Transient etiolation: protochlorophyll(ide) and chlorophyll forms
in differentiating plastids of closed and breaking leaf buds of horse chestnut (Aesculus hippocastanum)†

KATALIN SOLYMOSI,1 KÁROLY BÓKA1 and BÉLA BÖDDI1,2

1 Department of Plant Anatomy, Eötvös University, Pázmány P. s. 1/C, Budapest, H-1117, Hungary
2 Corresponding author (bbphotos@ludens.elte.hu)

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Summary An accompanying paper reports the accumulation of photoactive protochlorophyllide (Pchlide) in the innermost leaf primordia of buds of many tree species. In this paper, we describe plastid differentiation, changes in pigment concentrations and spectral properties of bud scales and leaf primordia of horse chestnut (Aesculus hippocastanum L.) from January until the end of bud break in April. The bud scales contained plastids with grana, stroma thylakoids characteristic of chloroplasts and large dense bodies within the stroma. In January, proplastids and young chloroplasts were present in the leaf primordia, and the fluorescence spectra of the primordia were similar to those of green leaves except for a minor band at 630 nm, indicative of a protochlorophyll(ide). During bud break, the pigment concentrations of the green bud scales and the outermost leaf primordia increased, and Pchlide forms with emission maxima at 633, 644 and 655 nm accumulated in the middle and innermost leaf primordia. Depending on the position of the leaf primordia within the bud, their plastids and their pigment concentrations varied. Etio-chloroplasts with prolammellar bodies (PLBs) and prothylakoids with developing grana were observed in the innermost leaves. Besides the above-mentioned Pchlide forms, the middle and innermost leaf primordia contained only a Chl band with an emission maximum at 686 nm. The outermost leaf primordia contained etio-chloroplasts with well-developed grana and small, narrow-type PLBs. These outermost leaves contained only chlorophyll forms like the mature green leaves. No Pchlide accumulation was observed after bud break, indicating that etiolation of the innermost and middle leaves is transient. The Pchlide forms and the plastid types of the primordia in buds grown in nature were similar to those of leaves of dark-germinated seedlings and to those of the leaf primordia of dark-forced buds. We conclude that transient etiolation occurs under natural conditions. The formation of PLBs and etio-chloroplasts and the accumulation of the light-dependent NADPH:protochlorophyllide oxidoreductase are involved in the natural greening process and ontogenesis of young leaf primordia of horse chestnut buds.

Keywords: bud scale, etio-chloroplast, leaf primordia, LPOR, NADPH:protochlorophyllide oxidoreductase, prolamellar body, proplastid, prothylakoids.

Introduction

The transformation of protochlorophyllide (Pchlide) to Chlide, catalyzed by NADPH:Pchlide oxidoreductase (LPOR), is a light-dependent reaction in angiosperms (for review see Sundqvist and Dahlin 1997, Schoefs 2001, Masuda and Takamiya 2004). As a consequence, Pchlide accumulates in dark-germinated plants. The LPOR-catalyzed reaction is usually studied in leaves of etiolated seedlings; however, in the accompanying paper (Solymosi and Böddi 2006) we showed that, under natural conditions, protochlorophyll (Pchl) and Pchlide forms accumulate in buds covered with large, dark-pigmented and firmly closed bud scales. A similar phenomenon has been observed in the inner leaves of the head of white cabbage (Brassica oleracea L. cv. ‘capitata’), which is considered to be a modified bud. In this organ, because the outer leaves do not transmit light to the inner leaf layers, the latter develop in the dark (Solymosi et al. 2004). The fluorescence emission spectra of the innermost leaves of cabbage heads have a maximum at 633 nm and a shoulder of low intensity at around 655 nm (Solymosi et al. 2004). Similar spectra were observed for the innermost leaf primordia of closed bud of common ash (Fraxinus excelsior L.; Solymosi and Böddi 2006). No chlorophyll (Chl) was detected in these innermost leaf primordia. Similar spectral properties are typical for leaves of very young dark-germinated seedlings (Klein and Schiff 1972, Schoefs and Franck 1993, He et al. 1994, Schoefs et al. 2000a) and for dark-germinated or dark-forced stems or stem-related organs (McEwen et al. 1994, Böddi et al. 1994, 1998, Skribanek et al. 2000, Skribanek and Böddi 2001). Ultrastructural analysis showed that the innermost leaves of cabbage head (Solymosi et al. 2004), epicotyls of etiolated pea (Pisum sativum L.; Böddi et al. 1994) and hypocotyls of etiolated bean (Phaseolus vulgaris L.; McEwen et al. 1994) contain proplastids with poorly developed and often concentri-

† This and the accompanying paper are dedicated to Professor István Gyurján on the occasion of his 70th birthday.
cally arranged membrane vesicles. Occasionally prolamellar bodies (PLBs), which are characteristic of etioplasts of etiolated leaves, are also present in the plastids (Böddi et al. 1994, McEwen et al. 1994, Solymosi et al. 2004). The PLBs of dark-germinated seedlings have a highly regular inner membrane lattice consisting of hexagonal units. Depending on species and developmental stage, different types of PLBs can be distinguished. For example, in maize (Rascio et al. 1976) and oat (Gunning 1965), paracrystalline PLBs are characteristic, hereafter called narrow-type PLBs. The PLBs in barley have a looser, less regular structure and are referred to as open (Gunning 2001) or wide-type PLBs (Robertson and Laetsch 1974). However, narrow- and wide-type PLBs can develop in etioplasts of the same plant, and even within the same etioplast (Ryberg and Sundqvist 1991).

The intermediary leaves of the cabbage head and the outer, older leaf primordia of common ash buds contain Chls and Pchlide (Solymosi et al. 2004, Solymosi and Böddi 2006). The presence of the Chl form with an emission maximum at 682–688 nm indicates that the leaves must have been irradiated at some stage in their earlier development, whereas the accumulation of photoactive Pchlide forms confirms that the leaves grew in darkness at a later stage. This Chl form and Pchlide are also present in the innermost leaf primordia of the buds of several species, e.g., common walnut (Juglans regia L.), flowering ash (Fraxinus excelsior L.) and tree of heaven (Ailanthus altissima P. Mill.), and the ratio of the photoactive to the non-photoactive Pchlide forms is relatively high in these tissues (Solymosi and Böddi 2006).

Despite the spectral similarities between certain cabbage leaves and leaf primordia in leaf buds of several trees, the leaves of the cabbage head are special structures modified for storage. Therefore, plastid development in a cabbage leaf cannot be considered typical of leaves in general. Generally, chloroplast development includes several distinct plastid types, from proplastids through plastids with developing grana and perforated stroma lamellae to chloroplasts with well-developed grana (Whatley 1977). Etioplasts contain a special inner membrane structure with PLBs and prothylakoids. These membranes are considered to be a result of a development route characteristic only of dark-germinated seedlings. The PLBs in leaves of dark-germinated seedlings contain flash-photoactive Pchlide forms (Klein and Schiff 1972, Sperling et al. 1998). Because there is no comparable information on the development of leaf primordia under natural conditions, we followed changes in pigment concentrations, spectral properties and plastid development of horse chestnut leaf primordia from January until the end of bud break in April. Horse chestnut buds were chosen because of their relatively large size and easy accessibility.

Materials and methods

Plant material

Horse chestnut twigs with leaf buds were collected between January and April in 2003 and 2004 from parks in Budapest, Hungary. Excised twigs were put in jars containing tap water and transported directly to the laboratory under ambient light and temperature conditions. All buds were measured within 2–3 h of collection. Bud size varied between 1.5 cm (winter) and 3–4 cm (breaking buds in April). However, within a tree, the developmental stage of the buds varied, depending on position and ontogenesis (i.e., auxiliary or terminal buds). Consequently, closed buds (which predominate in January) can be found even at the end of March, when most buds are opened. The developmental stages of whole buds, their longitudinal sections and dissected leaf primordia are shown in Figure 1. The January and February buds, hereafter referred to as closed bud, are tightly compact and the outermost, brown bud scales overlap and cover the inner leaves (Figure 1A). The characteristic buds at the end of March and beginning of April, designated opening buds, are swollen with bud scales separating to reveal the expanding leaf primordia (Figure 1B). All leaf types can be observed in longitudinal sections of the opening buds. Below the outermost, brown bud scales there are several overlapping layers of inner, thin green bud scales which cover the developing leaf primordia. The leaf arrangement is opposite and decussate, and two or three pairs of leaf primordia can be observed in the buds (Figure 1B), hereafter referred to as outermost, middle and innermost leaves.

Buds were dissected under a dim green safelight, which did not cause phototransformation. Bud dissections were completed within 2–5 min. For artificial etiolation (dark-forcing) of the leaf primordia, twigs with closed buds were collected during March, placed in tap water, and kept in the dark at 20 °C. In parallel, horse chestnut seeds were collected in March and germinated in the dark for 3 weeks.

![Figure 1. Isolated leaf primordia (lp), side-view and longitudinal section of horse chestnut (Aesculus hippocastanum) leaf buds. (A) Closed buds characteristic of winter and early spring; (B) opening buds characteristic of the end of March and beginning of April in Hungary. Observe the arrangements of the brown bud scales (bbs), the leaf primordia and the green bud scales (gbs). The numbers refer to outermost (1), middle (2), innermost (3) leaf primordia and the shoot apex (4). Because of the low chlorophyll concentrations, the innermost and middle leaf primordia are pale. The outermost leaf primordia of opening buds are dark-colored. The primordia are densely covered by trichomes. The 5–7 leaflets can already be distinguished during the winter.](https://academic.oup.com/treephys/article-abstract/26/8/1087/1638461)
Pigments in leaf primordia and in green bud scales were analyzed. Pigments were not extracted from the brown bud scales, which were lignified and densely covered with resin. After measuring fresh mass, the samples were homogenized in 80% (v/v) acetone. The amount of esterified pigments was determined by petroleum ether phase separation method. The Chl and Pchl(ide) contents of the extracts were determined as described by Solymosi and Böddi (2006).

Spectroscopy

Whole bud scales and the leaflets of the primordia were used for the spectroscopic measurements. Ten closed buds (collected in January, February and March) and 10 opening buds (collected at the end of March and beginning of April) were analyzed. The absorption and transmission spectra were measured with a Shimadzu UV-2101 PC spectrophotometer as described by Solymosi and Böddi (2006). Mean values of pigment analyses of three buds are presented.

For fluorescence measurements the samples were halved; the first sample was cooled to 77 K in the dark, the other sample was irradiated for 10 s with white light of 70 µmol m–2 s–1 and immediately cooled to 77 K. After the measurements of the dark samples, they were warmed to ~10°C and irradiated for 10 s, before being reimmersed in liquid nitrogen. The 77 K fluorescence spectra were measured with a Fluoromax-3 (Jobin Yvon-Horiba, France) spectrofluorometer. The excitation and emission slits were 2 and 5 nm, respectively. The excitation monochromator was set at 440 and 460 nm. For the pigment extracts, 430 and 450 nm excitation wavelengths were also used. The integration time was 0.2 s. The means of three spectra are presented.

The SPSERV V3.14 program (C. Bagyinka, Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary) was used for baseline correction and 3- and 5-point linear smoothing of the spectra. All emission spectra were corrected for wavelength-dependent sensitivity variation of the signal detection.

Electron microscopy

Fluorescence emission spectra of the bud scales and leaf primordia collected for electron microscopic studies were measured. Tissue pieces were then fixed in 2% glutaraldehyde for 3 h and post-fixed in 1% OsO4 for 2 h. A 70 mM Na-K phosphate buffer (pH 7.2) was used in the fixatives and for rinsing. After dehydration in an alcohol series, the samples were embedded in Durcupan ACM resin (Fluka Chemie AG). Ultrathin sections were cut with Reichert Jung ULTRACUT E microtome (Austria). Sections were stained with uranyl acetate and lead citrate (Reynolds 1963), and examined with a Hitachi 7100 TEM (Japan) at 75 kV accelerating voltage.

Results

At the end of May, when the buds of the next vegetation period started to develop, all bud scales were green; however, by September or October, the outermost scales became brown. In parallel with the color change, colleters appeared on the abaxial side of the brown scales that became lignified. The buds also contained green bud scales, which were relatively thin. The leaf primordia, which were surrounded and protected by the overlapping bud scales (Figure 1), were small (10–15 mm in diameter) at this stage, and densely covered with trichomes. Between five and seven leaflets could be distinguished. This developmental stage was observed from September–October until the beginning of bud break in March and April.

By the end of March, elongation growth of the leaf primordia was rapid and the buds opened. The primordia had green and yellow areas, the ratio of which depended on their position; the outer parts were usually green (Figure 1). The leaflets were easily distinguishable and folded: the mid-vein was on the abaxial side (outside) and the blades turned to the adaxial side (toward the inside of the buds). After bud break, the petiole elongated rapidly in parallel with the growth of the leaf blade until the leaves reached their final size by the end of April or the beginning of May. In the meantime the bud scales dried and abscised.

Light filtering by bud scales and leaf primordia

To study how the bud scales and the different leaf primordia absorb light and shade the tissues beneath them, the leaf layers were dissected and placed one by one over the light sensor. The transmitted light was decreased to 6% by a single isolated brown bud scale, 35% by a thin, green bud scale and to 5–10% by a hairy leaf primordium. The leaflets were folded, thus the same blade can form several layers. The scales and leaf primordia together transmitted 1% of the incident light to the center of the closed buds, and this value decreased to 0.5–0.8% or to 0% in certain regions in the more developed, breaking buds collected at the end of March.

The transmission spectra of the bud scales (Figure 2) were similar to those observed for bud scales of other species (Solymosi and Böddi 2006); they had a local minimum at ~678 nm, where the transmission was about 6–10% relative to that at 800 nm. There was a small increase in transmittance between 678 and 630 nm, then a decrease at shorter wavelengths. Between 600 and 400 nm, the transmission was around 5%. Similar transmission spectra were obtained with leaf primordia and green bud scales, only the transmittance values varied. The transmittance of the brown bud scales did not change during the studied period, whereas the transmittance of leaf primordia and green bud scales increased greatly during bud break (data not shown).
Changes in pigment concentrations

Given the strong light filtering by the scales and outer leaf primordia, we determined if chlorophyll precursors accumulated in the innermost leaves. The Chl a and Chl b concentrations in green bud scales increased in March and decreased in aging green bud scales in April, 1 week after the end of bud break. No Pchl(ide) accumulation was observed in the green bud scales (Table 1).

As the buds developed, the pigment concentration of the leaf primordia also changed (Table 1). In January, the primordia contained Chl a and Chl b, but Pchl(ide) pigments were not detected. The Chl concentration of the primordia increased at the end of March (Table 1). At the same time, Pchl(ide) pigments accumulated in the primordia. The amount of Pchl increased only slightly, whereas Pchlide accumulated significantly at the beginning of April (Table 1). Thus the Pchl/Pchlide ratio decreased from 0.8 to 0.3 during bud break. The Chl concentration of leaf primordia increased from January until April (Table 1). At the end of bud break, the young, green leaves contained 273 and 77 µg g⁻¹ of Chl a and Chl b, respectively. No Pchl(ide) pigments were detected in green leaves (Table 1).

Table 1. Pigment concentrations (µg g⁻¹) of green bud scales and leaf primordia of horse chestnut (Aesculus hippocastanum) buds. The samples were taken at the end of January, end of March and beginning of April. Mean values of three measurements from different buds are shown. The difference between the three samples was always less than 10%. An asterisk (*) indicates that the 77 K fluorescence emission spectra showed the presence of the pigment, but its concentration was below the sensitivity of the assay. Abbreviations: Chl = chlorophyll; Pchl = protochlorophyll; and Pchl(ide) = protochlorophyllide.

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<th>Chl a (µg g⁻¹)</th>
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Spectral characteristics of the bud scales and leaf primordia

To study the distribution of the Chl and Pchl(ide) pigments, we analyzed the 77 K fluorescence emission spectral properties of bud scales and leaf primordia. Two Chl bands were detected in the fluorescence emission spectra of the brown and the green bud scales: one at 730–740 nm and the other at 685 nm (data not shown). There was a shoulder at about 695 nm on the latter band. The spectral properties of the bud scales did not change greatly during the studied period and were similar to those of fully developed green leaves.

In January, February and at the beginning of March, the spectra of the leaf primordia were similar to those of the bud scales. The Chl bands characteristic of PSI and PSII dominated the spectra (Figure 3, curve A). However, a minor band was observed at about 630 nm and a shoulder was detected at around 655 nm when the 600–660 nm region of spectrum A was magnified 50× (Figure 3, curve A*50). These two bands probably represent Pchlide pigments; however, they were not prominent and were not detected in the pigment extracts (Table 1). In March, at the beginning of bud flushing, the spectral properties of the inner leaf primordia changed. Fluorescence bands at 633 and 655 nm became more pronounced (Figure 3, curve B). The spectra of the innermost leaf primordia often comprised only three main bands at 633, 655 and 686 nm (Figure 3, curve C). The ratio of the Pchlide and Chl bands varied.

Figure 3. 77 K fluorescence emission spectra of leaf primordia of horse chestnut (Aesculus hippocastanum) buds. Excitation wavelength: 440 nm. (A) Innermost leaf primordia of closed buds collected and measured in January; (A*50 dashed line) the 600–660 nm region of the “A” spectrum multiplied by 50; (B) spectrum of a middle leaf primordium of opening buds, collected in March; (C) spectrum of innermost leaf primordium of opening buds, collected in March; and (D) spectrum of outermost leaf primordium of opening buds, collected in March.
among the different middle and innermost leaves and with time. The relative contribution of the Pchlide bands was highest in opening buds at the end of March and decreased at the beginning of April (data not shown). In parallel, the $F_{655}/F_{633}$ ratio ($F =$ fluorescence intensity) gradually increased. The outermost leaf primordia of the opening buds and the young uncovered leaves after bud break lacked Pchlide bands, but had the spectral characteristics of mature green leaves (Figure 3, curve D). However, at the beginning of bud break, the spectral properties of young leaves varied even within the same leaflet; the fluorescence emission spectrum of the outer part of the leaflet (i.e., the mid-vein) was similar to that of a mature green leaf, whereas Pchlide bands could be observed in the spectra of the leaf blades. Because fluorescence spectra show the integrated fluorescence properties of a leaf area of about 10 × 2 mm, they cannot reveal small scale changes across the leaf blade.

The presence of photoactive Pchlide forms suggests that the inner (adaxial) parts of the middle primordia and the innermost leaves developed in the dark. The photoactivity of the Pchlide forms was tested by irradiating the leaves with white light. As an example, the spectra of the innermost leaves are presented in Figure 4. Following 10 s of irradiation, the band at 655 nm disappeared, but the bands at 633 nm and 644 nm remained almost unchanged (Figure 4). Although peak values of the spectra cannot be compared, the shape of the band at 686 nm changed, indicating the formation of Chlide forms.

**Plastid differentiation**

To determine how plastid differentiation was affected by the absence of light and by the accumulation of Pchlide forms in the buds, we performed an ultrastructural analysis of brown and green bud scales and leaf primordia. In January, the plastids of the brown bud scales (Figure 5A) were relatively large (5–10 µm), and contained grana with usually 5–10 thylakoids. The abaxial epidermis and the cell layer below the epidermis of the brown bud scales contained large plastids with electron-dense content. Beneath these layers, grana were observed in the plastids that contained the same dense material (Figure 5A).

Proplastids with dense stroma were present in the epidermis of the green scales. The cell layers below the epidermis contained young chloroplasts (2–5 µm in size), the number of stroma thylakoids was relatively small (Figure 5B). Many plastids contained electron-dense material as seen in brown bud scales (Figure 5A). Only small proplastids (same size as mitochondria) were present in the plastids that contained the same dense material (Figure 5A).

The leaf blades of the primordia collected in January contained mostly meristematic cells with proplastids or chloroplasts at an early developmental stage. In these plastids only a few, parallel thylakoids were observed and almost no stacking (Figure 5C). These small plastids (about 1 µm in size) were characteristic of the closed buds collected between January and the beginning of bud break in March. Near the vascular system, the stroma of the plastids was light and the plastids were relatively well developed, containing small developing grana (Figure 5D). Plastid divisions were also observed in the primordia.

The plastid differentiation process speeded up at the end of March. The plastids of the innermost primordia of opening buds contained regular PLBs with (pro)thylakoids connected to them (Figure 5E). Unlike the etioplasts, the PTs had developing grana stacks, in which 2–3 lamellae were overlapping. These typical etio-chloroplasts were characteristic of the mesophyll areas and were relatively large (2–4 µm). Considerable ultrastructural heterogeneity was observed among plastids of the epidermis, the intervein part of the leaves and close to the mid-vein. Proplastids were observed in the epidermis, however, their stroma was dense. Proplastids were also observed in the proximity of the veins.

The outermost leaf primordia of opening buds and the young green leaves present directly after bud break also contained etio-chloroplasts, but the stacking of the thylakoids was more pronounced (Figure 5F) and the size of the plastids was 2–4 µm. In these primordia, the PLBs were regular narrow-type PLBs. The epidermis of these leaves also contained proplastids.
Dark-forcing of leaf buds

Photoactive Pchlide forms accumulated in relatively high amounts in opening buds, accompanied by the formation of etio-chloroplasts. To compare these etiolation symptoms occurring under natural conditions within the buds with artificial dark forcing of the young leaves, we placed closed buds in the dark for 1 week at room temperature (20 °C) at the beginning of March. During this period, the buds opened and the accumulation of the photoactive Pchlide forms was observed. The measured fluorescence emission spectra (not shown) were similar to those observed in the innermost primordia of opening buds (Figure 3, curve B).

Plastids (1–4 µm) with well-developed PLBs were found in all leaf primordia. The innermost primordia contained young, differentiating plastids with PLBs and poorly developed thylakoids (Figure 6A) in their meristematic cells. These plastids were similar to usual etioplasts present in dark-germinated seedlings. The cells of the outermost primordia were differentiating and had large vacuoles. The plastids of these primordia were etio-chloroplasts with grana stacks and PLBs (Figure 6B). Sometimes several thylakoid lamellae lay parallel to each other along almost their entire length. Osmiofil plastoglobuli were often observed in the plastids. Plastids containing PLBs were also observed in the epidermis of these leaves, but we found no differences in the plastid ultrastructure of different parts of the same leaflet.

The fluorescence emission spectra of the brown and green bud scales did not change during the dark period, and there were no significant changes observed in their plastid structure. The plastids of the bud scales contained no PLBs, but contained well-developed grana. The plastids of the brown bud scales had large plastoglobuli, dense bodies and grana, but almost no stroma thylakoids, and they exhibited symptoms of senescence. The plastids of the green bud scales did not change during the artificial dark period (Figure 5B).

Comparison with leaves of dark-germinated horse chestnut seedlings

Previous studies on Chl biosynthesis and etiolation symptoms have been carried out exclusively with dark-germinated seed-
lings (for review see Sundqvist and Dahlin 1997, Schoefs 2001). To compare the transient etiolation symptoms of leaf primordia observed under natural conditions within buds with those in leaves of dark-germinated seedlings, horse chestnut seeds were germinated in the dark for 3 weeks. The fluorescence emission spectra of the leaves of etiolated horse chestnut seedlings indicated only Pchlide pigments with main bands at 655 nm and 633 nm (Figure 7). A shoulder of low intensity appeared at 672 nm. In the spectra, recorded with 460 nm excitation, the presence of a band at about 642 nm was also observed (Figure 7B). In response to 10 s of irradiation, the short wavelength forms did not change, but the band at 655 nm dis-

Discussion

The etiolation process has generally been studied in dark-germinated seedlings of herbaceous plants (Gunning 1965, Henningsen and Boynton 1969, Robertson and Laetsch 1974, Rascio et al. 1976, 1986, Whatley 1977). In the accompanying paper, Solymosi and Böddi (2006) provide the first evidence that the transformation of etioplasts to chloroplasts observed on illumination of dark-germinated seedlings in the laboratory also occurs in nature in closed and opening buds collected

Figure 6. Plastids in leaves of horse chestnut (Aesculus hippocastanum) in the innermost (A) and outermost (B) leaf primordia after 1 week dark forcing of closed buds collected in March. Developing grana can be observed only in the etio-chloroplasts of the outermost leaves. Etioplast characteristic of the first leaf of a 3-week-old, dark-germinated seedling (C). Note the perforation on the prothylakoids. The bars indicate 1 µm. Abbreviations: Cw = cell wall; G = grana; M = mitochondrion; P = peroxisome; Pg = plastoglobuli; PLB = prolamellar body; PT = prothylakoids; and V = vacuole.

Figure 7. 77 K fluorescence emission spectra of etiolated leaves of 3-week-old, dark-germinated horse chestnut (Aesculus hippocastanum) seedlings. Spectra of the dark samples (solid lines) and spectra recorded after 10 s of irradiation with white light (70 µmol m$^{-2}$ s$^{-1}$; dashed lines). The excitation wavelength was 440 nm (A) or 460 nm (B).
from a variety of tree species. We have now conducted a detailed study on horse chestnut buds to determine the circumstances leading to transient etiolation and to characterize the stages of pigment synthesis and plastid differentiation.

Spectroscopic measurements showed that the brown bud scales (Figure 2) together with the green bud scales and leaf primordia absorb light in the spectral region that is needed for photoreduction of Pchlide to Chlide and for plastid differentiation. The transmission spectra of the brown bud scales resembled those of optical cut-off filters (Figure 2), raising the possibility that bud development is light regulated. Light filtering by the scales and outermost leaf primordia was most efficient during bud break in March, when the pigment concentrations of these organs increased greatly (Table 1).

Based on our results, we categorized the leaves of the buds into three groups: the scales, the inner leaf primordia and the outer leaf primordia. The scales (both the outer, brown and the inner, green) developed and differentiated almost completely in the previous vegetation period; they were exposed to light and developed the entire photosynthetic apparatus as in green leaves (Figure 3). These leaves also received light at later stages in their development and so they had fully developed chloroplasts or later gerontoplasts throughout the studied period. These leaves had Chl a and Chl b forms but did not contain Pchl or Pchlide (Table 1).

The innermost leaf primordia comprising the second group were also exposed to light at a certain early period of their development but later they were fully covered by the outermost primordia and the scales. During their early development in light, the entire photosynthetic apparatus formed, with Chl emission bands at 684, 694 and 740 nm and only traces of non-photoactive Pchl(ide) were present (Figure 3, curves A and A^*50). During this period (Figure 1), the meristematic tissues of these leaves contained proplastids with a poorly developed inner membrane system (Figure 5C and D, Marinos 1967). Small amounts of Pchlide have also been detected in greening tissues developing in light (Treffry 1973, Franck and Strzałka 1992, Franck et al. 1993, Schoefs et al. 2000a, 2000b). During bud break the innermost primordia became covered by outer leaves and scales; consequently, little if any light reached these primordia at the time of bud break. At this stage, the innermost primordia had etio-chloroplasts (Figure 5E) and only one Chl form having a sharp emission band at 684–686 nm but no other 0-0 transition bands of Chl (Figure 3, curve C). The emission bands of Pchl or Pchlide forms, or both, could be observed (Figure 3, curve C); furthermore, the flash phototactic activity 655 nm form was present in these primordia (Figures 3 and 4). We postulate that the large amount of this form indicates etiolation of the innermost leaves. Small amounts of the 655-nm-emitting Pchlide form have also been identified in leaves developing at low irradiances (Franck and Strzałka 1992, Franck et al. 1993, Schoefs et al. 2000a, Amirjani and Sundqvist 2004). The occurrence of PLBs is in agreement with the presence of LPOR and the photoactive Pchlide form. Overexpression of LPOR restores PLBs and photoactive complexes in photomorphogenetic Arabidopsis mutants (Sperling et al. 1998) and LPOR has a photoprotective role against photooxidation caused by free, non-photoactive Pchlide pigments (Sperling et al. 1997). The non-photoactive Pchlide forms with emission maxima at 633 and ~642 nm are also characteristic of dark-germinated plant material (McEwen et al. 1991, 1994, Böddi et al. 1992, Kis-Petik et al. 1999, Skribanek et al. 2000). Simultaneous presence of Pchlide and Chl forms has been reported for buds of other tree species (Solymosi and Böddi 2006) and also for re-etiolated plant materials (Minkov et al. 1988, Skribanek et al. 2000, Amirjani and Sundqvist 2004). After bud break, the horse chestnut leaves contained only Chl pigments (Table 1), thus etiolation was only transient in these leaves.

The outermost leaf primordia belong to the third category of leaves. Like the inner leaf primordia, the outer leaf primordia were exposed to light in winter. They contained proplastids, and Chl bands, characteristic of fully green leaves, were present in their fluorescence spectra. During bud break, the primordia started to develop, the cells elongated, large vacuoles developed (Figure 5E) and the leaf blades started to differentiate (Figure 1). In parallel with the growth and thickening of the leaf primordia, their absorbance and shading effect increased, but the shape of their fluorescence emission spectra did not change much (Figure 3, curve D). Despite the similarity of their spectra with photosynthetic green leaves, the outermost primordia contained well-developed etio-chloroplasts with large grana stacks (Figure 5F). This type of plastid is found in young leaves developed in low irradiances (Wrisher 1966, Henningsson and Boynton 1969, Laetsch and Price 1969, Treffry 1973, Rascio et al. 1976, Wellburn et al. 1982, Casadoro and Rascio 1987) and in young etiolated leaves subjected to low irradiance (Weier et al. 1970, Wrisher 1973, Ginrth et al. 1978). Treffry (1973) described etio-chloroplasts in leaves irradiated with dim red light, which is in agreement with our results, because dim red and far-red enriched light might be present under the bud scales and the outermost leaf primordia (Figure 2). Plastid differentiation and thylakoid formation as well as leaf growth are controlled by phytochromes (Bradbeer et al. 1974, Ginrth et al. 1978). Plant hormones, e.g., cytokinin, also influence plastid ultrastructure and LPOR gene expression (Kuroda et al. 1996, Chatfield et al. 2000, Seyedi et al. 2001).

The middle leaves represent an intermediary developmental stage between the inner and outer leaf primordia. In the leaf blades (turning towards the inside) Pchlide accumulated, and the Chl bands of PSI and PSII were also present (Figure 3, curve B). Depending on the position, the wrapping and the degree of shading, in some leaflets the mid-vein region (which is turned to face outward) contained no Pchlide pigments and had a spectrum similar to that of fully developed green leaves.

The closed buds, collected in March and transferred to the dark, opened within a week at room temperature, indicating that the primordia were in a quiescence stage. Temperature and not illumination was important in the initialization of sprouting. The spectra of the different leaf primordia of dark-forced buds were similar to those observed in the innermost primordia of opening buds (Figure 3, curve C). The outermost primordia of dark-forced buds contained etio-chloroplasts.
with developing grana (Figure 6B) and sometimes parallel (pro)thylakoid membranes. These parallel arrangements of the thylakoids are characteristic of the PTs of maize etioplasts (Rascio et al. 1986). The innermost leaves had small, PLB-containing plastids with poorly developed thylakoids (Figure 6A), such plastids are characteristic of both etiolated leaves (Gunning 1965) and re-etiolated leaves (Henningsen and Boytont 1969, Bradbeer et al. 1974, Rascio et al. 1984, Minkov et al. 1988).

The leaves of dark-germinated horse chestnut seedlings contained regular etioplasts, no grana were observed, but single perforated PT lamellae were present (Figure 6C); and the spectra comprised mainly Pchlide forms, characteristic of dark-germinated seedlings (Figure 7; Gunning 1965, Böddi et al. 1992). However, a minor band was observed at 672 nm. A similar long wavelength band has previously been observed in the epicotyl of dark-grown horse chestnut seedlings (Skribanek et al. 2000) and can probably be attributed to residual Chls present in the seeds that are transferred to the developing tissues as in pea (Böddi et al. 1999). Alternatively, this long wavelength band could be the long-wavelength Pchl(ide) form with a maximum at 670 nm (Böddi et al. 1999).

In conclusion, we have demonstrated that the highly regular membrane structure of the PLBs—which is usually regarded as a feature of etioplasts of dark-grown leaves—occurs under natural conditions. Depending on the position of the leaf primordia, two types of etio-chloroplasts could be distinguished in horse chestnut buds during sprouting (Figures 5E and 5F). The first type of etio-chloroplasts contain wide-type PLBs and developing grana only (Figure 5E) and resemble etioplasts. The second type of etio-chloroplasts had narrow-type PLBs with well-developed grana (Figure 5F). These PLB types are common in etio-chloroplasts of plants grown in low light (Wrischer 1966, Weier et al. 1970, Treffry 1973, Rascio et al. 1980). No Pchlide(ide) accumulation was observed in the low-light or light-grown leaves having the second type of plastids (Table 1; Figure 3, curve D). This indicates that the paracrystalline membrane structure can be maintained in the absence of significant amounts of Pchlide(ide) (Figure 5F; Figure 3, curve D). We conclude that the bud structure of horse chestnut causes transient formation of etioplasts and etio-chloroplasts under natural conditions. The Pchlide forms and etioplast inner membrane structures observed in the innermost leaves of a horse chestnut bud are similar to those found in leaves of etiolated seedlings.

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