

NOTE

PREPARATION AND CHARACTERIZATION OF PROTOPLASTS OBTAINED FROM THE PRASINOPHYTE *SCHERFFELIA DUBIA* (CHLOROPHYTA)¹

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The prasinophyte genera *Scherffelia* and *Tetraselmis* are the only genera that form a cell wall by an extracellular fusion of scales called a theca. We established a protocol for the production of protoplasts from *Scherffelia dubia* Pascher emend. Melkonian et Preisig using 3 mM Ellman's reagent (5,5'-dithio-bis-2-nitrobenzoic acid [DTNB]). Protoplasts analyzed by EM lacked flagella and thecae but were otherwise similar to control cells. In response to treatment with DTNB, many protoplasts synthesized new thecal scales in the Golgi apparatus, indicating that cells attempted to regenerate new cell walls. However, complete regeneration of the thecae only occurred once DTNB was washed out from the medium. At higher DTNB concentrations (5 mM), two protoplasts were found within the parental cell wall and scales accumulated between the plasma membrane of the protoplasts and the original theca but failed to form a new theca.

Key index words: cell wall; Ellman's reagent; protoplast; *Scherffelia*; theca

Abbreviations: DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; LV, large vacuole

Most prasinophytes are characterized by the presence of small structures of distinct size and shape on their cell surface called scales (Sym and Pienaar 1993). A cell wall-like structure has only evolved in the prasinophyte genera *Ostreococcus*, *Pycnococcus*, *Scherffelia*, and *Tetraselmis* (Sym and Pienaar 1993, Chretiennotdinet et al. 1995). The cell wall and its mode of biogenesis is not well characterized in *Ostreococcus* and *Pycnococcus*, however, several studies showed that in *Tetraselmis* and *Scherffelia*, the cell wall is derived ontogenetically from scales that fuse extracellularly to form a rigid cell wall (Manton and Parke 1965, Domozych et al. 1981, McFadden et al. 1986, Melkonian and Preisig 1986). For this reason, this special type of cell wall has been called a theca.

Prasinophyte scales consist of 90% polysaccharide (dry weight) with the unusual 2-keto sugar acids Kdo

(3-deoxy-2-manno-octulosonic acid), 5-O-methyl Kdo, and 3-deoxy-2-lyxo-heptulosaric acid as major constituents (Becker et al. 1989, 1990, 1991). Chemical analysis of isolated scales from various prasinophytes showed that all three 2-keto-sugar acids occurred in roughly equal molar amounts (Becker et al. 1991). In contrast, Kdo (50 mol% of total carbohydrates) and galacturohic acid (15 mol% of total carbohydrates) were the dominant monosaccharides in thecae isolated from *Scherffelia* and two different *Tetraselmis* strains and only minor amounts of 3-deoxy-2-lyxo-heptulosaric acid and 5-O-methyl Kdo were found (Becker et al. 1989, 1991). Based on these findings and structural data on oligosaccharides isolated from the theca of *Tetraselmis striata* (Becker et al. 1995b, 1998), a model for the extracellular fusion of scales into a theca has been proposed (Becker et al. 1994). Briefly, during theca biogenesis, thecal scales are synthesized with a chemical composition comparable with other scale types. Scales are converted into a theca by the addition of long Kdo chains substituted with sulfated galacturohic acid disaccharides and extensive cross-linking by Ca²⁺ ions (Becker et al. 1994).

Biogenesis of flagellar scales has been analyzed in great detail in *Scherffelia dubia* (McFadden and Melkonian 1986, Becker et al. 1995a, Perasso et al. 2000). Scales are synthesized in the Golgi apparatus and represent the most compelling example of cargo transport through the Golgi stack via the cisternal progression model; therefore, *Scherffelia* has played a major role in the renaissance of the cisternal progression model of intra-Golgi transport (Nebenführ 2003). To further develop *Scherffelia* as an experimental system, we developed a method to obtain protoplasts of *S. dubia* to aid in the development of a transformation system and analysis by fluorescence microscopy. In addition, protoplasts capable of regenerating a new cell wall would represent an ideal experimental system to address the interesting question how individual scales are assembled into a cell wall.

Here, we show that treatment of *S. dubia* cells with 5,5'-dithio-bis-2-nitrobenzoic acid (Ellman's reagent, DTNB) converts cells within a few days into protoplasts. We characterize the protoplasts by TEM and show that protoplasts have a normal ultrastructure and begin to synthesize thecal scales.

¹Received 23 March 2004. Accepted 19 August 2004.

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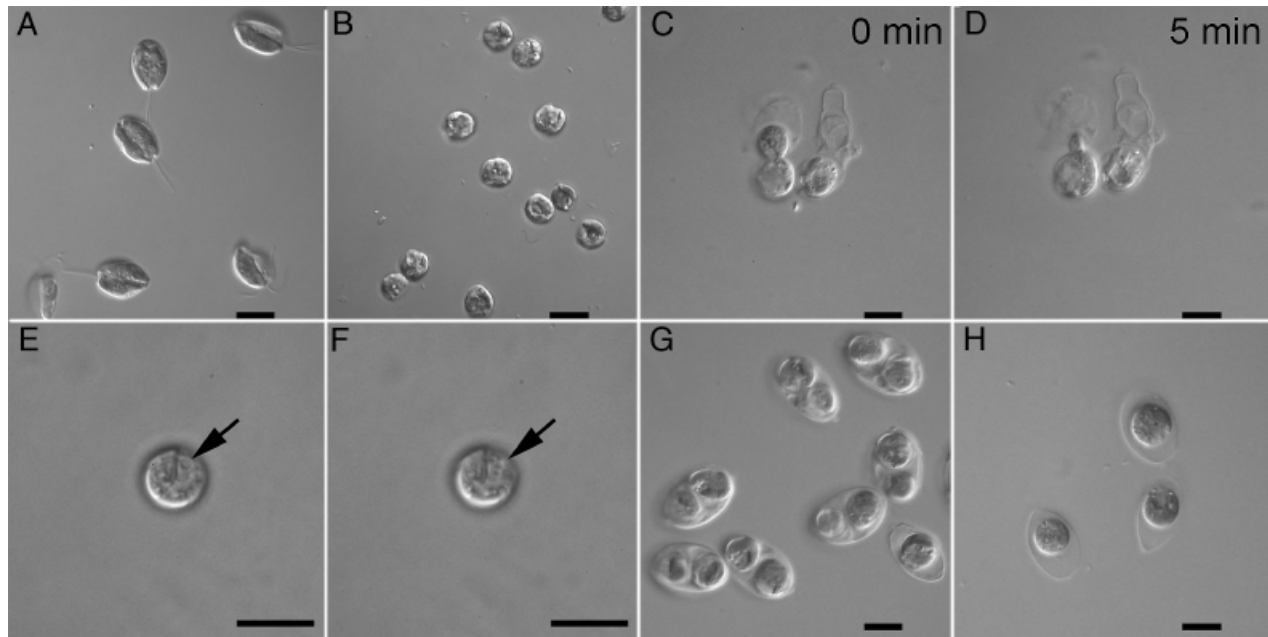


FIG. 1. Light microscopic analysis of the effect of DTNB on cells of *Scherffelia dubia*. (A) Control cells. (B–F) Cells treated with 3 mM DTNB. (B) Overview. (C and D) A protoplast escapes from a cell wall. Pictures were taken 5 min apart. (E and F) The same protoplast at higher magnification illustrating the presence of contractile vacuoles (arrows). Pictures were taken about 1 min apart. (G) Two-cell stage of *S. dubia* protoplasts after treatment with 5 mM DTNB. (H) Protoplasts that remained within the parental cell wall. Scale bar, 10 μ m.

Algal strain. Cells of *S. dubia* Pascher emend. Melkonian et Preisig were obtained from the Culture Collection of Algae at the University of Cologne (strain CAAC0019). The cultures were grown in a modified WARIS solution as described in Grunow et al. (1993).

Inhibitor experiments. For the preparation of a 50-mM stock solution, DTNB was dissolved in distilled water and the pH adjusted to 7.0 by the addition of aqueous 1 M NaOH. The solution was sterilized using a 0.2- μ m Membrex 25 syringe filter (Membra Pure, Bodenheim, Germany) and stored at 4° C until use (maximal 7 days). Best results were obtained when DTNB was added to a log-phase culture at a cell density of 2.0×10^6 cells \cdot mL⁻¹ about 2 h into the light cycle. Cells were cultured with DTNB in a 100-mL Erlenmeyer flask without agitation and aeration, at 15° C and with a 14:10-h light:dark cycle.

LM. Cells were fixed with 1% osmium tetroxide (OsO₄) in culture medium and observed with an IM 35 inverted microscope (Zeiss, Oberkochen, Germany) operating in the phase contrast mode. Micrographs were taken using a 50 E camera (model EOS, Canon) and Fuji Sensia 100 films (Düsseldorf, Germany).

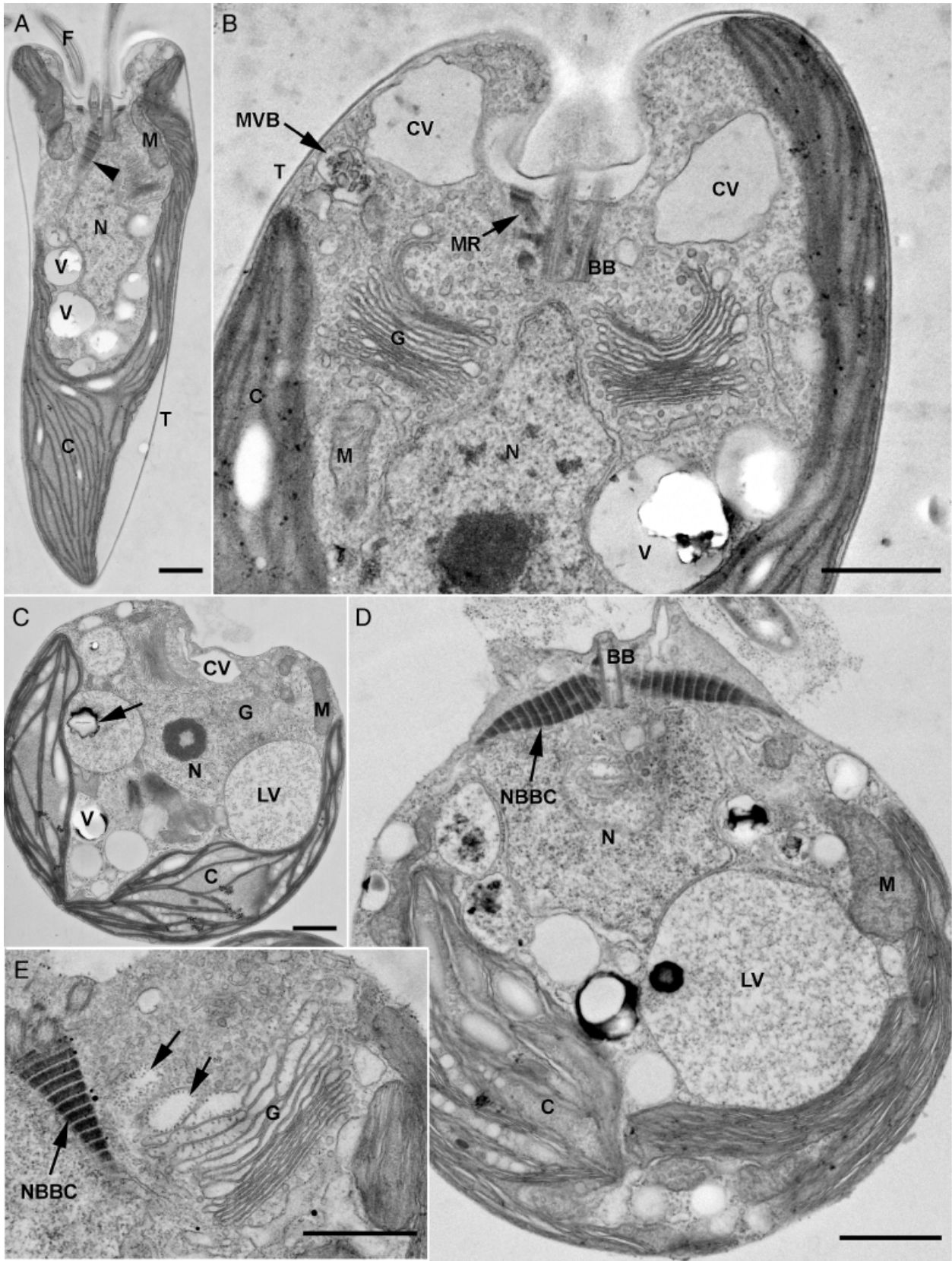
TEM. Cells were fixed for EM 6, 11, and 18 days after addition of DTNB by addition of 7 mL of cell culture to an ice-cold mixture of 2.5 mL 2% OsO₄ and 0.5 mL 25% glutaraldehyde (final concentration of fixatives, 0.5% OsO₄ and 1.25% glutaraldehyde). After 20 min at 0° C the cells were washed twice with culture medium. Further processing for TEM was standard. Ultrathin sections were cut with an MT-6000 microtome (RCM, Tucson, AZ, USA), stained

with uranyl acetate and lead citrate (Reynolds 1963), and micrographs were taken with a CM 10 electron microscope (Philips, Eindhoven, The Netherlands).

Cells were oval in face view (8–10 μ m wide and 13–17 μ m long, Fig. 1A) and narrower (5–6 μ m wide) and wedge shaped (Fig. 1A) in side view. When 3 mM DTNB was added to the culture medium, protoplasts of *S. dubia* (Fig. 1B) appeared in the culture medium within a few days. A closer inspection of the first hours upon addition of DTNB showed that cells shed their flagella and the protoplasts rounded up (data not shown) before being released into the medium through openings at the posterior end of the theca (Fig. 1, C and D), leaving the cell wall behind.

We then started a systematic investigation of the effect of DTNB on *S. dubia*. Concentrations of 3 mM or higher DTNB inhibited cell division in *S. dubia* (data not shown). Systematic variation of the concentration of DTNB, cell density, age of the starting culture, and time point of addition during the light:dark cycle led to the optimized protocol as described above. Using this protocol, we reproducibly obtained 50%–90% protoplasts within 5 days. It is noteworthy that higher concentrations of DTNB (5 mM) led to an accumulation of two protoplasts within the parental cell wall (two-cell stage, Fig. 1G), most likely representing cell division stages where the progeny cells failed to produce a new theca and mature. In some experiments we encountered protoplasts that failed to escape from the cell wall (Fig. 1H).

Protoplasts of *S. dubia* had a spherical cell shape (Fig. 1B) with an average diameter of 7.8 ± 0.6 μ m ($n = 10$). Protoplasts in the two-cell stage (Fig. 1G) were



noticeably smaller ($7.2 \pm 0.5 \mu\text{m}$, $n = 10$), whereas the single protoplasts still residing within the theca had the same diameter ($7.8 \pm 0.3 \mu\text{m}$, $n = 10$) as free protoplasts. Within the protoplasts, the chloroplast occupied most of the internal space and made it difficult to observe any additional internal structures with the light microscope. As in control cells, contractile vacuoles were present (Fig. 1, E and F, arrows) and discharged fluid with a period of about 20 seconds. Protoplasts were viable in DTNB containing medium for at least 5 weeks (maximum time tested). Upon removal of DTNB, cells regenerated new thecae that were indistinguishable from cell walls of control cells (not shown). Theca regeneration started about 24 h after removal of DTNB and continued over 4 days when the number of motile cells indistinguishable from untreated cells reached 80% of total cells. Only a few protoplasts could be detected in the medium at this time point. Measurement of the cell density of the culture after washout of DTNB indicated that the first cells started to resume cell division not before 48 h after removal of DTNB.

EM confirmed that the spherical cells observed by LM were indeed protoplasts (Fig. 2, C and D). For comparison, electron micrographs of control cells are presented in Figure 2, A and B. As observed in the light microscope, protoplasts were naked and the flagella were missing. Rarely, we observed a few individual scales on the cell surface of the protoplasts. The nucleus occupied the central region and two Golgi stacks were associated with the nucleus as in control cells (Fig. 2, B and C). In many cells the Golgi apparatus produced a large number of thecal scales (Fig. 2E), indicating that cells tried to regenerate a new covering. Except for the presence of scales in the cisternae and large secretory vesicles and the more concave appearance of the *trans* cisternae in non-scale-producing stacks (Fig. 2, D and E), no difference in the structure of the Golgi stacks in scale-producing or non-scale-producing protoplasts was observed. On average a Golgi stack contained 12.2 ± 1.3 cisternae ($n = 14$), which was significantly lower than the number of Golgi cisternae in control cells 16.2 ± 1.6 ($n = 7$) and less than the published value for the number of Golgi cisternae during cell division (16.1, McFadden et al. 1986) when thecal scales are synthesized *in vivo*. However, a recent reinvestigation of cell division in *S. dubia* showed that the number of Golgi cisternae varies between 10 and 14 during cell division depending on the division stage (Wustman et al. 2004); therefore, the number of Golgi cisternae in protoplasts and dividing cells of *S. dubia* is comparable.

Although protoplasts do not possess any flagella, the structure of the basal bodies seemed to be unaffected and were found in a wide hollow invagination of the plasma membrane reminiscent of the flagellar groove (Fig. 2, C and D), suggesting that the presence of a flagellar groove is independent from the presence of scales or a theca. Interestingly, the basal bodies were still docked to the plasma membrane (Fig. 2D) and two prominent nucleus basal body connectors (rhizoplasts, system II fibers) connected the basal bodies with the nucleus and stretched further to the plasma membrane (Fig. 2D). However, we did not find any evidence for a microtubular root system and rhizankyrae, which in untreated cells connect the basal apparatus to the theca (Fig. 2B).

In addition to the putative polyphosphate containing vacuoles (Melkonian and Preisig 1986) present in control cells (Fig. 2, A and B), many protoplasts accumulated large vacuoles (LVs) containing granular material of unknown origin. We believe the granular material in the LVs might be scales mistargeted to vacuoles, where they start to degrade. LVs were generally larger than the normal putative polyphosphate containing vacuoles (Fig. 2, C and D), but in some cells the same granular material was also present in some typical putative polyphosphate containing vacuoles (Fig. 2C, arrow).

We also used TEM to investigate the two-cell stage structure obtained by treatment of cells with 5 mM DTNB (Fig. 3). In this stage, two protoplasts were surrounded by a theca, and the space between the cells and cell wall was filled with individual scales (Fig. 3A). Sometimes the scales seemed to be arranged in multiple layers around the protoplasts (not shown), indicating that the cells tried to form a new cell wall. The structures of the Golgi stacks and basal apparatus at this stage were identical to the structures observed in free protoplasts (12.8 ± 1 cisternae per stack, $n = 7$). At this stage cells did not contain LVs as in free protoplasts. Instead, the LVs observed were filled with vesicular membrane material (Fig. 3B) similar to multivesicular bodies. Therefore, we call these structures multivesicular vacuoles (MVBs). However, multivesicular vacuoles were much larger than normal multivesicular bodies (compare with Fig. 2B, multivesicular body labeled MVB).

Ellman' reagent DTNB is commonly used in biochemical laboratories to determine the number of free sulfhydryl groups in proteins (Ellman 1958, Eyer et al. 2003). It was shown to inhibit the cross-linking of glycoproteins in the extracellular matrix of *Volvox*

FIG. 2. TEM analysis of protoplasts obtained from *Scherffelia dubia* by treatment with 3 mM DTNB. (A and B) Control cells. The arrowhead in A labels a rhizoplast stretching from a basal body to the nucleus. Arrows in B point to the microtubular root system (labeled MR) and a multivesicular body (labeled MVB). (C–E) Protoplasts of *S. dubia*. The arrow in C points to putative polyphosphate containing vacuole filled with granular material. The arrows in D and E labeled as NBBCs indicate rhizoplasts also known as nucleus basal body connectors or system II fibers. Arrows in E point towards *trans*-Golgi cisternae and a secretory vesicle filled with thecal scales. BB, basal body; C, chloroplast; CV, contractile vacuole; F, flagellum; LV, large vacuoles; N, nucleus; M, mitochondrion; T, theca (cell wall); V, putative polyphosphate containing vacuole. Scale bars, 1 μm .

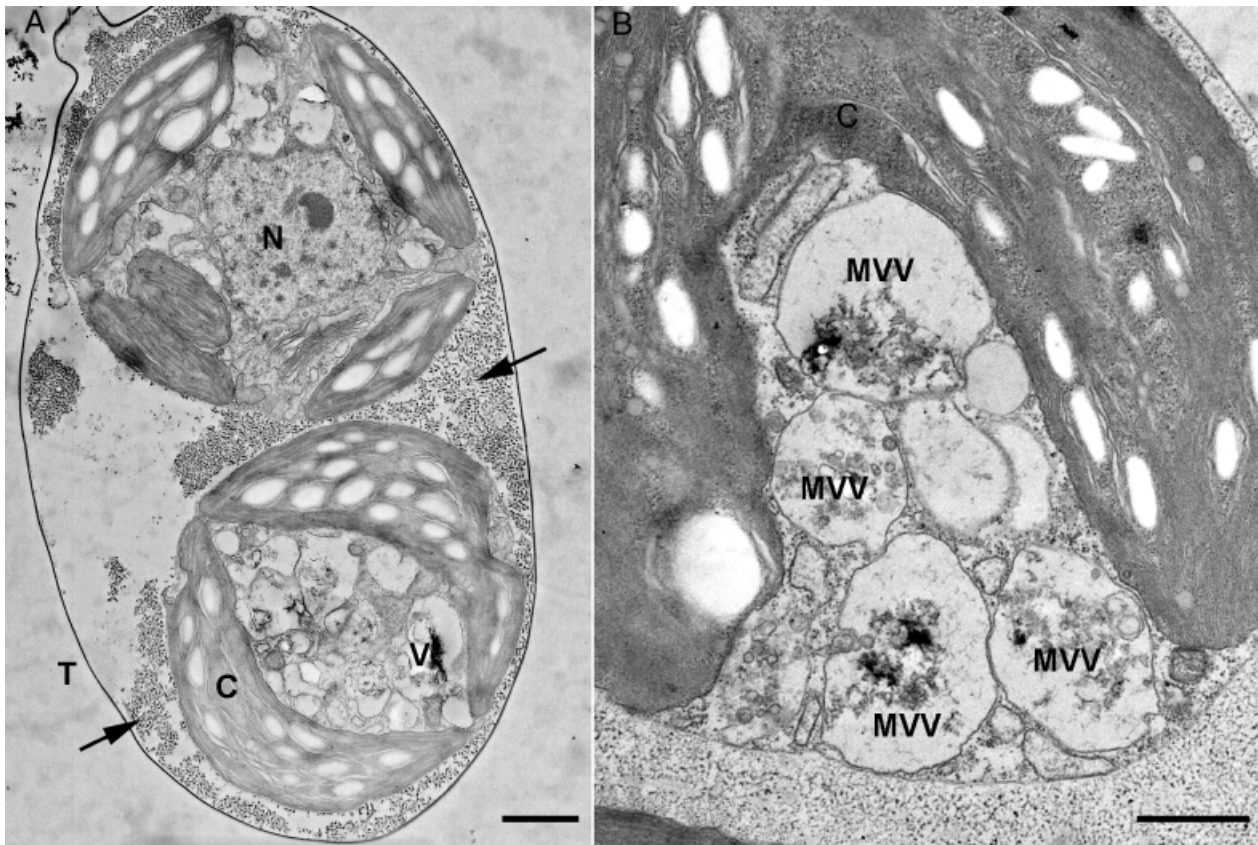


FIG. 3. TEM analysis of protoplasts in the two-cell stage obtained from *Scherffelia dubia* by treatment with 5 mM DTNB. (A) The arrows indicate the large numbers of scales accumulating between plasma membrane and cell. (B) The large vacuoles, labeled MVV, contain multivesicular membrane material. C, chloroplast; N, nucleus; T, theca (cell wall). Scale bars, 1 μ m.

carteri (Sumper et al. 2000). When DTNB was applied to isolated *Volvox* embryos, the embryos failed to form an extracellular matrix and develop properly. Instead, individual cells were set free (Sumper et al. 2000). DTNB is nontoxic, probably due to its charged nature that makes DTNB unable to penetrate the plasma membrane (Sumper et al. 2000). However, DTNB can most likely be taken up by endocytosis, allowing it to reach the lumen of all organelles of the endomembrane system, including the endoplasmic reticulum where the formation of disulfide bridges takes place. We showed that flagella scales are linked to the plasma membrane by high-molecular-weight protein complexes, where the individual proteins are cross-linked by disulfide bridges (Becker et al. 1996). Therefore, we reasoned that DTNB might also have an effect on the structure of the theca of *Scherffelia*. Indeed, upon incubation with DTNB we observed the formation of protoplasts, and the number of protoplasts within the medium increased over several days. The presence of protoplasts was confirmed by TEM analysis. Depending on the concentration, we obtained either free protoplasts or two protoplasts within the parental theca (two-cell stage). These results suggest that protoplasts might be obtained by mitosis when the progeny cells fail to mature and form a new cell wall.

In agreement with this, we observed in some experiments a temporary increase in the number of two-cell stages followed by an increase in the number of protoplasts. This is further supported by our observation that protoplasts leave the theca through an opening in the cell wall at the antapical end reminiscent of the situation during cell division (Wustman et al. 2004). To address this question more specifically, we counted the number of cells before the addition of DTNB and the number of protoplasts formed after several days. If protoplasts are produced by mitosis and the failure to develop a new theca, one would expect the number of protoplasts to be significantly higher (depending on the yield of protoplasts) than the number of cells at the beginning of the incubation with DTNB. However, we observed no increase in the number of cells during the incubation with DTNB. Therefore, the formation of protoplasts was not necessarily preceded by mitosis.

TEM analysis showed that many protoplasts synthesized thecal scales in their Golgi apparatus. We expected that the thecal scales would be secreted into the medium; therefore, it might be possible to isolate thecal scales from the culture medium of protoplasts as previously demonstrated for flagellar scales (Becker et al. 1989, 1990, 1991, Becker and Melkonian 1992). However, our preliminary experiments were

not successful. Many protoplasts contained LVs with a unknown granular material. It is possible that this material was degraded scales, and the question whether thecal scales are secreted or targeted for degradation remains open. In contrast, the situation is clearer regarding synthesis and exocytosis of thecal scales in the two-cell stage. TEM analysis showed that in all two-cell stages analyzed, thecal scales accumulate between the plasma membrane of the protoplasts and the parental cell wall, indicating that thecal scales were indeed secreted. The two-cell stage can be easily isolated from the culture medium; therefore, these cells might represent the ideal system for the large-scale isolation of thecal scales from *S. dubia* for biochemical analysis in the future.

This work was supported by the Deutsche Forschungsgemeinschaft.

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