The chlorophyll biosynthesis of the under-soil shoot of bean (*Phaseolus vulgaris* L.): etiolation and photosynthesis in the soil

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Introduction

The process of chlorophyll (Chl) biosynthesis is one of the primary subjects of plant biochemistry. Previous studies were predominantly done on laboratory-grown plants cultivated in darkrooms, in most cases using hydroponics. Systems “synchronized” in dark environments are favorable models for studying the ultrastructure of etioplasts and the initial steps of the greening process. Our research work however led us to question if these results are also characteristic for plants grown in the nature. Studies showed that in such plants “shaded” cells and prochlorenchymatic tissue segments can differentiate in layered or under-soil organs. If those tissues are partly shaded, special transition states can be observed. Most studies published were conducted on the above-soil organs, but only a few studies were concerning the under-soil tissues, hence we focused our experiments on this topic. Lighting conditions of the upper layers of soil can be characterized by a light gradient, while no light can penetrate to the deeper layers. As plant tissues are able to conduct light, the above-soil shoot region may transfer photons towards the under-soil shoot segment which is never exposed to direct sunlight. Since the development of plants grown in soil and under natural illumination conditions takes a long time (compared to the laboratory-grown etiolated plants), plastids with special structure and function were expected in their under-soil shoot.

Our study involved bean plants cultivated in soil and grown under natural light/dark cycles. Samples from the under-soil hypocotyl section were collected in the darkroom in several developmental stages of the plants, then pigment analysis, spectroscopic, structural and functional studies were performed on the samples. In order to test the light piping ability of the shoot a spectrofluorometer was used, the sample compartment of which was specially modified for the task. It allowed us to measure the intensity and spectral composition of the light piped through the hollow shoot.

Materials and methods

Seeds of bush bean (Phaseolus vulgaris L. cv. Magnum, Rédei Kertimag Ltd., Réde, Hungary) were pre-germinated in darkness for 3 days at room temperature (23°C). The seedlings were planted into pots at 4 cm depth using common potting soil under dim green light. The plants were grown under natural light/dark cycles close to the laboratory window. Samples were collected in the darkroom under dim green light from the following segments: the above-soil, green hypocotyl region (AS), the under-soil 2-cm-long hypocotyl section directly under the soil surface (US-1), the under-soil segment at 2-4 cm depths (US-2) and the
under-soil region between 4 and 6 cm depths (US-3). In the case of 14-day-old plants, samples were collected from the leaves for physiological studies, as well.

The light piping ability of the shoot was measured with a Fluoromax-3 (Jobin Yvon-Horiba, Paris, France) spectrofluorometer the sample compartment of which was specifically modified for the task.

Morphological features were examined using an Olympus BH2 microscope (Olympus, Tokio, Japan); the surface of the shoot was detected with a Hitachi S-2360N (Hitachi, Tokio, Japan) scanning electron microscope. Samples were collected from the under-soil hypocotyl segments and plastids were examined with a Hitachi 7100 (Hitachi, Tokio, Japan) transmission electron microscope using 75 kV accelerating voltage.

The 77 K fluorescence emission spectra were recorded with the Fluoromax-3 spectrofluorometer; the samples were in liquid nitrogen. The data were exported and the SPSERV V3.14 software (© Csaba Bagyinka, Institute of Biophysics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary) was used for computer analysis of the spectra. Photoactivity of the pigment forms was tested under different illumination conditions.

The pigments were extracted with 80% (v/v) acetone. In order to separate the esterified pigments, phase separation was performed using petroleum ether. To determine the pigment concentrations, calibration curves were used (Skribanek and Böddi 2001); quantitative calculations were done according to Brouers and Michel-Wolwertz (1983) and Porra et al. (1989).

Plastids and thylakoids were isolated then the pigment-protein complexes and their apoprotein compositions were determined with first dimension Blue-native polyacrylamide gel electrophoresis (Kügler et al. 1997) and second dimension denaturing (Laemmli 1970) gel electrophoresis (Bio-Rad, Hercules, USA). Gels were scanned using an Epson Perfection V750 PRO (Epson, Suwa, Japan) gel scanner then were analyzed using the Phoretix 4.01 software (Phoretix International, Newcastle upon Tyne, UK). Some proteins were identified through western-blot and mass spectrometry.

Photosynthetic electron transport and non-photochemical energy dissipation were assessed by measuring Chl fluorescence using pulse-amplitude modulated (PAM) fluorometry on 14-day-old plants. Variable Chl fluorescence parameters were measured either as average yields (PAM 101-102-103, Heinz Waltz, Effeltrich, Germany) or as fluorescence images (Imaging-PAM with MAXI-head, Heinz Waltz, Effeltrich, Germany). The parameters were calculated
according to Klughammer and Schreiber (2008), Hendrickson et al. (2005) and Solti et al. (2009).

**Results and discussion**

- **The hollow shoot of bean can pipe light**
  The light piping properties of roots and shoots were studied in detail by Sun et al. (2003, 2005) although the experiments in the mentioned works were carried out on thin tissue segments and the surface of the cut samples was directly illuminated. In our work, the intact shoot was illuminated and the intensity and spectral composition of the incident light was measured by a spectrofluorometer. The spectrum of the whole intact shoot showed that the transmission was high in the UV region then it gradually decreased to 10 % and remained constant through the visible light and infrared regions. Light piping depends on the anatomy of the shoot and the shape of its cells (Vogelmann 1993 – review). The pea epicotyl has a dense pith region (Hideg et al. 2010) thus the soil-grown plants contained Chl only in the first 1 cm of the under-soil shoot segment (Vitányi et al. 2013). In parallel, the hollow shoot of bean can pipe light as far as several cm down in the soil and therefore, Chl accumulation was expected in the under-soil hypocotyl regions to a greater degree.

- **Light piping ensures chlorenchyma formation under the soil surface**
  The 7 and 14 days old plants had an above-soil shoot which piped light downwards thus Chl biosynthesis and chlorenchymatic tissues were observed in the US-1 and US-2 parts of the under-soil hypocotyl. The US-1 section was green and its fluorescence emission spectra showed maxima at 685, 695 and 730-740 nm. This segment contained only Chl, no protochlorophyllide (Pchlide) or protochlorophyll (Pchl) was detected. The Chl\((a+b)\) content gradually increased with the age of the plants. Chloroplasts were present in the xylem, phloem and pith parenchyma cells in the US-1 of 7- and 14-day-old plants. Large starch grains were usually found and the grana were low and broad. This feature of the chloroplasts can be due to a “double feeding” of the cells; besides the sugars transported from the above-soil shoot the under-soil hypocotyl may use its own photosynthetic products. Fluorescence microscopic studies showed that the hollow hypocotyl piped light suitable for chlorenchymatic tissue development. A light gradient was observed along the hypocotyl in parallel with decreasing Chl content of the tissues. In the US-2 sections a transition state was observed: Chl-protein complexes were still dominant, however, very low intensity emission bands of Pchlide forms appeared at 632 and 652-654 nm. In the US-3 section the molar ratio of Pchlide and Pchl was 1:1 in the 7 and 14 days old plants as well, which can be a consequence of special
differentiation processes. As the US-3 hypocotyl segment is never exposed to direct sunlight and only a few photons can reach this region by light piping the detected small amount of Chl can be of embryonic origin (Böddi et al. 1999).

- **Tissue regions with photosynthetic activity are present at 0-2 cm soil depths**

  Only Chl was present in the thylakoids of the AS and US-1 segment. Interestingly, despite of the great differences in the amounts of Chls and thus the Chl-protein complex contents of these segments, only minor differences were found in the Chl-protein complex ratios. The Chl-\(a/b\) ratios of leaves and AS sections were similar. The relative amounts of photosystem (PS) II were higher in supercomplexes in the US-1 region than in the AS segment. This kind of arrangement is important for efficient energy transfer under low-light conditions (Kouřil és mtsai 2013). The far-red wavelengths are mainly absorbed by PSI thus the relative increase in the light harvesting complex (LHC) II to PSII ratio along with the lower Chl-\(a/b\) ratio in low-light adapted plants is suggested to ensure an efficient electron supply of PSI (Walters 2005 – review).

  An interesting feature of the under-soil hypocotyl section was the 66 kD protein detected in the thylakoids. It showed the highest coverage with a plasmamembrane intrinsic protein (PIP)-type aquaporin. Aquaporin family proteins facilitate not only water transport but they are also transporters of CO\(_2\) (Terashima and Ono 2002) and H\(_2\)O\(_2\) (Bienert and Chaumont 2014). In the light sensitive hypocotyl sections, the transport of H\(_2\)O\(_2\) may have an important role not only in protecting the thylakoid membranes by regulating the redox state in its environment but also in the signalization of the oxidative stress processes (Maruta et al. 2012).

  The Chl-\(a\) fluorescence induction measurements demonstrated that the observed structural similarities between US-1 and photosynthetic AS tissues were reflected in PSII photochemistry. Hypocotyls adapted to very low light in the upper region of soil showed smaller but significant PSII electron transport. There was a significant enhancement in the yield of regulated non-photochemical quenching mechanisms \([Y(NPQ)]\) in US-1 segments when higher light intensity was used. It indicated that the photosynthetic apparatus present in the US-1 segment was capable for light acclimation and xanthophyll-cycle based quenching processes. These results show that the general concept regarding the source role of the above-soil shoot and sink function of the under-soil tissues should be modified.

- **In the lowermost under-soil hypocotyl region special plastids develop in the dark**

  The young seedlings were fully covered by a few cm thick soil layer and as light does not penetrate into these depths (Woolley and Stoller 1978) they were similar to the etiolated
plants kept in darkness (McEwen et al. 1994), with respect to the pigment content, the spectral pigment forms and plastid structure. Under-soil shoots (Vitányi et al. 2013) were shown to have these characteristics, as well. However, the morphology of the etiolated bean hypocotyl is basically different: it was 24 cm long in 7-day-old dark-grown laboratory plants (McEwen et al. 1994) and only 7-8 cm long in the soil- and light-grown plants. Also the pigment contents were strikingly different; while the lowermost segment of the etiolated hypocotyls contained only 0.004 nmol Pchl(ide) g⁻¹ fresh weight (McEwen et al. 1994), the similar segment of soil-grown plants had several order higher pigment content. This difference in the pigment contents may be explained by the strong elongation of the hypocotyl of the artificially etiolated seedlings. The dominance of the Pchlide₆₃₃ was not as significant as in etiolated plants grown in the laboratory (McEwen et al. 1994). The variance between the bean cultivars and/or the age difference of the tissues may be the cause for this inequality.

As the seedlings broke through the soil surface the transformation of Pchlide-chlorophyllide (Chlide) became continuous. As a consequence, only Chl pigments could be detected in the US-1 segment in each developmental stage and Chl-protein complexes characteristic for green leaves were present. The etioplasts transformed into chloroplasts at the beginning of development then the amount of thylakoids gradually decreased in the 60-day-old plants. However, the number of plastoglobules did not change as in gerontoplasts (Lichtenthaler and Sprey 1966). The starch content was about 70% in young and older plants as well thus the chloroplasts mainly transformed into amyloplasts. In the US-3 section, the pigment forms changed by the age. The intensity of the photoactive Pchlide₆₅₅ gradually decreased after 35 days of development then it disappeared from the spectra of 60-day-old plants; however, a new maximum appeared at 641 nm. This form was not phototransformable when flash or continuous illuminations were used. Pchl accumulation, in parallel with the loss of Chl biosynthesis ability and the disappearance of etioplast inner membranes were described in pumpkin seed coats (Sundqvist and Ryberg 1979). Pchl accumulating plastids were described in other members of the Cucurbitaceae family in which extreme amounts of Pchl were found, in some cases in crystalloid-like structures (Sundqvist et al. 1980). The plastids described in our work resemble the appearance of Pchl accumulating plastids. High Pchl (i.e. various Pchlide esters) content was also found in several roots (McEwen et al. 1991) indicating that preferential accumulation of Pchl is typical for non-photosynthetic under-soil organs. The development of adventitious roots further indicates that the primary function of the under-soil hypocotyl segment is water and mineral uptake. Therefore, the under-soil hypocotyl develops root-like features during its differentiation.
Based on the results of our work, a new developmental pathway of plastids can be added to the generally accepted plastid transformation scheme (Solymosi and Schoefs 2010 - review). The plastids found in old tissues of bean hypocotyls originate from amyloplasts or to a far lesser extent etioplasts which developed in constant darkness during at least 60 days and lose their ability for greening. However, the plastid metabolism is complex: they have essential role in secondary metabolism (Durango et al. 2014, Singh and Sharma 2015) and function as starch, amino acid and fatty acid biosynthesis centers (Neuhaus and Emes 2000 - review). The importance of Pchl accumulation is not clear. Upon illumination, this pigment is a sensor of reactive oxygen species (ROS) production and bleaching (Erdei et al. 2005) – the latter was observed also in this work. Some of the ROS molecules (for instance H₂O₂) have complex regulatory function including plant organogenesis (Cheeseman 2007- review) and retrograde signaling (Maruta et al. 2012).

Summary

Most seeds germinate in the soil, thus the under-soil part of the developing shoot remains in the shade of soil particles during the whole life span. As only a few works could be found concerning the chlorophyll (Chl) biosynthesis of the under-soil shoots, our study was focused on this shoot segment. We used bean plants cultivated in soil and grown under natural light-dark cycles. The light piping properties of the hollow shoot and its effects on the under-soil regions were examined using pigment analysis, spectroscopic and microscopic methods. The photosynthetic ability of the under-soil hypocotyl was tested with proteomics and fluorescence induction studies.

We were able to measure the spectrum of the transmitted light of the intact plant using a spectrofluorometer with a specifically modified sample compartment. Photons were piped into 4-6 cm depths through the plant tissues. Due to the light gradient, the under-soil shoot regions showed different etiolation symptoms. The segment close to the soil surface contained only Chl; chloroplasts and extensive chlorenchyma were observed. The 77 K spectra of this region showed Chl-protein complexes typical for green leaves; the photosynthetic activity was similar to that of the lowest, 1 cm above-soil hypocotyl segment. Using higher light intensity the reaction centers became inactivated and photochemical quenching processes took place. The hypocotyl segment in 2-4 cm soil depths showed transient features, it contained Chl, protochlorophyllide (Pchlide) and protochlorophyll (Pchl). The lowermost hypocotyl section developed in full darkness; it contained Pchlide and Pchl, the amount of the latter gradually increased by age. After 60 days of development plastids were still present in this region but
their inner membrane content decreased or disappeared, plastoglobules and phytoferritin were observed. After flash or continuous illumination only bleaching of the pigments was detected, no Chl biosynthesis appeared.

Our results show that plants can pipe light downwards to the under-soil tissues. Based on the morphological features of the shoot, a low light adapted photosynthetic apparatus can differentiate in a few cm soil depths. Therefore, the under-soil shoot is capable of contributing to its own carbon supply and it does not only depend on the photosynthetic compounds transported from the above-soil shoot. In the lowermost hypocotyl segments special plastids differentiate, the pigment content of which increases (particularly the amount of Pchl), while they lose the ability to greening; they most probably take part in the processes of secondary metabolism.

References

Bienert GP, Chaumont F (2014) BBA Gen Subjects 1840: 1596-1604
Cheeseman JM (2007) Plant Stress 1: 4-15


Sundqvist C, Ryberg H (1979) Physiol Plant 47: 124-128


List of publications

Publications directly related to the dissertation, published in peer reviewed international journals:


Conferences directly related to the dissertation:


