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The change of chloroplast nucleoids during zygote formation in *Spirogyra*

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The change of chloroplast nucleoids during zygote formation in *Spirogyra* was observed by fluorescence microscopy using a DNA-specific fluorochrome 4′, 6-diamidino-2-phenylindole (DAPI). Every chloroplast of the vegetative cells included numerous nucleoids distributed throughout it and, usually, densely around pyrenoids. In each of the chloroplasts in young zygotes many nucleoids were contained. At seven days or so after conjugation about a half of the chloroplasts included in a zygote began to disintegrate, and they seemed to contain nucleoids at high density as compared with the other half of the chloroplasts that persisted in the zygote. Every disintegrating chloroplast subsequently became fragments of various sizes, each with nucleoids. The nucleoids were generally smaller but, frequently, some of them were larger than those in the persisting chloroplasts. The chloroplast fragments were finally destroyed within two weeks after conjugation. In the persisting chloroplast many nucleoids were present and adjacent ones situated around pyrenoids often seemed to connect together at their opposite ends. The present results suggest that the disintegrating chloroplast contains nucleoids until it is destroyed.

Key Index Words: *Chloroplast DNA*; *chloroplast nucleoids*; *DAPI-staining*; *disintegration of chloroplast*; Spirogyra; *zygote formation.*

In recent years fluorescence microscopy using 4′, 6-diamidino-2-phenylindole (DAPI), a fluorochrome sensitive and highly specific to duplex DNA (HAJDUK 1976), made it possible to detect plastid DNA readily (JAMES and JOPE 1978, COLEMAN 1978, 1979, KUROIWA *et al.* 1981). The plastids of various plants examined, except for *Acetabularia* (COLEMAN 1979, 1985, LÜTTKE 1981) and *Volvox* (COLEMAN and MAGUIRE 1982), have been demonstrated to possess nucleoids, and the size, shape, and number and distribution in a plastid of them vary from species to species and at the developmental stages of the plastid (COLEMAN 1979, 1985, KUROIWA *et al.* 1981). DAPI-staining method revealed the preferential disappearance of chloroplast nucleoids of the male gamete origin after mating in *Chlamydomonas reinhardii* (KUROIWA *et al.* 1982, 1985, COLEMAN 1984).

DNA fibrils have been shown to be localized in one or more of electron transparent areas of plastid stroma in some green plants (RIS and PLAUT 1962, HERRMANN and KOWALLIK 1970, KOWALLIK and HERRMANN 1972), and, according to KUROIWA *et al.* (1981), numerous (150-300) nucleoids are dispersed throughout the chloroplast of the vegetative cell in a species of *Spirogyra*. It is known that in *Spirogyra* about a half of the chloroplasts brought into the zygote through both the male and female gametes decrease in volume, becoming many fragments, and finally disintegrate during zygote maturation (CHMIELEVSKY 1890). An electron microscopic investigation demonstrated that in the disintegrating chloroplast thylakoid membranes vesiculated and degraded, starch grains almost disappeared, and plastoglobuli increased in size and number (OGAWA 1982). It is therefore probable that, accompanying

the marked changes in the internal structures of the disintegrating chloroplast, the size and shape of nucleoids and their distribution in the chloroplast consequently alter. As to the change of nucleoids in the disintegrating chloroplast of *Spirogyra*, however, little information has been obtained.

The aim of the present study is to clarify the change of nucleoids during zygote formation in *Spirogyra* using DAPI as a probe for chloroplast DNA.

Materials and Methods

Spirogyra verruculosa JAO, the vegetative cell of which had generally five ribbonlike chloroplasts, was used for the present study. Many vegetatively growing filaments of the species were collected from a pond in Sendai and allowed to grow in a pot under natural environments until conjugation took place. The filaments, which had initiated to form conjugation tubes, were cultivated in a vessel half-filled with pond water which had passed through a filter paper, under white fluorescent lamps (ca. 2,000 lux) on a 16 hour light-8 hour dark regime at 25°C.

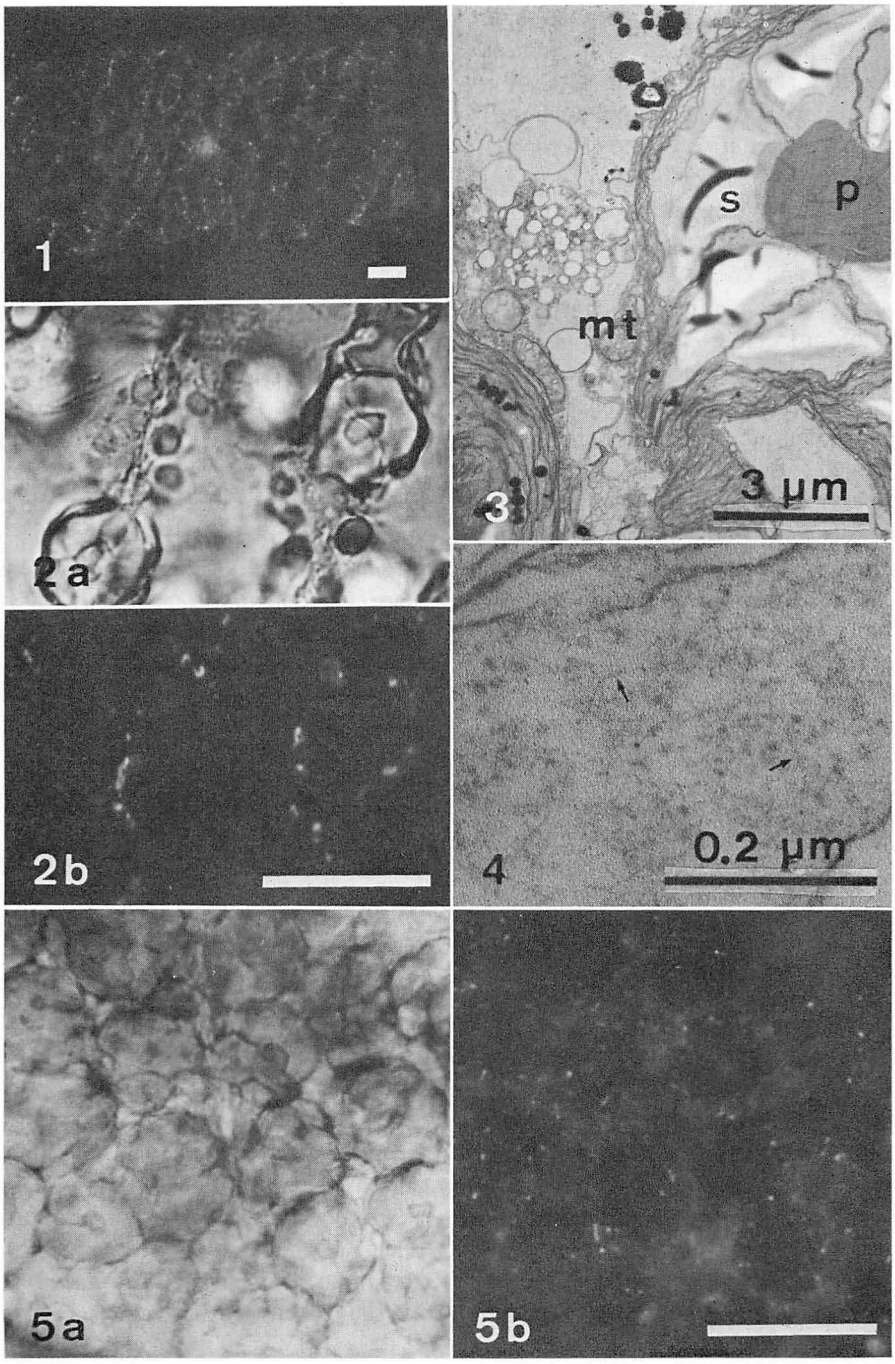
For staining with DAPI, the materials were fixed in phosphate-buffered 2.5% glutaraldehyde (pH 7.2) for 30 min at 4°C and rinsed in cold distilled water (DW) for 2 hr, and then, they were exposed to DAPI (0.5 μg/m*l* in DW) at 4°C for 10–20 min (vegetative cells and young zygotes) or for 20 hr (zygotes three or more days old), with or without DNase treatment. They were then washed in DW for 2 hr, mounted in citrate-phosphate buffer (pH 5.6) containing 1% 2-mercaptoethanol, and examined with an Olympus MMSP fluorescence microscope, using a UV excitation filter (UG-1) in combination with a 420 nm supression filter (L420) and SIApo 40 or SIFL 100 objective.

For electron microscopy, the materials were fixed with glutaraldehyde and osmium tetroxide, dehydrated in an ethanol series, and embedded in epoxy resin, following the method described previously (OGAWA 1982). Silver sections stained with lead acetate were examined with a Hitachi HS-9 electron microscope.

Results

After staining with DAPI, the nucleus emitted bright blue-white fluorescence characteristic of DNA-DAPI complex, and, as reported by KUROIWA *et al.* (1981), numerous fluorescent particles of blue-white color could be seen over the entire chloroplast (Fig. 1). The particles were located densely around pyrenoids and sometimes arranged like a line of beads along the midrib of the chloroplast, and each of them was about 1.0 μm in size and was usually spherical or oval and sometimes dumbbell-shaped (Figs. 2a and 2b). Treatment with DNase before dying with DAPI completely abolished fluorescence such as mentioned above, but not with RNase or trypsin. A rough estimation indicated that several hundred or more of fluorescent particles were present over each chloroplast. The electron micrographs of sections through vegetative cells showed that many mitochondria lay in the vicinity of the chloroplast (Fig. 3), suggesting that some of the blue-white particles seen over the chloroplast might be attributed to them. But, cytoplasmic threads suspending the nucleus, where mitochondria were present, did not contain clear fluorescent bodies of blue-white color, and the fractioned chloroplast fragments, nearly free from mitochondria, had numerous fluorescent particles of blue-white color. These results suggest that almost all of the fluorescent particles are associated with the chloroplast. Fine fibrils, each about 2 nm thick, could be seen in stroma near pyrenoids (Fig. 4).

Every chloroplast of the one-day-old zygote had many nucleoids like that of the vegetative cell (Figs. 5a and 5b). The male and female pronuclei came close to each other and occupied the central portion of the zygote. The zygote wall gradually thickened centripetally, and at several days after conjugation the fluorescence from the pronuclei as well as from the chloroplast



Figs. 1–5. Light and electron micrographs of *S. verruculosa*. 1: Fluorescence micrograph of a vegetative cell stained with DAPI, showing fluorescing particles over chloroplasts. ×250. 2a and 2b: The same view of chloroplasts of a vegetative cell after DAPI-staining. Fluorescing particles are located densely around pyrenoids. ×1,100. 3: Electron micrograph of a longitudinal section of a vegetative cell, showing mitochondria (mt) lying near chloroplasts. Pyrenoid (p), starch grain (s). ×6,600. 4: Chloroplast stroma with fine fibrils (arrows) about 2 nm thick. ×134,000. 5a and 5b: The same view of a one-day-old zygote after staining with DAPI. Each chloroplast includes many nucleoids. ×1,100. Figs. 1, 2, and 5. Scale=20 μm.

could not be detected, probably because some components deposited in the wall almost completely absorbed ultraviolet light for excitation. In order to examine the changes of the size and distribution of the chloroplast nucleoids, some of the older zygotes were squashed and their cytoplasmic contents were pushed away from their thick zygote walls (Figs. 6a and 6b).

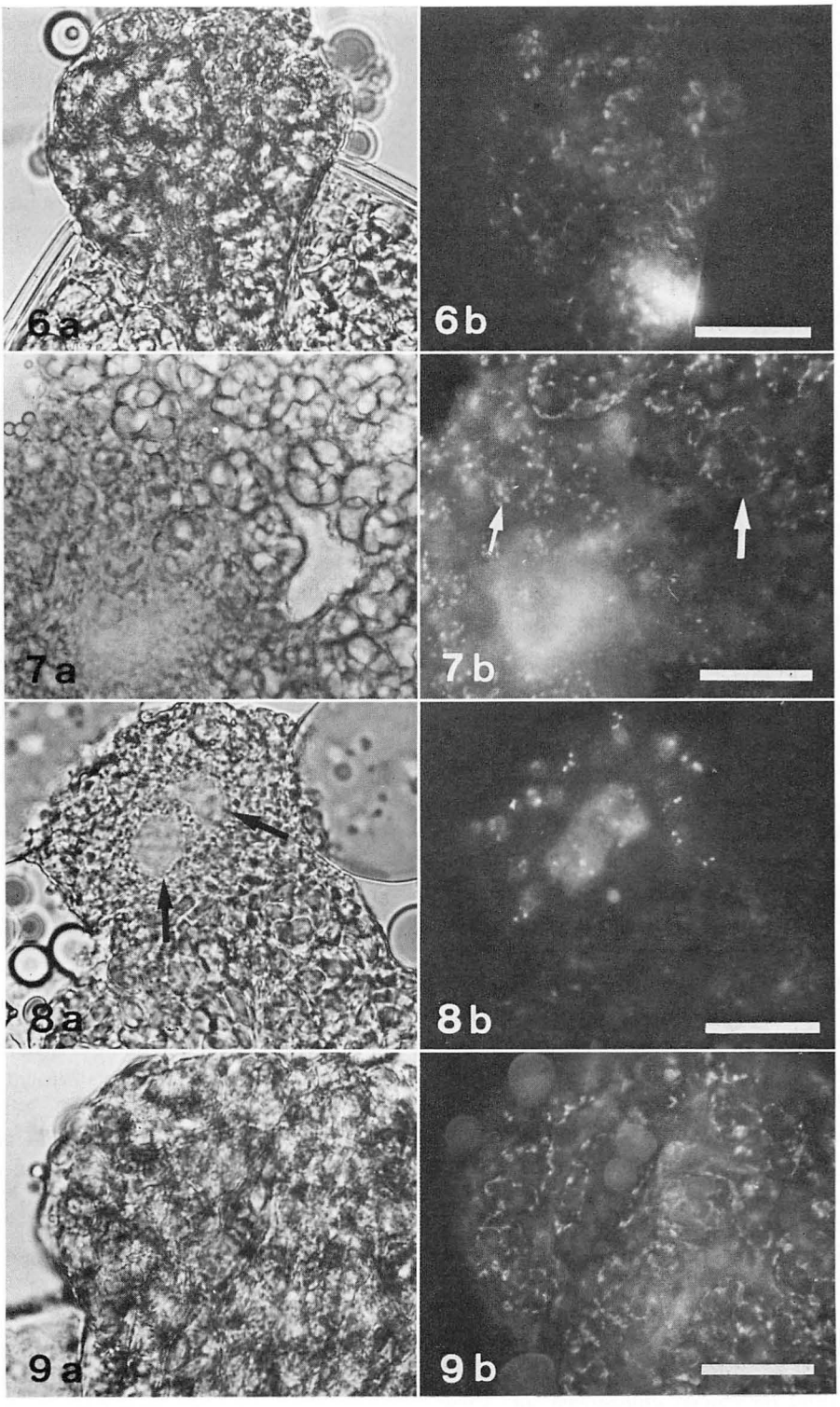
At about seven days after conjugation the disintegration of chloroplast commenced, and about a half of the chloroplasts included in a zygote turned yellow probably due to chlorophyll degradation, while the other half remained green. The density of nucleoids per unit area of the disintegrating chloroplast seemed to be higher than that of the persisting one (Figs. 7a and 7b), probably because the disintegrating chloroplast decreased in volume accompanying the gradual reduction of starch. The persisting chloroplast contained many oblong nucleoids (Fig. 7b). The disintegrating chloroplast then became many round fragments of various sizes, each with numerous spherical nucleoids. These chloroplast fragments seemed to move gradually to the central portion of the zygote near the two pronuclei, which lay close to each other but did not completely unite together (Figs. 8a and 8b). Each of the relatively large chloroplast fragments included nucleoids. Generally, the nucleoids were smaller than those of the persisting chloroplast, but, frequently, in some of the chloroplast fragments larger and brighter nucleoids were observed (Fig. 8b). As seen in Fig. 8b, one or two spherical, slightly dark bodies without fluorescence of DNA-DAPI complex were present in the chloroplast fragment. In terms of size, number, and shape, the respective body might be equivalent to a pyrenoid core free from starch grains. The chloroplast fragments were destroyed within two weeks after conjugation. The persisting chloroplast contained many nucleoids. The boundary between adjacent nucleoids localized around pyrenoids often became obscure, and they seemed to join together (Figs. 9a and 9b).

Discussion

Chloroplast DNA of *Spirogyra* could not be always detected successfully by such conventional cytochemical methods as Feulgen, methyl green, and acridine orange staining, and, hence, the results on the presence of DNA reported by some workers were contradictory to one another (STOCKING and GIFFORD 1959, MEYER 1966, MASUBUCHI 1968). The present author employed these cytochemical methods first, but they were not effective in demonstrating the existence of chloroplast DNA in any *Spirogyra* species so far examined, though, occasionally, the chloroplasts were observed to include many dark green granules after dying with methyl green. DAPI-staining method was most useful for demonstrating chloroplast DNA in *Spirogyra* with certainty. The chloroplast of the present *Spirogyra* species seems to contain so small quantity of DNA as not to be visualized by the conventional cytochemical methods.

As compared with the persisting chloroplast, the disintegrating one seemed to include nucleoids at high density (Fig. 7b), and the resulting chloroplast fragments often had relatively large nucleoids (Fig. 8b). The actual development of these larger nucleoids in the disintegrating chloroplast remains

Figs. 6-9. Light micrographs of *S. verruculosa*. 6a and 6b: The same view of a DAPI-stained zygote of five days old. Nucleoids can be seen in the chloroplasts pushed away from the thick zygote wall but not in those enveloped in the wall. ×780. 7a and 7b: The same view of a nine-day-old zygote, showing the distribution of nucleoids in disintegrating chloroplasts (small arrow) and in persisting ones with abundant starch (large arrow). ×780. 8a and 8b: Pronuclei (arrows) and fragments of disintegrating chloroplasts with larger nucleoids in a zygote of ten days old. ×780. 9a and 9b: Persisting chloroplasts of a 14-day-old zygote. Some of the chloroplast nucleoids localized around pyrenoids seem to connect together. ×780. Figs. 6-9. Scale=20 μm.


6a
6b
7a
7b
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unclear, but it is possible that the peripyrenoidal nucleoids come close to one another as starch grains decrease in volume, and then some of them gradually unite together into larger ones.

In *Chlamydomonas reinhardii* the chloroplasts from both the male (mating type minus) and female (mating type plus) gametes fused together after mating (CAVALIER-SMITH 1970). The chloroplast nucleoids of the male gamete origin disappeared before chloroplast fusion, but those of the female gamete origin persisted and united together into a single or a few larger nucleoids in the fused chloroplast (KUROIWA *et al.* 1982, COLEMAN 1984). In *Spirogyra* the chloroplast nucleoids seemed to be isolated from one another in the vegetative cell (Fig. 2b) and in the young zygote (Fig. 5b), but the adjacent nucleoids located around pyrenoids in the persisting chloroplast of the 14-day-old zygote often appeared to connect together at their opposed edges (Fig. 9b). However, the assembly of the chloroplast nucleoids into larger ones as found in *Chlamydomonas* could not be seen in the persisting chloroplast at least by 14 days after conjugation.

As observed by COLEMAN (1985), the chloroplast nucleoids were distributed densely around pyrenoids in *Spirogyra* (Figs. 1 and 2b). The chloroplast nucleoids were spherical or oval in the vegetative cells (Fig. 2b) and in young zygotes (Fig. 5b), but they were oblong or narrow oblong in the persisting chloroplasts of older zygotes (Fig. 7b). The dense distribution of chloroplast nucleoids at peripyrenoidal regions and their change in shape from spherical to oblong may lead to the aspect of lateral union of the chloroplast nucleoids located around pyrenoids in the persisting chloroplast of older zygotes (Fig. 9b). The process and cause of the alteration in shape of the nucleoids of persisting chloroplasts are unclear.

The fully developed chloroplast of *Vicia faba* included many nucleoids, but each of the degenerated chloroplasts in the older leaves had only a few small nucleoids (KUROIWA *et al.* 1981). In *Spirogyra* the disintegrating chloroplast, though decreased markedly in volume, contained numerous nucleoids (Fig. 7b). The nucleoids of the disintegrating chloroplast fragments were usually smaller but, frequently, some of them were larger than those of the persisting chloroplasts (Fig. 8b).

It has been repeatedly suggested that in *Spirogyra* the chloroplast destroyed during zygote formation is of the male gamete origin (CHMIELEVSKY 1890). However, no direct evidence was presented because an applicable method for inducing sexual reproduction of *Spirogyra* had not been established until comparatively recently (GROTE 1977, SIMON *et al.* 1984) and because the chloroplasts of both gametes were indistinguishable from each other in their submicroscopic structures (OGAWA 1982). The present fluorescence microscope study was not successfull in demonstrating the preferential elimination of the chloroplast from the male gamete since the chloroplast nucleoids of both gametes were very similar in size, shape, and distribution. Further investigations would be required for demonstrating the selective disintegration of chloroplast in *Spirogyra*.

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小川　茂：アオミドロ（*Spirogyra*）の接合子形成期における葉緑体核様体の変化

接合藻アオミドロの接合子形成期における葉緑体核様体の変化を，DNA に特異的な蛍光色素 4′, 6-diamidino-2-phenylindole (DAPI) で染色して蛍光顕微鏡で観察した。栄養細胞の各葉緑体は多数の核様体を有していた。核様体は葉緑体全体にみられたが，特にピレノイド周辺部に密に分布していた。若い接合子のすべての葉緑体にも多数の核様体がみられた。接合後 1 週間程経過すると接合子内の約半数の 葉緑体が黄色に変わり退化を始める。一方，残り半数の葉緑体は接合子内に維持される。退化葉緑体は，維持される葉緑体に比較して高密度で核様体を有する様相を示した。退化葉緑体は，続いて大小，多数の断片となる。各断片には，普通，多数の比較的小さな核様体がみられたが，しばしば，維持される葉緑体に比べて大きな核様体がみられた。接合後 2 週間以内に退化葉緑体の断片は崩壊する。維持される葉緑体では，ピレノイド周辺部の隣りあった核様体の境界がしばしば不明瞭となり，互いに連なる様相を示した。今回の観察から，退化葉緑体は崩壊直前まで核様体を有することが示唆された。

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