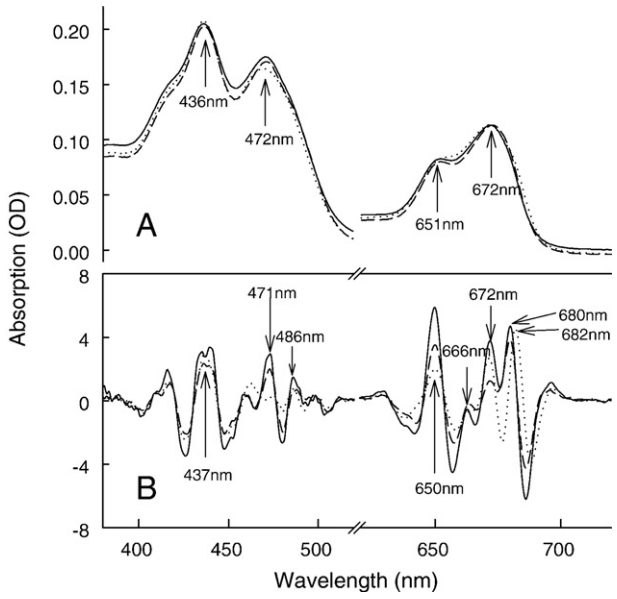

Fig. 2. Absorption spectra of Lhcb1–3 complexes at room temperature (A) and their fourth-deviation spectra (B). The spectra were normalized at red-most absorbance. Lhcb1 (solid line), Lhcb2 (dashed line) and Lhcb3 (dotted line).

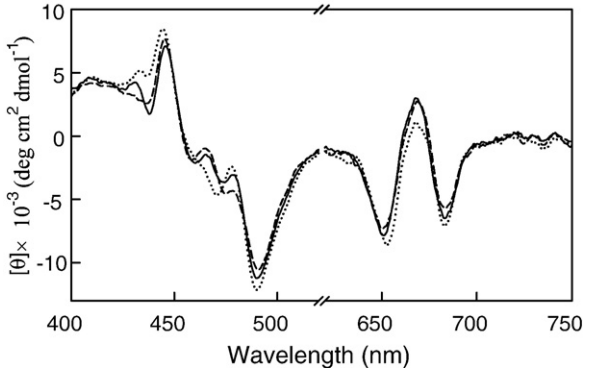

Fig. 4. Circular dichroism (CD) spectra of different isoforms measured at room temperature. The spectra were normalized to the same Chl concentration; Lhcb1 (solid line), Lhcb2 (dashed line) and Lhcb3 (dotted line).

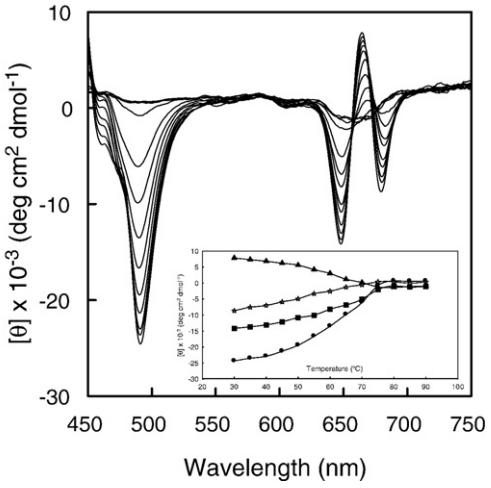

Fig. 5. Changes of CD spectra of different isoforms during the temperature gradient. The temperature was raised from 20 to 90 °C at a rate of 1 °C min^{-1}. The spectra were measured at 10 °C intervals. The insert shows the tendencies of different peaks during the temperature treatment. CD 490 nm: (circle); CD 648 nm: (square); CD 664 nm (triangle); CD 680 nm: (star).

3.6. Thermal stability

Thermal stabilities of different isoforms of LHCIIb were examined by monitoring the diminishment of CD signal at 492 nm and changes of fluorescence emission spectra during a temperature gradient, and the decay kinetics of sensitized Chl a fluorescence emission in the complexes at 37 °C.

Fig. 5 shows the changes of the CD spectra of Lhcb1 during the temperature gradient. Because the changes of CD spectra of Lhcb 1–3 upon raising the temperature show similar tendencies, only those of Lhcb 1 are presented here. From Fig. 5 we can see that the conformation of the monomeric LHCIIb was sensitive to the changing ambient temperature. The CD spectra of different isoforms exhibited a small lag as the temperature rose from 20 °C to 30 °C, and then began to vanish as the temperature increased from 30 to 70 °C, and reached the minimal CD signal at 75 °C. Comparing the decreases of different CD bands (Fig. 5 inset), we can see that the changes of the CD spectra were characterized by a synchronous diminishment of all four major CD signals.

The time-course of the CD signal at 492 nm during the temperature treatment was observed at time intervals of 0.2 min, since this is a good indicator for dipole–dipole interaction of binding chromophores [41]. The observed data could be fitted very well with sigmoid curves ($Y=a/(1+\exp(-(X-b)/c))$) and the inflection point (b) was taken as T_m. As mentioned previously, the T_m observed here can only be regarded as a relative measure because no attempt has been made to reach the equilibrium state at individual temperatures [20]. The results are presented in Table 2, from which, we can see that the T_m of different isoforms of LHCIIb measured here were around 65 °C. No difference in thermal stability could be observed in different isoforms regarding the conformation of the complexes.

Table 2
Thermal stability of different isoforms of LHCIIb

Lhcb	T_m(°C)
Lhcb1	65.7±3.0
Lhcb2	66.3±3.8
Lhcb3	65.8±3.7

All values presented here are the average of at least 6 individual measurements± standard derivation. The data represent the dissociation temperature (°C) at which half of the signal disappeared during the temperature profile.

Fig. 6 presents the changes of fluorescence emission spectra of Lhcb1 after different temperature treatments. Those of Lhcb2 and Lhcb3 showed similar tendencies during the temperature treatments (data not shown). From Fig. 6A, we can tell that the emission at 680 nm upon 480 nm excitation decreased and the emission at 660 nm increased when the temperature was raised from 20 to 70 °C, indicating that the energy transfer from Chl b to Chl a disrupted gradually upon raising the ambient temperature. The changes of the maximal fluorescence emission around 680 nm of the three isoforms during the temperature gradient (Fig. 6B) presented a sigmoid-like tendency. In order to compare thermal stabilities of different isoforms in this viewpoint quantitatively, the changes in the maximal fluorescence emission during the temperature treatment were fitted with asymmetric sigmoid curves and the apparent denaturing temperatures (T_{mF}) were calculated as 42.06 °C, 32.54 °C and 41.61 °C for Lhcb1, Lhcb2 and Lhcb3, respectively (Fig. 6B).

The measured T_{mF} value of Lhcb2 is much lower than the other isoforms (Fig. 6B). In order to verify this observation, we assayed the dissociation kinetics of different isoforms with regard to energy transfer within the complexes by monitoring the decay kinetics of the Chl b-sensitized Chl a fluorescence emission at 37 °C with the method described in Yang et al [27]. The course of fluorescence decay could be fitted into two-exponential kinetics. Table 3 lists the lifetime constants

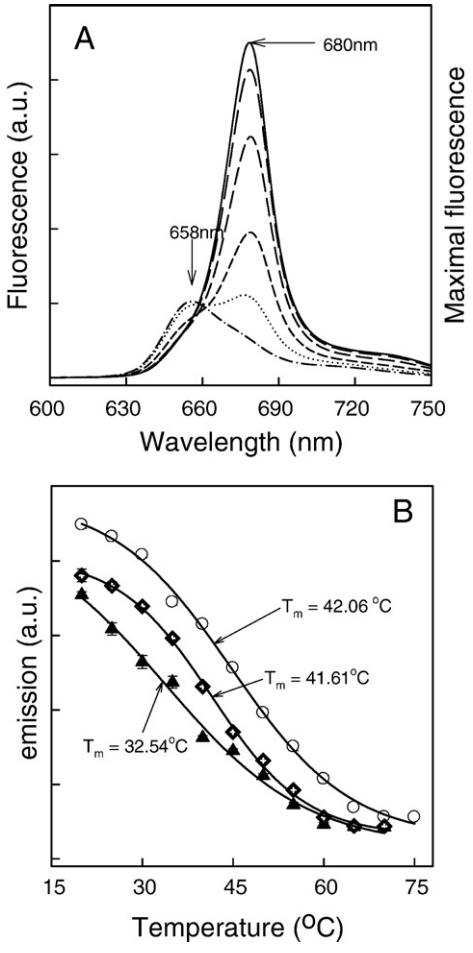

Fig. 6. Temperature-dependent change in fluorescence emission spectra. A, Changes of fluorescence emission spectra of Lhcb1 from 20 to 70 °C. The spectra were measured on the basis of the same Chl concentration. 20 °C: (solid line); 30 °C: (long dashed line); 40 °C: (medium dashed line); 50 °C: (short dashed line); 60 °C: (dotted line); 70 °C: (dot-dashed line); B, Change of the maximal fluorescence emission of Lhcb1–3 after the temperature gradient treatment according to method and material. The samples were diluted to same Chl concentration for different isoforms. Lhcb1: (circle); Lhcb2: (triangle); Lhcb3: (diamond and X-hair).

Table 3
Lifetimes of energy transfer from Chl b to Chl a of different isoforms of LHCIIb incubated at 37 °C

Lhcb	τ_1(s)	τ_2 (min)	A_1/A_2
Lhcb 1	40.3±0.5	29.5±5.5	1.1±0.1
Lhcb 2	19.8±0.7	18.3±2.1	0.7±0.0
Lhcb 3	62.8±1.9	39.4±10.6	1.4±0.4

The data represent the lifetime constants τ_1 and τ_2 and amplitudes A_1 and A_2 derived from the Two-exponential fitting of the time-resolved measurements of the decrease in the energy transfer from Chl b to Chl a of different isoforms at 37 °C. All values are given as the average of 3 individual measurements±standard deviation.

of different isoforms of LHCIIb at 37°C, which revealed that the lifetime of the Chl b-sensitized Chl a fluorescence emission in Lhcb2 at 37 °C was significantly shorter than Lhcb1 and Lhcb3.

Comparing the temperature dependent structural changes of the three isoforms in view of conformations of the complexes (diminishment of CD signals at 492 nm) and those regarding energy transfer in the complexes (the Chl b sensentized Chl a fluorescence emission at 680 nm), it is obvious that the disruption of energy transfer in the complexes occurred at lower temperatures than the breakdown of the complex structure.

3.7. Photobleaching

The highly reactive singlet O_2^*, which appears under strong light conditions, is dangerous to Chls since it oxidizes the Chls very quickly. Spatial configuraton and interaction of the pigments in LHCIIb make it possible that the over-excited energy being dissipated quickly via carotenoid under strong light conditions, so that the pigment in LHCIIb could be protected from strong illumination. Here, photostability properties of different isoforms of LHCIIb were investigated by monitoring the decrease in Chl absorption in the Qy region (600–700 nm) of the complexes under strong illumination according to the method described in [11].

Figs. 7A, B, and C present the changes of the absorption spectra of different isoforms (Lhcb 1-3) of LHCIIb respectively. Analysis of the difference absorption spectra of all the isoforms (insets of Fig. 8) revealed a similar two-stage pattern as observed to native LHCIIb made by Olszówka et al [42] in all the isoforms: At the initial stage, decrease of the absorbance of Chl a was centered at 670 nm, blue-shifted by about 3 nm compared to the original Chl a absorption, and at 650 nm, ascribable to Chl b; At the deeper bleaching stage, decrease of Chl was less selective towards Chl a or Chl b, producing a pattern similar to the absorption spectrum before the treatment.

The time course of Chl b/a ratio was plotted during the whole duration of strong illumination (Fig. 8). The Chl b/a ratio increased during the whole treatment, which was characterized by a two-stage process. During the initial photobleaching stage (1st–4th seconds), the Chl b/a ratio changed at a similar rate in all the three isoforms, while in the later stage (after 5th second), the change of Chl b/a in Lhcb3 was much slower (Δ(Chl b/a)/min=0.0053) than in Lhcb1 and Lhcb2 (Δ(Chl b/a)/min=0.0092 or 0.0086), which implied a lower bleaching rate of Chl a in Lhcb3. Comparing the changes of absorption spectra of Lhcb3 under strong illumination, we can see that the pigments absorbing at 675 nm were more sustainable than those absorbing at 650 nm.

In order to compare the bleaching rates of Chls in different isoforms, the time courses of the photobleaching were plotted by integrating the area under the absorption spectra of the three different isoforms during the whole strong illumination process (Fig. 9). At the earlier stage, all three isoforms showed similar rates of Chls photobleaching, while at the later stage, Lhcb3 showed a higher photostability than Lhcb1 or Lhcb2 under strong illumination.

4. Discussion

The major light-harvesting Chl a/b complex of PSII is not unique in composition. It consists of various combinations of three different isoforms encoded by Lhcb1–3 genes. The three different isoforms occur in a ratio of about 8:3:1 [43], which varies in thylakoid membranes according to different growth conditions [44,45]. It has been suggested that the three isoforms of LHCIIb have particular

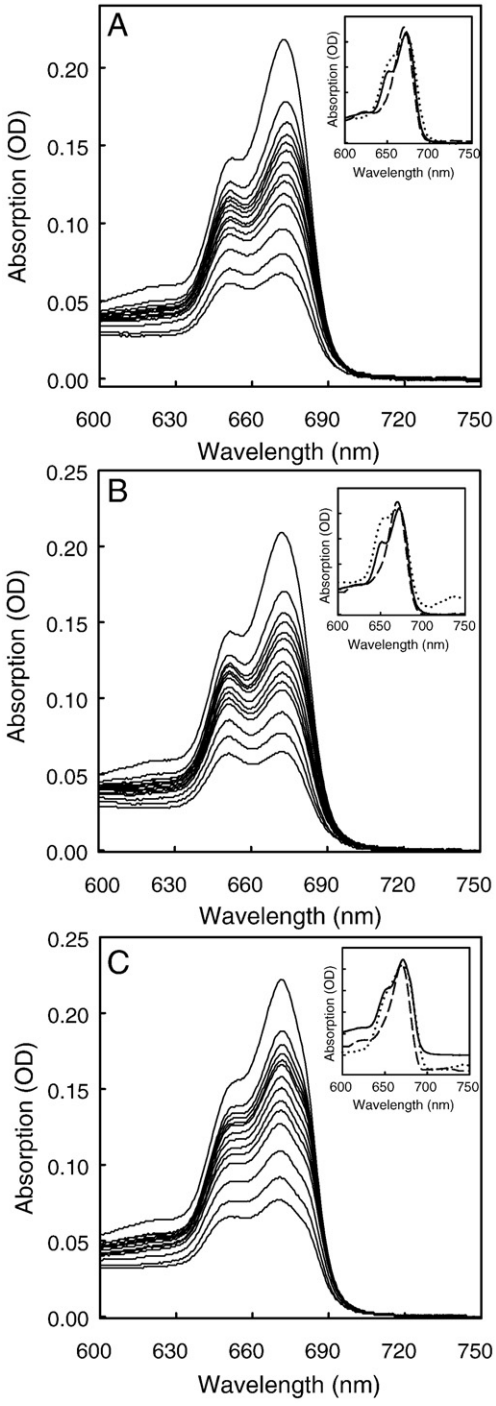

Fig. 7. Change of the absorption spectra during photobleaching process. The spectra were recorded at 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 20, 25, and 30 min under the strong illumination. The insets in the grafics present the difference absorption spectra at the initial (after 1 min) and advanced (after 30 min) stages of the photo-oxidation (solid and dashed line respectively), and the absorption spectrum of the Lhcb before strong illumination treatment (dotted line): (A): Lhcb1, (B): Lhcb2, (C): Lhcb3.

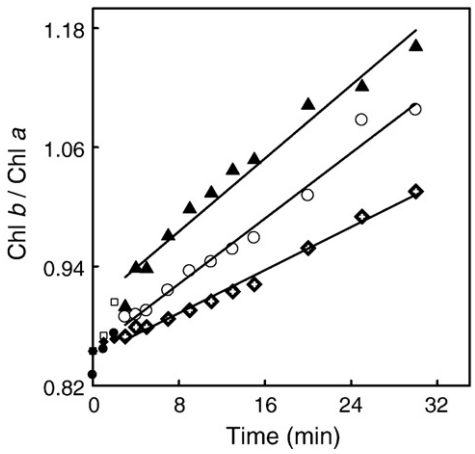

Fig. 8. Change of Chl b/Chl a ratio during the photobleaching process. Lhcb1: (circle), Lhcb2: (triangle), Lhcb3: (diamonds and X-hair). The lines present the regression of the trace in which the slopes are 0.0092, 0.0086 and 0.0053 for Lhcb1, Lhcb2, and Lhcb3 respectively.

functions in harvesting and utilizing solar energy [1]. However, due to the difficulties in purifying each of the components to homogeneity, it was difficult to identify their specific structural and functional characteristics. It is well established that the in vitro reconstituted LHCII is essentially identical to the native complexes [25,28,46]. In this work, we have refolded different isoforms of monomeric LHCIIb with thylakoid pigment in vitro, investigated their spectroscopic properties. Comparing the characteristics of the CD spectra[25] and the absorption and fluorescence emission spectra [47] of the reconstituted isoforms of Lhcbs with those of native monomeric complexes, we can see that the reconstituted Lhcbs showed many special common features like the native ones, which indicated their similar structural organizations. The fluorescence emission spectra of different Lhcb isoforms also demonstrated, that all the reconstituted complexes are functionally intact because the complexes showed complete energy transfer from Chl b to Chl a (Fig. 3). Based on these evaluations, we have investigated the structural stabilities under elevated temperatures or strong illumination, and analysed their possible physiological functions.

4.1. Sequence comparison in different isoforms and different species

The alignment of mature LHCIIb apoprotein sequences of pea, *Arabidopsis*, and barley indicates that some divergences, which are located mostly at loop regions, are present in different isoforms. Among all the three isoforms, the sequence of Lhcb3 is the most highly conserved. On the contrary, the sequence of Lhcb2 is the most divergent, not only among different species (Fig. 1), but also under different light conditions, e.g. the expression of Lhcb2 in vivo increases 2–3 times under limited light conditions [48].

Comparing the results obtained from the pea in our experiment and those from *Arabidopsis* [26] and barley [27], we found that the slight divergences in the sequences from different species have not resulted in pronounced changes in the complexes. On the contrary, there were more variations among different isoforms in the given species, e.g. higher Chl a/b ratio, and lower Qy electric transition in Lhcb3 (Table 1 and Fig. 2).

1. Phosphorylation sites — Phosphorylation of LHCIIb triggers migration of LHCIIb from PSII to PSI, which regulates the energy distribution between different PSs [49]. Our alignment results showed that the proposed phosphorylation motifs [33,34] were conservative in Lhcb1 and Lhcb2, but were missing in Lhcb3 (Fig. 1). It was proposed that the main function of Lhcb2 is the adaptation to different light conditions, because it is phosphorylated several times more than Lhcb1 [50], it undergoes the most variable structural changes via phosphorylation under strong light conditions [50,51] and it is located in a peripheral position of PSII particles [52].
2. Trimerization motifs — Trimerization is an important characteristic of LHCIIb. It has been observed that both Lhcb1 and Lhcb2 can form homotrimers in vitro, but not Lhcb3 [26,27]. Lhcb3 can only form heterotrimers with Lhcb1 or Lhcb2, or present as monomers tightly bound to PSII [53], although all the motifs needed for trimer formation, including the residues binding PG and DGDG [35] are conservative in Lhcb3 (Fig. 1). From the results of sequence alignment, we can see that the most important divergence of Lhcb3 is that it lacks the peptide R1–S14 in its N-terminal. It might be possible that the peptide (R1–S14) in the N-terminal influences the conformation of the S14–D54 which takes part in the trimerization [2], because the conformation of the N-terminal domain of LHCIIb is flexible for regulating different functions of LHCIIb [54].

4.2. Thermal stability of the structure of different isoforms

A plant lives in an environment which undergoes substantial changes. The light intensities and leaf temperatures vary dynamically with diurnal and seasonal changes. Light, one of the most important environmental factors influencing plant growth, directly increases the leaf temperature. Therefore it is necessary for plants to have some basic protective mechanisms against strong illumination, or high-temperature stress conditions. As the main light-harvesting apparatus, LHCIIb plays a crucial role in regulating harvesting energy or dissipating over-excited energy. Accordingly, the thermal- and photostabilities of LHCIIb isoforms may be related to their functions in vivo.

Our observation reveals that the T_m on Lhcb1–3 from the viewpoint of complexes conformation could be estimated as around 65°C (Table 2). The upper limits of leaf temperatures, which are transiently reached at high ambient temperatures and intense illuminations without irreversible damage, are around 60 °C. These temperature limits seem seldom to be reached, although the temperatures of sunlit leaves can be well over 10 °C above the ambient temperatures [55]. It has been found that the macro-aggregate of LHCIIb are ss up to about 40 °C, and small aggregates up to about 45 °C [56]. A high-temperature-induced trimer–monomer transition appears at 55–65 °C [16,18,19].

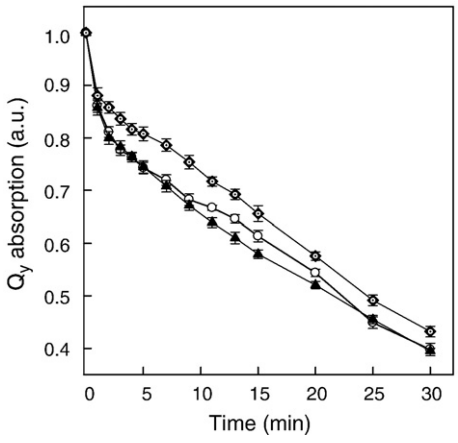

Fig. 9. Time course of the photobleaching processes. The decay curves show the whole pigment absorptions, measured upon integration of the absorption spectra in the Qy region, relative to the initial value (the initial value was set to 1). Each point represents the average of 5 individual experiments. Lhcb 1: (circle), Lhcb 2: (triangle), Lhcb 3: (diamond and X hair).

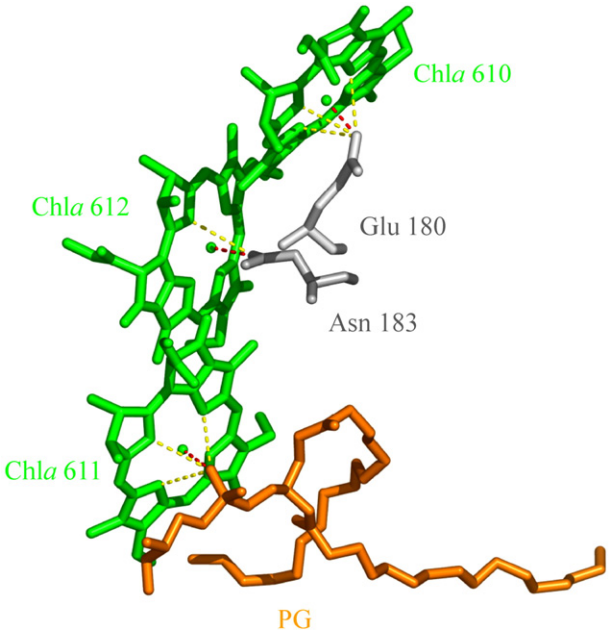

Fig. 10. Representation of the structure of the Chl cluster Chla 610–Chla 611–Chla 612 of LHCIIb based on the crystal structure of Liu et al [2]. The figure elucidates the ligands of Chl cluster Chla 610–Chla 611–Chla 612. The Chls are represented only with their porphyrin (in green) and labelled according to the nomenclature of Liu et al [2]. The H-bonds are presented as dashed lines. The figure is based on PDB 1RWT [2].

Above 65 °C, the monomers can be completely denatured. Our results agree very well with the former observations. The temperature-induced dissociation of LHCIIb seems to be a progressive process, in which the macro-domains are dissociated at first, followed by the trimer–monomer transition and at last the denaturing of the monomeric complexes. Our result reveals no significant differences in thermal stabilities, regarding the changes of the LHCIIb conformations in different isoforms. These results imply that it is the macro-domain structure of LHCIIb that is sensitive to outer heat and light conditions, rather than the different monomeric structures, under high temperatures.

Lhcb 2 is the most sensitive to elevated temperature regarding energy transfer in the complexes. It phosphorylates several times higher than Lhcb1 [50]; It is the most divergent, not only among different species (Fig. 1), but also under different light conditions [48]; and it locates in the peripheral position of PSII [52]. All these imply that Lhcb2 possibly does not play an important role in energy transfer at high temperature, but rather in the adaptation process of LHCIIb under strong light conditions.

4.3. Transition energy of different isoforms could be altered by slight sequence differences in the loop regions

Although the divergence in the sequence does not influence the pigment stoichiometry of the complexes, it affects the spectroscopic properties of the complexes.

The absorption spectra analysis revealed three peaks in the Chl a absorbance in the Q_y region of Lhcb1 and Lhcb2, which were centered around 666 nm, 672 nm and 680 nm. These are consistent with the model, proposed by van Grondelle et al [57] on the basis of the new crystal structure [2,3], that the Chl a bound in LHCIIb are organized into three clusters in the energy transfer pathway: Chla 602–Chla 603, absorbing at 672 nm, Chla 610–Chla 611–Chla 612, absorbing at 680 nm, and Chla 613–Chla 614, absorbing at 666 nm. The red-most absorption of Lhcb3 has been shifted from 680 nm to 682 nm, which confirms the previous observations [26,27]. The crystal model of LHCIIb at the atomic level [2] shows that the pigment ligands of Chla 610–Chla 611–Chla 612 are E180–phospho diester-N183 (Fig. 10). E180 and N183 are conservative in all the three LHCIIb isoforms (Fig. 1), which means that changes in the third ligand for this cluster, the phospho-diester bond, may be the reason for the red-shift associated with the red-most absorbance in the Q_y region. As we know from the result of sequence alignment, the most striking difference of Lhcb3 from the other two isoforms is its lack of R1–S14 peptide in the N-terminal, which leads to the loss of the trimerization possibility of the Lhcb3 [26]. Since R1–S14 is close to PG [2, 3], it is reasonable to infer that the shortage of this peptide in Lhcb3 influences the position of PG, which in turn results in changes of the pigment cluster Chla 610–Chla 611–Chla 612 and then changes of the transition energy of chromophores.

The photobleaching experiment has revealed that the Chls in Lhcb3 survive better under strong illumination than the other two isoforms (Fig. 9), which should be attributrd to higher photostability of the bound Chl a (Fig. 8). It is plausible to suggest that the environment of Chl a, which might be related to red-shift of the red-most Qy absorbance, should be important in preventing the bleaching of Chl a under strong illumination.

Lhcb3 is the most conservative isoform, and is the most resistant of the three isoforms to bleaching under strong illumination. Lhcb3 cannot migrate between different PSs via phosphorylation because it lacks the phosphorylation site. It has been found in *Arabidopsis* that Lhcb3 is located closest to PSII core and does not take part in the NPQ [7]. These observations lead to the conclusion that Lhcb3 may act more like an inner antenna and functions as a bridge between the PSII core complexes and outer antenna [26,27]. But the results of femtosecond transient absorption and time-resolved fluorescence measurements at 77K do not support this proposal [58]. More experiments are still needed to determine the function of Lhcb3.

Acknowledgements

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