Confocal microscopy of the green-algal chloroplast

Využití konfokální mikroskopie při studiu chloroplastu zelených řas

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Abstract

The confocal microscopy proves useful in the investigations of chloroplast morphology and ontogeny of several coccal green algae. The three-dimensional images allow the reconstruction of the chloroplast morphology and the exact determination of the chloroplast number in the cell. This character is essential for species determination in numerous genera of coccal green algae. The simple method of pyrenoid visualisation by confocal images is demonstrated. Within the pyrenoid we can recognise the structure and shape of the invaginated thylakoids. In comparison with the methodically difficult transmission electron microscopy, the confocal microscopy provides a simple and appropriate method for the morphological and taxonomic studies of various algal taxa.

Introduction

The structure of the chloroplast is considered to be one of the most important features for taxonomic determination of green algae. The detailed structure, changes of morphology and the number of chloroplasts during the cell cycle are important for distinguishing of individual species (ANDREYEVA 1998, ETTL & GÄRTNER 1995). The ontogeny and the modification of the chloroplast structure were investigated by light microscopy in several studies (ETTL 1969, 1975). During the cycle flow and before the cell division starts, the chloroplast structure is often radically changed and its form is simplified in shape. Detailed performance of the chloroplast structure during these processes is unknown in most species. Furthermore, in the species containing complicated three-dimensional chloroplast (e.g. *Kentrosphaera*, *Dictyochloropsis*) the observation with use of convential light microscopy is difficult.

Application of the transmission electron microscopy provided exact information about chloroplast ultrastructure, but it does not allow the

observation of its 3-D structure and ontogeny. Moreover, the investigation of the chloroplast structure at the majority of algae requires a large number of ultrathin serial sections.

As an alternative way for the chloroplast structure examination is to apply laser scanning confocal microscopy, which enables the chloroplast examination in living cells using the autofluorescence of the algal chlorophyll. This method has been repeatedly applied for the investigation of the chloroplast morphology in higher plants (GUNNING & SCHWARTZ 1999, SARAFIS 1998). However, confocal microscopy has been only sporadically used in the investigations of algal chloroplasts so far (KREIMER et al. 1991, ZAKRYS et al. 2002).

Material and methods

The following strains were used in the study:

1. *Dictyochloropsis splendida* GEITLER - Strain isolated from the soil sample at the top of the Boreč hill in České Středohoří Mts., Czech Republic.
2. *Coelastrella vacuolata* (SHIHIRA & KRAUSS) HEGEWALD & HANAGATA – Strain isolated from the surface of the stone at the top of the Boreč hill in České Středohoří Mts., Czech Republic.
3. *Muriella zofingiensis* (DÖNZ) HINDÁK – Strain isolated from the bark sample of unidentified tree in the secondary tropical rain forest in the Kelantan province, Malaysia. The strain was kindly provided by Mgr. Jiří Neustupa, PhD. (Department of Botany, Charles University, Prague).
4. *Muriellopsis sphaerica* BROADY (originally labelled as *Muriella* sp.) – Strain isolated from the soil sample in Anchorage Island, Antarctica. It was kindly provided by Ing. Alena Lukešová, CSc. (Institute of Soil Biology, Academy of Sciences of the Czech Republic, České Budějovice).
5. *Chlorella kessleri* FOTT & NOVÁKOVÁ (type culture). Strain maintained in Culture Collection of Algae of Charles University of Prague (CAUP H1901), originally isolated by Winokur (1945) from freshwater biotope, NY, USA.

All strains were cultivated on agar-solidified BBM medium (BISCHOFF & BOLD 1963) in the temperature 25 °C, under the illumination of about 2500 lux (light source: Philips TLD 18W/33, cool white). The samples of the algae were investigated by a laser scanning confocal microscope Bio-Rad MRC-600 equipped with an Argon-Krypton laser using the 488 nm excitation line. The Nikon 100x oil immersion objective fitted on the Nikon Diaphot inverted fluorescent microscope was used. The living cells were placed on the object glass together with a thin layer of agar-solidified medium to prevent the motion of the cells in the time of the observation. The cells were screened in numerous subsequent 0.5 μm thick optical layers to obtain the serial focal plane sections of the chloroplasts. The fluorescence of the chlorophyll was used for visualisation of the chloroplast structure. For the final processing of the confocal images, the Confocal Assistant programme, version 4.02 (TODD CLARK BRELJE, University

of Minnesota, USA) was used. The 3-D reconstruction images were created by computer programme Amira™ 2.3 (Indeed - Visual Concepts GmbH, Berlin, Germany).

Results and discussion

Chloroplast morphology and ontogeny in *Dictyochloropsis splendida*
(Figs 1, 2, 4-6, 13, 14)

One of many advantages of confocal microscopy is the possibility to obtain a number of subsequent serial optical sections of the chloroplast in a very short time. Thanks to the possibility of analysing living cells, we can study chloroplast structure during the cell ontogeny in detail.

Individual stage of chloroplast ontogeny is visible on Figs 4-6. The pictures show the proliferation of chloroplast strands, which gradually fill up the inner space of the cell. Fig. 6 records the cell division and separation of the daughter cells. The chloroplast surface close to the cell wall is visible in Fig. 13. Next picture (Fig. 14) represents the cross section of the same cell. The both pictures are transformed in 3-D reconstruction, achieved by application of the software Amira (tm), which enables the image processing of serial sections.

Number of chloroplasts in *Muriella zofingiensis*
(Figs 7-9)

Number and shape of the chloroplast is particularly important for determination of species. (ETTL & GÄRTNER 1995). However, the number of chloroplasts (or the lobes of the single chloroplast) often varies in the course of the life cycle. The exact counting of chloroplasts is sometimes difficult.

The species of *Muriella* are characterized by the presence of numerous chloroplasts in the cell. No pyrenoid is present. On the other hand *Chlorella* species have only one chloroplast containing pyrenoid. However, before the cell division, the chloroplast of *Chlorella* is often divided into several parts and such cells resemble *Muriella* species.

In accordance with the description, the chloroplast of young cells of *Muriella* is band-shaped or pot-shaped, later it forms a hollow sphere. In the adult cells chloroplast disintegrates into polygonal parts, which differ in size. (ETTL & GÄRTNER 1995).

Confocal microscopy simply provides numerous serial sections through the whole investigated cell. These sections were used to determine the chloroplast number and their position in the cell. Fig. 7 shows one section containing 5-6 chloroplast segments in two investigated cells. For accurate estimation of chloroplast position, we applied maximum intensity projection of serial sections to show 3-D image. After 3-D reconstruction we establish five (Fig. 8), or six (Fig. 9) chloroplasts in the cells.

In spite of our observation, some uncertainty remains concerning the morphological independence of the chloroplast lobes. This question was not solved unequivocally using the TEM and serial ultrathin sections.

Presence of the pyrenoid in *Muriellopsis* aff. *sphaerica*
(Figs 3, 10, 16)

Presence or absence of the pyrenoid in the chloroplasts of coccal green algae is considered as an important specific or generic feature (KOMÁREK 1987, ANDREYEVA 1998). However, in some species the pyrenoid is difficult to observe. Particularly difficult is the detection of the naked pyrenoid without starch sheath, because the iodine containing reagents don't work. The staining with cotton blue (KALINA 1994) was also negative. It is the reason for what the naked pyrenoid of *Stichococcus* was overlooked until it was discovered by means of TEM (PICKETT-HEAPS 1975).

Confocal microscopy confirms the presence of the pyrenoid in one of the chloroplast (or in one of chloroplast lobes) of the investigated strain. The pyrenoid fills up the whole body of the chloroplast (or chloroplast lobe; Figs 10, 16). 3-D reconstruction permits the counting of five or six single chloroplasts (or lobes) in the cell (Fig.16). These findings support the new determination of the strain, which was originally labelled as *Muriella* sp. New name of the studied strain is *Muriellopsis sphaerica*.

In comparison with the TEM, the application of a confocal microscope for pyrenoid distinguishing is faster and more efficient. 3-D imaging of the confocal microscope pictures facilitates the study of the pyrenoid morphology and its position within the chloroplast. The confocal microscopy can't solve the question, whether or not the single chloroplast is divided into lobes, or the cell of *Muriellopsis* contains numerous independent chloroplasts. Single parietal chloroplast, which is dissected into anastomosing lobes, is widely distributed among chlorococcal algae, e.g. *Coelastrella*, numerous species of *Scenedesmus*, *Tetraedron* etc. (KALINA & PUNČOCHÁŘOVÁ 1987). In all enumerated genera one of the chloroplast's lobe contains one pyrenoid.

Inner structure of pyrenoid in *Chlorella kessleri* and *Coelastrella vacuolata*
(Figs 11, 12, 15, 17, 18)

Inner structure of the pyrenoid of coccal green algae was used for species determination in *Chlorella*, *Trebouxia* (IKEDA & TAKEDA 1995, FRIEDL 1989) and further genera. One of the structures under consideration is the number and shape of the thylakoids penetrating through the pyrenoid stroma. In *C. kessleri,* the pyrenoid consists of two symmetrical starch grains separated by single penetrating thylakoid (Figs 11, 12). The 3-D reconstruction confirms this observation (Fig. 18). Such structure corresponds with the pictures observed in TEM using ultrathin sections (NĚMCOVÁ & KALINA 2000). Fig. 17 represents the

3-D reconstruction of the whole chloroplast of *C. kessleri,* the pyrenoid is deposited inside of the chloroplast.

The next picture represents the spatial reconstruction of chloroplast of *Coelastrella vacuolata* (Fig.15). Part of the chloroplast was removed. The almost spherical pyrenoid has homogeneous structure. This finding corresponds with the TEM pictures published by KALINA & PUNČOCHÁŘOVÁ (1987).

Conclusions

The confocal microscopy enables the accurate and rapid observation of chloroplast morphology and its metamorphoses in the course of the cell cycle. The artefacts are minimized, because all observations are made using the living cells. The confocal microscopy complements the laborious methods of staining and reconstruction based on serial sectioning and subsequent observation in TEM. At the same time the further confirmation of the observed structure using alternative methods is advisable. The only disadvantage of the described method is the problem of accessibility of the confocal microscope, which represents expensive and rare equipment in the biological labs.

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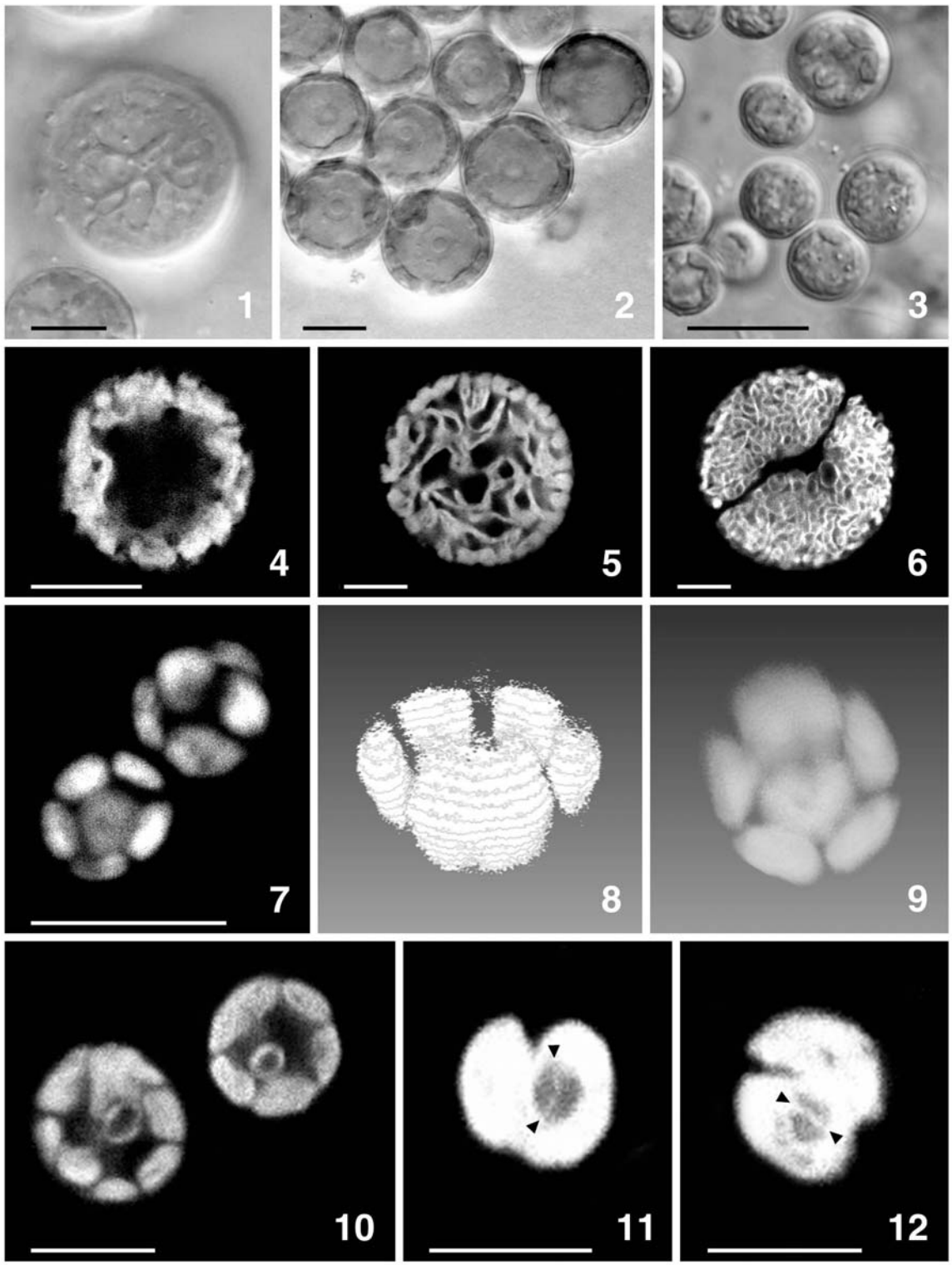
Tab. 1: Fig. 1 - *Dictyochloropsis splendida*, the adult cell with net-shaped chloroplast. Fig. 2 – the young cells of *D. splendida*. Fig. 3 – *Muriellopsis sphaerica*. Figs 4-6 – The confocal images of *D. splendida* in different ontogenetic stages. Fig. 7 – The confocal image of *Muriella zofingiensis*. Figs 8, 9 – 3-D reconstruction images of the chloroplasts of *M. zofingiensis*. Fig. 10 – The confocal image of *Muriellopsis sphaerica.* Figs 11, 12 – The confocal sections of *Chlorella kessleri*. Note the thylakoids that penetrated into the pyrenoid matrix. Scale bars 10 μm (Figs 1-7) or 5 μm (Figs 10-12).



Tab. 2: Figs 13, 14 – Three-dimensional chloroplast reconstructions of *Dictyochloropsis splendida* (Fig. 13 – outer view, Fig. 14 – inner view. The chloroplast is divided to the three, 5 μm thick sections of different coloration). Fig. 15 – The chloroplast morphology of *Coelastrella vacuolata*. Small segment of the chloroplast was removed to visible the central positioned pyrenoid. Fig. 16 – The 3-D reconstruction image of *Muriellopsis sphaerica* chloroplasts. Figs 17, 18 – The chloroplast reconstruction of the *Chlorella kessleri*. Fig. 18 – The morphology and position of the pyrenoid, divided by thylakoids into two equal parts.