Photosynthesis Research **82:** 141–150, 2004.
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Regular paper

Tissue specific protochlorophyll(ide) forms in dark-forced shoots of grapevine (*Vitis vinifera* L.)

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Received 29 September 2003; accepted 13 May 2004

Key words: chlorenchyma, dark-forcing, etioplast, fluorescence, grapevine, plastid, protochlorophyllide

Abstract

Cuttings of grapevine (*Vitis vinifera* L. cv. Chardonnay) were dark-forced at least three weeks. Pigment contents, 77 K fluorescence emission and excitation spectra of the leaves, petioles and stems and transmission electron micrographs of the etioplasts from leaves and the chlorenchyma tissues of the stems were analysed. The dark-grown leaves and stems contained 8 to 10 and 3 to 5 μg/g fresh weight protochlorophyllide and its esters, respectively. HPLC analysis showed that the molar ratio of the unesterified and esterified pigments was 7 : 3 in the shoot developed in darkness. The dark-forced leaves contained carotenoids identified as: neoxanthin, violaxanthin, antheraxanthin, lutein and β-carotene. Detailed analyses of the fluorescence spectra proved that all tissues of the dark-forced shoots had protochlorophyllide or protochlorophyll forms with emission maxima at 628, 636, 644, 655 and 669 nm. The 628 and 636 nm emitting forms were present in all parts of the dark-forced shoot, but dominated in the stems, which may indicate an organ specificity of the etioplast development. Variations in the distribution of the pigment forms were even found in the different tissues of the stem. The subepidermal layers were more abundant in the 655 nm form than the parenchyma cells of the inner part of the cortex and the pith. In the latter cells, the plastid differentiation stopped in intermediary stages between proplastids and etioplasts. The plastids in the subepidermal layers had developed prolamellar body structures, which were similar to those of etiolated leaves. The results highlight the importance of organ- and tissue specificity of plastid differentiation for chlorophyll biosynthesis and greening of different plant organs.

Introduction

Protochlorophyllide (Pchlide), as a precursor in chlorophyll (Chl) biosynthesis has a regulative role, via a feed-back mechanism, on its own accumulation (Schiff and Epstein 1966). In higher plants the Pchlide transformation into chlorophyllide (Chlide) is catalysed by the light-dependent enzyme NADPH:protochlorophyllide oxidoreductase (LPOR) (Fujita 1966). Leaves of 8- to 15-day-old, dark-germinated seedlings usually contain etioplasts with the inner membranes forming prolamellar bodies (PLB) and prothylakoids (PT) (Gunning 1975). LPOR form ternary complexes with Pchlide and NADPH (Griffiths 1975), which are embedded in the PLB membranes (Ryberg and Dehesh 1986). Detailed spectroscopic studies (Böddi et al. 1992, 1993) and experiments with crosslinkers (Wiktorsson et al. 1993) have shown that the LPOR is aggregated with a geometry placing the Pchlide molecules close enough to each other to form excitonic interactions among their π-electronic systems (Böddi et al. 1989). Pchlide and LPOR are thus forming a macrodomain structure showing red-shifts of the absorption (A) and fluorescence (F) spectra with maxima at A638/F644 and A650/F657 nm (Shibata 1957; Böddi et al. 1989). The enzyme

activity is unique as the reaction proceeds even at low temperatures (below 0 °C) (Sironval and Brouers 1970) and at flash illumination (Madsen 1963; Dobek et al. 1981). Furthermore, the macrodomain ensures photoprotection for both Pchlide and Chlide (Armstrong et al. 2000). During the phototransformation process, the oxidised NADP molecules are continuously re-reduced, creating Pchlide and Chlide micro-cycles (Franck and Inoue 1984; Franck et al. 1999; Schoefs 2001). Due to these cycles, the excitation energy drives rather photoreduction than photooxidation reactions.

Epicotyls from dark-grown pea plants (Böddi et al. 1994) and dark-forced stems of a number of woody plant species (Skribanek et al. 2000) have Pchlide in different amounts with various native organisations as shown by the 77 K fluorescence spectra. In some species, the Pchl and/or Pchlide amount is about one-tenth of that of leaves, or 1 to 3 μg/g fresh mass. In the 77 K fluorescence emission spectra the bands are found at 628 and 636 nm slightly blue-shifted compared to those of leaves. Pchlide having these bands are not flash photoactive, but can be transformed into chlorophyllide (Chlide) with continuous illumination (Böddi et al. 1996). The analysis of the 0–0 transitions of the bands demonstrated that the 629 and 636 nm Pchlide forms contain pigment molecules in a monomeric state. In addition to the monomeric forms, flash photoactive 644 and 653–655 Pchlide forms are present in minor amounts. Stems dominated of monomeric Pchlide forms are photosensitive. Ordinary daylight causes bleaching and severe damages (Skribanek and Böddi 2001).

Dark forcing is a well-known method used in grapevine propagation. In this work, the pigments were analysed in dark-forced grapevine cuttings. The spectral properties of the native Pchlide or Pchl forms were investigated with 77 K fluorescence spectroscopy and the etioplast ultrastructures were studied in various parts of the dark-forced shoots.

Materials and methods

Experimental material

Cuttings of grapevine of 20–30 cm long (*Vitis vinifera* L. cult. Chardonnay) collected for propagation, were soaked in running tap water for 2 days. The cuttings were put into Erlenmeyer flasks containing tap water and were forced in the dark at 20 °C for usually 14 to 42 days, but occasionally up to 100 days. The tap water was changed every second day. The first dark-forced shoots appeared after 2 weeks, and after 6 weeks their length was about 20 cm. Leaves, petioles and 2-cm long pieces of stems were studied. In some experiments, longitudinal sections were cut from the stems and the fluorescence spectra of the subepidermal cortex, the inner cortex and the pith tissue layers were separately measured.

Fluorescence spectroscopy

The 77 K fluorescence spectra were measured with an SLM 8000C spectrofluorometer or a Fluoromax 3 Jobin Yvon Horiba spectrofluorometer, both equipped with liquid nitrogen accessories. The samples were immersed into liquid nitrogen during the measurement. The excitation and emission slits were set to 4 and 8 nm, respectively. The emission spectra were corrected for the wavelength dependent sensitivity variation of the detector. The excitation spectra, measured between 400–500 nm, were corrected for the Xenon lamp profile, while those measured above 600 nm, were not corrected. Three spectra were recorded and averaged from each sample.

HPLC

Pigments were extracted from the plant materials with 80% acetone. The pigments were transferred into diethyl ether and then into methanol. HPLC was carried out using two Consta Metric pumps (Milton Roy Co., Riviera Beach, Florida) and a C_{18} reversed-phase column, 20 cm long, packed with Nucleosil 5 μm (Macherey-Nagel Co, Düren, Germany). The procedures are described in detail in Böddi et al. (1989).

Computer analyses of the fluorescence spectra

All spectra were processed with the software SPSERV V3.14 (copyright: C. Bagyinka, Inst. Biophys. Biol. Res. Cent. of the Hungarian Acad. Sci., Szeged, Hungary). The spectra were smoothed with three and five point linear smoothing to get rid off the electronic noise. This procedure was tested not to modify the fine

structure of the spectra. Baseline correction was done for light scattering using spectra recorded from the empty sample holder. In case of leaves and petioles, spectra of 10 samples, in the case of stems, spectra of 25 samples were averaged and these average spectra were used for calculations of the second and fourth derivative spectra. The derivative spectra and the amplitude changes of the emission bands in spectra recorded with different excitations provided input information for the Gaussian deconvolution. The deconvolution was performed in a wave number function of the spectra. The results shown in the Figures were then transformed into wavelength function.

Transmission electron microscopy (TEM)

Leaf and stem pieces were collected for TEM studies. In order to distinguish plastids from different tissue layers, the stem pieces were oriented. The tissue pieces were fixed in 2% glutaraldehyde for 48 h and were post-fixed with 1% OsO_4 for 3 h. The fixing solutions were diluted and then rinsed with 70 mM Na-K-phosphate buffer (pH 7.2). The samples were dehydrated in ethanol series and embedded in Durcupan epoxi resin (Fluka Chemie AG, Buchs, Switzerland). The 50 nm ultrathin sections were stained for 4 min with 5% uranil-acetate dissolved in methanol and treated with lead citrate solution for 6 min (Reynolds 1963). The sections were investigated with a Hitachi 7100 TEM (Tokyo, Japan) at 75-kV accelerating voltage.

Results and discussion

Etiolated leaves of dark-germinated seedlings are often used in works studying the last steps of chlorophyll biosynthesis. The age of the seedlings usually does not exceed 14 days because of the senescence starting when the limited amount of storage material is exhausted in the seed. The cuttings studied in this paper contained a larger pool of storage materials than most seeds; allowing long growth periods in the dark. Consequently, samples used in this work could be older (samples were collected even after a growth period of 100 days in the dark) than the leaves of etiolated seedlings. The study of all parts of the dark-forced shoots (i.e., leaves, petioles and stems) was expected to deliver new information about the pigment forms at a mature stage of the tissue.

The dark-forced shoots were yellow all along the stems and so were the leaves. At a shoot age of 6 weeks, the leaves were 1.5 to 2 cm in diameter and had an approximately 1-cm-long petiole. After sampling, thin secondary shoots developed. These shoots were white and were not studied in this work.

All parts of the shoots contained Pchl/ide pigments (i.e., Pchlide and Pchl, designated as Pchl/ide if not separately distinguished) and carotenoids. The amounts of Pchl/ide were around 8–10 and 3–5 μg/fresh weight in the leaves and stems, respectively. The pigment content of stems is about 10 times higher than that of epicotyls of dark-germinated pea seedlings (Böddi et al. 1994). However, similar amounts were found in dark-forced stems of several woody plants (Skribanek et al. 2000). There was no remarkable gradient in the pigment content along the stem and the content decreased very slowly with age. However, the presence of Pchl/ide was clearly seen in basal region of even 100-day-old dark-forced stems (results not shown). The slow decrease of Pchl/ide pigments shows that the dark-forced grapevine stems have basically a different developmental pattern than the dark-germinated seedlings. In leaves of oat seedlings, the Pchl/ide content increased until 7 days then it gradually decreased to 20% of the maximal value by the 11th day (Bergweiler et al. 1984).

The HPLC analysis of the pigments showed that around 30% of the Pchl/ide pigment was Pchlide-ester (i.e., Pchl) and 70% was Pchlide. Besides the Pchl/ide pigments, the following carotenoids were found: neoxanthin, violaxanthin, antheraxanthin, lutein and β-carotene with relative molar ratios of 26.8, 4.2, 14.0, 5.3 and 1, respectively. Similarly to our results, neoxanthin and violaxanthin are the dominating carotenoids in roots of dark-grown wheat, pea and maize seedlings (Mc Ewen and Lindsten (1992). The etiolated leaves, however, have different carotenoid ratios, which change remarkably at illumination under different light and temperature conditions (Virgin 1966, 1967; Albrecht and Sandman 1994; Król et al. 1999). On the other hand, violaxanthin is a dominating and neoxanthin is only a minor carotenoid in potato tubers (Breithaupt and Bamedi 2002).

To study the native arrangement of the Pchl/ide pigments 77 K fluorescence spectra of the different plant materials were measured and analysed. The spectra of the 6-week-old leaves (average of spectra of 10 leaves) were similar to those of 16-day-old etiolated barley leaves (Avarmaa et al. 1984). At 440 nm excitation, a band at 632 nm was dominating and a distinct band of lower amplitude was found at 655.5 nm. When the excitation was shifted to 460 nm, the main band appeared at 641 nm and the peak at 655.5 nm became more pronounced (Figure 1). In spectra of leaves of small sizes (with 0.5 cm diameter or smaller), collected from young regions of the shoot, the 655.5 nm band was less pronounced (not shown).

Also the tips of the 100-day-old shoots contained leaves; they grew on the young segments and had similar spectra as the leaves 1.5 to 2 cm in diameter described above. Other leaves from more basal regions of this old shoot were dry and were not studied.

The spectral properties of the petiole were similar to those of the leaves but the relative amplitudes of the long wavelength bands were higher than those in the spectra of leaves. However, the bands were slightly blue-shifted compared to those in the spectra of leaves. They were found to have maxima at 640 and 654 nm (Figure 2).

An interesting feature of these samples was the high contribution of the distinct band at 640–641 nm. A corresponding band at 644 nm can be detected in 77 K emission spectra of etiolated leaves (Böddi et al. 1992), but it is very small and strongly overlapped. The band in the spectra of grapevine leaves had an especially narrow maximum when the spectra were recorded with 460 nm excitation. Considering the age of the grapevine shoot after the long dark forcing period, the form with this band around 640 nm can be a result of senescence resulting in a structural change of the PLB membranes (Gounaris et al. 1983). In case of leaves of etiolated seedlings, the arrangement of the PLB membranes goes through a developmental succession. In the youngest cells there are PLBs with a tubular membrane network, which develops into 'hexagonal rings'. In a later stage they can be narrow or wide spaced and finally in old tissues still regular but loose membrane structures are formed (Robertson and Laetsch 1974; Rascio et al. 1986).

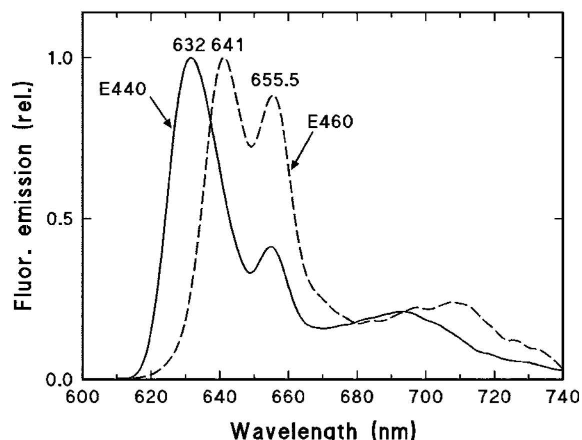

Figure 1. Fluorescence emission spectra (measured at 77 K) of 6-week-old leaves from dark-forced grapevine shoots. E440 and E460, respectively, indicate the excitation wavelength values when measuring the spectra.

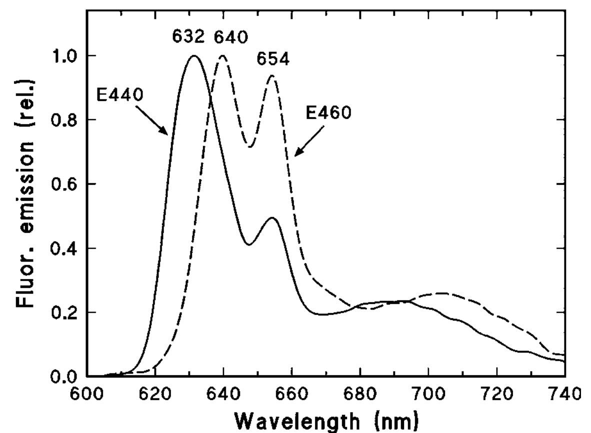

Figure 2. Fluorescence emission spectra (measured at 77 K) of petioles of 6-week-old leaves from dark-forced grapevine shoots. E440 and E460, respectively, indicate the excitation wavelength values when measuring the spectra.

A detailed study was done on the stems. Two-centimeter long pieces of stem from six-week-old shoots were used. The spectra of 25 different stems were measured, their average was calculated and analysed. The stem segments did not show remarkable variance from the tip to the base neither in their pigment contents nor in the amplitude ratios of bands in their fluorescence spectra. The emission spectrum measured with 440 nm excitation exhibited its maximum at 630 nm. This band was asymmetric on the red side and a shoulder was observed around 655 nm. In spectra recorded with 450 nm excitation, the main band shifted to 634 nm and the emission increased on the red side

of the peak. When the excitation was shifted to 460 nm, the maximum appeared at 638 nm and the emission band at 654 nm was distinct and intense (Figure 3).

The multiplicity and complexity of the Pchl/ide forms was seen in the Soret region of the excitation spectra measured at 625, 640 and 655 nm. The excitation spectrum recorded at 625 nm had a maximum at 441 nm and a broad shoulder around 445 nm, the one recorded at 640 nm had maximum at 447 nm and a complex shoulder with inclinations around 455 and 465 nm. The main Soret band of the excitation spectrum measured at 655 nm was at 448 nm and the contribution of the 455 nm excitation band increased (Figure 4). Although the maxima of these spectra are in similar positions as those of pea epicotyls (Böddi et al. 1994) the spectra are more complex indicating a different composition of the pigment forms and/or different energy migration efficiencies among the different pigment forms.

To study the spectral forms of Pchl/ide in stems more in detail, the Qy(0–1) absorption bands at 575, 585 and 595 nm were used for excitation to record emission spectra. These absorption bands are usual in spectra of porphyrins and are present also in spectra of pea epicotyls (Böddi et al. 1998). With 575 nm excitation, the main emission band appeared at 629 nm. The spectrum recorded with 585 nm showed maximum at 635 nm. When the 595 nm excitation was used, the 654.5 nm band became the maximum of the spectrum and the short-wavelength band showed a complex structure with shoulders around 630 and 640 nm and a local maximum at 635 nm (Figure 5). However, we could not find an excitation wavelength, which could separately excite the 644 nm emission band reported in the 10 K emission spectra of pea epicotyls (Böddi et al. 1998). The existence of this band was shown only by the complexity of the shorter wavelength band in the spectrum measured with 595 nm excitation (Figure 5).

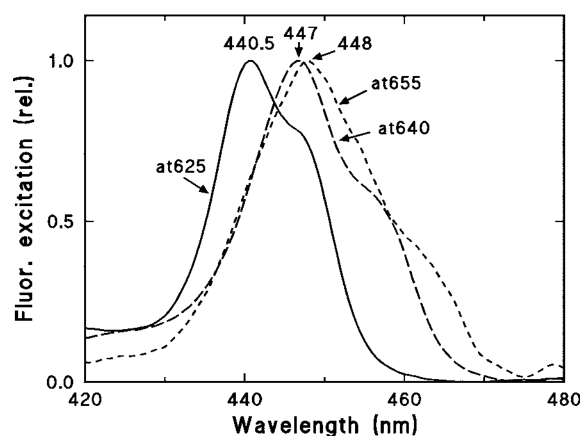

Figure 4. Fluorescence excitation spectra (measured at 77 K) of stem segments of 6-week-old dark-forced grapevine shoots. The spectra were recorded at 625, 640 and 655 nm (as labelled in the figure).

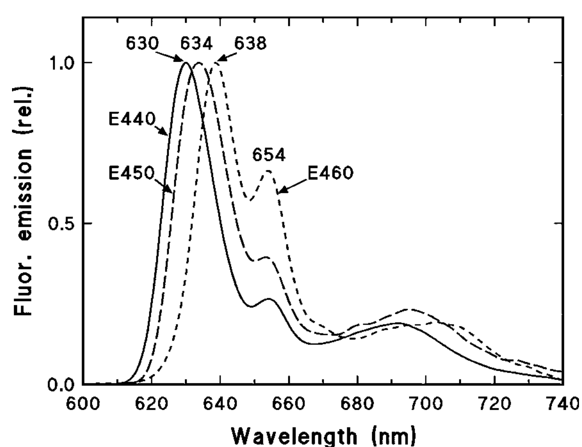

Figure 3. Fluorescence emission spectra (measured at 77 K) of stem segments of 6-week-old dark-forced grapevine shoots. The spectra of 25 stem pieces were averaged. E440, E450 and E460, respectively, indicate the excitation wavelength values when measuring the spectra.

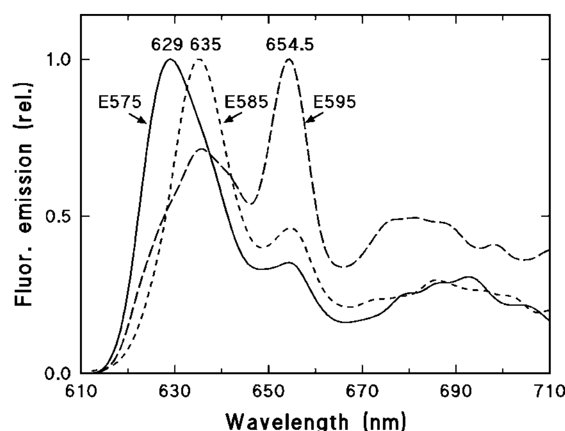

Figure 5. Fluorescence emission spectra (measured at 77 K) of petioles of 6-week-old stems of dark-forced grapevine shoot. E575, E585 and E595, respectively, indicate the excitation wavelength values when measuring the spectra.

Additional information was provided by the excitation spectrum recorded in the 600–660 nm region containing the main, Qy(0–0) absorption bands of Pchl/ide forms. In this spectrum, the maximum was at 629 nm with shoulders around 635 and 644 nm and a band of small amplitude at 655 nm. The fourth derivative of this curve clearly showed the presence of bands at 628.5, 634.5, 644 and 656 nm (Figure 6).

With the help of this information, the 600–740 nm region of the emission spectra of stems shown in Figure 3 were deconvoluted into Gaussian components. The spectra with 440, 450 and 460 nm excitation (Figure 7, a, b and c, respectively) had the same Gaussian components localised at 628, 636, 644, 655, 669, 684, 698, 711 and 726 nm, only the relative amplitudes of the components varied. The first four bands are attributable to Pchl/ide forms, i.e., to their main, 0–0 electronic transitions (Böddi et al. 1993, 1994). The 669 nm band a result of the overlap of a vibronic satellite band and the 0–0 transition of an independent Pchl/ide form (Kis Petik) et al. 1999). The other longer wavelength emission bands are regarded as vibronic or satellite bands of the Pchli/ide forms having 0–0 transitions below 669 nm (Böddi et al. 1993; Kis-Petik et al. 1999).

Due to the high amount of storage material in the cuttings, the etiolated tissues of the dark-forced stems showed only slight ageing symptoms. The basal region of 100-day-old stems – which contained the oldest tissues – had Pchlide forms similar to those in the 3-week-old samples. The amplitude of the 655 nm band in the emission

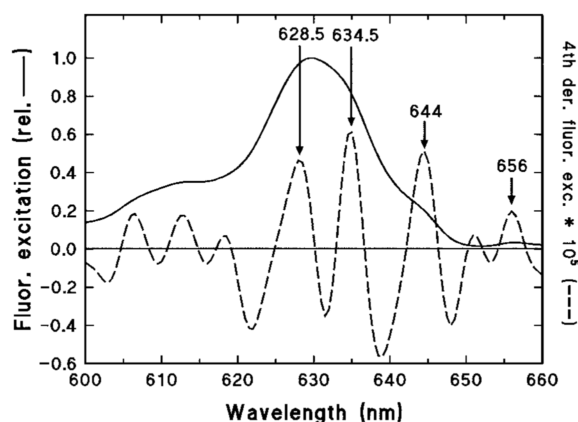

Figure 6. Fluorescence excitation spectrum (measured at 77 K) (solid line) and its fourth derivative (dashed line) of stems of 6-week-old dark-forced grapevine shoots. The spectrum was measured at 670 nm.

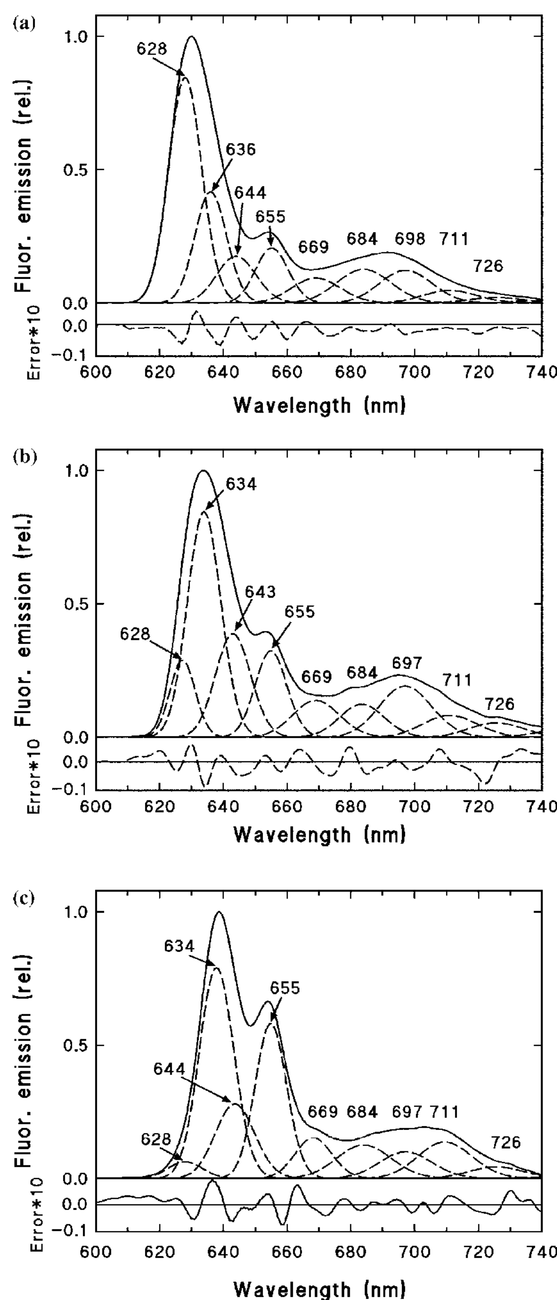

Figure 7. Gaussian components (dashed lines) of the 77 K fluorescence emission spectra of stems of dark-forced grapevine shoots. The spectra are averages of 25 independent measurements. (a): Excitation at 440 nm. (b): Excitation at 450 nm. (c): Excitation at 460 nm. Bottom panels: difference spectra between the experimental and fitted (sum of the Gaussian components) curves.

spectra was, however, lower than in those of 6-week-old stems. In spectra collected from the tips of 60-cm-long dark-forced stems, the amplitude of the 655 nm band was, however, significantly higher. In fact, the amplitude ratio of the 633 nm to the 655 nm band was similar to that of the 6-week-old stems.

The complex spectral properties of the dark-forced grapevine stem showed similarities to the epicotyls of dark-grown pea seedlings: the short-wavelength 628–629 and 634–636 nm forms were dominating in the spectra of both samples. The similarities to the forms found in pea epicotyls were shown by similar positions of the main, 0–0 and vibronic 1–0 transition band pairs; the 629–684 and 636–700 nm band pairs in the spectra of pea epicotyls and the 628–684 nm and 636–698 in young or 634–697 nm band pairs in spectra of old grapevine stems. The specific characteristic of the grapevine stem is the relatively high contribution of the 643–644 nm band. This band was not found in the 77 K spectra of the pea epicotyl. It was detected only as a small Gaussian component in the 10 K spectra (Böddi et al. 1998) but is generally present as an overlapped Gaussian component in 77 K spectra of leaves of etiolated seedlings (Böddi et al. 1992). It is worth mentioning that the appearance of this band did not depend on the age of the stem. It was present in spectra of young and rather old stems. Compared to spectra of the grapevine leaves, the amplitude of the 653–655 nm band was low in spectra of stems, a property shared with the spectra of pea plants. The low amplitude of the 653–655 nm band of stems, however, was a general property of the grapevine stems, independently on the age. It can be regarded as a general feature of dark-forced stems (Skribanek et al. 2000). Similarly to spectra of grapevine stems, the dominance of the short wavelength Pchl or Pchlide forms is characteristic for cells of meristematic or parenchymatic tissues in early developmental stage. A single emission band at 633 nm was found in room-temperature fluorescence spectra of tobacco cell cultures (Kamiya et al. 1981); the 633 nm band is dominant in 77 K emission spectra of 2- to 3-day-old wheat leaves (Younis et al. 1995), in maize and oat mesocotyls (Virgin 1996), in middle sections of hypocotyls (Mc Ewen et al. 1994) and in subperidermal layers of potato tubers (Virgin and Sundqvist 1992).

The contribution of the 653–655 nm band varied in spectra of the different tissue layers of the stems. It was the highest in the outer layers of the cortex, which corresponds to a subepidermal chlorenchyma in green stems (Figure 8, spectrum A), and was only a weak shoulder in spectra of the pith (Figure 8, spectrum C). The inner cortex layer was intermediary in this respect (Figure 8, spectrum B). The distribution of Pchlide fluorescence in the grapevine stem thus resembled that of red fluorescence from thin sections of wild-type pea and its lip1 mutant fluorescing from the pith and the sub-epidermal cortex layer (Seyedi et al. 2001).

In parallel with the gradual decrease of the contribution of the 653–655 nm emission band, a developmental difference of the PLBs was found towards the inner tissue layers of the stems. The etioplasts of the subepidermal cells contained typical PLBs with regular membrane structure (Figure 9b) similar to those in leaf etioplasts (Figure 9a). The mean size of the stem PLBs was smaller than that of the leaf etioplasts. The cells of the inner cortex layers had similarly smaller etioplasts than the subepidermal cells. The PLBs were distinct but their inner membrane system was less organised than that of PLBs of leaves or subepidermal stem cells. Only loose membranes without organisation into a regular network were found (Figure 9c). The etioplasts of pith cells were even less developed; their size was around 50% of that of subepidermal cells. No PLBs were present but only a few separate inner membranes were found (Figure 9d).

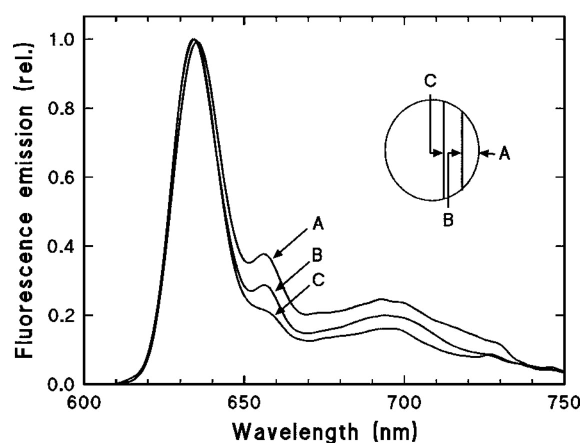

Figure 8. 77-K fluorescence emission spectra of different tissue layers of 6-week-old dark-forced grapevine stem. (A): Subepidermal layer. (B): Deep cortex layer. (C): Pith. The arrows in the inset show the surface facing towards the excitation light in the spectrofluorometer. Excitation wavelength 440 nm.

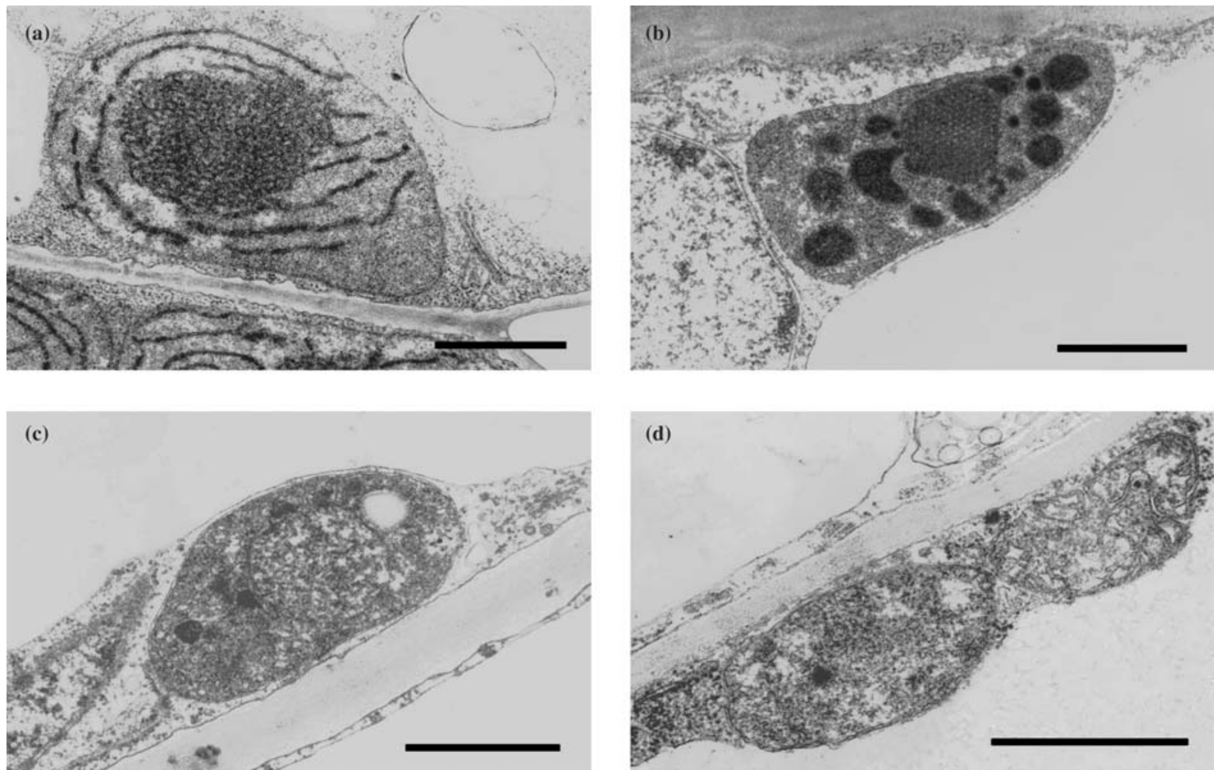

Figure 9. Transmission electron micrographs of etioplasts from different cells of a 6-week-old dark-forced grapevine shoot. (a): Leaf etioplast. (b): Etioplast from the outer, subepidermal cortex layer. (c): Etioplast from the inner cortex layer. (d): Etioplast (lower in the left) and a mitochondrion (on the right) from the pith. The bar is 1 μm.

The results of this paper show that the plastid development is organ specific. In dark-forced stems the etioplasts differentiation is less developed than in leaves. Moreover, probably depending on the destination of the tissue, various plastids appear. In subepidermal cell layers, which will transform into chlorenchyma in light, the plastids are more similar to those of leaves. However, in the cortex and stele only certain parenchyma cells develop chloroplasts. Here, plastids are present with less organised membranes, which contain mainly monomeric Pchl and Pchlide forms. Finally, the pith cells, which gradually degrade in developed stems, contain the less developed etioplasts resembling proplastids with few single membranes and predominantly monomeric pigments. Similarly to our results, the various research materials having dominant short-wavelength, 628–633 nm emitting Pchl(ide) forms, mentioned above had proplastids or etioplasts with PT membranes but no or small PLBs.

Spectra recorded from *in situ* tissue pieces or from homogenates prepared from organs usually are the average spectra of many, differently developed cells or tissues. A combination of spectroscopy and ultrastructural analysis may provide important information about the developmental routes of the organs or the different tissues within them.

The result of this paper shows that dark-growth maintains the meristematic developmental stage of tissues causing a slow or incomplete plastid development. As a consequence, the etioplasts do not contain PLBs or the PLBs have loose structure and the majority of Pchl(ide) pigments is in monomeric form.

Acknowledgements

This work has been supported by the IVA, Sweden, the Hungarian Academy of Sciences and the

Ministry of Education, Hungary (FKFP 0302/2000). We are grateful to Agneta Lindsten for doing the HPLC analyses.

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