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Investigations of cell morphology and reproduction in *Macrochloris radiosa* Ettl & Gärtner (Stephano-sphaerina, Chlorophyta) by light- and WUDQVPLVLRQ electron microscopy

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Abstract

Cell division and reproduction of a cultivated strain of *Macrochloris radiosa* (Stephanosphaerina clade) were studied by light- and transmission electron microscopy. Multinucleate cells were frequently observed allowing description of the cell structure and details of the reproduction process. Nuclear staining revealed the position of the multiple polymorphic nuclei between the chloroplast lobes. Ultrastructure of coenocytic cells showed no signs of cleavage of the protoplast in the cytoplasm, although basal bodies were already present within the multinucleate cells. In the further course of the reproduction, biflagellate zoospores were developed that retained their flagella during sporulation. Zoospores were subsequently released from the sporangia.

Keywords

coenocytic green algae; reproduction; multinuclear cell

Introduction

Among coccal green algae, only a few genera show multinucleate (coenocytic) vegetative cells. The occurrence of such stages is likely due to a prolonged cell cycle, where after nuclear division no simultaneous sporogenesis takes place. Coenocytic coccal green algae are mainly isolated from soil and aeroterrestrial habitats, and reports on their development in cultures are sparse (e.g., Ahmadjian et al. 1990, Uzunov et al. 2010). Molecular data of the 26S and 18S ribosomal RNA gene from *Macrochloris radiosa* (UTEX 1964) only recently became available (GenBank: KC196723.1; KC 196735.1). *M. radiosa* has been screened for genetic stability after cryopreservation, by amplified fragment length polymorphism (AFLP), which showed a maximum of 3.6 % non-reproducible fragments, demonstrating the

successful application of this technique for detecting alterations caused by different cryopreservation procedures (Müller et al. 2007).

A recent phylogenetic analysis by Buchheim et al. (2013) indicated that *Macrochloris* groups with the Stephanosphaerina clade placed in the Chlamydomonadales within the class Chlorophyceae (Buchheim et al. 2013; the species was erroneously assigned to *M. 'radiata'* in their Fig. 6). *Macrochloris radiosa* has been traditionally classified within the family Actinochloridaceae (Ettl & Gärtner 1988), which turned out to be an artificial group, since *Macrochloris* (a member of the Stephanosphaerina clade) and *Actinochloris* are unrelated (Leliaert et al. 2012, Nakada et al. 2008).

Morphology of multinucleate cells has been documented by light microscopy (Ettl & Gärtner 1988). The life cycle of *Macrochloris* has not been described so far and sexual reproduction is not observed in this genus. In the present study, we focused on the ultrastructure of *M. radiosa*, which was not studied until now to add knowledge to the phenomenon of multinucleate vegetative cells.

Material and methods

Algal material

Macrochloris radiosa was collected and isolated by F. Mainx under the name *Chlorococcum infusionum* (Prague culture collection number A 91, Baslerová & Dvořáková 1962, Ettl & Gärtner 1988) and has been assigned strain number 213-2a in the Culture Collection of Algae at Göttingen University, Germany (SAG, Schlösser 1994). The habitat of *M. radiosa* remains unclear, the culture was probably isolated from soil (Schlösser 1994).

The strain was cultivated on Bold's Basal Medium (BBM, Bischoff & Bold 1963) solidified with 1.5 % agar in culture tubes (light intensity 30–35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ under a light/dark cycle of 12:12 h L/D; light sources OSRAM Daylight Lumilux® Cool White lamps 36 W).

Light microscopy

For light microscopy, either an Olympus BX 40 or a Zeiss Axiovert 200 M microscope were used for bright-field and differential interference contrast (DIC). The images were captured with a JENOPTIK ProgRes CT3 Camera and further processed with ProgRes® CapturePro v2.8.0. software (with the Olympus BX 40) or a Zeiss AxioCam MRc5 camera.

Transmission electron microscopy

Transmission electron microscopy (TEM) was performed according to Holzinger et al. (2006). Briefly, cells were fixed chemically in 50 mM cacodylate buffer (pH = 6.8) containing 2.5 % glutaraldehyde for 1 h. For further processing, samples were embedded in 3 % agarose. The samples were washed in 50 mM cacodylate buffer and postfixed in the same buffer containing 1 % OsO_4 for 14 h at 4° C. After several rinsing steps, samples were dehydrated in increasing ethanol concentrations and embedded in a modified Spurr's low viscosity embedding kit (Cat. No. 14300, Science Services, Munich, Germany) according to Ellis (2006). For TEM, ultra-thin sections (~60 nm) were prepared with a Leica

Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany), counterstained for 10 min with 2 % uranyl acetate and 2 min with Reynold's lead citrate, and investigated in a Zeiss LIBRA 120 transmission electron microscope at 80 kV. Images were captured with a ProScan 2k SSCCD camera (Proscan Electronic Systems, Lagerlechfeld, Germany) and further processed using Adobe Photoshop software (Adobe Systems Inc., San José, CA, USA).

Results

Light microscopy

Young cells of *Macrochloris radiosa* were globular to ellipsoidal with a parietal chloroplast with a pyrenoid and one nucleus (Fig. 1a). After 20 days, nuclear division occurred in the globular cells. Adult vegetative cells had thick cell walls and the chloroplast was complexly lobed and corrugated (Fig. 1b). At this stage, more than one pyrenoid was visible, each enveloped with a continuous starch sheath. With increasing cell size, the number of nuclei successively rose in the vegetative stage (Fig. 1b). Most adult cells hosted between 8–16 nuclei, rarely the number raised to 32–64 nuclei. Subsequently, the chloroplasts divided and (their diameter was 2.8–3.0 μm). At this stage, the diameter of adult vegetative cells was between 10 and 40 (50) μm (Fig. 1c). Under favorable conditions, simultaneous protoplast division took place (Fig. 1c). Protoplast division started with chloroplasts fragmentation (Fig. 1c); at the final stage, each reproduction unit contained one part of the plastid and one nucleus (Fig. 1d). Inside the mother cell acting as sporangium, reproduction units developed into a zoospore. The number of reproduction units per sporangium was mostly between 8 and 16, but could be much higher, depending on the preceding multiple nuclear division. The zoospores (Fig. 1e–g) had two isokont flagella, one nucleus, a parietal lobed chloroplast and two apical contractile vacuoles. Immediately after loss of the flagella, the ellipsoidal cell body started to grow and finally became spherical. Then the first nuclear division started, leading to the formation of the multinucleate vegetative cells. The light-microscopical findings are summarized in a scheme for better overview (Fig. 2).

Transmission electron microscopy

Most cells of *Macrochloris radiosa* investigated by transmission electron microscopy showed thick cell walls (approx. 1.5 μm), several chloroplast lobes, and a single nucleus (Fig. 3a, c). The chloroplasts contained parallel thylakoid membranes (Fig. 3b, c), and depending on the orientation of the section, pyrenoids were visible (Fig. 4a, e). The cytoplasm contained small vacuoles (Fig. 3b) and numerous electrondense particles with a diameter of approx. 0.5–0.8 μm , possibly representing phenolic compounds. The cytoplasm contained golgi bodies (Fig. 3c) and dense accumulations of ribosomes (Fig. 3c). Individual cells contained numerous nuclei (Fig. 4a, b, c), the cytoplasm was not compartmented (Fig. 4a). The individual nuclei were clearly visible (Fig. 4b, N₁, N₂), without any signs of cleavage furrows. Between the nuclei, basal bodies of flagella were observed (Fig. 4c, d). In these structures, no central pair of microtubules was visible and they appeared to be oriented clockwise (Fig. 4c). Zoosporangia contained numerous zoospores covered by an extremely thin cell wall (Fig. 4e). Depending on the position of the section, either the chloroplasts or the individual nucleus were visible in the zoospores (Fig. 4e). Several cross sections of

flagella were visible in the zoosporangia, characterized by the typical 9+2 pattern of microtubules (Fig. 4e, insert).

Discussion

In the present study, parts of the life cycle of *Macrochloris radiosa* were investigated by different microscopy techniques, revealing stages with multinucleate cells termed coenocytia. Multinucleate cells are not only found in the Chlamydomonadales (where *M. radiosa* belongs to; Buchheim et al. 2013), but also in genera of the closely related Sphaeropleales, e.g., *Bracteacoccus*, *Chromochloris*, *Dictyococcus*, *Follicularia*, *Pseudomuriella*, *Rotundella*, *Tumidella*, *Bracteamorpha* (Fučíková et al. 2013). Reproduction in *M. radiosa* proved to be asexual, as reported previously by Ettl & Gärtner (1988).

The size of adult cells in *Macrochloris* is exceptional within coccoid green genera. *M. multinucleata* (Reisigl) Ettl & Gärtner can reach a diameter of more than 110 µm, and *M. radiosa* may exceed 50 µm (Ettl & Gärtner 1988). The exact number of nuclei present during the development of adult vegetative cells was difficult to determine especially by light microscopy, because of the complex architecture of the chloroplast. In many cases, the ribs and lobes of the chloroplast masked the nuclei, which were more or less scattered throughout the cell lumina, but were sometimes concentrated between the lobes of the chloroplast. Additionally, pyrenoids with their starch sheaths impeded observation of the nuclei in light microscopy. The number of nuclei varied between 4 and 64 in different coenoblasts of the same culture and about the same age.

The formation of multinucleated cells in algae has been investigated from the cell-biological perspective in various groups (e.g., Hildreth & Ahmadjian 1981, Ahmadjian 1990, McNaughton & Goff 1990) and was usually found to be related to large cell sizes. Microtubules play a crucial role in spacing the nuclei, e.g., in *Hydrodictyon* (McNaughton & Goff 1990).

Uninucleate coccoid green algal taxa usually show *Chlamydomonas*-like chloroplast morphology and a clear cellular polarity, where contractile vacuoles and other organelles are oriented in a distinct pattern. Contrarily, multinucleate cells of the Stephanosphaerina clade including *Macrochloris* do not show such an inner polarity. Additionally, the development of vegetative stages and reproduction proceed in a very special mode, as earlier shown by Geitler (1934), Schussnig (1954), Ettl (1964, 1980) and Ettl & Gärtner (1988, p. 146, fig. 107). As described by Ettl & Gärtner (1988), nucleus division starts during the growth of young cells, but is not followed by protoplast separation. This is a particularly interesting observation, which was also observed by TEM, where multinucleate cells with no signs of protoplast division were clearly visible. In contrast, in the early stages of protoplasm division in the Ulvophyceae *Scotinosphaera gibberosa* Klebs, small cleavage furrows were already visible together with multiple nuclei, which became more obvious in the cytoplasm of later stages (Škaloud et al. 2013). The process of protoplast separation has been studied by TEM, e.g., in Trebouxiophyceae, where in combination with the nuclear division cleavage furrows are visible, as is autospore formation (Sluiman & Lokhorst 1988, Friedl

1993). In *Trebouxia jamesii* (originally described as *Pseudotrebouxia jamesii* Hildreth & Ahmadjian), the formation of multinucleate cells was interpreted to be a consequence of the culture conditions, as it occurred in media that contained BBM with peptone (Ahmadjian 1990). In these media enriched with organic substances, the cells were large, the nuclei divided before the chloroplasts, and some cells were found to contain 12 or more nuclei and an undivided chloroplast (Ahmadjian 1990), similar to the observations on *Macrochloris* in this study.

It was particularly evident in *Macrochloris* that in cells where protoplasmic separation had not yet taken place, basal bodies were already found in the cytoplasm. It remains unsolved, how long this multinucleate state persists and how zoospore formation is triggered; at some point sporulation takes place and multiple zoospores are formed that retain the flagella during sporulation. The formation of flagellated zoospores is shared by the closely related *Haematococcus* and *Stephanosphaera* (Droop 1956a, b). The orientation of the basal bodies in the flagellar apparatus has been used as an evolutionarily conserved character (e.g., Melkonian 1984, Watanabe & Floyd 1989), and has proven to concord with molecular data in zoosporic chlorococcalean green algae (e.g., Lewis et al. 1992, Wilcox et al. 1992). The clockwise orientation of the basal bodies in *M. radiosa* supports the phylogenetic position proposed by Buchheim et al. (2013). Interestingly, the directly opposite orientation of the basal bodies (an extreme form of the clockwise orientation) has been linked to the occurrence of multinucleate cells in *Neochloris* sp. group II by Watanabe & Floyd (1989).

The present study introduces the cytological characteristics of *M. radiosa*, which in the future might serve as a research object for physiological processes involved in the interesting phenomenon of uncoupling nuclear division and cytokinesis.

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Fig. 1. Light microscopy of *Macrochloris radiosa*. **a**, young vegetative cell, **b**, adult cell with dense chloroplast containing pyrenoids and multiple nuclei (arrows), **c**, adult cell with already divided chloroplasts, **d**, zoosporangium with multiple zoospores, pyrenoids are clearly visible, **e–g**, released zoospores with two flagella. **a, c–g**, differential interference contrast. Abbreviations: Chl chloroplast, N nucleus, Py pyrenoid, V vacuole. Bars a–d = 10 μm, e–g = 5 μm.

Fig. 2.

Vegetative life cycle of *Macrochloris radiosa*. **a**, young cell with nucleus (symbolized by dotted line with nucleolus) and ovoid cell shape likely caused by a rather flexible cell wall, **b**, cell increased in size, **c**, further cell size increase, **d**, nuclear division leads to multinucleate vegetative cell, **e**, separation of the protoplast, **f**, formation of zoospores, **g**, release of biflagellated zoospores.

Fig. 3. Transmission electron micrographs of *Macrochloris radiosa*, uninucleate cells. **a**, overview showing chloroplast lobes, nucleus and cell wall, **b**, detail in the cell center, electrondense particle is marked by arrow, **c**, detail in the cell periphery, with a large number of ribosomes, golgi body and vacuoles. Abbreviations: Chl chloroplast, CW cell wall, G golgi body, N nucleus, V vacuole. Bars a = 2 μm , b–c = 1 μm .

Fig. 4.

Transmission electron micrographs of *Macrochloris radiosa*, a–d multinucleate cells, e zoosporangium. **a**, overview, numerous nuclei are visible in one cell, **b**, detail showing two of the multiple nuclei (N_1 , N_2), **c**, detail of basal body (arrows) between two nuclei, **d**, microtubule rings lacking the central pairs of microtubules (arrows), **e**, zoosporangium containing numerous zoospores, cross section of flagella (arrow); insert, cross section of flagellum with typical 9+2 pattern of microtubules. Abbreviations: Chl chloroplast, CW cell wall, N nucleus, Py pyrenoid, ZS zoospore. Bars a = 10 μm , b = 1 μm , c–d = 500 nm, e = 1 μm , insert 100 nm.