Chlorina and *viridis* mutants of barley (*Hordeum vulgare* L.) allow assignment of long-wavelength chlorophyll forms to individual Lhca proteins of photosystem I in vivo

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Abstract The isolated subcomplex LHCI-730 of plant photosystem I (PSI) chlorophyll (Chl) *a/b* binding antenna is a heterodimer of Lhca1 and Lhca4 and has a 77 K fluorescence emission peak at 730 nm (F730). Recently, three Chl spectral forms with 77 K fluorescence emission peaks at 720 nm, 730 nm and 742 nm were identified in native PSI. In an attempt to assign the two longest wavelength emission maxima to peripheral PSI antenna proteins, we performed 77 K fluorescence emission spectroscopy on intact leaves of *chlorina* and *viridis* mutants from barley which lack individual LHCI-730 proteins. This approach indicates that F732 is found only in Lhca1 and F742 only in Lhca4, when these proteins are associated with the PSI reaction centre.

Key words: *Chlorina*; Long-wavelength chlorophyll; Photosystem I; *Viridis*; *Hordeum*

1. Introduction

The 77 K fluorescence emission spectrum obtained from intact photosystem (PS) I shows a characteristic long-wavelength band at 735 nm (F735) [1–3]. Removal of peripheral chlorophyll (Chl) *a/b* binding proteins (LHCI) leads to PSI reaction centre complexes that exhibit 77 K emission of core antenna Chl at 720 nm (F720) [1–3]. The peripheral LHCI antenna can be isolated and was shown to fluoresce at 730 nm at 77 K [4]. LHCI could be fractionated into two subcomplexes with different fluorescence characteristics [2,5,6]. In a more recent characterisation, LHCI in barley could be separated into three subcomplexes providing a nomenclature based on the 77 K fluorescence emission of each subcomplex as follows [3]. The LHCI-730 complex was a heterodimer composed of a 21-kDa (Lhca4) and a 22-kDa protein (Lhca1). Each of the LHCI-680 complexes had one type of apoprotein. LHCI-680A consisted of the 25-kDa Lhca3, LHCI-680B of the 23-kDa Lhca2 protein. Electron microscopy, chemical cross-linking of plant PSI and pigment stoichiometries used to obtain structural information revealed that the subunits associate independently with the reaction centre proteins PsaA/B, that two of each of the four Lhca proteins bind to PsaA/B, that they are organised as dimers, and that they surround the reaction centre forming a monolayer [7–9].

In cyanobacterial PSI lacking LHCI proteins, F720 emission could be attributed to a small number of Chl *a* molecules that connect the bulk of core antenna Chl through excitation energy transfer to P700 and which may act as a sink to focus excitons near P700 [10,11]. The origin of F735 in plants is LHCI [1–3,5,12] and time-resolved fluorescence relaxation measurements on isolated LHCI [13,14] and femtosecond absorption spectroscopy on LHCI and two of its subcomplexes, LHCI-730 and LHCI-680, were undertaken [15] to derive models for energy transfer events in the complexes.

Two physiological functions for F735 are discussed. Stahl et al. [16] argued that the pericentral long-wavelength Chl is not essential for excitation energy transfer, but has a protective role against over-excitation. Trissl [17], on the other hand, showed that PSI can afford antenna pigments absorbing at lower energy than P700 and thereby increase its absorption cross-section.

Despite the great interest in the long-wavelength chlorophylls responsible for F735, the antenna proteins harbouring this special Chl form have not been determined unambiguously. Antenna subfractions isolated from spinach PSI using anion exchange perfusion chromatography led to the localisation of a Chl *b* pool responsible for an enhancement of F730 on Lhca4 [12], consistent with the conclusion of Mukerji and Sauer [13,14] that Chl *b* must be specifically associated with emission around 730 nm at 77 K. The authors concluded that the origin of this long-wavelength emission is Lhca4 or that it emanates from an interaction between Lhca1 and Lhca4 subunits [12]. In vitro reconstitution experiments showed that Lhca1 monomers fluoresce around 685–687 nm, whereas Lhca4 monomers had a 77 K fluorescence maximum at 730–732 nm [18]. Thus, in vitro the Lhca4 monomer has a long-wavelength fluorescence peak similar to the *isolated* LHCI-730 heterodimer. The latter experiments clearly showed that LHCI-730 is a heterodimer consisting of Lhca1 and 4 and, most remarkably, changes in the organisation of pigments, i.e. assembly of additional Chl *b*, could be observed upon heterodimerisation [18].

Very recently, Croce et al. [19] identified *three* Chl spectral forms in native PSI from maize thylakoids with 77 K fluorescence emission maxima at 720, 730 and 742 nm. In the present report, the combination of in vivo 77 K fluorescence emission measurements on leaves of *chlorina* and *viridis* mutants of barley with immunological analyses that reveal the lack of individual Lhca antenna proteins, allows us to assign all three emission maxima to individual pigment proteins. We show that the two emission peaks at 732 nm and 742 nm emanate

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Abbreviations: Chl, chlorophyll; clo, *chlorina*; LHC/LHCP, light-harvesting chlorophyll *a/b* protein; Lhca, light-harvesting chlorophyll *a/b* protein of photosystem I; Lhcb, light-harvesting chlorophyll *a/b* protein of photosystem II; LHCII, light-harvesting chlorophyll *a/b* protein II; PS, photosystem; Psa, photosystem I subunit; vir, *viridis*; WT, wild type

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from Lhca1 and Lhca4, respectively, when these LHCI-730 proteins are bound to the reaction centre of PSI and, thus, represent a remarkable example of the influence of protein environment on spectral properties of Chl.

2. Materials and methods

2.1. Plant material

Chlorina (clo-$f2^{f2}$, clo-$f2^{101}$, clo-a^{126}, clo-b^{125}) and *viridis* mutants (vir-k^{23}, vir-s^{44}, vir-zb^{63}) of barley (*Hordeum vulgare* L.) from the Carlsberg mutant collection (Department of Physiology, Carlsberg Laboratory, Copenhagen, Denmark) were grown at 22°C and a light intensity of 75 μmol photons/m^2/s in a 14-h light/10-h dark cycle for 7–8 days [20,21].

2.2. Isolation of thylakoid membranes and immunoblot assays

Intact chloroplasts of *chlorina* and *viridis* mutants were isolated according to Nielsen et al. [22], subsequently broken and the thylakoid membrane proteins applied to denaturing SDS-PAGE on 16% gels [23]. Proteins were transferred to a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA) with a Mini-Trans-Blot-Transfer Cell (Bio-Rad, Munich, Germany) at 20 V for 12 h.

Polyclonal monospecific antibodies against the proteins Lhca1, Lhca3, and Lhca4 [24], and a monoclonal antibody detecting Lhca2 and Lhcb4 (CP29) [25] were used.

2.3. 77 K fluorescence emission spectroscopy

Low-temperature emission spectra were obtained after excitation at 435 nm of whole leaves with a Hitachi F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Spectra were not corrected for light source or photomultiplier response.

3. Results and discussion

3.1. Barley mutants lack individual proteins of the peripheral photosystem I antenna

Among the barley *chlorina-f2* alleles, clo-$f2^{f2}$, clo-$f2^{101}$ and clo-$f2^{108}$ are free of Chl *b* [21]. Using monospecific antibodies against all plant light-harvesting proteins, Bossmann et al. [26] identified the individual proteins affected by Chl *b* deficiency and showed that only three out of 10 antenna proteins fail to accumulate in the absence of Chl *b* in these alleles. Fortunately, only one out of the four Chl *a/b* proteins of PSI, Lhca4, was missing, although Lhca1, Lhca2 and Lhca3 were present in wild-type amounts and, thus, seemed to be fully stable in the absence of Chl *b*. Another group of clo mutants had unusual 77 K fluorescence properties (high F685/F742 ratio) which could be due to the absence of PSI antenna proteins [21]. Of this group, the allelic clo-a^{117}, clo-a^{126} and clo-a^{134} lacked Lhca1 and had only traces of Lhca4, while in clo-b^{125} only traces of Lhca1 and Lhca4 were detectable, whereas the levels of the LHCI-680 proteins Lhca2 and Lhca3 were only slightly reduced compared with barley wild-type (WT) (Fig. 1A–D). In this group of mutants Chl *b* is only slightly depleted, and there is no obvious depletion of

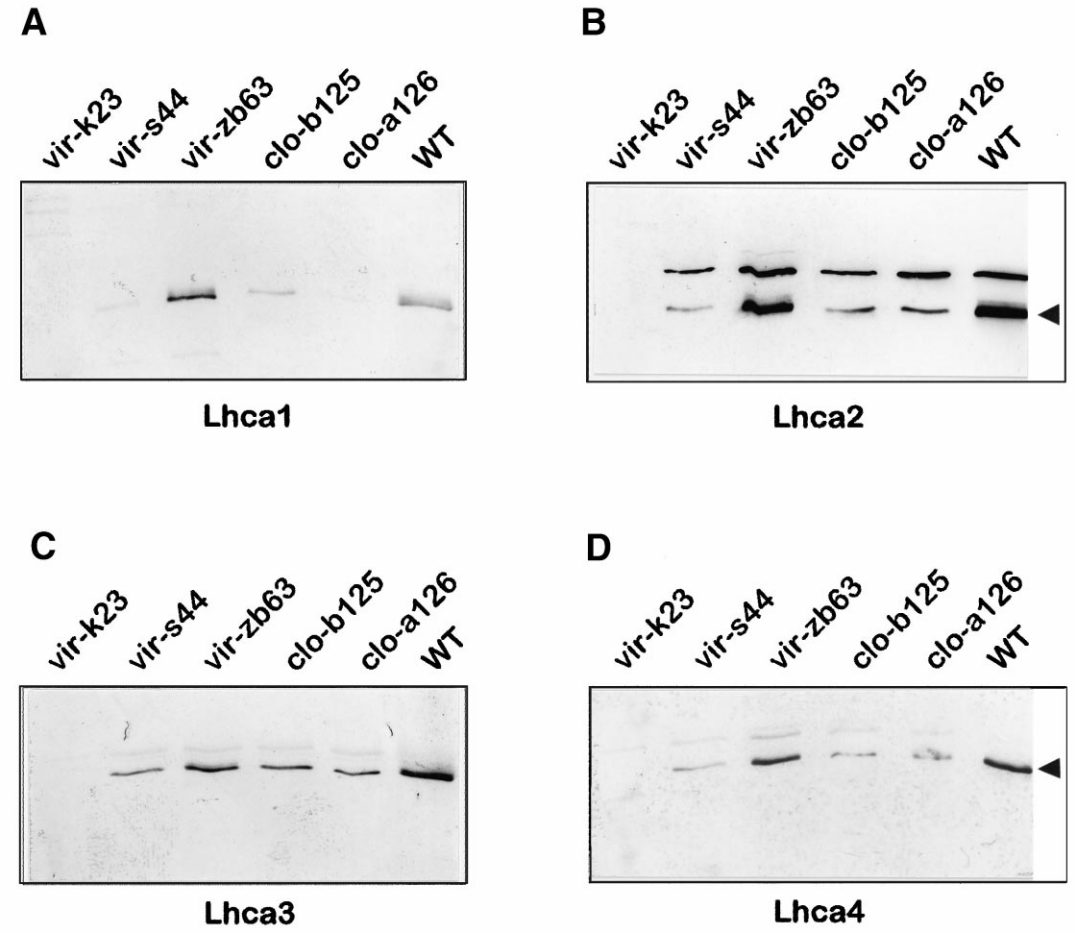

Fig. 1. A–D: Immunoblot analysis. Thylakoid membranes were isolated from barley wild-type (WT), the chlorophyll *b*-deficient *chlorina-a*^{126} (clo-a^{126}), clo-b^{125}, and the *viridis-k*^{23} (vir-k^{23}), vir-s^{44} and vir-zb^{63}. 6 μg of protein was loaded onto each track, separated by 16% SDS-PAGE and blotted onto PVDF membrane. Proteins were detected with monospecific antibodies against Lhca1, Lhca2, Lhca3 and Lhca4.

any other pigment [21]. All four Lhca proteins were found in WT amounts in vir-zb^{63} (Fig. 1A–D), which is almost devoid of PSI core polypeptides [27]. On the other hand, vir-k^{23} lacks all Lhca proteins (Fig. 1A–D). Vir-s^{44} has slightly reduced levels of Lhca2 and Lhca3, but lacks Lhca1 and has only trace amounts of Lhca4.

Thus, to analyse 77 K long-wavelength fluorescence characteristics of the LHCI-730 heterodimer, we have clo-*f*2 mutants lacking Lhca4, clo-*a* mutants and vir-s^{44} lacking Lhca1 and having trace amounts of Lhca4, and clo-*b* with trace amounts of Lhca1 and 4. In all these mutants, the levels of Lhca2 and Lhca3 are almost unaffected. In addition, one *viridis* mutant, vir-k^{23}, is devoid of all PSI antenna proteins whereas in vir-zb^{63}, PSI reaction centre proteins are reduced to 1–5% of WT levels [27].

3.2. Low-temperature fluorescence emission measurements on intact mutant leaves

The 77 K fluorescence emission spectra obtained from intact leaves after excitation with 435 nm and normalised to 680 nm emission are shown in Fig. 2A,B. F742 can be assigned to Lhca4 on the following basis. In barley WT, F742 is present but in clo-$f2^{101}$ which lacks only Lhca4, there is a blue shift of the long-wavelength fluorescence emission from 742 nm to 732 nm (Fig. 2A). Similarly, we deduce that F732 originates from Lhca1. Clo-$f2^{101}$ accumulates WT levels of Lhca1 in the absence of the second LHCI-730 protein Lhca4 and fluoresces at 732 nm (Fig. 2A). The blue shift is not a consequence of Chl *b* depletion in the clo-*f*2 mutant. Recent studies using an *Arabidopsis* antisense mutant lacking Lhca4 show a shift in 77 K fluorescence emission from 728 nm to 722 nm [28].

In clo-b^{125}, clo-a^{126} and vir-s^{44}, where LHCI-730 proteins are almost absent, a drastic reduction of long-wavelength fluorescence emission is observed (Fig. 2B). Interestingly, the emission spectrum obtained from leaves of vir-s^{44} shows a double maximum at 721 nm and 735 nm indicating that the different Chl forms exist in vivo. And finally, as expected, F720 can be attributed to Chl of the PsaA/B reaction centre in the LHCI-depleted vir-k^{23}.

3.3. Interactions between Lhca1, Lhca4 and reaction centre determine long-wavelength characteristics

The 77 K fluorescence emission of vir-zb^{63} leads us to conclude that pigment-pigment and pigment-protein interactions between the LHCI-730 antenna proteins and the reaction centre determine the spectroscopic properties of the long-wavelength pigments. Vir-zb^{63} contains all Lhca protein in WT amounts, but in the absence of PSI core polypeptides the 77 K emission maximum is at 732 nm (Fig. 2A). This resembles the 77 K long-wavelength emission of in vitro reconstituted Lhca4 [18] and of isolated Lhca1/4 heterodimer [3]. When associated with the reaction centre, Lhca4 shows a 77 K emission at 742 nm, as can be seen from the comparison of the fluorescence spectra from intact barley WT leaves with those from clo-*f*2. Thus, in an isolated form, Lhca4 certainly binds the longest wavelength emitting chlorophylls of all the Lhca proteins. But it seems that binding to the reaction centre rather than heterodimerisation leads to a further red shift from 732 nm to 742 nm. Isolated Lhca1 organises Chl which fluoresces around 685–687 nm at 77 K [18]. A 77 K fluorescence at 732 nm can be attributed to this protein bound to the PSI reaction centre, as can be seen from the spectrum in which clo-*f*2 leaves lacking Lhca4 were used. Consistent with this, in vitro reconstitutions using recombinant tomato Lhca1 showed that its protein environment, especially homodimerisation, had pronounced effects on the spectral properties of ligated pigments [18]. However, it is not known whether Lhca1 monomers or homodimers bind to the PSI reaction centre in clo-*f*2.

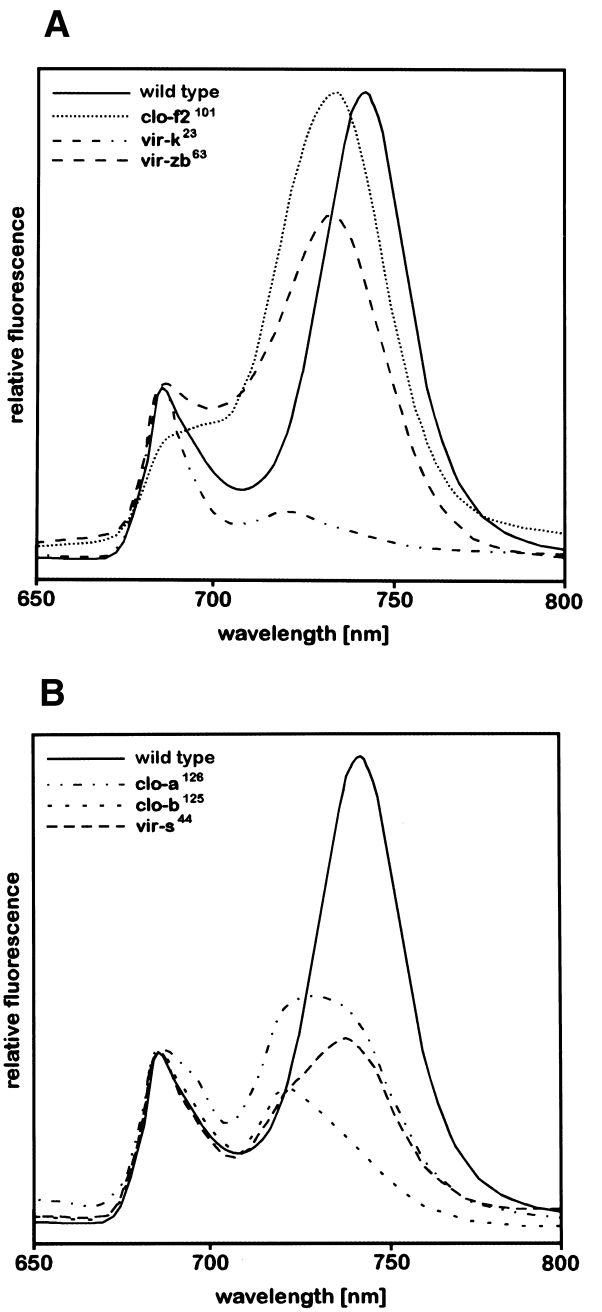

Fig. 2. A: 77 K fluorescence emission spectra upon 435 nm excitation of whole leaves of barley wild-type (WT), clo-$f2^{101}$ lacking Lhca4, vir-k^{23} lacking all four Lhca proteins, and vir-zb^{63} with full Lhca antenna protein complement, but almost totally devoid of PSI reaction centres. B: 77 K fluorescence emission spectra upon 435 nm excitation of whole leaves of barley wild-type (WT), clo-a^{126} and vir-s^{44} both lacking Lhca1 and having trace amonts of Lhca4, and clo-b^{125} with trace amounts of Lhca1 and Lhca4.

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