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Red region excitation spectra of protochlorophyllide in dark-grown leaves from plant species with different proportions of its spectral forms

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Abstract

Etiolated leaves of three different species, maize, wheat, and pea, as well as a pea mutant (*lip1*) were used to compare the excitation spectra of protochlorophyllide (Pchlide) in the red region. The species used have different composition of short-wavelength and long-wavelength Pchlide forms. The relation between different forms was furthermore changed through incubating the leaves in 5-aminolevulinic acid (ALA), which caused an accumulation of short-wavelength Pchlide forms, as shown by changes in absorption and fluorescence spectra. This is the first time a comprehensive comparison is made between excitation spectra from different species covering an emission wavelength range of 675–750 nm using fluorescence equipment with electronic compensation for the variations in excitation irradiance. The different forms of Pchlide having excitations peaks at 628, 632, 637, 650, and 672 nm could be best measured at 675, 700, 710, 725, and 750 nm, respectively. Measuring emission at wavelengths between 675–710 nm gave an exaggeration of the short-wavelength forms and measuring at longer wavelengths gave for the pea leaves an exaggeration of the 672 nm peak. In general, an energy transfer from short-wavelength Pchlide forms to long-wavelength Pchlide forms occurred, but such an energy transfer sometimes seemed to be limited as a result of a discrete location of the Pchlide spectral forms. The excitation spectra resembling the absorption spectrum most were measured at an emission wavelength of 740 nm. Measuring the excitation at 710 nm gave higher intensity of the spectra but the short-wavelength forms were accentuated.

Additional key words: chlorophyllide; etioplast; fluorescence spectrum; mutants; *Pisum*; species differences; *Triticum*; *Zea*.

Introduction

Dark grown plants contain different spectral forms of protochlorophyllide (Pchlide) with characteristic low-temperature fluorescence emission and fluorescence excitation (absorption) spectra (Böddi *et al.* 1992, Sundqvist and Dahlin 1997, Lebedev and Timko 1998). Depending on their photo-transformability to chlorophyllide (Chlide) with short (ms) flashes, two types of Pchlide, *i.e.* photoactive and non-photoactive Pchlide, can be defined. Photoactive Pchlide is regarded to be an aggregate of ternary complexes, composed of the photoenzyme, NADPH-protochlorophyllide oxidoreductase (POR), the substrate Pchlide, and the cofactor NADPH (Rüdiger 1997, Schoefs 1999, Townley *et al.* 2001). POR can be present (*e.g.* in *Arabidopsis*), as three differently regulated isoforms: PORA, PORB, and PORC (Su *et al.* 2001). Non-photoactive Pchlide is considered not to bind directly to the active site of the photo-enzyme, and be present mostly in monomeric forms (Böddi *et al.* 1998). Aggregated forms having NADP$^+$ instead of NADPH also lack photoactivity (Schoefs *et al.* 2000).

In etiolated leaves three main spectral forms of Pchlide are present with fluorescence emission maxima at 633, 645, and 656 nm, respectively (Böddi *et al.* 1992). The 633 nm band of non-photoactive Pchlide can further be split into four bands with peaks around 625, 631, 637, and 642 nm (Schoefs *et al.* 2000), but the number of bands and peak positions can vary (Kis-Petik *et al.* 1999). The 625 and 631 nm emission components are regarded as not being bound to protein, due to the similarity of their peak position to that of Pchlide in solution (Kotzabasis *et al.* 1990). However, an association to a protein or a lipid is highly probable as judged from

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Abbreviations: ALA – 5-aminolevulinic acid; Chlide – chlorophyllide; COP1 – constitutive photomorphogenesis; Pchlide – protochlorophyllide; Pchl(ide) – protochlorophyllide and/or protochlorophyll; Pchlide$_{x\text{-}y}$ – Pchlide form having the absorption maximum at x nm and the fluorescence maximum at y nm at 77 K; PLB – prolamellar body; POR – NADPH-protochlorophyllide-oxidoreductase; PT – prothylakoids.

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fluorescence lifetime measurements (Myśliwa-Kurdziel *et al.* 2003). The 642 nm form is suggested to be located in the prothylakoids (PTs) (Böddi *et al.* 1989). The component with a peak position at 645 nm is usually scarce, which can be explained by an energy transfer to the 656 nm form (Kahn *et al.* 1970, Böddi *et al.* 1992). Treatment of leaves with the Pchlide precursor 5-aminolevulinic acid (ALA) increases the content of mainly short-wavelength Pchlide in plants. Energy transfer from short-wavelength Pchlide to newly formed Chlide is originally low (Brouers and Sironval 1974) but increases when its amount increases after ALA treatment and it becomes a part of the energy transfer units (Sironval 1972). Isolation experiments show that the ALA induced Pchlide is mainly localized in the PTs (Ryberg 1983). The predominant form of photoactive Pchlide with emission at 656 nm has a basic role in the formation of Chlide during greening of etiolated leaves. The fluorescence band at 656 nm is composed of two components, which emit at 652 and 657 nm, respectively (Schoefs *et al.* 2000). The relationships between different spectroscopic Pchlide species, their possible association with the POR enzyme, and the intraplastidic localisation of possible pigment-protein complexes is far from being completely defined.

Low-temperature fluorescence spectra of etioplasts contain additional minor very long-wavelength bands. Low intensity bands around 670, 690, 713, and 728 nm were described in fluorescence emission spectra by Sironval *et al.* (1968). De-convolution into Gaussian components of this spectral region has indicated bands at 669, 682, 692, 712, and 730 nm (Litvin and Stadnichuk 1980, Böddi *et al.* 1992). These minor spectral Pchlide forms (here called far-red forms) have been interpreted as vibration satellites of short-wavelength Pchlide forms (Böddi *et al.* 1992, Kis-Petik *et al.* 1999) but also as separate spectral forms (Ignatov and Litvin 2002, Stadnichuk *et al.* 2005). The measurements of excitation spectra within the red region using the far-red forms for emission can contribute to the understanding of the nature of the far-red Pchlide forms and also reveal energy transfer possibilities. Such measurements can also be an aid for the future use of excitation spectroscopy when studying pigment conversions during plastid development.

The relationship between short-wavelength and long-wavelength Pchlide forms varies between species (Böddi *et al.* 1992, Stadnichuk *et al.* 2005). The aim of the present work was to identify different Pchlide forms in the red region excitation spectra to determine their variations in different plant species with different ratios between short-wavelength and long-wavelength Pchlide forms before and after a treatment with ALA. A similar comparison has not earlier been performed. An important consideration was to find emission wavelengths, where the excitation spectrum showed a large similarity with the absorption spectrum and to evaluate the possibility of energy transfer between Pchlide forms.

Materials and methods

Plants: Seeds were soaked for 12 h at 298 K in tap water, and then sown in commercially available seed-sowing compost (*Yrkesplantjord*, *Svalöf-Weibull AB*, Landskrona, Sweden) in 15 cm wide pots. The plants used were wheat (*Triticum aestivum*, cv. Kosack), maize (*Zea mays* cv. Sundance), pea (*Pisum sativum* L. cv. Kelwedon wonder; *Svalöf-Weibull AB*, Landskrona, Sweden), and the *lip1* mutant of the pea cv. Alaska (described by Frances *et al.* 1992). The seedlings were grown for 7 d in the dark at 298 K. The wheat and maize leaves were cut 1.5 cm from the tip and the following 2 cm were used for measurements. From pea the bud of primary leaves and from *lip1* the first fully developed leaves were used. Some samples were denatured by heating for 2 or 6 min at full effect (800 W) in a microwave oven (*Electro Helios*, Stockholm, Sweden). All handling of the seedlings was done under dim green light.

ALA treatment: The ALA solution used for treatment of the plants was 10 mM in 50 mM phosphate buffer, pH 6.7. ALA was given immediately after the leaves were cut. During the treatment the sections were submersed in the ALA solution.

Fluorescence and absorption measurements: Fluorescence emission and excitation spectra were recorded using a *Fluorolog-3* spectrofluorimeter (*Spex Instruments*, New Jersey, USA). Data from the fluorimeter were retrieved over a PC interface using the *GRAM/32* program (*Galactic Industries Co.*, Salem, USA). Emission and excitation spectra were electronically corrected for the spectral sensitivity of the photomultiplier and the lamp emission spectrum, respectively. In both the excitation and emission monochromator, the slit width was 2 nm. Emission spectra were measured with the excitation set at 440 or 460 nm. Fluorescence excitation spectra were recorded at different wavelengths of emission given in the text. Samples of intact etiolated tissues were placed into small cylindrical glass cuvettes and kept immersed in liquid nitrogen. The re-absorption of emitted fluorescence was regarded as negligible.

In vivo absorption spectra were recorded with a *Perkin Elmer Lambda 900* UV/VIS spectrophotometer. The leaves were placed in a double layer on transparent plastic in front of an Ulbricht integrating sphere. In the reference beam an ordinary glass cuvette was placed with the light scattering sides in the beam. The recordings were performed with 2 nm optical slits, collecting data on each nm with an integration time of 0.04 s and a recording speed of 12.5 nm s^{-1}. The results are the averages of at least 5 repetitions of each experiment.

Pigment extraction and estimation of Pchl(ide) content: Pigments were extracted with 80 % acetone as described by Brouers *et al.* (1983). The treated and untreated leaves (1.0–1.5 g) were ground to a fine powder in liquid nitrogen using a mortar and pestle. The pigments were extracted with 3 cm^3 of cold 80 % acetone. The acetone extracts were centrifuged at 30 000×*g* for 10 min and the resulting pellet was extracted with cold 80 % acetone. This operation was repeated 3 times. The successive supernatants were pooled and clarified by centrifugation at 40 000×*g* for 5 min. The absorbance spectra of the extracts were measured and the total amount of Pchl(ide) was determined with equations recommended by Brouers *et al.* (1983).

Results

Absorbance spectra: The room temperature *in vivo* absorbance spectra were different for the different plant species used (Fig. 1). Treatment with ALA increased the Pchl(ide) content in the leaves (Table 1). For wheat and maize leaves the increase was about 5 and 7 times, respectively, and for pea and *lip1* leaves it was about 2 times. Mainly the contents of short-wavelength components increased. In wheat and maize the long-wavelength Pchlide form absorbing at 650 nm was dominating before ALA treatment and this form was still visible as a shoulder after the ALA treatment when the main peak was found at 634 nm. In untreated control pea leaves there were two absorption peaks of about equal height, one around 638 nm and one at 650 nm. After ALA treatment the dominating peak was at 634 nm with the 650 nm form seen just as a weak shoulder. In *lip1* the short-wavelength peak at 634 nm dominated in both the untreated and ALA-treated leaves.

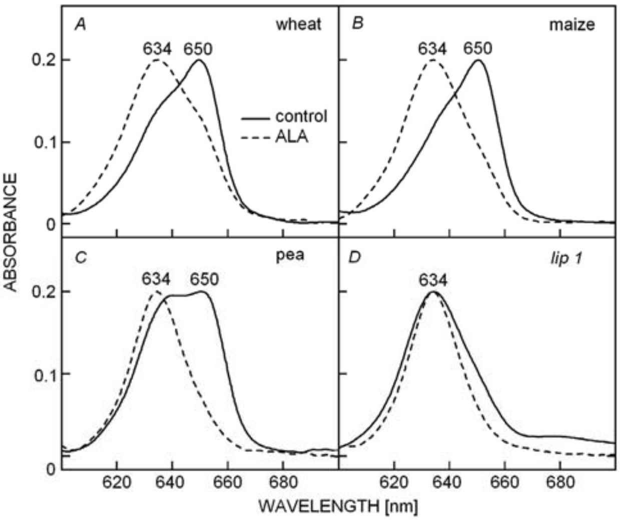

Fig. 1. Absorption spectra of dark-grown leaves from different plant species. Leaves treated with ALA for 5 h are compared with non-treated control leaves. The spectra are normalised to the highest peak.

Table 1. Total amount of Pchl(ide) [µmol kg^{-1}(FM)] measured in 80 % acetone extract of dark-grown untreated and ALA treated leaves of different plant species. The leaves were treated with ALA for 5 h in darkness.

	Maize	Wheat	Pea	*lip1*
Control	13	17	6	16
ALA	90	75	14	30

Fluorescence emission spectra: The low temperature (77 K) fluorescence emission spectra of etiolated leaves from the different plant species had a species dependent ratio between the fluorescence peaks around 631 and 656 nm (Fig. 2). With excitation at 440 nm the spectra of maize and wheat leaves had a minor short-wavelength band at 631 nm, a main peak present at 656 nm, and bands of low intensity at 671, 685, 710, and 728 nm (Fig. 2*A*,*B*). The maize leaves had an extremely low content of the 631 nm fluorescing form. Excitation at 460 nm increased the dominance of the peak at 656 nm (Fig. 3*A*,*B*). After ALA treatment the short-wavelength peak was increased and in fact became the dominating peak with 440 nm excitation. At the same time a slight red shift occurred giving a peak position of 634 nm (Fig. 2*A*,*B*). The fluorescence in the interval 670–690 nm also increased in relation to the fluorescence at 710 and 728 nm. With 460 nm excitation the ALA treated leaves of wheat and maize had the main peak at 653 nm but also an evident short-wavelength peak red-shifted to 641 nm (Fig. 3*A*,*B*). A similar red shift was not obtained in ALA-treated pea and *lip1* leaves for which the peak was found at 634 nm. This was probably dependent on the low amount of Pchlide accumulated in pea and *lip1* leaves (Table 1).

A short-wavelength peak at 631 nm dominated the fluorescence emission spectra of pea and *lip1* leaves after excitation at 440 nm (Fig. 2*C*,*D*). When the fluorescence was excited at 460 nm, the short-wavelength peak was found close to 639 nm for both pea and *lip1* (Fig. 3*C*,*D*). After excitation at 460 nm the fluorescence spectra of wild-type pea leaves and *lip1* leaves had a prevailing peak or shoulder at 654 and 653 nm, respectively, and also distinct emission bands at 671, 685, 710, and 728 nm similar to wheat (Fig. 3*C*,*D*). These bands were less resolved during excitation with 440-nm radiation, probably due to the overlapping of vibronic bands belonging to the intensive short-wavelength forms.

In ALA treated pea leaves the short-wavelength fluorescence was displaced to 634 nm and was considerably increased compared to the 654 nm fluorescence that was seen just as a shoulder. After excitation with 460-nm radiation the position of the short-wavelength peak shifted from 631 to 639 nm in untreated leaves, but remained at 634 nm in ALA treated leaves (Fig. 3*C*). In the long-wavelength region the peak at 690 nm was prominent after 440 nm excitation and even more so in the ALA-treated leaves (Fig. 2*C*). In the control and ALA treated leaves the 460 nm excitation accentuated the peak around 710 nm (Fig. 3*C*). In the *lip1* leaves the emission spectra were even more dominated by the emission of short-wavelength forms, especially after ALA-treatment, when the peak was strong enough to mask all 654 nm fluorescence. The peak at 695 nm was not specifically increased but slightly blue-shifted.

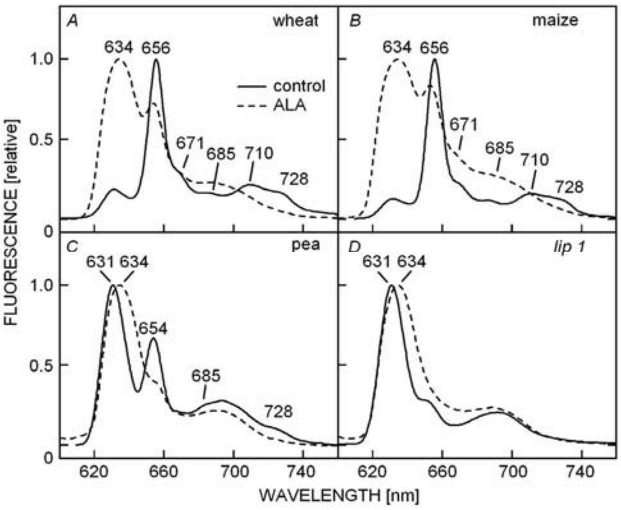

Fig. 2. Low temperature (77 K) fluorescence emission spectra of dark-grown leaves from different plant species. Spectra are shown for leaves treated with ALA for 5 h and for non-treated control leaves. Excitation with 440 nm. The spectra are normalised to the highest peak.

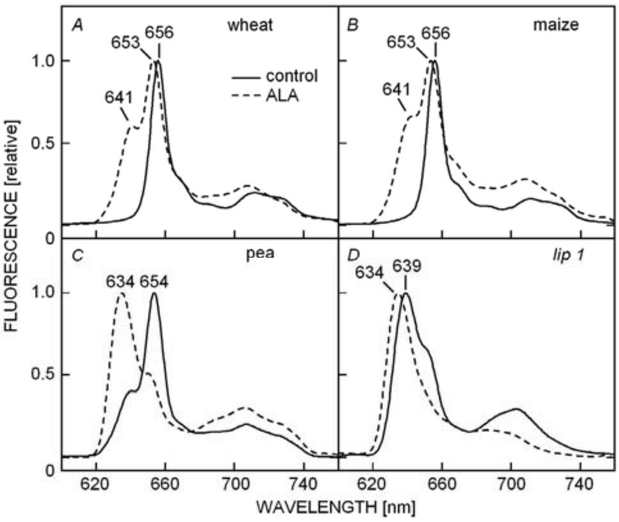

Fig. 3. Low temperature (77 K) fluorescence emission spectra of dark-grown leaves from different plant species. Spectra are shown for leaves treated with ALA for 5 h and for non-treated control leaves. Excitation with 460 nm. The spectra are normalised to the highest peak.

Fluorescence excitation spectra: A series of excitation spectra was recorded with the emission wavelength set at different positions from 675 to 750 nm. The variations between plant species and between ALA treated and untreated leaves were accentuated in the so recorded spectra from the red region. The variation in fluorescence intensity as well as wavelength position is shown in Fig. 4. For the *lip1* leaves the excitation peak at 631 nm was the most intense when the emission monochromator was set to 700 nm. For wheat the peak at 649 nm was the dominant one when the emission was recorded at 675 nm. Spectra recorded with the emission monochromator set to 740 or 750 nm were comparatively weak. To facilitate comparisons the following spectra are shown adjusted to the same peak height.

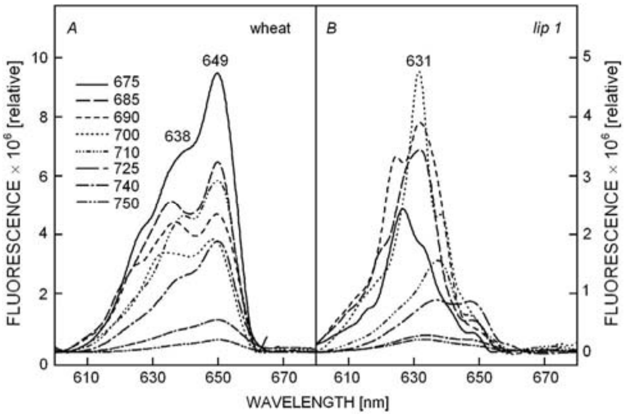

Fig. 4. Low temperature (77 K) fluorescence excitation spectra of dark-grown leaves from wheat and *lip1* in the red region. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated.

For wheat leaves the peak position and the relative peak height of the excitation spectra were thus dependent on the emission wavelength used. When the emission monochromator was set at different wavelengths within the region 675–700 nm the short-wavelength part was accentuated compared to the case when the emission was measured at longer wavelengths (710–750 nm) (Fig. 5*A*,*B*). The emission wavelengths 685 and 690 nm gave an evident peak at 636 nm. With the longer emission wavelengths of 710–750 nm a peak or a shoulder were seen at 638 nm but in all cases the 649 nm peak was the most conspicuous. When the emission was measured at 740 and 750 nm an excitation peak at 672 nm was also evident.

For ALA-treated wheat leaves the short-wavelength peaks were dominating when the emission monochromator was set within the region 675–710 nm (Fig. 5*C*,*D*). At an emission of 675 nm the maximum position was at

628 nm with a shoulder at 634 nm and a peak at 649 nm. A peak shift occurred from 628 to 634 nm when the emission was increased from 675 to 700 nm. When the emission was recorded in the region 725–750 nm the peak at 649 was evident, but it was dominating only at an emission wavelength of 725 nm (Fig. 5*D*). The 672 nm band was all the time very low, but could be best recognised when the emission was measured at 740 and 750 nm.

The fluorescence emission spectra of maize indicated that the long-wavelength form Pchlide$_{650\text{-}656}$ was a dominating form in these leaves (Fig. 2*B*). The peak position of the excitation spectra was also independent of the emission wavelength used. Within the whole emission region examined, from 675 to 750 nm, the excitation peak was found at 649 nm (Fig. 6*A*,*B*). Minor variations occurred in the intensity of the shoulder at 636 nm. This shoulder was especially prominent when using 710 nm as emission wavelength. Also for maize the 672 nm peak was most conspicuous at an emission wavelength of 750 nm.

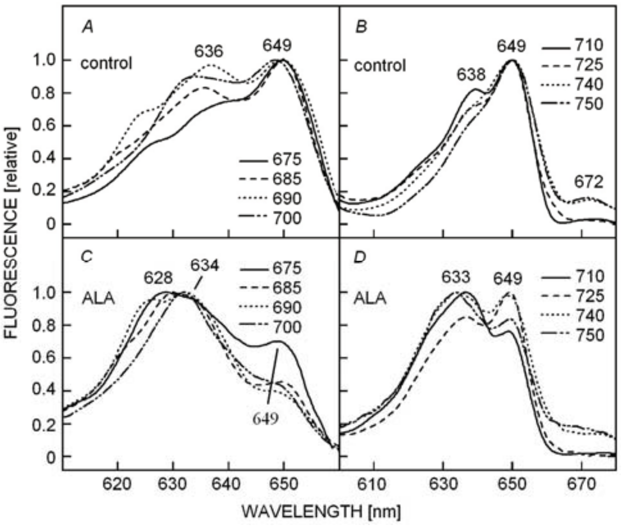

Fig. 5. Low temperature (77 K) fluorescence excitation spectra of dark-grown untreated (*A*, *B*) and ALA treated (*C*, *D*) leaves from wheat in the red region. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated. The spectra are normalised to the highest peak.

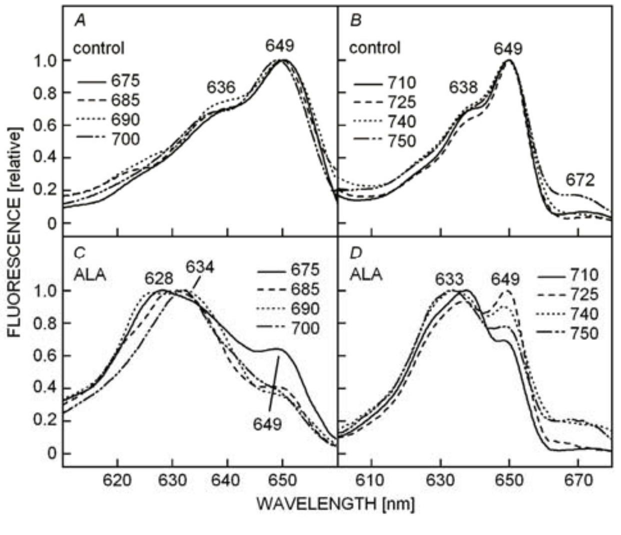

Fig. 6. Low temperature (77 K) fluorescence excitation spectra of dark-grown untreated (*A*, *B*) and ALA treated (*C*, *D*) leaves from maize in the red region. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated. The spectra are normalised to the highest peak.

For ALA-treated maize leaves short-wavelength peaks dominated when the emission was recorded within the region 675–710 nm (Fig. 6*C*,*D*). At an emission of 675 nm the maximum position was at 628 nm with a shoulder at 634 nm and a peak at 649 nm. When the emission monochromator was set at 725 nm the peak at 649 nm was dominating but then again at 740 and 750 nm short wavelength peaks were the most conspicuous (Fig. 6*D*). The 672 nm band was, as for wheat, all the time very low, but could be recognised when the emission was measured at 740 or 750 nm.

For pea leaves short-wavelength peaks dominated the excitation spectra when the emission monochromator was set within the region 675–710 nm (Fig. 7*A*,*B*). A peak shift occurred from 628 to 638 nm in connection with an increase of the 649-nm peak when the emission was recorded at successively longer wavelengths. The emission at 675 nm was nearly exclusively induced by a 628 nm absorbing form and this form had also a stronger contribution to the 690 nm emission than to the 685 nm emission. Similar but not so obvious results were obtained for the other species tested. When the emission monochromator was set in the region 725–750 nm the peak at 649 nm was dominating and there was a consecutive increase in the peak at 672 nm with increasing emission wavelength. At 750 nm the peak at 672 nm was nearly as strong as the 649 nm peak but the short wavelength peak at 638 nm also highly contributed (Fig. 7*B*).

After ALA treatment of the pea leaves the short-wavelength peaks were even more emphasized when the emission monochromator was set within the region 675–710 nm (Fig. 7). The peak shift from 628 to 638 nm occurred but the relative increase in the 649 nm peak was less conspicuous. In many cases the peaks were broadened after ALA-treatment. At 725 nm the peak at 649 nm became dominating but the short-wavelength peaks were all the time prominent in contrast to the case of untreated leaves. There was a consecutive increase in the peak at 672 nm but the long-wavelength form at 672 nm never reached a similar height as the 649 nm peak did in the ALA-treated leaves (Fig. 7*C*,*D*). Measuring the emission at 750 nm again gave dominance to the peak at 633 nm.

For *lip1* leaves, the short-wavelength peaks all the time dominated the excitation spectra when the emission monochromator was set within the region 675–750 nm (Fig. 8*A*,*B*). Peak shifts occurred from 628 to 637 nm when the emission was recorded at successively longer wavelengths. When the emission was measured in the region 725–750 nm there was an evident broadening of the spectrum in the 650 nm region.

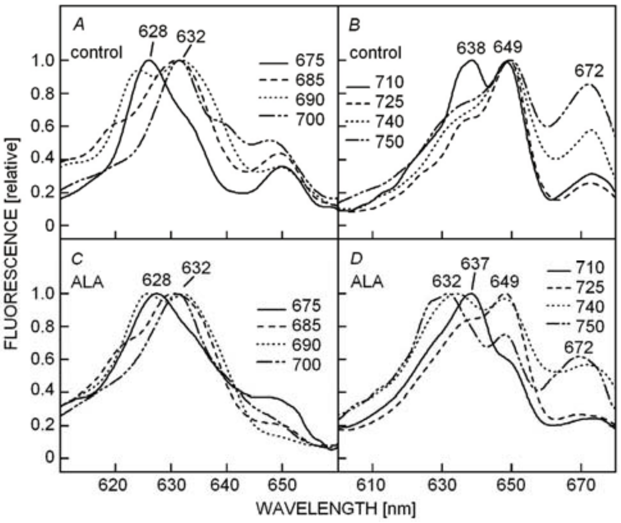

Fig. 7. Low temperature (77 K) fluorescence excitation spectra of dark-grown untreated (*A*, *B*) and ALA treated (*C*, *D*) leaves from wild type pea in the red region. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated. The spectra are normalised to the highest peak.

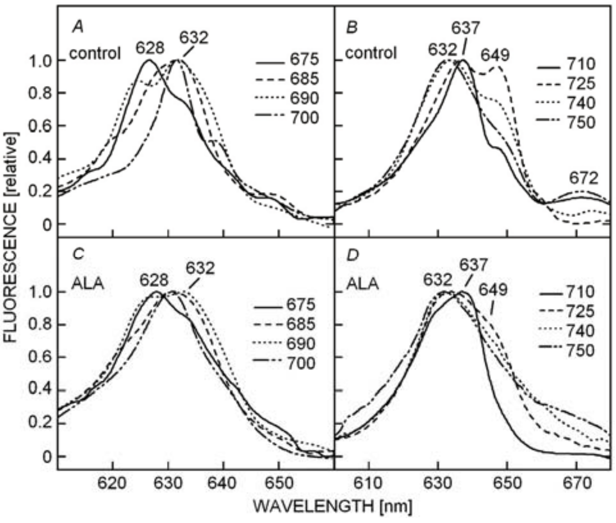

Fig. 8. Low temperature (77 K) fluorescence excitation spectra of dark-grown untreated (*A*, *B*) and ALA treated (*C*, *D*) leaves from the *lip1* mutant pea in the red region. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated. The spectra are normalised to the highest peak.

After ALA treatment of *lip1* leaves the short-wavelength excitation peaks were even more accentuated (Fig. 8*C*). The 675 nm emission gave a peak at 628 nm whereas the 690 nm emission gave a peak at 632 nm. A certain broadening occurred also after ALA-treatment in the 650 nm region, especially evident with the emission monochromator set to 725 nm. The peak at 672 nm was most evident when recording the emission at 750 nm (Fig. 8*D*).

The ALA treatment seems to induce specifically the Pchlide 632 nm fluorescing form (Fig. 9). This was also confirmed by a time-series with wheat leaves, showing more Pchlide [17, 27, 35, 60, and 127 μmol kg^{-1}(FM)] accumulated the longer (0, 1, 2, 3, and 6 h, respectively) the ALA treatment persisted. In the excitation spectrum measured with the emission monochromator set to 690 nm, the relative height of the 632 nm peak increased until, at

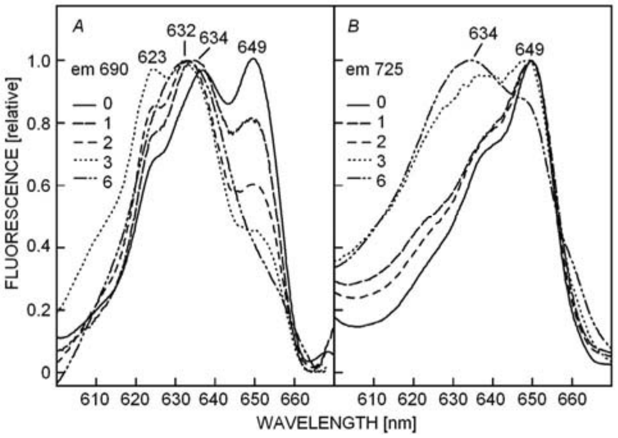

Fig. 9. Low temperature (77 K) fluorescence excitation spectra of dark-grown leaves from wheat treated with ALA for different periods of time. The spectra were measured with the emission set to 690 (*A*) or 725 (*B*) nm. The spectra were normalised to the highest peak.

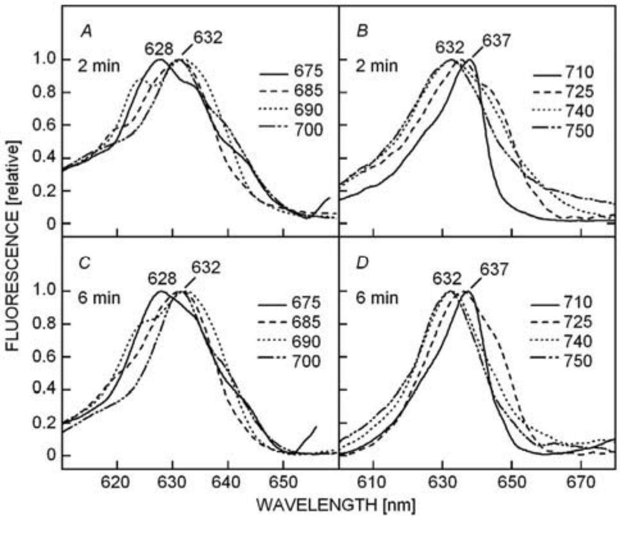

Fig. 10. Low temperature (77 K) fluorescence excitation spectra of heat denatured dark-grown leaves of wheat. The leaves were heat denatured for 2 or 6 min. The spectra were measured with the emission registered at different wavelengths (675–750 nm) as indicated. The spectra were normalized to the highest peak.

a very high Pchlide content, this peak became fully dominant and other peaks were hardly seen even as shoulders. Measured at 725 nm the peak at 649 nm was dominating. Only after 6 h treatment with ALA the short-wavelength peak became dominating.

After heat treatment the long-wavelength Pchlide fluorescing at 656 nm is lost (Dujardin and Sironval 1970, Stadnichuk *et al.* 2005). A short heat treatment for 2 min (Fig. 10*A*,*B*) was enough and a treatment prolonged to 6 min did not change the appearance of the spectra more than marginally (Fig. 10*C*,*D*). Short-wavelength peaks dominated the excitation spectra of heat-treated wheat leaves. At an emission of 675 nm the maximum position was at 628 nm with a shoulder at 632 nm. A peak shift occurred from 628 to 632 nm when the emission wavelength was increased from 675 to 700 nm. There was a split into two peaks when the measurement was done at 690 nm. Also when the emission monochromator was set in the region 710–750 nm, short wavelength peaks were dominating. With 710 nm as the emission wavelength a peak at 637 nm, mainly free of other bands, dominated the spectrum. At an emission wavelength of 725 nm a shoulder at 645 nm was conspicuous.

Discussion

Characteristic properties of Pchlide spectral forms: Pchlide in etiolated leaves is spectrally heterogeneous. The proportion of the different forms varies between different plant species. Often the fluorescence emission spectra of dark-grown leaves have been used to characterize the different Pchlide forms (Sironval *et al.* 1968, Böddi *et al.* 1992, 1993, Franck *et al.* 1999, Schoefs *et al.* 2000). More than ten fluorescence emission components have been characterised through their main emission and vibration bands (Böddi *et al.* 1993, Schoefs *et al.* 2000, Stadnichuk *et al.* 2005). The red region excitation spectra have been less examined, partly because of the lack of suitable substances for excitation correction in this wavelength region. The appearance of the excitation spectra depends on the emission wavelength used for its determination and reflects the contribution of fluorescence from the different forms to this wavelength. Energy transfer complicates the interpretation and in addition the exact wavelength position for one and the same form of Pchlide can differ between different plant species.

The absorbance spectra of whole leaves of maize, wheat, and pea all had a long-wavelength peak at 650 nm (Fig. 1). However, the proportion between the different Pchlide forms varied in the different species, which was more evidently unveiled by the fluorescence emission spectra (Figs. 2 and 3). The maize leaves had an extremely low content of short-wavelength Pchlide forms. In wheat leaves the content of short-wavelength forms was greater while in pea leaves a large amount of short-wavelength Pchlide forms was present (Figs. 2 and 7). The short-wavelength forms appeared as strong peaks in the excitation spectra between 628–637 nm when measured at the emission wavelengths of their vibration bands in the interval 675–700 nm. The contribution from the short-wavelength bands was small when the excitation spectra were measured at emission wavelengths in the interval 710–750 nm (cf. Fig. 5*B*, 6*B*, 7*B*). This indicated that the short-wavelength Pchlide did not transfer much of the energy to the long-wavelength form in untreated peas.

The *lip1* leaves had an absorption peak at 634 nm and a dominating fluorescence emission at 631 nm. The apparent lack of a Stokes shift has been discussed earlier (McEwen *et al.* 1991). The *lip1* leaves exhibited low contents of long-wavelength Pchlide forms. In the excitation spectra of the *lip1* mutant leaves at least four short-wavelength bands at about 619, 628, 632, and 637 nm, in addition to a peak at 649 nm, could be identified (Fig. 8*A*,*B*). The presence of a form with an excitation peak slightly shorter than 628 nm was also indicated when measuring at 690 nm. This form was also seen in pea and wheat but its closeness to the 628 nm form requires a more detailed analysis for its separation and identification.

Specific Pchlide forms after ALA-treatment: During ALA-treatment the amounts of short-wavelength forms increased. The results from the time series (Fig. 9) indicated that it was mainly the excitation band at 632 nm, which increased even if increased intensity was seen also at 628 nm (Fig. 9). With a high accumulation of Pchlide the excitation peak at 632 nm dominated the spectrum. The Pchlide$_{638\text{-}644}$ form seemed less affected as such a clear peak never appeared in the spectra from the untreated samples as in the spectra from ALA-treated leaves. This differential increase in specific excitation bands contributes to the identification of separate short-wavelength forms.

In maize, short-wavelength forms were scarce in untreated leaves, but never the less the content of Pchlide$_{628\text{-}633}$ form increased with ALA-treatment. Its absence thus might be attributed to a low formation of ALA and indicate a tight control of the Pchlide biosynthesis in maize. In pea and *lip1* large amount of Pchlide$_{628\text{-}633}$ was present already before ALA treatment. The accumulation of Pchlide was low in pea and *lip1*. The reason for the low amount of Pchlide accumulated could, however, be attributed to a decreased penetration of ALA into the leaves of pea and *lip1* and might not be related to Pchlide biosynthesis.

The presence in *lip1* of an extensive system of PTs and a lack of PLBs (Frances *et al.* 1992) could have influenced the spectral properties of the Pchlide accumulated after ALA-treatment. However, the peak position of the short-wavelength Pchlide after ALA treatment was the same in all plant species (Figs. 5–8). This pointed to a specific location of the Pchlide formed after ALA-treatment. The active site of POR is probably filled with Pchlide formed from endogenous ALA. The additional Pchlide formed after ALA-treatment could participate in the formation of aggregates through a pigment-pigment interaction. However, the content of long-wavelength Pchlide form, which is regarded as an aggregated form, was not increased. This indicates that most of the Pchlide formed after ALA-treatment is not participating in aggregate formation together with the Pchlide$_{650\text{-}656}$. Furthermore, it shows that the pigment is *per se* not the limiting factor for the formation of the aggregated long-wavelength Pchlide form. In fact this is probably the POR (Franck *et al.* 2000) and possibly another factor such as the abundance of membranes with a specific lipid composition (Seyyedi *et al.* 1999, Klement *et al.* 2000). The availability of POR and not the availability of Pchlide might then regulate the size of the aggregates. A prolonged treatment with ALA causes a red shift of the short-wavelength Pchlide from 633 to 640 nm (Sundqvist and Klockare 1975), a shift which might, however, represent an aggregation of the Pchlide molecules not bound to POR.

Energy migration between Pchlide forms: If an efficient energy transfer occurs between short- and long-wavelength forms of Pchlide, the short wavelength forms should be visible in the excitation spectra measured at wavelengths where mainly the long-wavelength forms emit. The maize leaves are nearly free from short-wavelength forms, but the wheat leaves contain a small amount. The excitation spectra measured at 740 and 750 nm look very similar for the two species. If an efficient energy transfer had occurred the short wavelength peaks should have been more accentuated in the excitation spectra of wheat. The evident shoulder between 630–640 nm appearing in both maize and wheat can be regarded as an intrinsic property of the Pchlide$_{650\text{-}656}$ form (Virgin 1975), even if it might point to some degree of energy transfer. Even the pea samples have at the emission measuring wavelengths 740 and 750 nm excitation spectra similar to that of maize. Thus in pea, with a large amount of short-wavelength Pchlide, most of this pigment does not participate in energy transfer. In the ALA-treated leaves the short-wavelength forms of Pchlide became dominant when the emission was measured at 740 and 750 nm. In ALA-treated samples of all species there was also a dominance of an excitation peak of Pchlide at 637 nm when the emission was set to 710 nm. This strongly favours a vibration band of the Pchlide$_{637\text{-}642}$ form at this wavelength but it can also indicate an energy transfer to Pchlide$_{650\text{-}656}$, which is suggested to have a vibration band here. The energy transfer from short-wavelength to long-wavelength forms in especially pea and *lip1* is thus normally low, but it can increase after ALA-treatment. The lack of energy transfer from some of the short-wavelength Pchlide indicates that the pigments can be differently located in the leaf. In fact, some short-wavelength Pchlide has been found in the plastid envelope (Pineau *et al.* 1993, Barthélemy *et al.* 2000) and has also been found to co-purify with a microsomal fraction (Gross *et al.* 1979). A division of the spectral forms between different tissues such as mesophyll cells and vascular strands is also possible (Seyyedi *et al.* 2001, Marchand *et al.* 2004). When the excitation spectra from pea and *lip1* were measured in the region 720–750 nm, there was a strong increase of the Pchlide with absorption at 672 nm, which indicated the presence of a vibration band in this region.

Spectral forms after heat denaturation: A heat denaturation causes the loss of long wavelength Pchlide forms (Dujardin and Sironval 1970, Böddi *et al.* 1993). However, there was still a considerable structure in the excitation spectra after such a treatment (Fig. 10). When the excitation was measured at an emission of 675 nm, a clear peak was obtained at 628 nm with only small shoulders at 632 and 640 nm. In a similar manner the emission at 710 nm was strongly dominated by the excitation peak at 637 nm. This peak was clearly distinguished in non-heated wheat and pea but in that case it was combined with a strong band of Pchlide$_{650\text{-}656}$. The emission at 710 nm is thus composed of contributions from more than one form. After heat denaturation the pattern of the short-wavelength forms is still comparable to that of non-heated leaves, with peaks at 628, 632, and 637 nm, indicating that the surrounding of the pigment molecules giving rise to these absorption events probably has not changed completely. Primarily the aggregated long-wavelength forms disappeared when the POR protein was denatured. This showed the significance of the POR protein in constituting the aggregates. The long-wavelength forms are generally correlated to a regular PLB structure (Ryberg and Sundqvist 1988, Franck *et al.* 2000) and this structure is also destroyed during heat-denaturation (Henningsen 1970).

Localization of Pchlide forms within the plastid: A specific connection of Pchlide to PORA or PORB does not affect the spectral properties (Armstrong *et al.* 1995, Franck *et al.* 2000). This implicates that both PORA and PORB can participate in formation of aggregates and that they both in a similar way interact with lipids in the PLB membranes (Selstam and Wigge 1993, Klement *et al.* 2000). The existence of different pigment forms points to the binding of some of the Pchlide to other proteins or a difference in interaction between some of the Pchlide molecules with the lipid structure of the plastid membranes. Different Pchlide forms containing POR complexes have also been isolated (Wiktorsson *et al.* 1992, Chahdi *et al.* 1998). Variations in the lipid composition in the surroundings of the POR aggregates might then contribute to the presence of different spectral forms. The esterified Pchlide has spectral properties identical to the un-esterified form and will not directly contribute to the spectral diversity. However, the formation of aggregates and the interaction with lipids might be different and the esterification can therefore cause spectral modifications (Ryberg *et al.* 1980, Sundqvist *et al.* 1980).

Each POR polypeptide binds only one pigment molecule (Klement *et al.* 1999) and makes, together with NADPH, a ternary complex. Such complexes can assemble into multi-molecular aggregates (Böddi *et al.* 1993). The ternary complex of POR can then be arranged so close to each other that the chromophore molecules interact through the porphyrin rings of the Pchlide protruding from the POR molecule (Klement *et al.* 1999, Townley *et al.* 2001). The formation of pigment aggregates can be of an order of dimers and of higher orders. A value of eight to nine pigments in an energy transfer unit has been suggested (Schoefs and Franck 2003). Pchlide aggregates in solution or in artificial films have strongly red shifted bands (Böddi and Láng 1984, Kotzabasis *et al.* 1990). The long-wavelength Pchlide forms are thus *per se* indicative of a possible aggregational pigment state. In addition to Pchlide located at the POR active site a second competitive binding site has been suggested (Klement *et al.* 1999). This second site might well be the Pchlide pigment molecule itself. Normally only POR-bound Pchlide molecules can be thought to form flash-photoactive Pchlide$_{650-657}$ but when other Pchlide molecules bind to a POR for the formation of a pigment dimer the phototransformation is diminished; this can be observed after prolonged treatment with ALA. Pchlide molecules can also be associated with a transfer protein during the insertion into POR or be stored within the framework of the PLB membranes as short-wavelength forms.

In conclusion, the fluorescence excitation spectra within the red region seem to be an excellent tool to determine the presence of different Pchlide forms. By choosing different emission wavelengths the form of interest can be exaggerated and its presence can be determined even in minute amounts, something which will be important for disclosure of its function. The measurements showed that the different forms absorbing at 628, 632, 637, 650, and 672 nm could be best measured at 675, 700, 710, 725, and 750 nm, respectively. The spectrum most resembling the absorption spectrum was measured at 740 nm. However, the signal at this wavelength was rather week. An emission wavelength, which gave a spectrum of a reasonable intensity, was 710 nm even if the short-wavelength forms were slightly accentuated at this wavelength. After ALA-treatment the 649 nm form was too strong when measuring at 725 nm and the best fit of the excitation spectrum to the absorption spectra was found when the emission was measured at 700 nm.

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