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Protochlorophyllide photo-transformation and chlorophyll(ide) formation in barley etioplast fractions

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Abstract

Localization of protochlorophyll(ide) (Pchlide) forms and chlorophyllide (Chlide) transformation process were studied by using comparative analyses of de-convoluted 77 K fluorescence spectra of barley etioplast stroma and different membrane fractions obtained by sucrose gradient centrifugation. Non-photoactive 633 nm Pchlide form was mainly located in the envelope-prothylakoid membrane mixture while the photoactive 657 nm Pchlide was dominant pigment in the prolamellar body membrane and in the soluble etioplast fraction (stroma). When these fractions were exposed to a saturating flash, conversion of photoactive Pchlide into 697 nm Chlide was preferential in the prolamellar body and in the stroma, while the 676 nm Chlide was dominant pigment form in the envelope-prothylakoid fraction. These spectral characteristics are considered to reflect molecular composition and organization of the pigment-protein complexes specific for each etioplast compartment.

Additional key words: fluorescence emission spectra; *Hordeum*; NADPH:protochlorophyllide oxidoreductase.

Introduction

Etioplast is an organelle in leaves of dark-grown angiosperm plants which accumulates protochlorophyllide (Pchlide) serving as precursor for chlorophyll (Chl) synthesis when plants are irradiated (Klein and Schiff 1972, Kolossov and Rebeiz 2003). Pchlide in etioplasts is associated with the photoenzyme NADPH:protochlorophyllide oxidoreductase (POR) and cosubstrate NADPH forming photoactive ternary complex (Apel *et al.* 1980, Oliver and Griffiths 1980). Two barley POR isoforms have been identified, PORA as predominant form in etiolated seedlings and PORB in green leaves (Holtorf *et al.* 1995). The aggregation state of the ternary complex and the redox state of the co-substrate were suggested to affect spectral properties of Pchlide whereas POR isoform appears not to have an effect on the Pchlide spectra (reviewed by Schoefs and Franck 2003). In etiolated leaves, two photoactive Pchlide forms are indicated by two fluorescence emission bands at 645 nm ($Pchlide_{645}$) and 657 nm ($Pchlide_{657}$) (Böddi *et al.* 1989). Non-photoactive Pchl(ide) is indicated by the fluorescence emission band at 633 nm which remains unchanged when etiolated leaves are exposed to a short saturating flash (Granick and Gassman 1970).

Exposure of etiolated leaves to a short saturating flash leads to photo-conversion of photoactive Pchlides into different Chlide spectral forms (reviewed by Sundqvist and Dahlin 1997). Primary Chlide form, $Chlide_{690}$, is considered to represent Chlide-POR-$NADP^+$ complex (Oliver and Griffiths 1982). This complex has been suggested to serve as a precursor of both $Chlide_{695}$ and $Chl(ide)_{676}$ appearing within seconds following the flash (Mathis and Sauer 1973). The $Chl(ide)_{676}$ is a stable protein-unbound pigment, whereas for $Chlide_{695}$ it has been suggested to represent aggregated Chlide-POR-NADPH ternary complex undergoing further transformation into $Chlide_{682}$ (Litvin and Belyaeva 1971, Franck *et al.* 1999). This transformation is indicated by the blue spectral shift, called the Shibata-shift (Shibata 1957). Subsequently, Chlide is released from POR and esterified to Chl which serves for structural and functional assembly of the photosynthetic apparatus (Kim *et al.* 1994, Rassadina *et al.* 2004). When Chlide is released from the catalytic site,

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Abbreviations: Chl – chlorophyll; Chlide – chlorophyllide; Pchlide – protochlorophyllide; PLB – prolamellar body; POR – protochlorophyllide-oxidoreductase; PS2 – photosystem 2; PT – prothylakoid.
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POR enzyme is reused for regeneration of photoactive Pchlide-POR-NADPH complex. This pattern of Chlide transformation and Pchlide regeneration is considered to represent a part of continuous process in etiolated leaves exposed to irradiation (Sironval 1981). Chlide transformation and Pchlide regeneration processes may occur under *in vitro* conditions in either intact etioplasts or isolated etioplast inner membranes (El Hamouri *et al.* 1981, Ryberg and Sundqvist 1988, Klement *et al.* 2000).

Both photoactive $Pchlide_{657}$ and non-photoactive $Pchl(ide)_{633}$ are localized in the inner etioplast membrane fraction where $Pchlide_{657}$ is mainly situated in the prolamellar body (PLB) and $Pchl(ide)_{633}$ in prothylakoids (PT) (Ryberg and Sundqvist 1982a). Localization of Pchlide and POR in the chloroplast envelope and thylakoid membrane has been reported by Joyard *et al.* (1990) and Barthélemy *et al.* (2000). This multiple localization of POR and Pchlide raises questions on structural and functional properties of POR-pigment complexes located in different plastid compartments. The Chlide transformation process in PLB and PT fractionated from wheat etioplasts has been investigated earlier (Ryberg and Sundqvist 1982a,b). In this study, by using a de-convolution method, we further investigated Pchlide and Chl(ide) spectral forms occurring during Chlide transformation process in PLB, PT, and stroma fractions of barley etioplast. In order to reveal the dependency between specific Chlide forms and different plastid compartments, we isolated etioplast fractions on a sucrose gradient, containing native Pchlide-POR complexes, which have been exposed to a short flash. De-convolution of 77 K fluorescence spectra was used to identify different Pchl(ide) and Chl(ide) forms.

Materials and methods

Plants: Barley (*Hordeum vulgare* var. Chapais) seedlings were grown in the dark at 24 °C for 6 d on vermiculite moistured with tap water.

Isolation and fractionation of intact etioplasts was done by the modified method of Cline *et al.* (1981): Leaf tissue of 150 g was homogenized by using blender at low speed (two 5 s bursts) in 1 500 cm^3 of 25 mM HEPES-NaOH, pH 7.5 buffer containing 0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM aminocaproic acid, and 1 mM benzamidine. The homogenate was filtered through two layers of *Miracloth* (*Calbiochem*) and then centrifuged at 3 500 rpm for 10 min using *Beckman JA 14* fixed angle rotor. The obtained crude etioplast pellet was re-suspended in 6 cm^3 of the homogenization medium. The etioplast suspension adjusted to 25 % (v/v) *Percoll* was layered on 40 % (v/v) *Percoll* cushion and intact etioplasts were sedimented by centrifugation at 6 500 rpm using *Beckman JA 20* fixed angle rotor. Etioplasts were washed twice with 10 cm^3 of the homogenization buffer and then recovered by centrifugation at 3 500 rpm for 5 min. The washed etioplast pellet was re-suspended in 0.12 cm^3 of the homogenization buffer and lysed during 15 min with 1.4 cm^3 of 25 mM HEPES-NaOH, pH 7.5 buffer containing 1 mM $MgCl_2$, 1 mM EDTA, 5 mM aminocaproic acid, 1 mM benzamidine, and 0.3 mM NADPH. During lysis procedure NADPH was employed to preserve native state of membrane-associated Pchlide-POR complexes. The lysate was adjusted to 0.26 M sucrose and then placed on a sucrose step gradient made of equal volumes (3 cm^3) of 1.20, 1.00, and 0.46 M sucrose in the lysis buffer. The gradient was then centrifuged at 38 000 rpm in the *SW41* swinging-bucket rotor for one hour in order to separate etioplast fractions. Fractions of 1 cm^3 were finally collected from the sucrose gradient and stored at –80 °C. All the manipulations were performed at 4 °C. During the fractionation, stroma remained at the top of the gradient (fractions 1 and 2) while the membrane material migrated to different positions along the gradient (see Fig. 1*A*). Low-temperature fluorescence spectra revealed three Pchlide-containing bands corresponding to fractions 2, 5, and 9. The fractions 5 and 9 may be considered as envelope-PT mixture- and PLB-containing fraction, respectively, based on buoyant densities of etioplast PLB and PT membranes and chloroplast envelope membrane (Cline *et al.* 1981, Ryberg and Sundqvist 1982b). Therefore, we refer to the fractions 2, 5, and 9 as stroma, envelope-PT, and PLB fractions, respectively.

Fluorescence spectroscopy at 77 K: Aliquot (100 mm^3) of each sucrose gradient fraction was introduced into glass tube of 2 mm internal diameter and frozen at 77 K. Recordings of spectra were done between 600 and 780 nm by using a spectrofluorometer (*LS 50-B*, *Perkin Elmer*, Norwalk, USA) equipped with a low temperature attachment. In order to minimize the effect of stray radiation, a low-pass green filter (λ<580 nm, model *59070*, *Oriel*) was placed in front of the excitation window and a high-pass filter (λ>600 nm, model *59512*, *Oriel*) was used in front of the emitting window. The excitation wavelength was set at 440 nm and both excitation and emission slits were 5 nm. Samples showing fluorescence of Pchlide (aliquots of fractions 2, 5, and 9) were first allowed to warm up to 253 K by keeping them for 1 min at ambient temperature and then exposed to a 20 ms saturating flash in order to trigger photoactive Pchlide transformation into Chlide. The samples were allowed to gradually warm up to room temperature (20 °C) during the course of experiment. Their low temperature fluorescence spectra were recorded after 30 s, 2, 15, and 30 min dark-incubation, that followed the exposure of etioplast fractions to the flash. Spectra were corrected concerning the baseline and the wavelength-

dependent sensitivity of the fluorometer. Spectra were normalized as indicated.

Spectrum analysis: The fluorescence spectra were de-convoluted into Gaussian components by using the *Microcal Origin 6.0*® peak fitting module (*OriginLab*). The used starting parameters of the components were according to Böddi *et al.* (1993) and Böddi and Franck (1997). Results obtained by de-convolution procedure were used only when the difference between the sum of the Gaussian components and the experimental spectra did not exceed 1 %.

SDS-PAGE and Western blot analysis: All fractions of the sucrose gradient were analyzed by SDS-PAGE using 10 to 20 % polyacrylamide precast gels (*Bio-Rad*) (Laemmli 1970). After electrophoresis the gels were either silver stained or blotted to a PVDF membrane (*Roche*). Immunodetection was carried out by using antibodies raised against barley POR (Barthélemy *et al.* 2000) and alkaline phosphatase-conjugated secondary antibodies (*ECL*, *Amersham*).

Results and discussion

Distribution of Pchlide and POR in etioplasts of dark-grown (etiolated) barley leaves: Etioplast fractions' stroma, envelope-PT, and PLB were separated by sucrose gradient ultracentrifugation. Identification of these fractions was based on their buoyant densities (see Materials and methods; Fig. 1*A*). Low-temperature fluorescence spectra revealed the presence of Pchl(ide) in all these etioplast compartments (Fig. 1*B*). However, the presence of Pchl(ide) spectral forms in the separated fractions was different. Photoactive Pchlide emitting at around 657 nm was major Pchlide form in the stroma (fraction 2; 0.26/0.46 M sucrose interface) and the PLB (fraction 9; 1.2/1.0 M sucrose interface) whereas non-photoactive Pchl(ide) 633 was a dominant pigment form in the envelope-PT mixture (fraction 5; 0.46/1.00 M sucrose interface). Our results concerning PLB and PT are in agreement with previously reported data showing preferential accumulation of $Pchlide_{657}$ in PLBs and $Pchl(ide)_{633}$ in PTs and envelope membrane isolated from wheat etioplasts and pea chloroplasts, respectively (Ryberg and Sundqvist 1982a, Pineau *et al.* 1986). To our knowledge, the predominant presence of $Pchlide_{657}$ in the stroma of etioplasts is reported here for the first time. This finding may indicate the presence of a soluble and highly aggregated Pchlide-POR-NADPH complex in etioplasts. One may consider that the stroma-located Pchlide-POR complex is a result of its accidental release from PLB sub-membrane fraction during etioplast lysis procedure. However, this release is prevented due to the presence of NADPH in the isolation media and also the use of only osmotic shock as mild treatment to rupture etioplasts. Similar conditions were used earlier to preserve association of POR with sub-membrane fractions of etioplast (Ryberg and Sundqvist 1988).

In the protein profiles of stroma, envelope-PT, and PLB fractions the band of 36 kDa was identified as POR protein by using POR-antibody (Fig. 2). In this study POR was considered as the sum of PORA and PORB isoforms which exist in barley (Holtorf *et al.* 1995). It is reasonable to believe that the differences in the distribution of PORA and PORB in etioplast compartments (Reinbothe *et al.* 2003) would not cause difficulties for interpretation of POR-pigment complex fluorescence spectra since these isoforms form complexes having very similar spectral characteristics (Franck *et al.* 2000). Contribution of POR to total protein content was the highest for the PLB, while in the envelope-PT and the stroma fraction it was 45 and 94 % lower, respectively (Fig. 2*B*). Wheat and squash etiolated seedlings have majority of POR located in PLB but much less in PT (Ikeuchi and Murakami 1983, Lindsten *et al.* 1988). Previous findings also indicated the presence of the envelope membrane-bound POR in (etio)chloroplasts of barley and in mature chloroplasts of pea (Joyard *et al.* 1990, Barthélemy *et al.* 2000). Therefore, high amount of POR protein that we found in the envelope-PT mixture of etioplast suggested that POR was a highly abundant protein in the etioplast envelope membrane system.

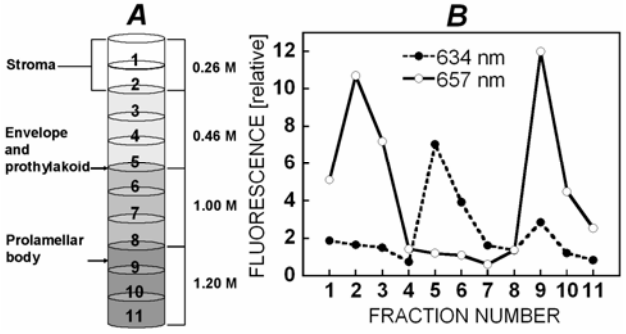

Fig. 1. (*A*): Sucrose gradient column used for the separation of etioplast fractions. Steps of sucrose concentration are indicated on the right and the positions of separated etioplast fractions on the left side. Numbers 1–11 indicate the collected and analyzed sucrose gradient fractions (see Materials and methods for further details). (*B*): Distribution of non-photoactive $Pchl(ide)_{633}$ and photoactive $Pchlide_{657}$ are represented by the fluorescence emission intensities at 634 and 657 nm, respectively.

Fluorescence spectra of etioplast fractions exposed to short irradiation: The non-irradiated stroma, envelope-PT, and PLB fractions contained two main emission bands, one at 634–636 nm and another at 655–658 nm which correspond to non-photoactive $Pchl(ide)_{633}$ and photoactive $Pchlide_{657}$ forms (Fig. 3*A*), as similarly seen earlier in wheat etioplasts by Ryberg and Sundqvist

(1982a). However, we may assume that bands after 670 nm represent vibrational bands of these Pchlide forms according to previous interpretations (Böddi *et al.* 1992). Some recent evidence showed for this spectral region to indicate also some far-red Pchlide forms (Stadnichuk *et al.* 2005).

After a 20-ms saturating flash-irradiation followed by dark-incubation for 30 s, stroma, envelope-PT, and PLB fractions showed the presence of bands at 692, 693, and 695 nm, respectively, representing newly formed Chlide (Fig. 3*B*, spectra 1a, 2a, 3a). Same bands have been identified also within one second after the flash (results not shown). Similar spectral bands have been found earlier in etiolated leaves and it was considered to represent highly aggregated POR-Chlide-NADP$^+$ ternary complex (Oliver and Griffiths 1982, Franck *et al.* 1999). Slight differences between spectral maxima of the etioplast fractions may be interpreted as caused by different concentrations of sucrose in the surrounding medium. Similar effect of sucrose was found earlier for PLB membrane isolated from wheat leaves (Zhong *et al.* 1996).

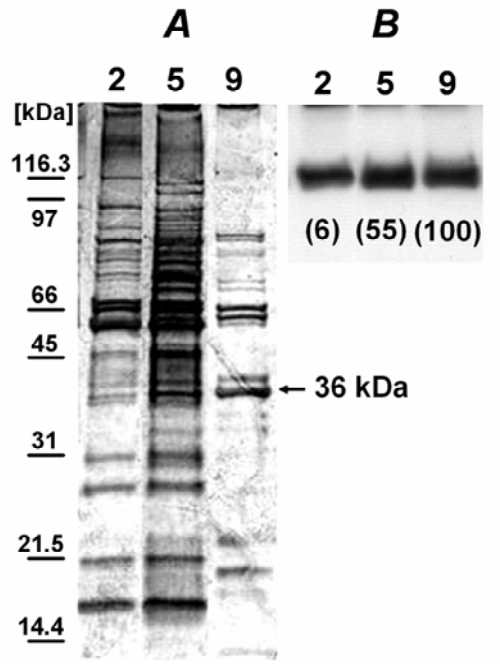

Fig. 2. (*A*) Protein profiles of gradient fractions 2 (stroma), 5 (envelope-PT), and 9 (PLB). Molecular mass standards (broad range, *Bio-Rad*) [kDa] are indicated by their molecular masses (*left*) and a 36 kDa polypeptide (*right*). Equal amount of proteins (0.5 μg per lane) from each fraction was separated by SDS-PAGE and silver-stained. (*B*) Western blot analysis of the stroma (fraction 2) and membrane fractions: envelope-PT (5) and PLB (9). The membrane fractions were washed with 3 volumes of the lysis buffer, pelleted by centrifugation at 100 000×*g*, and re-suspended in the sample loading buffer. Unequal amounts of proteins (5.0, 0.6, and 0.3 μg for stroma, envelope-PT, and PLB, respectively) were loaded per lane to enable densitometric evaluation of their POR band intensities. These intensities (shown in parentheses) were expressed per μg of total protein and then normalized by POR band intensity specific for PLB considering this intensity as 100 %.

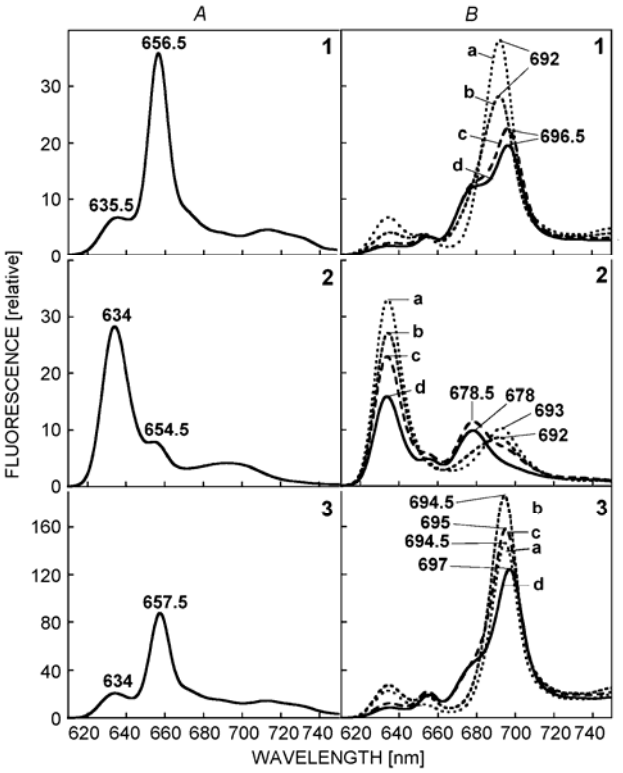

Fig. 3. Irradiation-induced modifications of 77 K fluorescence spectra of (1) stroma, (2) envelope-PT, and (3) PLB etioplast fractions. *A*: Spectra of non-irradiated etioplast fractions. *B*: Spectra of etioplast fractions irradiated by a short saturating flash (20 ms) and incubated in the dark for (a) 30 s, (b) 2 min, (c) 15 min, and (d) 30 min. The spectra have been normalized on reflected stray radiation at 607 nm. For more details, see Materials and methods.

Spectra recorded two min following the flash indicated slight differences between stroma, envelope-PT, and PLB fractions. Envelope-PT showed a broadening and a slight blue-shift giving the band at 692 nm (Fig. 3*B*, spectrum 2b). At the same time, the stroma fraction showed less pronounced broadening while the PLB fraction did not undergo any substantial spectral change (Fig. 3*B*, spectra 1b and 3b). However, at 15 min following the flash more important changes of Chlide spectra were noticed. In the envelope/PT membrane, these changes were manifested by the blue-shift from 692 to 679 nm and by the formation of a long-wavelength shoulder at around 695 nm (Fig. 3*B*, spectrum 2c). In the stroma, a short-wavelength shoulder at around 680 nm appeared concomitantly with a red-shift from 692 to 697 nm (Fig. 3*B*, spectrum 1c). However, the change in the PLB fraction was manifested only by a formation of a shoulder at around 680 nm (Fig. 3*B*, spectrum 3c). At 30 min, the Chlide emission maximum of the PLB fraction shifted to 697 nm while the shoulder at 695 nm of

the envelope-PT fraction disappeared (Fig. 3*B*, spectra 3d and 2d). We noticed for the fluorescence emission spectra described above for PLB and envelope-PT fractions under *in vitro* conditions to be similar to those reported for PLB and PT fractions obtained from etiolated wheat leaves being continuously irradiated (Lindsten *et al.* 1993).

Etiolated wheat leaf shows red shift from 690 to 695 nm after a short irradiation. This shift may reflect the formation of highly aggregated POR-Pchlide-NADPH complex within seconds following the flash (Franck *et al.* 1999). However, the corresponding red shift in the stroma and the PLB that we found occurred with a delay of several minutes. This delay is caused by the low-temperature effect when stroma and PLB were incubated in the dark similarly as we reported earlier for etiolated barley leaf (Eullaffroy *et al.* 1995). However, differences in the rate of the red shift between PLB and stroma fractions were assumed to result from the effect of specific immediate environments on the Pchlide-POR complexes. Regeneration of photoactive $Pchlide_{655}$ at the expense of non-photoactive one was observed in all etioplast fractions. This process was indicated by the increase of the $Pchl(ide)_{655/633}$ ratio (see Fig. 3, spectral region 620–660 nm). These regeneration processes are comparable to those found *in vivo* for etiolated barley leaves (Böddi *et al.* 2003).

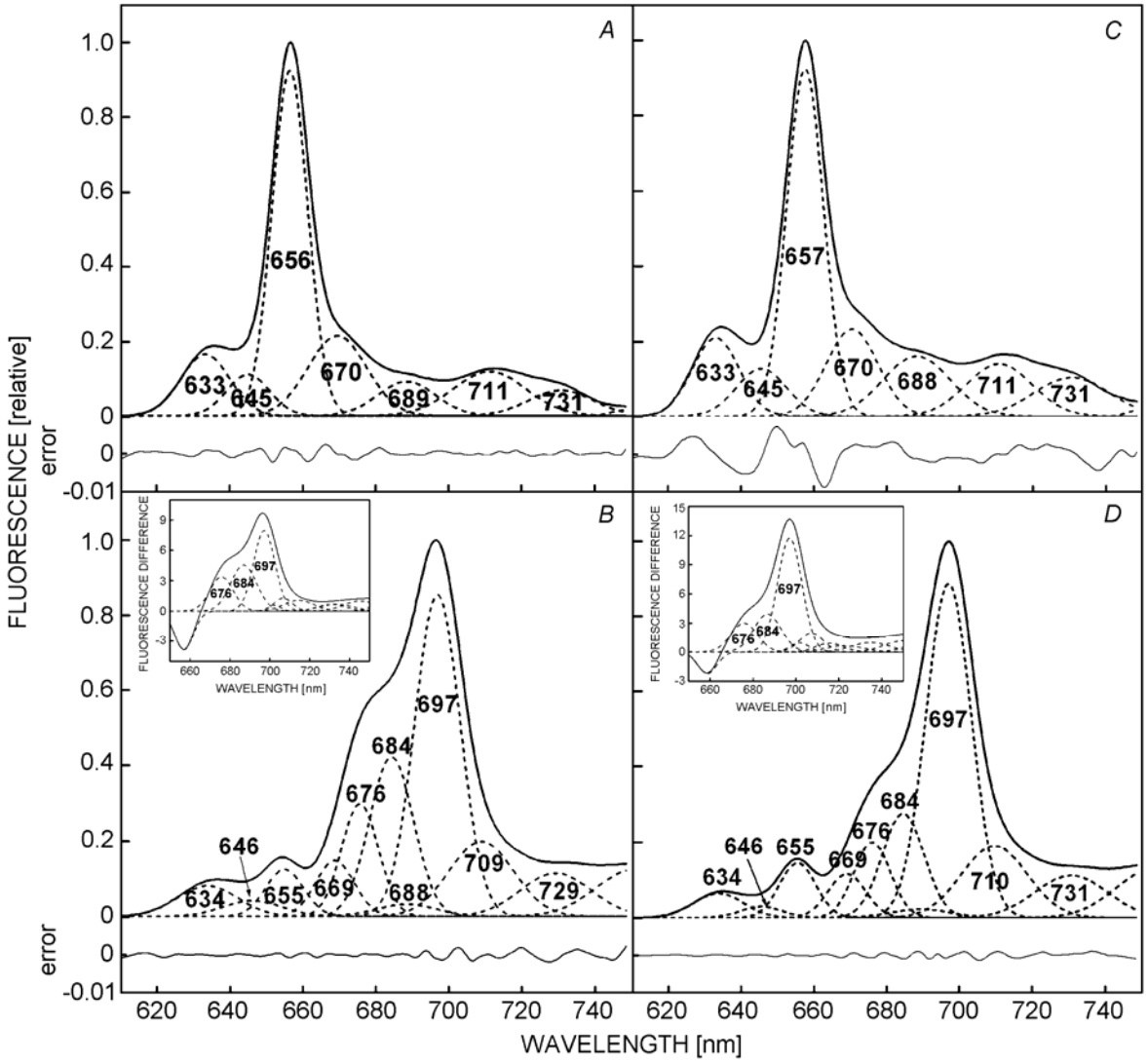

Fig. 4. De-convolution of 77 K fluorescence spectra of the stroma (*A, B*) and PLB (*C, D*) fractions. *A*: non-irradiated and *B*: incubated for 15 min in the dark after the flash-irradiation. The spectra were normalized at their maxima. *Solid line*: experimental spectrum; *broken lines*: Gaussian components; *numbers* indicate wavelengths [nm] of their maxima. *Bottom panels*: error of the fit of the sum of the components to the experimental spectrum. *Insets*: De-convolution of the spectrum obtained after subtracting the spectrum presented in *A* or *C* (irradiated sample) from the spectrum in *B* or *D* (non-irradiated sample). Before the subtraction the spectra were normalized at their 633 nm band maxima.

Composition of fluorescence spectra of etioplast compartments exposed to short irradiation: De-convolution of the fluorescence spectra of etioplast fractions after being irradiated showed the presence of different Chlide forms resulting from *in vitro* Pchlide photo-conversion process. Pchlide region of the spectra obtained for non-irradiated stroma fraction was de-convoluted into seven Gaussian components (Fig. 4*A*). Major components found in the spectra of non-irradiated stroma had short- and long-wavelength maxima at 633, 645, 656, and 670 nm corresponding to non-photoactive 633 and 670 nm and to photoactive 645 and 657 nm Pchlide forms, respectively. Minor components found at 689, 711, and 731 nm correspond to vibrational Pchlide bands according to previous studies with higher plants (Böddi *et al.* 1992). These bands that we detected by using de-convolution method may represent approximations of overlapping main bands of far-red Pchlides and vibrational bands of short- and long-wavelength Pchlides. Indeed, the spectral region 670–730 nm indicates the presence of far-red Pchlides emitting at 666, 680, 690, 698, and 728 nm (Stadnichuk *et al.* 2005). When the stroma was exposed to a short saturating flash and maintained in the dark for 15 min, the same short- and long-wavelength Pchlide forms were found (Fig. 4*B*) compared to non-irradiated stroma fraction (Fig. 4*A*). However, spectra of regenerated photoactive Pchlide pool were slightly blue-shifted. Similar shift was found also when wheat etioplast membranes and leaves of wheat were irradiated shortly (Franck *et al.* 1999). The major Chlide forms in the stroma were at 676, 684, and 697 nm. The component at 676 nm was earlier suggested to represent rapidly released "free" Chl(ide)$_{676}$ in etiolated leaves exposed to flashes (Litvin and Belyaeva 1971). Major component found at 697 nm may indicate the presence of Chlide$_{695}$ known to be the precursor of Chlide$_{682}$ (Oliver and Griffiths 1982). In the Chlide region of the stroma fraction, the 684 nm Chlide component may correspond to the Chlide$_{682}$. In this study, de-convolution analysis was also done after subtraction of Pchl(ide) contribution (*inset* in Figs. 4, 5, and 6) to avoid contribution of vibrational bands of Pchlide$_{633}$ and Pchlide$_{656}$ forms to bands originating from Chlide$_{676}$, Chlide$_{684}$, and Chlide$_{696}$. De-convoluted spectra of non-irradiated envelope-PT membranes showed main components at 625, 631, 637, 644, 655, and 669 nm (see Fig. 5*A*), which have been found previously in dark-grown bean leaves. The components at 631, 637, and 644 nm may represent monomeric, dimeric, and oligomeric forms of Pchlide-POR complexes, respectively, and the band at 625 nm was proposed to be a "free" Pchl(ide) form (Böddi and Franck 1997, Schoefs *et al.* 2000). When the envelope-PT fraction has been irradiated, we noticed that Chlide forms show similarity in the position of their emission maxima by comparing to those of stroma and PLB (Fig. 4*B*,*C* and 5*B*). However, participations of these forms in the total fluorescence emission spectra of the stroma and the PLB were very different compared to the envelope-PT.

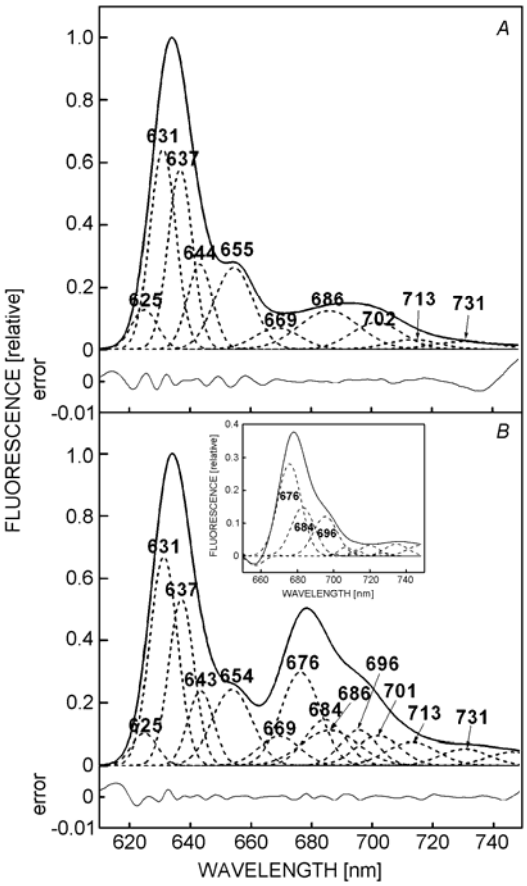

Fig. 5. De-convolution of 77 K fluorescence spectra of the envelope-PT fraction. *A*: non-irradiated and *B*: incubated for 15 min in the dark following the flash. Other indications are the same as in Fig. 4.

Based on these results we assume that the functional Pchlide-POR complex exists in the soluble etioplast fraction. Physiological reasons for the existence of stroma-located highly aggregated Pchlide-POR complexes are not yet clear. One may propose that after translocation of POR protein into etioplasts' assembly of the ternary complex takes place in the stroma prior to its association with the PLB membrane. This possibility is supported by the recent results obtained *in vitro* showing that PORA was transiently accumulated in the stroma of etiochloroplasts when translocation of its precursor was induced by 5-aminolevulinic acid (Reinbothe *et al.* 2004). When isolated etioplast fractions of barley were shortly irradiated, Chlide transformation process, controlled by POR enzyme, indicated differences despite demonstrated similarity of de-convoluted spectra (see Figs. 4–5). During this process, we noticed that Chlide$_{690}$ form was present in all fractions. However, the subsequent Chlide$_{695}$ formation was much more pronounced in the stroma and the PLB than in the envelope-PT fraction. On the other hand, the envelope-PT fraction was characterized by the preferential formation of Chlide$_{676}$. These spectral

properties may reflect molecular composition and organization of pigment-POR complexes characteristic for each etioplast compartment. Spectral similarity between the stroma- and the PLB-located complexes suggest that the intrinsic structure of these complexes rather than their immediate environments may have dominant effect on their spectral properties.

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