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Optical properties of bud scales and protochlorophyll(ide) forms in leaf primordia of closed and opened buds†

KATALIN SOLYMOSI[1] and BÉLA BÖDDI[1,2]

[1] *Department of Plant Anatomy, Eötvös University, Pázmány P. s. 1/C, Budapest, H-1117, Hungary*

[2] *Corresponding author (bbfotos@ludens.elte.hu)*

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Summary The transmission spectra of bud scales of 14 woody species and the 77 K fluorescence emission spectra of the innermost leaf primordia of closed and opened buds of 37 woody species were studied. Pigment concentrations were determined in some species. Bud scales had low transmittance between 400 and 680 nm with a local minimum around 680 nm. Transmittance increased steeply above 680 nm and was > 80% in the 700–800 nm spectral region. Significant protochlorophyllide (Pchlide) accumulation was observed in leaf primordia of tightly packed, closed buds with relatively thick, dark bud scales. In common ash (*Fraxinus excelsior* L.) and Hungarian ash (*Fraxinus angustifolia* Vahl.), the innermost leaf primordia of the closed buds contained protochlorophyll (Pchl) and Pchlide (abbreviated as Pchl(ide)), but no chlorophyll. We observed Pchl(ide) forms with emission maxima at 633, 643 and 655 nm in these leaves. Complete transformation of $Pchlide_{655}$ (protochlorophyllide form with maximum emission at 655 nm) into $Chlide_{692}$ (chlorophyllide form with maximum emission at 692 nm) occurred after irradiation for 10 s. The innermost leaf primordia of the buds of four species (flowering ash (*Fraxinus ornus* L.), horse chestnut (*Aesculus hippocastanum* L.), tree of heaven (*Ailanthus altissima* P. Mill.) and common walnut (*Juglans regia* L.)) contained $Pchl(ide)_{633}$, $Pchl(ide)_{643}$ and $Pchlide_{655}$ as well as an emission band at 688 nm corresponding to a chlorophyll form. The $Pchlide_{655}$ was fully photoactive in these species. The outermost leaf primordia of these four species and the innermost leaf primordia of 28 other species contained all of the above described Pchl(ide) forms in various ratios but in small amounts. In addition, Chl forms were present and the main bands in the fluorescence emission spectra were at 690 or 740 nm, or both. The results indicate that Pchl(ide) accumulation occurs in leaf primordia in near darkness inside the tightly closed buds, where the bud scales and the external leaf primordia function as optical filters.

Keywords: chlorophyll biosynthesis, etiolation, LPOR, NADPH:protochlorophyllide oxidoreductase

Introduction

In angiosperms, a key step in chlorophyll (Chl) biosynthesis is the photoreduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide; for review see Rüdiger 1997, Beale 1999, Willows 2003, Masuda and Takamiya 2004). In the absence of light, Chl biosynthesis is arrested at Pchlide; and instead of chloroplasts, etioplasts differentiate with inner membranes forming prolamellar bodies (PLBs) and prothylakoids (PTs; Gunning 1965). Protochlorophyllide, the photoenzyme NADPH:Pchlide oxidoreductase (LPOR) and NADPH accumulate (Griffiths 1978) mainly in the PLBs (Ryberg and Dehesh 1986). The protein units of LPOR, Pchlide and NADPH form ternary complexes (Griffiths 1978) that aggregate into macrodomains (Wiktorsson et al. 1993). This macrodomain structure determines the spatial arrangement of the Pchlide molecules, enabling interactions among their π-electronic systems (Böddi et al. 1989) and leading to complex spectral properties with red shifted bands in the absorption and fluorescence spectra of Pchlide in etiolated leaves (Shibata 1957, Böddi et al. 1989). Depending on the environment and the aggregational state of Pchlide, four populations of Pchlide molecules—denoted as spectral forms—have been described in leaves (Böddi et al. 1989, 1992). The monomeric form is probably not bound to the enzyme and is located mainly in the PTs. Its fluorescence emission maximum is at 633 nm, which resembles that of monomeric Pchlide in organic solvents (Myśliwa-Kurdziel et al. 2004). The pigment in this form can be Pchlide or esterified Pchlide, i.e., protochlorophyll (Pchl; Lindsten et al. 1988). Because Pchlide and Pchl cannot be distinguished spectroscopically in native samples, their forms are often denoted as "Pchl(ide)". The short-wavelength, monomeric Pchl(ide) form predominates in young seedlings (Klein and Schiff 1972, Schoefs and Franck 1993, He et al. 1994, Schoefs et al. 1994, 2000*a*). The pigment in this form is not flash photoactive; instead it regenerates the long-wavelength Pchlide forms (Kahn et al. 1970, Schoefs et al. 2000*b*). In the 644 and 655–657 nm emitting Pchlide forms, the pigments are bound in the active site of LPOR (Böddi et al. 1991, 1992) and they are flash photoactive (Böddi et al. 1991). Other, longer wavelength emitting Pchl(ide) forms having an emission maximum at 670 nm or above have been described, but they are not discussed in this paper as their structure is unclear (Kis-Petik et al. 1999, Ignatov and Litvin 2002).

Photoreduction of the photoactive, 644- and 655-nm-emit-

† *This and the accompanying paper are dedicated to Professor István Gyurján on the occasion of his 70th birthday.*

ting Pchlide forms to Chlide forms occurs on a micro- to millisecond time scale and the Pchlide–LPOR complexes are converted to Chlide–LPOR complexes (Dobek et al. 1981, Böddi et al. 2003, Heyes et al. 2003). After disaggregation of the LPOR macrodomain structure and the release of Chlide from the enzyme, Chlide is esterified and Chl is formed (Butler and Briggs 1966, Rüdiger et al. 1980). The structure of the etioplasts also changes after irradiation and thylakoids develop (Henningsen and Thorne 1974, Rascio et al. 1984).

Most studies on Pchlide photoreduction and the greening process have been carried out on dark-germinated plants, in which Chl synthesis is fully arrested at Pchlide (for review see Ryberg and Sundqvist 1991, Sundqvist and Dahlin 1997, Schoefs 2001), so that the reaction mechanism can be studied under controlled irradiation. The age of the dark-germinated seedlings used in these studies is usually between 2 and 15 days (Böddi et al. 1989, 1992, Schoefs and Franck 1993, He et al. 1994, Schoefs et al. 1994, 2000*a*). Such long-lasting continuous darkness during germination and early growth, however, usually does not occur in nature. Recently, Pchl(ide) accumulation was described in the inner leaves of the cabbage head, where the outer leaves function as optical filters, creating dark conditions at the center of the cabbage head (Solymosi et al. 2004). This observation led us to ask if this phenomenon was general in similar structures where the leaves are tightly packed and form layers. In this study, we examined the optical properties of bud scales of 14 species and the pigment forms in the innermost leaf primordia of closed and opened buds of 37 species.

Materials and methods

Plant material

Transmission spectra of bud scales were studied in 14 species (Table 1). Leaf primordia were studied in closed and opened buds of 37 plant species (Table 2). During March and April 2004 and 2005, twigs with buds and young leaves were collected in the Botanical Garden of the Corvinus University of Budapest, Hungary and in the woods in the hills around Budapest. The twigs were excised, put in jars containing tap water and transported directly to the laboratory. All buds were measured within 2–3 h of collection. Twigs were kept in natural light during transportation.

Sample preparation and treatment

The buds were dissected in dim green light, which does not cause phototransformation of Pchlide in dark-grown leaves. In all species, the inner leaf primordia were halved and put in sample holders for fluorescence measurements. All manipulations in dim green light took less than 2 min to perform. One sample was cooled to 77 K in the dark; the other was irradiated for 10 s with white light of 70 μmol m^{-2} s^{-1}. This sample was immersed in liquid nitrogen 10 s after irradiation. When the fluorescence spectra indicated a considerable accumulation of Pchl(ide), the outer leaf primordia and the bud scales were also studied. Each dark sample was warmed to –10 °C after measuring the 77 K fluorescence emission spectrum and was irradiated under the same conditions as above. The sample was then re-cooled to 77 K and remeasured. In most cases, three buds were measured per species, but in species with high Pchl(ide) concentrations, 7–10 buds were studied. Because of the way they were folded, light exposure varied from one part of a primordium to another, with the result that the ratio of the bands in the fluorescence spectra varied greatly and could not be evaluated quantitatively. Representative spectra are shown in the figures. The data in the tables were taken from spectra of samples with the highest or a characteristic Pchlide accumulation. When the innermost leaf primordia were small, they could not be separated from the shoot apex. In these cases, the spectra were recorded from the shoot tip, i.e., the combined cell layers of the apex and the leaf primordia.

Pigment determination

Fresh mass of the samples was measured and the samples were

Table 1. Transmission values (%) of bud scales taken from one representative spectrum of each of 14 species. The size of the buds used for the transmission measurements is also presented.

Species	Bud size (mm)	Transmission at 440 nm	Transmission at 650 nm	Transmission at 680 nm	Transmission at 750 nm
Ailanthus altissima P. Mill.	6–15	3.0	22.5	12.0	94.4
Malus domestica Mansf.	7–10	3.0	17.0	22.0	85.7
Prunus domestica L.	7	3.7	33.9	35.0	74.9
Juglans regia L.	7–14	3.7	12.8	7.2	79.7
Aesculus hippocastanum L.	17–27	3.7	10.9	6.3	85.8
Prunus persica (L.) Batsch.	6	4.8	19.2	10.2	97.2
Corylus avellana L.	17–24	8.9	24.5	17.0	93.9
Rosa canina L.	4–6	11.7	50.5	38.7	93.1
Fraxinus excelsior L.	5–12	16.1	19.2	16.1	65.6
Sambucus nigra L.	7–8	16.8	47.6	43.1	92.8
Acer pseudoplatanus L.	17–23	16.9	64.3	49.8	96.4
Prunus avium L.	8	18.7	56.0	39.2	96.3
Euonymus europaeus L.	7	26.7	74.1	66.3	98.0
Rubus idaeus L.	8	32.4	70.4	71.6	94.0

Table 2. Characteristics of the fluorescence emission spectra of the innermost leaf primordia of buds and young shoots (indicated by a single asterisk (*)) of 37 species. Values are fluorescence emission ratios at the indicated wavelengths (nm). A plus (+) sign indicates that no characteristic 0-0 band was at the wavelength of the denominator, but only vibronic bands of Pchl(ide) or Chl, or both, were observed. When the amplitude values (F_{633} and F_{655}) were around background values, or the bands were not specific, a minus (–) sign is given and the ratios were not calculated. Data are calculated from representative spectra and the spectra with highest Pchl(ide) accumulation were chosen for the calculations for each species. For the species labeled with an asterisk, the buds were already opening at the time of the measurements; however, several layers of older leaves covered the youngest leaf primordia of the shoot apex.

Species	Bud size (mm)	F_{633}/F_{682}	F_{740}/F_{682}	F_{633}/F_{655}
Fraxinus excelsior L.	5–12	3.978^{+}	0.405^{+}	2.191
Fraxinus angustifolia Vahl.	5–9	2.858^{+}	0.401^{+}	2.045
Fraxinus ornus L.	8–13	0.473	0.159	2.110
Juglans regia L.	7–14	0.423	0.229	2.297
Ailanthus altissima P. Mill.	6–15	0.277	0.165	2.522
Aesculus hippocastanum L.	17–27	0.116	0.269	1.369
Juglans nigra L.	8–11	0.240	1.946	2.449
Rhus hirta (L.) Sudw.	5–7, 10–11	0.216	1.332	3.193
Syringa vulgaris L.*	10–20	0.210	1.594	2.879
Acer pseudoplatanus L.	15–23	0.128	0.674	1.792
Melia azederach L.	4–7	0.098	1.893	3.491
Populus nigra L.	12–22	0.075	1.258	2.117
Acer platanoides L.	15–20	0.073	1.576	2.675
Rhus verniciflua Stokes	3–5	0.071	1.043	4.800
Platanus acerifolia Willd.	8–11	0.054	0.994	0.885
Sorbus aria (L.) Crantz	20–24	0.039	2.070	3.084
Quercus robur L.	5–8	0.036	2.476	3.425
Hedera helix L.*	7–8	0.026	1.385	2.017
Morus nigra L.	7–10	0.024	1.091	1.653
Quercus pubescens Willd.	6–8	0.018	2.630	3.118
Corylus avellana L.*	17–24	0.015	2.426	2.292
Cotinus coggygria Scop.*	10–12	0.012	3.016	2.987
Tilia platyphyllos Scop.	9–10	0.011	2.193	4.052
Tilia cordata Mill.	8–10	0.010	2.000	2.654
Carpinus betulus L.	10–12	0.009	3.787	1.649
Acer negundo L.*	10–15	0.008	2.667	2.113
Morus alba L.	6–8	0.006	3.019	1.957
Philadelphus coronarius L.*	19–21	0.006	0.944	1.998
Quercus petraea Liebl.	10–13	0.006	1.775	3.216
Populus canescens L.*	7–8	0.006	1.277	0.941
Magnolia stellata Max.	10–17	0.005	3.100	2.461
Salix cinerea L.*	13–16	0.004	2.730	2.196
Parthenocissus tricuspidata Planch. *	14–16	0.004	2.045	2.095
Liquidambar styraciflua L.*	14–16	0.002	2.175	10.726
Celtis australis L.*	10–13	–	2.443	–
Fagus sylvatica L.	20–25	–	2.147	–
Ficus carica L.	10–17	–	2.005	–

homogenized in 80% aqueous acetone. The pigment extracts were filtered through four layers of gauze. After determining the pigment content of the extract by absorption or fluorescence spectroscopy, petroleum ether and water were added to the aqueous acetone in a 1:1 (v/v) ratio, as described by Skribanek and Böddi (2001). When the phases separated, the upper petroleum ether phase was collected and measured. The efficiency of the pigment separation was checked with repeated phase separations. All manipulations were performed in dim green light. Pigment concentrations were calculated per gram of fresh mass according to the equations of Porra et al. (1989), Brouers and Michel-Wolwertz (1983) and data from Kahn (1983). For the fluorescence measurements, the excitation wavelengths were 430 and 450 nm. Calibration curves were determined with a series of Pchl, Chl a and Chl b solutions of known concentrations (determined by absorption spectroscopy). Only the linear region of each calibration curve was used for the calculations.

Spectroscopy of native samples

Absorption and transmission spectra were recorded with a Shimadzu UV-2101 PC (Japan) spectrophotometer, in the 400 to 800 nm region. The optical slits were 0.2 nm. The data frequency was 1 nm for measurements of native samples and

0.5 nm for pigment determination. For transmission measurements, the bud scales were fixed on the surface of a 2 × 15 mm window of a masked cuvette.

The 77 K fluorescence spectra were measured with a Fluoromax-3 (Jobin Yvon-Horiba, France) spectrofluorometer. The samples were immersed in liquid nitrogen during the measurements. Fluorescence emission spectra of leaf pieces were recorded with excitation wavelengths of 440 and 460 nm. The 440 nm excites predominantly the short-wavelength Pchl(ide) forms, whereas the 460 nm excitation is effective in exciting the long-wavelength forms (Böddi et al. 1993). The excitation and emission slits were 2 and 5 nm, respectively. The integration time was 0.2 s. The mean of three spectra was calculated automatically for each sample. The spectra were corrected for the wavelength-dependent sensitivity of the detector.

Analyses of spectra

Computer analyses were made with the SPSERV V3.14 program (copyright: C. Bagyinka, Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary). When necessary, baseline correction and a combination of 3-point and 5-point linear smoothing were performed.

Results

Optical properties of bud scales

Bud scales of different species differ in color, ranging from dark-brown or black (e.g., flowering ash, *Fraxinus ornus,* common ash, *Fraxinus excelsior*), to green (sycamore, *Acer pseudoplatanus*), brownish (common hazelnut, *Corylus avellana*), brownish-gray (common walnut, *Juglans regia*) to purple (tree of heaven, *Ailanthus altissima*). To determine how bud scales of different colors filter light, we measured the transmission spectra of bud scales of 14 species (Table 1; Figure 1). Although the shapes of these spectra were similar among species, the relative amplitudes of the transmission bands varied. Between 400 and 500 nm, the transmission was relatively low (less than 10%) in almost all spectra; it often increased slightly in the 500–600 nm region (Figure 1). The transmission usually had a local minimum around 680 nm. Above 680 nm, the transmission had a steep increase and transmission was 100% at 800 nm. Transmission values at 680 and 440 nm varied between 6.3–72% and 3–32%, respectively (Figure 1; Table 1). The transmission spectra of the bud scales varied widely even within the same species, depending on the position of the bud and on its developmental stage (or age). The spectra in Figure 1 and the data in Table 1 are representative of the qualitative characteristics of the bud scales. Transmittance values at 440, 650, 680 and 750 nm were used to characterize and compare the optical properties of the bud scales (Table 1). Light at 440 and 650 nm is effective in photoreducing Pchlide. The 680 nm band indicates the presence of Chl forms. In addition to these optical characteristics, the arrangement (phyllotaxy) of bud scales also influences their shading effect. In the case of overlapping scales, the optical path is multiplied and several layers of the scales can act as optical filters (e.g., *F. excelsior*). To examine the optical properties and pigment forms of the bud scales and the different layers of leaf primordia in buds in more detail, we measured their 77 K fluorescence emission spectra. As an example, the spectra obtained from the different bud parts of common ash are shown in Figure 2. In common ash, the inner and outer sides of the bud scale had different emission spectra. Despite the black coloration of the scale, the spectrum of its outer (abaxial) side showed fluorescence bands at 685, 695 and 742 nm, characteristic for green leaves (Figure 2, curve A). In

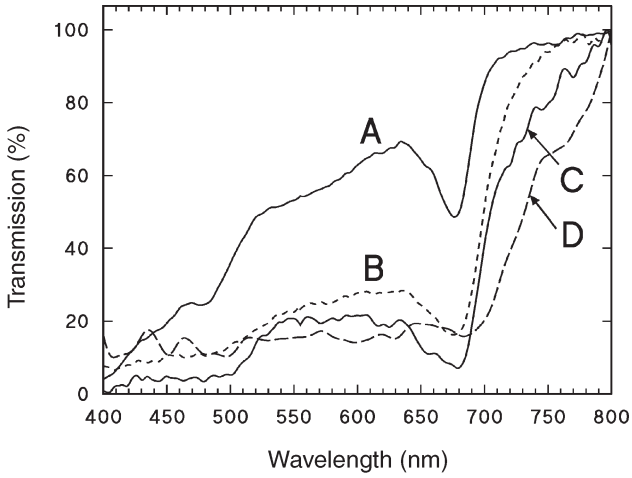

Figure 1. Transmission spectra of bud scales of sycamore (*Acer pseudoplatanus*; A), common hazelnut (*Corylus avellana*; B), common walnut (*Juglans regia*; C) and common ash (*Fraxinus excelsior*; D).

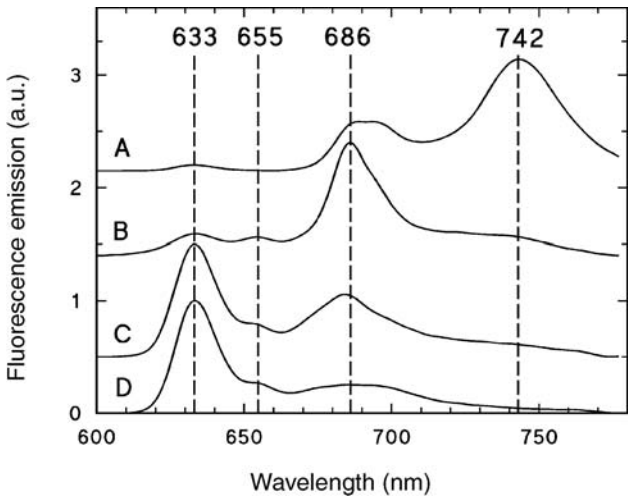

Figure 2. 77 K fluorescence emission spectra of bud scale (A, B), outer leaf primordium (C) and innermost leaf primordium (D) of common ash (*Fraxinus excelsior*). (A) outer (abaxial) side of the bud scale, covered by black hair; and (B) inner (adaxial) side of the bud scale. Excitation wavelength was 440 nm. The spectra are normalized at their maxima and shifted along the *y* axis for better presentation. Abbreviation: a.u. = arbitrary units.

addition, a minor band was present at 633 nm indicating the presence of protochlorophyllous pigments. The emission spectrum of the inner (adaxial) side of this bud scale showed a characteristic band at 686 nm, but the 742 nm band was absent. Minor bands were observed at 633 and 655 nm (Figure 2, curve B). The relative amplitude of the 686 nm band decreased and the peak at 633 nm increased in the spectra of leaf primordia under the scales and a shoulder at 655 nm was found. The spectrum of the primordium in the third layer (below two layers of completely overlapping and closed bud scales) is shown in Figure 2, curve C. The spectrum of the innermost leaf primordia contained only Pchl(ide) bands at 633 and 655 nm (and their broad satellite bands between 660 and 750 nm); no bands indicative of Chl or Chlide forms were detected (Figure 2, curve D).

We measured the 77 K fluorescence emission spectra of the innermost leaf primordia of 37 woody species to determine if Pchl(ide) accumulation occurred and to what extent Chl biosynthesis could proceed inside the buds. To characterize the spectra of the leaf primordia, the fluorescence amplitude ratios for different pairs of wavelengths are given in Table 2 (F_{633}/F_{682}, F_{740}/F_{682} and F_{633}/F_{655}, where subscripts indicate wavelengths in nm). Based on these ratios, three main categories of leaf primordia were distinguished.

Plants with innermost leaf primordia containing only Pchl(ide) (Category 1)

The completely closed buds of two species of ash (*F. excelsior* and *F. angustifolia*) belonged to this category (Table 2; Figure 2, curve D). The bud scales had low transmission values (Table 1; Figure 1, curve D). The spectra of the young leaves exhibited a narrow fluorescence band at 633 nm and a shoulder of very low amplitude at 655 nm at 440 nm excitation (Figure 2, curve D; Figure 3A, solid line). When the excitation was set at 460 nm, the main peak was at 643 nm and a shoulder was observed at 655 nm (Figure 3A, dashed line). The leaf primordia contained 4–6 and 1–3 μg g_{fm}^{-1} of Pchlide and Pchl, respectively (Table 3).

In response to 10 s of irradiation, the 655-nm-emitting form transformed to Chl(ide) with a broad band centered at 685 nm (Figure 3B, solid line). The 643 nm form remained unchanged as indicated by the spectra recorded with 460 nm excitation (Figure 3B, dashed line). The spectra recorded with 460 nm excitation revealed the complexity of the broad Chlide emission band; a maximum appeared at 692 nm and a small shoulder was found at 677 nm (Figure 3B, dashed line). The ratios of the Pchl(ide) forms varied with age of the primordia; in the younger innermost leaves the band at 643 nm predominated, but in older leaf primordia and in the inner (adaxial) side of the bud scale the photoactive 655 nm band became prominent.

When these buds started to sprout and opened, they had similar spectra to normally developed, fully green leaves. Thus, Chl emission bands appeared at 685, 695 and 730 nm in the spectra of the innermost leaf primordia (data not shown). These spectra were similar to those of the adaxial side of the bud scales in closed buds (Figure 2, curve B). The outer leaf primordia had fluorescence spectra similar to the abaxial part

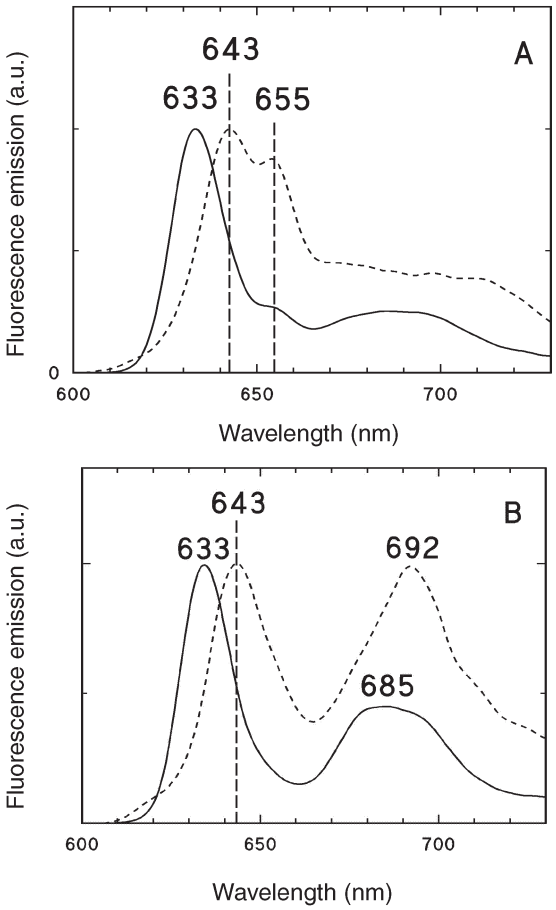

Figure 3. 77 K fluorescence emission spectra of innermost leaf primordia of common ash (*Fraxinus excelsior*). The buds were opened in the dark and the leaves were frozen in liquid nitrogen before (A) and after irradiation for 10 s with white light (70 μmol m^{-2} s^{-1}; B). Excitation wavelengths were 440 nm (solid lines) and 460 nm (dashed lines). Abbreviation: a.u. = arbitrary units.

of the bud scales (Figure 2, curve A). The bud scales contained almost no Pchl(ide) pigments.

Plants with innermost leaves containing Pchl(ide) and Chl (Category 2)

The innermost primordia of most buds and shoots contained Pchl(ide) and Chl. However, different spectral forms of Chl were present in these leaves, thus two subcategories were distinguished.

Plants with innermost leaves containing Pchl(ide) forms and a Chl form with emission maximum at 688 nm (Category 2a)

In four species—flowering ash, common walnut, tree of heaven and horse chestnut (*Aesculus hippocastanum*)—the innermost leaf primordia of the closed buds contained Pchl(ide) forms with emission maxima at 633, 643 and 655 nm and a Chl(ide) form with an emission maximum at 688 nm (Figure 4A, solid line; Table 2, rows 3–6). The 633/655 nm ratio was lowest in horse chestnut and varied within species (Ta-

Table 3. Pigment concentration (μg g_{fm}^{-1}) of inner leaf primordia of the closed buds of woody species determined from absorption and fluorescence measurements of acetone and petroleum ether extractions. The size of the buds is the same as in Table 2. The minus (–) sign indicates that the pigment was not detected in the samples. An asterisk (*) indicates that young shoots rather than closed buds were analyzed. For the species labeled with an asterisk (*), the bud scales or the buds had already opened at the time of measurement; however, several layers of older leaves covered the youngest leaf primordia of the shoot apex.

Species	Chlorophyll a (μg g_{fm}^{-1})	Chlorophyll b (μg g_{fm}^{-1})	Protochlorophyll (μg g_{fm}^{-1})	Protochlorophyllide (μg g_{fm}^{-1})
Fraxinus excelsior	–	–	3.4	5.9
Fraxinus angustifolia	–	–	0.6	3.6
Ailanthus altissima	24	10	1.8	2.2
Acer pseudoplatanus	41	18	2.7	3.3
*Syringa vulgaris**	200–270	66–80	2.2	2.1

ble 2, rows 3–6). As a representative of Category 2a, the fluorescence emission spectra of the innermost leaves of the buds of common walnut are shown in Figure 4. The band at 655 nm disappeared following a 10-s irradiation, but the bands at 633 and 643 nm were unaffected by the irradiation (Figure 4). Because the samples shown in Figure 4 originated from different leaves, the ratios of the bands cannot be compared. The presence of four fluorescence bands (633, 655, ~690 and ~740 nm) was characteristic of the outer leaf primordia (data not shown). The adaxial side of the cataphylls (leaves, covering the buds from outside like bud scales) contained only the Pchl(ide) form with an emission maximum at 633 nm in addition to the two main Chl peaks (data not shown). The outer side of the cataphylls did not contain Pchl(ide) pigments, but fluorescence bands corresponding to PSI and PSII were present (data not shown).

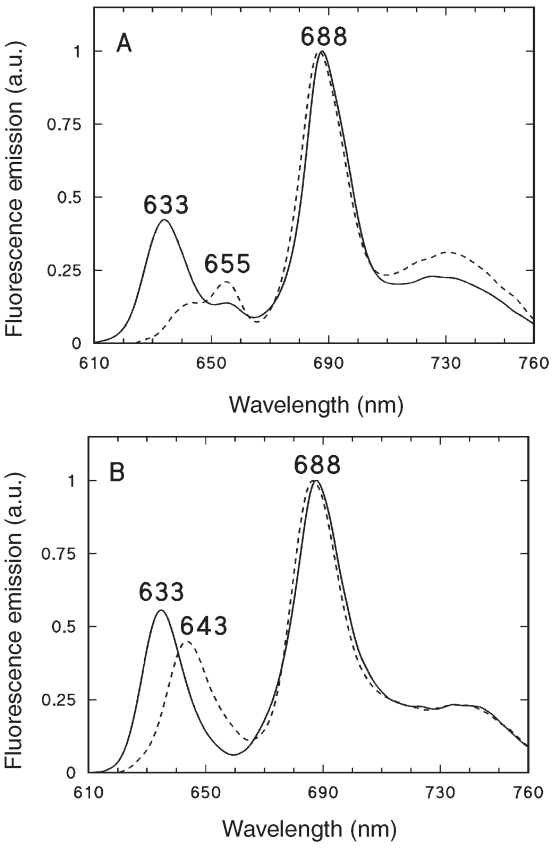

Figure 4. 77 K fluorescence emission spectra of the innermost leaf primordium of common walnut (*Juglans regia*) buds. The buds were opened in the dark and the leaves were frozen in liquid nitrogen before (A) and after (B) irradiation for 10 s with white light (70 μmol m^{-2} s^{-1}). Solid lines represent the spectra measured with 440 nm excitation and dashed lines represent spectra with 460 nm excitation. Abbreviation: a.u. = arbitrary units.

To further characterize the leaf primordia in Category 2a, the pigment concentration of the leaf primordia of closed buds of tree of heaven was determined (Table 3). The inner leaf primordia contained Pchl and Pchlide at concentrations of 1.8 and 2.2 μg g_{fm}^{-1}, respectively (i.e., a Pchlide:Pchl ratio of 1.2). The Chl concentration of the primordia was 34 μg g_{fm}^{-1} (Table 3).

All these features were characteristic only for the compactly closed buds and the fluorescence spectra altered when the buds started to open and the bud scales or outer cataphylls separated. The changes included the appearance of the 740 nm band in the innermost leaves and a species-specific decrease in the 633/655 nm fluorescence ratio (data not shown). During bud break, the inner primordia of opened buds of tree of heaven contained low amounts of pigments (about 1–1.5 μg g_{fm}^{-1} of Pchl and Pchlide and 100 and 30 μg g_{fm}^{-1} of Chl a and Chl b, respectively).

Plants with innermost leaves containing Pchl(ide) forms and Chl forms characteristic of green leaves (Category 2b)

Most of the studied species belonged to Category 2b; however, the species differed in the extent of Pchl(ide) accumulation and in the ratio of the 633/655 forms (Table 2, rows 7–34). It seems that the simultaneous presence of both Pchl(ide) bands at 633 and 655 nm and Chl bands characteristic of fully green leaves is common during the greening of leaf primordia. Both Pchl(ide) and Chl bands were also observed during ontogenesis of the primordia and in older, outer leaf primordia and bud scales belonging to Categories 1 and 2a (Figure 2, curves A–C).

The innermost leaf primordia of buds of four species (syca-

more, Norway maple (*Acer platanoides*), Japanese lacquer tree (*Rhus verniciflua*), staghorn sumac (*Rhus hirta*) accumulated relatively large amounts of Pchl(ide) forms. The ratio of the 633/655 forms was lowest in the spectra from sycamore (Table 2), which is shown as an example of Category 2b (Figure 5A). In the spectra recorded with 460 nm excitation (Figure 5A, dashed line), the band at 655 nm was higher than the band at 641 nm. In response to 10 s of irradiation, the 655 nm band disappeared, but the bands at 633 and 641 nm did not change (Figure 5B). The spectra of the outer leaf primordia contained only the Pchl(ide) band at 633 nm and Chl bands at 690 and 740 nm (data not shown). The spectral characteristics of the bud scales were similar to those of normally green leaves (data not shown). The Pchl(ide) concentration of the leaf primordia of sycamore was 6 μg g_{fm}^{-1} and the Pchl:Pchlide ratio was 0.8 (Table 3). Besides Pchl(ide), the leaf primordia contained 41 μg g_{fm}^{-1} of Chl a and 18 μg g_{fm}^{-1} of Chl b (Table 3).

The Chl concentrations and the accumulation of the different spectral forms showed high variability among species (Table 2; Figure 6). In the innermost leaves of lilac (*Syringa vulgaris*) shoots, the Pchl(ide) form with an emission maximum at 633 nm was predominant, and the long-wavelength Pchlide forms were present in only low amounts (data not shown). The innermost leaf primordia of lilac shoots contained about 2 μg g_{fm}^{-1} each of Pchl and Pchlide (Table 3). The Chl concentration of these leaves was the highest among the five species studied (Table 3). For most studied species, the intensity of the Chl bands obscured the Pchl(ide) bands, and they could be seen only when this region of the spectrum was magnified and analyzed separately (Figure 6). We were unable to quantify the trace amounts of Pchl pigments present in the samples because the amounts were below the sensitivity of the methods we used.

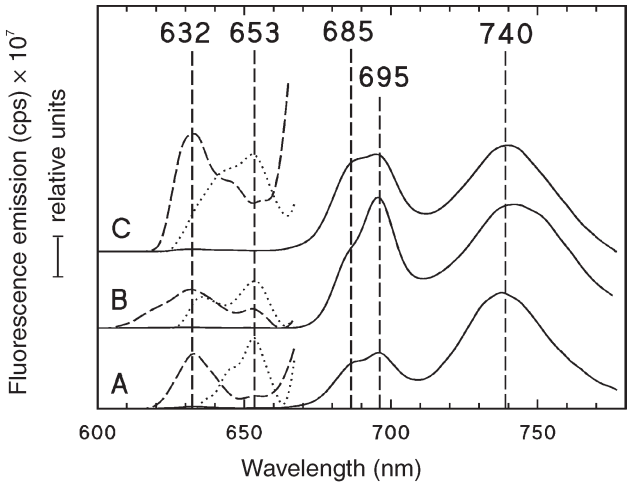

Figure 6. 77 K fluorescence emission spectra of innermost leaves of common whitebeam (*Sorbus* aria; A), mock orange (*Philadelphus coronarius*; B), black mulberry (*Morus nigra*; C). Excitation wavelength was 440 nm. In the 600–670 nm region, the corresponding spectra are also shown multiplied by 50 (dashed line: excitation wavelength at 440 nm; and dotted line: excitation wavelength at 460 nm). Abbreviation: cps = counts per second.

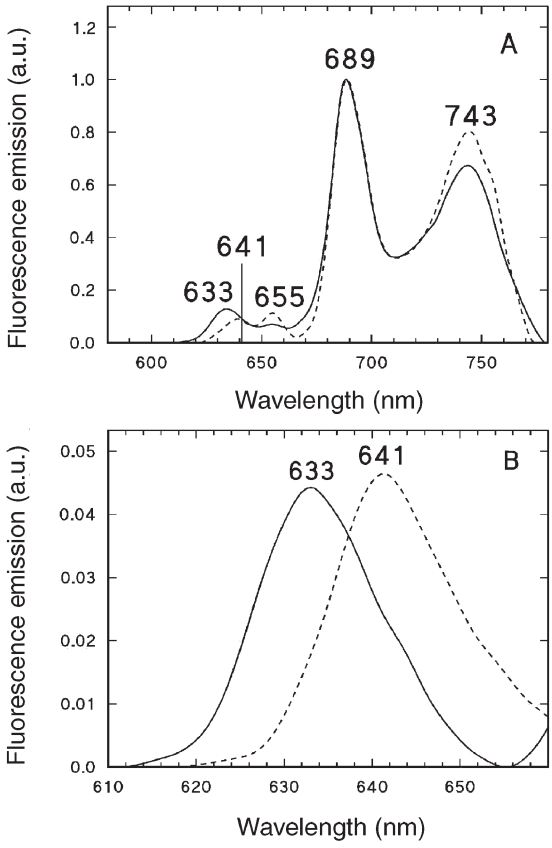

Figure 5. 77 K fluorescence emission spectra of the innermost leaf primordia of sycamore (*Acer pseudoplatanus*) buds before (A) and after (B) irradiation for 10 s with white light (70 μmol m^{-2} s^{-1}). The spectra are normalized. Excitation wavelengths were 440 nm (solid lines) and 460 nm (dashed lines). Abbreviation: a.u. = arbitrary units.

Plants showing no significant Pchl(ide) accumulation in the buds or young shoots (Category 3)

In some species, fluorescence spectra of the innermost leaf primordia of the buds or young shoots contained no bands characteristic of Pchl(ide), but only the fluorescence bands at 690 and 740 nm, indicating the presence of Chl–protein complexes of PSI and PSII (Table 2, rows 34–37; spectra not shown). These leaves had the spectral characteristics of fully green leaves and were not analyzed in detail. The concentrations of Chl a and Chl b in these leaf primordia varied between 200 and 400 and between 70 and 120 μg g_{fm}^{-1}, respectively.

Discussion

Although leaf development and greening are widely studied processes, there are few published data on the pigment forms and concentrations or on plastid differentiation of leaf primordia of buds. Raskin (1976) covered the closed leaf buds of lime (*Tilia* sp.) with black cloth and after bud burst he used the resulting etiolated leaves to study the late stages of greening. Recently, a natural long-lasting etiolated stage was described in the head of white cabbage, which is considered to be a mod-

ified bud (Solymosi et al. 2004). The innermost leaves in the cabbage head develop in the dark because of shading by the outer leaves. These leaves were at a primordial stage, accumulated Pchl(ide) and formed etioplasts. There are structural similarities between the cabbage head and closed buds; in the case of buds the innermost leaf primordia are covered by the outer leaf layers, and bud scales of various colors protect the whole bud. Therefore, we predicted the occurrence of Pchl(ide) accumulation and etioplast formation in the innermost leaf primordia and during the transitional stages of greening of the outer leaves. To test this prediction without encountering the experimental limitations associated with the study of greening in dark-germinated seedlings, we characterized the greening process in leaf primordia of closed and opened buds under natural conditions.

We demonstrated that bud scales filter light efficiently in the 400–700 nm range (Figure 1), whereas they exhibit high transmittance above 700 nm. According to Bradbeer et al. (1974), it is this optical property of the scales that underlies the light regulation of leaf primordium development. The species with leaf primordia belonging to Categories 1 and 2a (in which photoactive $Pchlide_{655}$ accumulated in leaf primordia to high concentrations) had relatively thick, large and dark bud scales or cataphylls (except sycamore, in which they were green). However, shading of leaf primordia is possible only in closed buds in which the scales and outer leaves are tightly arranged. The presence of bud scales alone is probably insufficient to create conditions for total etiolation of the leaf primordia. On the other hand, Pchl(ide) was detected on the adaxial sides of bud scales (Figure 2, curve B), indicating that the tissue structure and the pigments of scales provide local light filtering, i.e., conditions for "dark development," which may occur locally in certain tissue regions. The thickness of the scales is also important; no significant Pchl(ide) accumulation occurred in the buds of beech (*Fagus sylvatica*), common hazelnut, oak species (*Quercus* sp.) and common hornbeam (*Carpinus betulus*), which are covered by overlapping, brownish, but thin bud scales (Table 2). The shading effect of outer leaves or leaf primordia was also significant. Green tissues absorb wavelengths necessary for the photoreduction of Pchlide to Chlide. In lilac, the $Pchl(ide)_{633}$ form accumulated in the inner leaves of young shoots covered by outer, older leaves (Tables 2 and 3).

In addition to the optical properties of the scales, the arrangement of the scales is important. Species with leaf primordia in Categories 1 and 2a had compact buds, which ensured that some parts of the leaf primordia developed in the dark. The presence of the photoactive, 655-nm-emitting Pchlide form indicates that low irradiances could reach the innermost primordia (Figures 2–6). Small amounts of the photoactive Pchlide have been described in leaves exposed to low irradiances and the amount was inversely proportional to the irradiance (Franck and Strzałka 1992, Franck et al. 1993, Schoefs et al. 2000*a*, 2000*b*, Amirjani and Sundqvist 2004). Photoactive Pchlide is very sensitive and it is transformed to Chlide even at low temperatures and low irradiances (Sironval and Brouers 1970, Böddi et al. 1991, 2003, Heyes et al. 2003). In buds of trees, etiolation takes place under natural conditions and it is independent of the diurnal cycle of Chl biosynthesis observed in green leaves (Griffiths et al. 1985).

The innermost leaves of two ash species contained only Pchl(ide) and no Chl (Table 3), indicating that these primordia developed in complete darkness. The fluorescence emission spectra of these leaves (Figure 2, curve D; Figure 3), and the ratio of the different Pchl(ide) spectral forms resemble those of dark-germinated young leaves (Klein and Schiff 1972, Schoefs and Franck 1993, He et al. 1994, Schoefs et al. 2000*a*) and those of dark-grown stems and stem-related organs (McEwen et al. 1994, Böddi et al. 1994, 1998, 2004, Skribanek et al. 2000, Skribanek and Böddi 2001). They are also similar to those of the innermost leaves of cabbage heads (Solymosi et al. 2004). Based on our results and these studies, we conclude that the innermost leaves and the young shoot apex remained in an etiolated stage probably throughout the winter (or the Chl pigments were decomposed during this period), with greening of the etiolated leaf primordia occurring in the next growing season following bud break in the same way that greening occurs in etiolated plants (for reviews see Ryberg and Sundqvist 1991, Sundqvist and Dahlin 1997). In most species, bud formation starts at the end of the growing season in one year and is completed in the following growing season. Similar long periods of etiolation cannot be maintained in dark-germinated seedlings because of their limited amounts of storage material. Detailed spectroscopic analyses of the bud scales and the outer primordia of ash species indicated changing ratios of the Pchl(ide) forms and the presence of Chl in the outer primordia (Figure 2 and data not shown). The $Pchlide_{655}$ form increases with age in etiolated seedlings (Klein and Schiff 1972, Schoefs and Franck 1993, Schoefs et al. 2000*a*). The non-photoactive Pchl(ide) form with an emission maximum at 642 nm (observed as a separate band in the spectra recorded with 460 nm excitation; Figure 3 and in other species also in Figures 4–6) may be associated with Pchlide– $NADP^+$–LPOR complexes (Franck et al. 1999, Schoefs et al. 2000*a*, Schoefs 2001, Böddi et al. 2003).

The fluorescence spectra of the innermost leaves of the species having leaf primordia belonging to Category 2a (Figure 4), as well as the outer primordia and the inner part of the bud scales of common ash buds (Figure 2) had a Chl band at 688 nm in addition to the Pchl(ide) forms described earlier (Böddi et al. 1992). The presence of this band indicates that these leaves received light at the beginning of their development, but developed in the dark after complete closure of the buds and differentiation of the bud scales or cataphylls. Similar transient or retained Chl pigment forms are characteristic of the inner, intermediary leaf layers of white cabbage (Solymosi et al. 2004), re-etiolated potato tubers (Virgin and Sundqvist 1992), some dark-forced twigs (Skribanek et al. 2000) and the stipula and some parts of the epicotyls of dark-germinated pea (Böddi et al. 1999). In pea, the Chl pigments probably originated from the plastids of the green seeds (embryos). A Chl band with emission maximum at 680 nm was observed in stems of 25-day old dark-germinated horse chestnut seedlings and in this case the Chl may also have originated from the

seed (Skribanek et al. 2000).

In species in which the innermost leaf primordia belong to Category 2b (Table 3; Figures 5 and 6), photoactive Pchlide and non-photoactive Pchl(ide) accumulated during etiolation, but these leaf primordia also displayed spectral bands characteristic for PSI and PSII. Similar features were observed in the 8th leaf layer of white cabbage (Solymosi et al. 2004) and in re-etiolated plants (Minkov et al. 1988, Skribanek et al. 2000, Amirjani and Sundqvist 2004). The presence of Pchl(ide)$_{633}$ and the band at 650–653 nm is observed in almost all species, however, the strong fluorescence signal of the Chls hindered the detection of these bands. The simultaneous occurrence of Pchl(ide) bands, PSI and PSII may be the result of local high irradiances causing increased accumulation of Chl and a decreased steady-state concentration of Pchlide. Because the F_{740}/F_{682} ratio (Table 2) indicates the various amounts of Chl–protein complexes of PSI and PSII, it may indicate the developmental stage of the plastids (Amirjani and Sundqvist 2004); however, comparison of the band intensities of the fluorescence spectra provides precise information only at similar Chl concentrations or geometries (Weis 1985).

Based on our results, we conclude that Pchl(ide) accumulation is a common phenomenon in nature. The ratios of the different Pchl(ide) forms, and the amount and the ratio of Pchl to Pchlide depend on the species (Ryberg and Sundqvist 1991, Myśliwa-Kurdziel et al. 2003, Amirjani and Sundqvist 2004), developmental stage and age (Klein and Schiff 1972, Lancer et al. 1976, Schoefs and Franck 1993) and the physiological state of the dark-grown plants (Harsányi et al. 2002). Therefore, our data can be considered only in qualitative terms. Nevertheless, the Pchl(ide) concentration of the etiolated leaf primordia was similar to that in leaves of etiolated pea (Treffry 1970, Böddi et al. 1994) and bean (McEwen et al 1994). We have demonstrated that etiolation occurs transiently during normal ontogenesis of leaf primordia within the buds or within the young shoot apices covered by other, outer leaves. The experimental system is suitable for further studies of the greening process of etiolated plants and of the special features of the LPOR enzyme.

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References

Amirjani, R. and C. Sundqvist. 2004. Regeneration of protochlorophyllide in green and greening leaves of plants with varying proportions of protochlorophyllide forms in darkness. Physiol. Plant. 121:377–390.

Beale, S.I. 1999. Enzymes of chlorophyll biosynthesis. Photosynth. Res. 60:43–73.

Böddi, B., A. Lindsten, M. Ryberg and C. Sundqvist. 1989. On the aggregational states of protochlorophyllide and its protein complexes in wheat etioplasts. Physiol. Plant. 76:135–143.

Böddi, B., M. Ryberg and C. Sundqvist. 1991. The formation of a short-wavelength chlorophyllide form at partial phototransformation of protochlorophyllide in etioplast inner membranes. Photochem. Photobiol. 53:667–673.

Böddi, B., M. Ryberg and C. Sundqvist. 1992. Identification of four universal protochlorophyllide forms in dark-grown leaves by analyses of the 77 K fluorescence emission spectra. J. Photochem. Photobiol. B: Biol. 12:389–401.

Böddi, B., M. Ryberg and C. Sundqvist. 1993. Analysis of the 77 K fluorescence emission and excitation spectra of isolated etioplast inner membranes. J. Photochem. Photobiol. B: Biol. 21:125–133.

Böddi, B., B. McEwen, M. Ryberg and C. Sundqvist. 1994. Protochlorophyllide forms in non-greening epicotyls of dark-grown pea (*Pisum sativum*). Physiol. Plant. 92:706–713.

Böddi, B., K. Kis-Petik, A.D. Kaposi, J. Fidy and C. Sundqvist. 1998. The two spectroscopically different short wavelength protochlorophyllide forms in pea epicotyls are both monomeric. Biochim. Biophys. Acta 1365:531–540.

Böddi, B., A. Lindsten and C. Sundqvist. 1999. Chlorophylls in dark-grown epicotyl and stipula of pea. J. Photochem. Photobiol. B: Biol. 48:11–16.

Böddi, B., R. Popovic and F. Franck. 2003. Early reactions of light-induced protochlorophyllide and chlorophyllide transformations analyzed in vivo at room temperature with a diode array spectrofluorometer. J. Photochem. Photobiol. B: Biol. 69:31–39.

Böddi, B., K. Bóka and C. Sundqvist. 2004. Tissue specific protochlorophyll(ide) forms in dark-forced shoots of grapevine (*Vitis vinifera* L.). Photosynth. Res. 82:141–150.

Bradbeer, J.W., A.O. Gyldenholm, J.W. Smith, J. Rest and H.J.W. Edge. 1974. Plastid development in primary leaves of *Phaseolus vulgaris* IX. The effects of short light treatments on plastid development. New Phytol. 73:281–290.

Brouers, M. and M.R. Michel-Wolwertz. 1983. Estimation of protochlorophyll(ide) contents in plant extracts; re-evaluation of the molar absorption coefficient of protochlorophyll(ide). Photosynth. Res. 4:265–270.

Butler, W.L. and W.R. Briggs. 1966. The relation between structure and pigments during the first stages of proplastid greening. Biochim. Biophys. Acta 112:45–53.

Dobek, A., E. Dujardin, F. Franck, C. Sironval, J. Breton and E. Roux. 1981. The first events of protochlorophyll(ide) photoreduction investigated in etiolated leaves by means of the fluorescence excited by short, 610 nm laser flashes at room temperature. Photobiochem. Photobiophys. 2:35–44.

Franck, F. and K. Strzałka. 1992. Detection of the photoactive protochlorophyllide-protein complex in the light during the greening of barley. FEBS Lett. 309:73–77.

Franck, F., X. Barthélémy and K. Strzałka. 1993. Spectroscopic characterization of protochlorophyllide photoreduction in the greening leaf. Photosynthetica 29:185–194.

Franck, F., B. Bereza and B. Böddi. 1999. Protochlorophyllide-$NADP^+$ and protochlorophyllide-NADPH complexes and their regeneration after flash illumination in leaves and etioplast membranes of dark-grown wheat. Photosynth. Res. 59:53–61.

Griffiths, W.T. 1978. Reconstitution of chlorophyll formation by isolated etioplast membranes. Biochem. J. 174:681–692.

Griffiths, W.T., A.S. Kay and R.P. Oliver. 1985. The presence of photoregulation of protochlorophyllide reductase in green tissues. Plant Mol. Biol. 4:13–22.

Gunning, B.E.S. 1965. The greening process in plastids. 1. The structure of the prolamellar body. Protoplasma 60:111–130.

Harsányi, A., B. Böddi, K. Bóka, A. Almási and R. Gáborjányi. 2002. Abnormal etioplast development in barley seedlings infected with BSMV by seed transmission. Physiol. Plant. 114:149–155.

He, Z.-H., J. Li, C. Sundqvist and M.P. Timko. 1994. Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum*). Plant Physiol. 106:537–546.

Henningsen, K.W. and S.W. Thorne. 1974. Esterification and spectral shifts of chlorophyll(ide) in wildtype and mutant seedlings developed in darkness. Physiol. Plant. 30:82–89.

Heyes, D.J., C.N. Hunter, I.H. Van Stokkum, R. Van Grondelle and M.L. Groot. 2003. Ultrafast enzymatic reaction dynamics in protochlorophyllide oxidoreductase. Nat. Struct. Biol. 10:491–492.

Ignatov, N.V. and F.F. Litvin. 2002. A new pathway of chlorophyll biosynthesis from long-wavelength protochlorophyllide Pchlide 686/676 in juvenile etiolated plants. Photosynth. Res. 71: 195–207.

Kahn, A. 1983. Spectrophotometric quantitation of protochlorophyll-(ide): specific absorption and molar extinction coefficients reconsidered. Physiol. Plant. 59:99–102.

Kahn, A., N.K. Boardman and S.W. Thorne. 1970. Energy transfer between protochlorophyllide molecules: Evidence for multiple chromophores in the photoactive protochlorophyllide-protein complex in vivo and in vitro. J. Mol. Biol. 48:85–101.

Kis-Petik, K., B. Böddi, A.D. Kaposi and J. Fidy. 1999. Protochlorophyllide forms and energy transfer in dark-grown wheat leaves. Studies by conventional and laser excited fluorescence spectroscopy between 10 K-100 K. Photosynth. Res. 60:87–98.

Klein, S. and J.K. Schiff. 1972. The correlated appearance of prolamellar bodies, protochlorophyllide species, and the Shibata shift during development of bean etioplasts in the dark. Plant Physiol. 49:619–626.

Lancer, H.A., C.E. Cohen and J.A. Schiff. 1976. Changing ratios of phototransformable protochlorophyll and protochlorophyllide of bean seedlings developing in the dark. Plant Physiol. 57:369–374.

Lindsten, A., M. Ryberg and C. Sundqvist. 1988. The polypeptide composition of highly purified prolamellar bodies and prothylakoids from wheat (*Triticum aestivum*) as revealed by silver staining. Physiol. Plant. 72:167–176.

Masuda, T. and K. Takamiya. 2004. Novel insights into the enzymology, regulation and physiological functions of light-dependent protochlorophyllide oxidoreductase in angiosperms. Photosynth. Res. 81:1–29.

McEwen, B., C. Sundqvist and S. Younis. 1994. Protochlorophyll-(ide) forms in hypocotyls of dark-grown bean (*Phaseolus vulgaris*). Physiol. Plant. 90:396–407.

Minkov, I.N., M. Ryberg and C. Sundqvist. 1988. Properties of reformed prolamellar bodies from illuminated and redarkened etiolated wheat plants. Physiol. Plant. 72:725–732.

Myśliwa-Kurdziel, B., M.R. Amirjani, K. Strzałka and C. Sundqvist. 2003. Fluorescence lifetimes of protochlorophyllide in plants with different proportions short-wavelength and long-wavelength protochlorophyllide forms. Photochem. Photobiol. 78:205–215.

Myśliwa-Kurdziel, B., J. Kruk and K. Strzałka. 2004. Fluorescence lifetimes and spectral properties of protochlorophyllide in organic solvents in relation to the respective parameters in vivo. Photochem. Photobiol. 79:62–67.

Porra, R.J., W.A. Thompson and P.E. Kriedermann. 1989. Determination of accurate extinction coefficients and simultaneous equation for assaying chlorophyll-a and b extracted with different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975: 384–394.

Rascio, N., P. Mariani and G. Casadoro. 1984. Etioplast-chloroplast transformation in maize leaves: effects of tissue age and light intensity. Protoplasma 119:110–120.

Raskin, V.I. 1976. Mechanism of protochlorophyllide photoreduction in intact etiolated leaves. Vestsi Acad. Nauk BSSR, Ser. Biol. Sci. 5:43–46.

Rüdiger, W. 1997. Chlorophyll metabolism: from outer space down to the molecular level. Phytochemistry 46:1151–1167.

Rüdiger, W., J. Benz and C. Guthoff. 1980. Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur. J. Biochem. 190:193–200.

Ryberg, M. and C. Sundqvist. 1991. Structural and functional significance of pigment protein complexes of chlorophyll precursors. *In* Chlorophylls. Ed. H. Sheer. CRC Press, Inc., Boca Raton, Florida, pp 587–612.

Ryberg, M. and K. Dehesh. 1986. Localization of NADPH:protochlorophyllide oxidoreductase in dark-grown wheat (*Triticum aestivum*) by immuno-electron microscopy before and after transformation of the prolamellar bodies. Physiol. Plant. 66:616–624.

Schoefs, B. and F. Franck. 1993. Photoreduction of protochlorophyllide to chlorophyllide in 2-d-old dark-grown bean (*Phaseolus vulgaris* cv. Commodore) leaves. Comparison with 10-day-old dark-grown (etiolated) leaves. J. Exp. Bot. 44:1053–1057.

Schoefs, B., H.P. Garnir and M. Bertrand. 1994. Comparison of the photoreduction of protochlorophyllide to chlorophyllide in leaves and cotyledons from dark-grown bean as a function of age. Photosynth. Res. 41:405–417.

Schoefs, B., M. Bertrand and F. Franck. 2000*a*. Spectroscopic properties of protochlorophyllide analyzed in situ in the course of etiolation and in illuminated leaves. Photochem. Photobiol. 72:85–93.

Schoefs, B., M. Bertrand and C. Funk. 2000*b*. Photoactive protochlorophyllide regeneration in cotyledons and leaves from higher plants. Photochem. Photobiol. 72:660-668.

Schoefs, B. 2001. The protochlorophyllide-chlorophyllide cycle. Photosynth. Res. 70:257–271.

Shibata, K. 1957. Spectroscopic studies on chlorophyll formation in intact leaves. J. Biochem. 44:147–173.

Sironval, C. and M. Brouers. 1970. The reduction of protochlorophyllide into chlorophyllide. II. The temperature dependence of the $P_{657–647} \rightarrow P_{688–676}$ phototransformation. Photosynthetica 4:38–47.

Skribanek, A. and B. Böddi. 2001. Light- and cold-stress effects on the greening process in epicotyls and young stems of red oak (*Quercus rubra*) seedlings. Tree Physiol. 21:549–554.

Skribanek, A., D. Apatini, M. Inaoka and B. Böddi. 2000. Protochlorophyllide and chlorophyll forms in dark-grown stems and stem-related organs. J. Photochem. Photobiol. B: Biol. 55: 172–177.

Solymosi, K., K. Martinez, Z. Kristóf, C. Sundqvist and B. Böddi. 2004. Plastid differentiation and chlorophyll biosynthesis in different leaf layers of white cabbage (*Brassica oleracea* cv. capitata). Physiol. Plant. 121:520–529.

Sundqvist, C. and C. Dahlin. 1997. With chlorophyll pigments from prolamellar bodies to light-harvesting complexes. Physiol. Plant. 100:748–759.

Treffry, T. 1970. Phytilation of chlorophyll(ide) and prolamellar body transformation in etiolated peas. Planta 91:279–284.

Virgin, H.I. and C. Sundqvist. 1992. Pigment formation in potato tubers (*Solanum tuberosum*) exposed to light followed by darkness. Physiol. Plant. 86:587–592.

Weis, E. 1985. Chlorophyll fluorescence at 77 K in intact leaves: characterization of a technique to eliminate artifacts related to self-absorption. Photosynth. Res. 6:73–86.

Wiktorsson, B., S. Engdahl, L.B. Zhong, B. Böddi, M. Ryberg and C. Sundqvist. 1993. The effect of cross-linking of the subunits of NADPH:protochlorophyllide oxidoreductase on the aggregational state of protochlorophyllide. Photosynthetica 29:205–218.

Willows, R.D. 2003. Biosynthesis of chlorophylls from protoporphyrin IX. Nat. Prod. Rep. 20:327–341.