

The Lhcb1,2 and 3 gene products, component the trimeric antenna complex of higher plant Photosystem II, have distinct biochemical and spectroscopic properties.

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Introduction

The major antenna complex of Photosystem II (PSII) is composed by the Lhcb1,2 and 3 gene products organised into heterotrimers. It has been shown that heterotrimeric LHCII complexes are connected to the PSII core in distinct positions thus building up a supramolecular complex in which the structural and functional role of individual Lhcb1-3 proteins is still to be elucidated (Boekema et al. 1999). Lhcb1-3 gene are found in many copies within the genome of most higher plants. In *Arabidopsis thaliana*, LHCII protein moiety is encoded by 5, 4 and 1 genes respectively for Lhcb1, 2 and 3 (Jansson, 1999). Although highly homologous, they carry specific aminoacid substitutions typical for each group. It is not known if differences in structure/function are associated to this sequence heterogeneity. Functional specificity can, however, be hypothesised on the basis of the observed differential accumulation of individual Lhcb1-3 gene products depending on growth conditions. In addition, it has been shown that the different LHCII isoforms (De Luca et al. 1999) are differentially enriched in subsets of the supramolecular PSII complex (Bassi and Dainese, 1992) thus suggesting that the topological location of each individual Lhcb gene product is genetically determined. Although a common structural and functional organization is generally assumed for Lhc proteins on the basis of sequence homology, individual members of this gene family exhibits important differences in xanthophyll content (Bassi and Caffarri, 2000), occupancy of chlorophyll binding sites (Bassi et al. 1999, Remelli et al. 1999) and presence of regulative sites such as phosphorylation sites, DCCD binding sites and Ca⁺⁺ binding sites (Testi et al. 1996, Pesaresi et al. 1997, Jegerschold et al. 2000) which can significantly affect the functional role of the antenna complex as exemplified by the widely different fluorescence lifetimes properties ranging from 4 ns in LHCII and 0.5 ns in LHCI (Moya et al. submitted, Ihalaenen et al. 2000). In the attempt to gain information on the role of individual Lhc gene products, we have studied Lhcb1,2 and 3 proteins, either recombinant, from *in vitro* reconstitution of apoproteins expressed in bacteria, or purified from chloroplast membranes, with respect to their biochemical and spectroscopic properties. We found that the Lhcb3 component of LHCII has characteristics clearly distinct from Lhcb1 and 2. Trimeric LHCII complexes which contain Lhcb3 are spectroscopically distinct with respect to those lacking this gene product. The possibility that Lhcb3 may have a function in mediating excitation energy transfer from trimeric LHCII to neighbor Lhcb4 and Lhcb5 subunits is discussed.

Materials and Methods

Lhcb1-3 cDNA isolation

lhcb1, *lhcb2* and *lhcb3* expressed genes were isolated from barley by screening a cDNA library from leaves with a maize *lhcb1* probe and by analysing EST from the same library.

Overexpression in E.coli and Reconstitution in vitro of LHCII proteins

The *lhcb1-3* genes were cloned in a modified pQE50 expression vector containing a sequence coding for six histidine at the 3' end of the polylinker. The reconstitution of the proteins (purified as inclusion body from *E. coli*) was carried out both in Ni⁺⁺ column (Rogl et al. 1998) and in batch (Giuffra et al. 1996, Croce et al. 1999a).

Native LHCII purification

Thylakoid membranes of *Zea mays* plants were prepared and solubilised as described in Bassi et al. (1988) and Caffarri et al. (2001). IEF was performed following the protocol reported by Dainese et al. (1990).

Pigment analysis

The pigment composition of the complexes was analysed by fitting the absorption spectrum of the acetone extract with the spectra of individual pigments (Connelly et al. 1997) and by HPLC analysis (Gilmore and Yamamoto 1991).

Gel electrophoresis

SDS-6M-urea PAGE was performed with the Tris-sulphate buffer system as previously reported (Bassi et al. 1985).

Spectroscopy

Absorption spectra were obtained using a SLM-Aminco DW-2000 spectrophotometer at room temperature. Fluorescence spectra were recorded at room temperature using a Jasco FP-777 spectrofluorimeter. The Chl concentration was 0.1 µg/ml. The LD spectra were recorded upon samples orientation of the particles by the polyacrylamide squeezing technique according to Haworth et al. (1982).

Results

Samples.

Lhcb1,2 and 3 genes were expressed in *E. coli* and reconstituted *in vitro* with purified pigments. In all cases it was possible to obtain reconstituted complexes and the reconstitution yields were comparable.

Pigment composition.

The three reconstituted and the native complexes were characterised for the composition of their pigment moiety. The data obtained from the analysis of acetone extracts are reported in Table I. Lhcb3 binds a lower amount of xanthophylls (on a chlorophyll basis) with respect to Lhcb1 and 2, neoxanthin being the pigment which showed a stronger decrease.

The stoichiometric determination of Chl/polypeptide ratio showed that Lhcb1 and Lhcb2 bind 12 Chls per polypeptide while only 11 were found in Lhcb3 implying, together with the higher Chl *a/b* ratio, that a Chl *b* binding site was missing or empty. Sequence comparison between the three

proteins indicates that all the Chl binding residues previously identified (Kuhlbrandt et al. 1994, Remelli et al. 1999) are conserved thus suggesting that the missing Chl is not among those directly co-ordinated to an amino acid residue. Chls bound to sites B1, B2, A6, A7 are thus eligible. Within this shortlist, site A7 meets both the characteristics of being Chl *b*-only binding (Remelli et al, 1999) and that of tuning the bound porphyrin to a 651 nm absorption (Fig. 2), which is a major component of the Lhcb1 minus Lhcb3 difference spectrum. We thus suggest that the missing Chl binding site in Lhcb3 corresponds to Chl A7.

Absorption spectra.

The absorption spectra for the three recombinant pigment-proteins at RT are shown in figure 1. While the absorption spectra of Lhcb1 and Lhcb2 are similar, in the case of Lhcb3 an absorption component around 685 nm was found as a distinctive feature. Conversely, a decrease in the absorption is observed at around 675 nm and 651 nm (Fig. 2). As a tentative interpretation of these results we suggest that either Chl A4 or Chl A5 absorption is red-shifted in Lhcb3 with respect to Lhcb1 (Remelli et al.1999). Chl A5 is the most probable candidate since the spectroscopic features typical of the excitonic interactions involving Chl A2, B2 and A4, previously described in Lhcb1, are unaffected in Lhcb3. This indicates that, despite high homology within LHCII components, the pigment organisation can be significantly different. The red shift of the 685 nm absorption form typical of Lhcb3 suggests strong interactions between pigments or between pigments and protein, similar to what observed in LHCI (Morosinotto, 2001).

Fluorescence emission.

Fluorescence emission analysis confirms the analysis of the absorption spectra: Lhcb3 spectrum peaks at 683.5 nm, while Lhcb1 and Lhcb2 show fluorescence peaks at 681-681.5 nm, confirming the presence of a low energy absorption form in Lhcb3 (Fig. 3). LHCII trimers containing Lhcb3 also exhibited a red-shifted fluorescence peak. This imply that, when participating to a trimeric complex, Lhcb3 becomes the main emitter of the excitation energy and suggests this antenna protein might act in focusing excitation energy during transfer to neighbour photosynthetic subunits.

Linear dichroism.

The LD spectrum of Lhcb3 shows a positive contribution at low energy which is not present in Lhcb1 complex (Fig. 4). This indicates that the Chl *a* (685 nm) transition moment forms an angle of more than 54.5° respect to the normal to the membrane plane. In the Soret region, a signal around 488 nm, due to the neoxanthin molecule in N1 site (Croce et al. 1999a), is missing with respect to Lhcb1. This, in agreement with the pigment analysis which show lower amount of neoxanthin associated to Lhcb3 compared to others LHCII complexes, suggests that in Lhcb3 N1 site is partially empty. It cannot be excluded, however, that the N1 site is fully occupied *in vivo* but it is partially emptied during purification because of the de-stabilisation produced by the missing Chl.

Stability.

The denaturation temperature of Lhcb3 is 68°C, while this value is 77° for Lhcb1. Stability measurements performed on Lhcb1 reconstituted in the absence of neoxanthin, therefore with N1 site empty, indicates a melting temperature of 65°C (Croce et al. 1999a). This again supports the view of N1 site at least partially empty in Lhcb3 complex. However, the lower number of Chls bound to this complex can also account for a small decrease of the melting temperature.

Oligomerisation of Lhcb 1-3.

Reconstitution experiments in the presence of lipids shows that, while Lhcb1 and Lhcb2 readily form homotrimers *in vitro*, Lhcb3 complex does not. It is interesting to note that it was not possible to purify Lhcb3 homotrimers while pure monomeric Lhcb3 was obtained (Fig. 5), this indicates that the function of Lhcb3 is related to its interaction with Lhcb1,2 while the latter may well form functional trimers in the absence of Lhcb3.

Lhcb 3 did not form heterotrimers *in vitro* when co-reconstituted with Lhcb1 and/or Lhcb2 in conditions leading to trimerisation of the latter proteins. However, when recombinant Lhcb3 was mixed with monomeric Lhcb1+2+3 mixture isolated from thylakoids, it formed trimers. This result suggests that an unknown factor was present in the thylakoid extract which was needed for Lhcb 3 interaction with Lhcb1/2 subunits. Alternatively, it is possible that Lhcb 3 interaction is only possible with specific Lhcb1,2 isoforms which were present in the thylakoid extract but were not encoded by the clones we have used for *in vitro* reconstitution. Analysis of the Lhcb3-containing trimers is in progress for identification of the specific gene products involved in trimerisation.

Localisation of Lhcb3 in the PSII supramolecular complex.

Thylakoid fractionation shows that Lhcb3 is indeed present in heterotrimeric fractions and that is particularly abundant in “band four” which is a supercomplex composed by a LHCII trimer, CP29 and CP24 (Bassi and Dainese, 1992). In this band only a subset of Lhcb1-2 gene products is present thus suggesting Lhcb3 needs specific partners for trimerisation (Fig. 6).

Conclusions

Lhcb3 complex is the most different out of all LHCII gene products. It tightly binds 11 Chls and two xanthophylls in sites L1 and L2 while a third xanthophyll binding site (N1) is more loose with respect to the case of Lhcb1 and 2. On the basis of previous analysis on Lhcb1, which show N1 site to be hold in place by interaction with Chl *b* ligands, we suggest that the absence of a Chl *b* chromophore located in site A7 affects the Car binding properties of the complex thus decreasing the strength of the interaction with neoxanthin.

The presence of Lhcb3 in the CP29-CP24-LHCII supramolecular complex, suggests a localisation of this complex at the interface between the peripheral LHCII trimers and the minor antenna proteins within the PSII structure (Harrer et al. 1998, Nield et al. 2000). The presence of an absorption form at lower energy compared to all others LHCII may help in the focusing of excitation energy harvested by the outer antenna at the Lhcb3 site for further transfer to PSII RC.

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Tables and figures

Table 1. Pigment composition of recombinant (rec-) and native (nat-) LHCII complexes of different types.
N=neoxanthin; V=violaxanthin; L=lutein; errors \pm 0.05.

	Chl a/b	N	V	L	Chl b	Chl a	Σ Car	Σ Chls
rec-Lhcb1	1,39	0,94	0,28	1,72	5,04	6,96	2,94	12
rec-Lhcb2	1,41	0,96	0,32	1,8	4,99	7,01	3,08	12
rec-Lhcb3	1,71	0,68	0,25	1,53	4,07	6,93	2,46	11
nat-Lhcb1,2 (mix)	1,40	1,00	0,20	1,80	5,00	7,00	3,00	12
nat-Lhcb3	1,76	0,62	0,16	1,80	3,99	7,01	2,57	11

Fig. 1. Absorption spectra of recombinant Lhcb1-3 holoproteins normalised at the Qy peak value. *Inset*, the red-shifted absorption of the Lhcb3 complex in the Qy region.

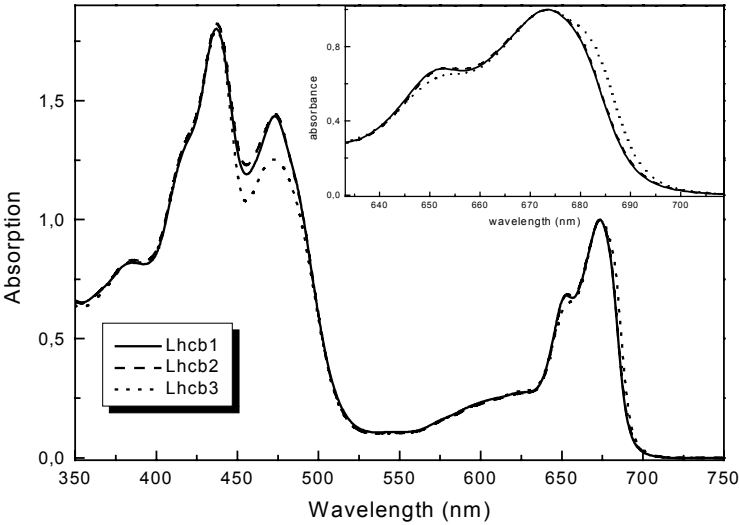


Fig. 2. The difference (Lhcb3 minus Lhcb1) absorption spectrum following normalisation according to the stoichiometry of bound chlorophyll. The positive contribution highlights the red-shifted Chl absorption form typical of Lhcb3 complex.

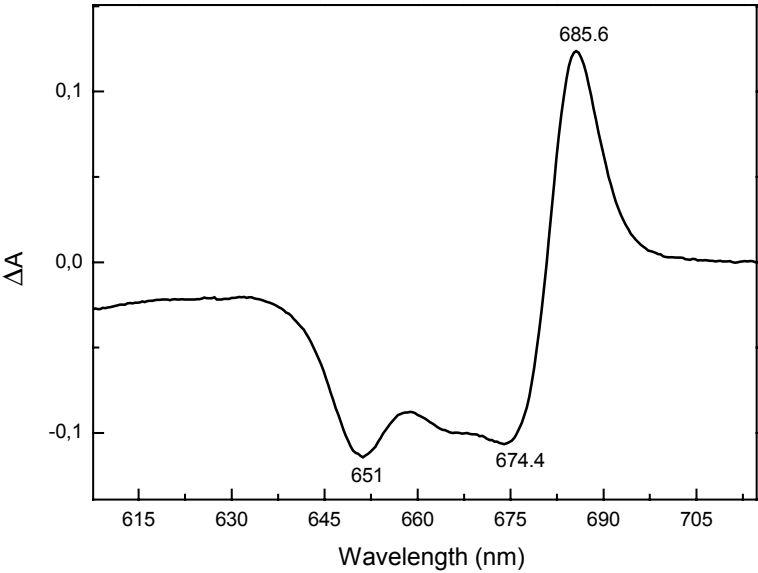


Fig. 3. Fluorescence emission spectra of Lhcb1-3 complexes (440 nm excitation). A red shift of the peak of the Lhcb3 emission is shown.

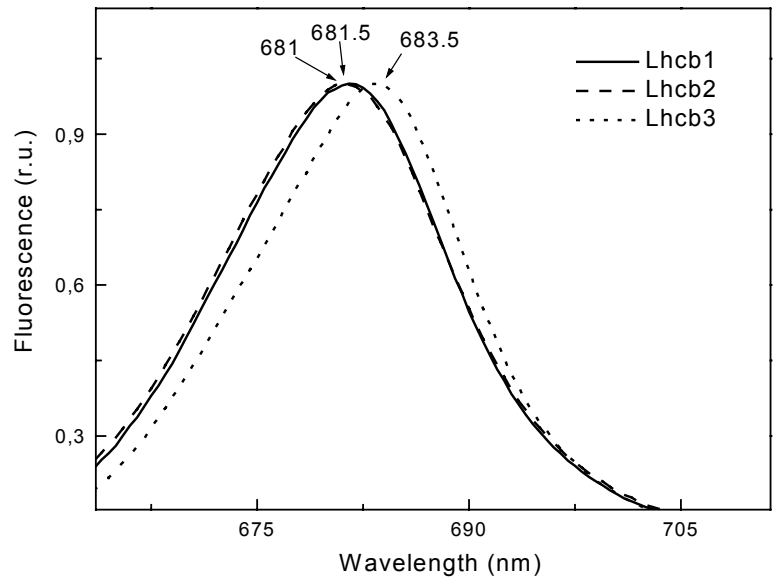


Fig. 4. LD spectra of Lhcb1 and Lhcb3 complexes. In the red region a strong LD signal is clearly visible corresponding to the the red-shifted Chl. In the Soret region, a signal around 488 nm, due to the neoxanthin molecule in N1 site, is missing with respect to Lhcb1.

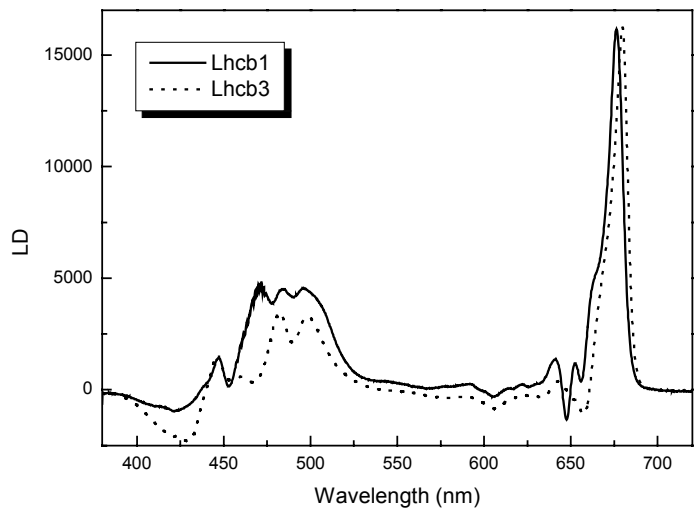


Fig. 5. Oligomerisation of Lhcb1-3 complexes is not the same. Lhcb1 and 2 complexes form homotrimers upon addition of lipid extract from thylakoids while Lhcb3 does not. Similar results are obtained with the native complexes. However, Lhcb3 can be purified in an heterotrimeric form with Lhcb1 and 2 (see text).

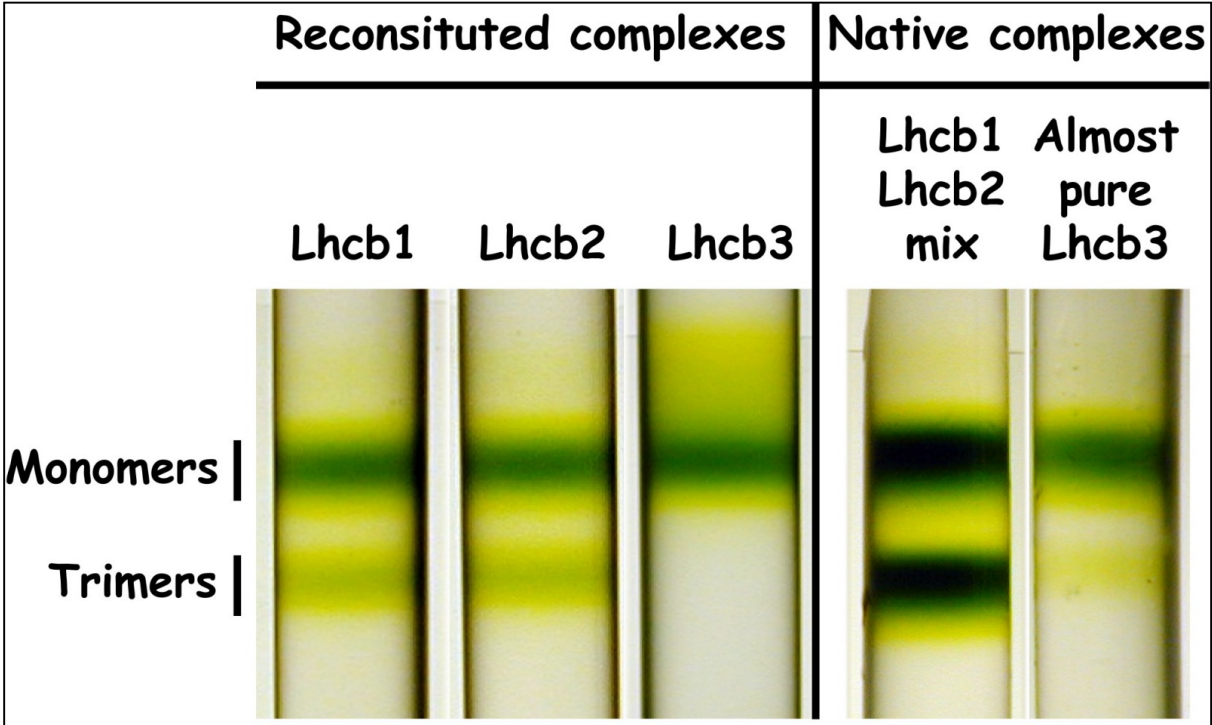


Fig. 6. Lhcb3 is found in an oligomeric form in the band 3 of a sucrose gradient of thylakoid membranes solubilised and it is particularly enriched in the band 4, a supercomplex containing LHCII-CP29-CP24. This results suggests a localisation of Lhcb3 at the interface between the peripheral LHCII trimers and the minor antenna proteins within the PSII structure and moreover the possibility that Lhcb3 needs a specific Lhcb1-2 partner for oligomerisation.

