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Effect of Light on the Chloroplast Division Cycle and DNA Synthesis in Cultured Leaf Discs of Spinach

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ABSTRACT

The effects of light on both the division cycle of chloroplasts and the synthesis of chloroplast DNA were investigated in cultured discs taken from the distal end of 2-centimeter spinach (*Spinacia oleracea*) leaves. Comparisons were made of discs cultured for a maximum of 4 days in a shaking liquid medium under continuous white light, darkness, and of discs cultured for 1 day in light following 3 days in darkness. In continuous white light the shortest generation time of chloroplasts observed in this study was 19.4 hours and the duration of spherical, ovoid, and dumbbell-shaped stages in the division cycle were 13.4, 2.8, and 3.1 hours, respectively. In darkness the generation times of chloroplasts extended to 51.5 hours. Under these conditions the duration of spherical, ovoid, and dumbbell-shaped stages were 22.8, 8.4, and 20.2 hours, respectively, suggesting that in darkness the separation of dumbbell-shaped chloroplasts may be the rate limiting step. When discs cultured in the dark were transferred to light, most dumbbell-shaped chloroplasts separated into daughter chloroplasts in less than an hour. Measurements of chloroplast DNA established that the cellular level of chloroplast DNA increased 10-fold over the 4 days of culture in continuous white light. Comparisons of the plastids of dark and light grown discs showed that the synthesis of chloroplast DNA was enhanced by light. Observations of DAPI stained dividing chloroplasts indicate that DNA partitioning can take place during the final stage of chloroplast division and that it does not precede plastid division.

It is generally accepted that plastids multiply by the constriction division of preexisting plastids. However, there are few confirmed direct observations of the process of chloroplast division and these are mainly confined to algae (1, 4). There are a number of indirect observations that suggest a relationship between the presence of dumbbell-shaped chloroplasts and increases in the number of chloroplasts in higher plants (2, 3, 5, 14). Calculations based on changes in both cell and chloroplast numbers in spinach (12) and wheat (2) have provided approximate generation times for these species.

In spinach it has been known that the division rate of chloroplasts is affected by environmental factors such as light, temperature and nutrition (13). In spinach leaf discs cultured in low intensity white or green light the relative frequency of dumbbell-shaped chloroplasts is increased and it has been suggested that the completion of the chloroplast division cycle becomes slower under these conditions (3).

In the present study we have measured the effect of light on the generation time and on the duration of the individual stages of the division cycle of chloroplasts using cultured discs of spinach leaves. As part of this study we have observed the process of DNA-nucleoid partitioning and measured changes in chloroplast DNA levels during the division cycle of spinach chloroplasts.

MATERIALS AND METHODS

Plant Material

Spinach plants (*Spinacia oleracea* Hybrid 102) were grown in aerated nutrient solution in a growth cabinet as described earlier (15).

Culture of Leaf Discs

Leaf discs 2.0 mm in diameter were taken from the distal end of 2-cm leaves at stem position 5. These were cultured in 5.5-cm diameter Petri dishes containing 10 mL of sterile liquid medium. This consisted of Murashige and Skoog (10) inorganic salts, supplemented with 5 g/L of sucrose and 0.5 mg/L of benzyladenine. The discs were cultured under continuous white light (60 μmol $m^{-2}s^{-1}$ PAR 400–700 nm) or in total darkness at 25°C on an oscillatory shaker at 80 rpm.

Measurement of Cell Number per Disc and Cell Area

Leaf discs were fixed with 3% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0). Cells were separated by digestion with chromic acid and counted using a Petroff-Hauser bacterial counter as described previously (14). Cell area was measured using light microscopy (15).

Measurement of Chloroplast Number per Cell and Chloroplast Area

This was made by phase contrast microscopy after individual cells were separated from glutaraldehyde-fixed leaf discs by treatment with 0.1 M EDTA (pH 9.0) at 60°C for 30 min (15).

Observations of Isolated Chloroplasts

Leaf discs were chopped with a razor blade into a precooled Honda *et al.* (7) medium containing 1% (w/v) glutaraldehyde.

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Chloroplasts in the slurry were observed by phase contrast microscopy and nucleoids in intact chloroplasts were stained with 0.3 μg/mL of DAPI[2] in the same medium and observed under epifluorescent UV illumination.

Measurement of Chloroplast DNA

Microspectrofluorometric measurements of absolute DNA amount per chloroplast were made by the method of Lawrence and Possingham (8, 9) with some modifications. Leaf discs were sliced into sections 0.5 mm wide and fixed on ice for 15 to 30 min in a medium containing 0.3% (w/v) glutaraldehyde, 0.7 M mannitol, 10 mM Tris-HCl (pH 7.6), and 5 mM MgCl. The fixed slices were chopped with a razor blade into the same medium, and the slurry was mixed with DAPI on a glass slide previously smeared with *Pediococcus damnosus* cells, the internal standard for DNA measurement. During the measurement of DAPI fluorescence of chloroplasts the red fluorescence due to Chl was screened out using a Zeiss KP560 barrier filter (6).

RESULTS

Growth of Leaf Discs

The overall growth of leaf discs was monitored on the basis of fresh weight, cell number per disc, and cell area (Fig. 1, a–c). The fresh weight of leaf discs increased 26-fold and the average cell area increased 12-fold after 4 d of culture under continuous white light. There was no increase in cell number per disc. In total darkness, both the fresh weight of discs and cell area increased only slightly. When discs cultured in the dark for 3 d were exposed to white light for 1 d, both the fresh weight of discs and cell area increased. Again there was no increase in cell number per disc. These results indicate that the growth of the discs from the distal end of leaves was light dependent and resulted from cell enlargement without concomitant cell division.

Chloroplast Division and Growth

Chloroplast number per cell in leaf discs increased 15-fold during 4 d of culture under continuous white light (Fig. 1, d and e). The rate of increase in chloroplast number per cell corresponded with the rate of chloroplast division as no cell division occurred in culture. Up to the second day of the culture under continuous white light the generation time of chloroplasts was 19.4 h. Chloroplast division rates were lower during the subsequent days of culture. The division rate of chloroplasts in darkness was constant between the 1st and 4th d of culture. The generation time of chloroplasts in the dark on d 3 was estimated to be 51.5 h, being 2.6 times longer than that in white light during d 1. When discs cultured for 3 d in darkness were transferred to white light, chloroplast division accelerated with a 2.2-fold increase in chloroplast number the day after transfer (Fig. 1, d and e).

Chloroplasts in different stages of the division cycle were observed by light microscopy (Fig. 2). They were classified into spherical (S type), ovoid (O type), dumbbell-shaped (D type), and deeply constricted chloroplasts with a narrow neck (Dn type), respectively. These categories were defined as follows: S type are chloroplasts which have a ratio of length to breadth of less than 1.5 (Fig. 2a). O-type chloroplasts have a ratio of length to breadth of more than 1.5, but do not have a constriction (Fig. 2c). Dn-type chloroplasts have a range of central constrictions (Fig. 2, e, g, and i). Dn type are D-type chloroplasts in which the ratio of diameter of the constricted neck to the width of two ends was less than 0.2 (Fig. 2, g and i). In most Dn-type chloroplasts the necks are both narrow and elongated, as shown in Figure 2, g (arrowhead) and i. It is assumed that chloroplast division takes place in sequence from stages S to O to D and finally to the Dn configuration. DAPI staining demonstrated the distribution and partitioning

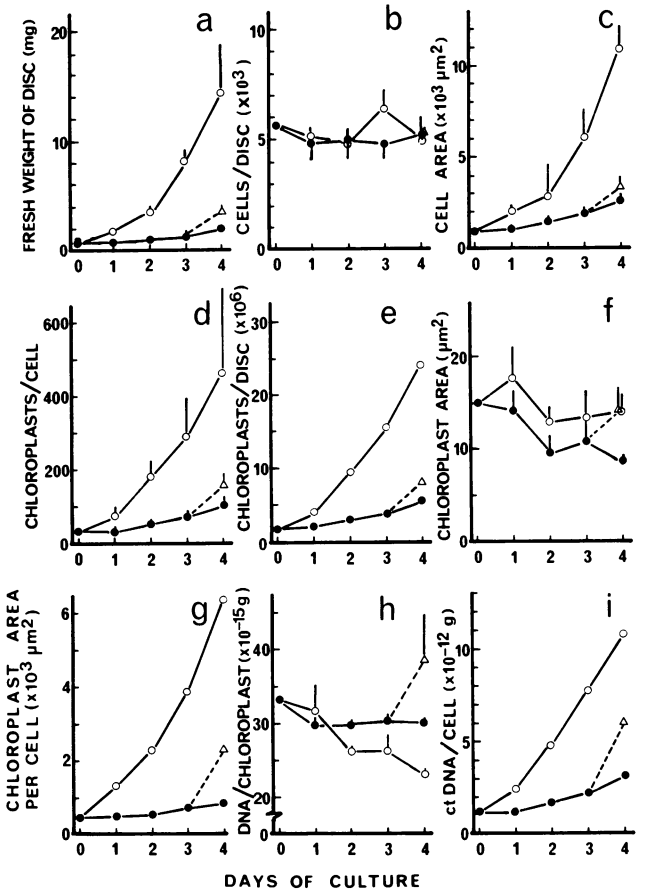

Figure 1. Changes in (a) fresh weight per disc, (b) cell number per disc, (c) cell area, (d) chloroplast number per cell, (e) chloroplast number per disc, (f) chloroplast area, (g) total chloroplast area per cell, (h) DNA amount per chloroplast, and (i) chloroplast DNA amount per cell in spinach leaf discs cultured for 4 d in continuous white light (○) or in darkness (●), or cultured for 3 d in the dark and then transferred to white light (△). Chloroplast number per disc (e) was calculated from the product of chloroplast number per cell and the mean cell number per disc. Total chloroplast area per cell (g) was calculated by multiplying chloroplast area and chloroplast number per cell. Chloroplast DNA per cell (i) was calculated by the multiplication of DNA amount per chloroplast and chloroplast number per cell. Each value for fresh weight per disc was the mean of three measurements; and for the other parameters the means of at least eight measurements. Vertical bars represent standard errors.

[2] Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole.

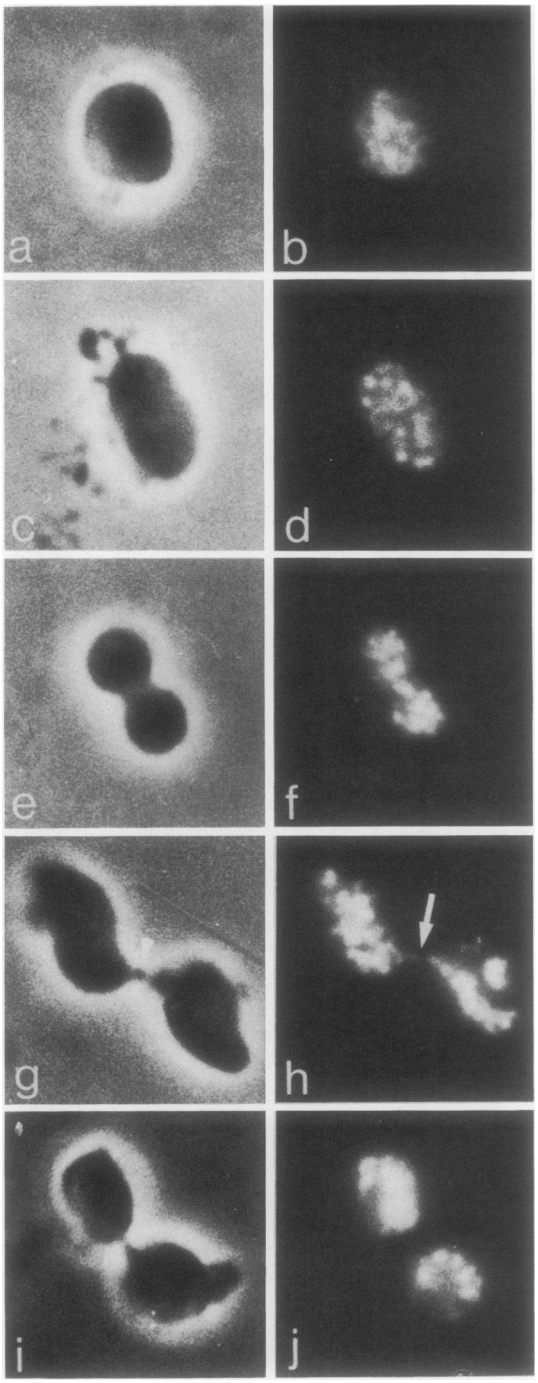

Figure 2. Phase contrast (left column) and fluorescence (right column) photomicrographs of DAPI-stained chloroplasts in different configurations: a and b, a spherical (S type); c and d, an ovoid (O type); e and f, dumbbell-shaped (D type); g and h, a deeply constricted chloroplast with a narrow neck (*arrowhead*) (Dn type) in which nucleoids can be seen in the neck (*arrow*); i and j, a deeply constricted chloroplast with a narrow neck (Dn type) in which nucleoids are not seen in the neck (magnification, × 35,000).

of chloroplast nucleoids during the division cycle (Fig. 2, b, d, f, h, and j). It is important to note that nucleoids were clearly observed in the narrow neck of some Dn chloroplasts (Fig. 2h, arrow) but not observed in others (Fig. 2j). This suggests that partitioning of chloroplast nucleoids is completed during the final stages of the chloroplast division.

The relative frequency (%) and the calculations of the duration of each chloroplast stage are shown in Table I. In light-grown discs after a single day of culture, the relative frequencies of D and Dn chloroplasts were 16.2% and 1.8%, which equate to their being at this stage for 3.1 and 0.3 h, respectively. In dark-grown discs after 3 d of culture chloroplasts remained in a D configuration 6.5 times longer than chloroplasts of light-grown discs at d 1. Difference in duration of the S and C stages between the light-grown and dark-grown discs were much smaller than the differences in the duration of the D stage (Table I). These results suggest that in the dark, separation of dumbbell-shaped chloroplasts is slower than the formation of dumbbells.

When leaf discs cultured for 3 d in the dark were transferred to white light, a cyclical change in the relative proportion of chloroplasts in each configuration was observed during the early period of illumination (Fig. 3, a–c). The proportion of S chloroplasts increased from 44% to 57% of the total in the 1st h, fell to 33% at the 2nd h, and gradually increased again. The frequency of O chloroplasts showed no significant change. The frequency of D chloroplasts varied with a pattern that was the mirror image of that for S chloroplasts. In the 1st h, D chloroplasts decreased from 39% to 29% of the total, increased to 51% at the 2nd h, and then gradually decreased. The frequency of Dn chloroplasts was almost constant between 2% and 4% throughout the 6 h period of illumination. These observations indicate that transferring dark-grown discs to white light caused the D chloroplasts to separate in less than an hour. In these discs the rate of separation of dumbbell-shaped chloroplasts was faster than their rate of formation.

The rate of increase in chloroplast numbers in cultured discs correlated with the time course of cell enlargement. In contrast, mean chloroplast size changed only slightly during the 4 d period of culture in light, but decreased in the dark (Fig. 1f). However the total area of chloroplasts in cells increased 12-fold during the 4 d of culture in continuous white light due to chloroplast multiplication (Fig. 1g). In darkness, mean chloroplast size decreased during culture due to continued chloroplast divisions. The total area of chloroplasts in cells cultured in darkness did not significantly alter. When discs were transferred from darkness to light, both chloroplast size and the total area of chloroplasts per cell increased (Fig. 1, f and g).

Chloroplast DNA Level

Chloroplast DNA amounts measured by microspectrofluorometry following DAPI staining are shown in Figure 1, h and i. Chloroplast DNA amount per cell increased 10-fold over the 4 d of culture in continuous white light. However, DNA amount per chloroplast gradually decreased during this period due to the rate of chloroplast division being higher than the rate of chloroplast DNA synthesis. Comparisons of chloroplast area (Fig. 1f) and DNA level (Fig. 1h) suggest that there is no correlation between these two parameters. In leaf discs cultured in darkness, there was a slight increase in total chloroplast DNA amount per cell. This was accompanied by

Table I. *Relative Frequency of Different Chloroplast Configurations, Generation Time, and Duration of the Length in Hours of the Individual Stages of Chloroplast Division Cycle in Spinach Leaf Discs Cultured under Continuous White Light or Darkness*

Generation time was calculated from the data shown in Figure 1e. Duration of the division stages was calculated from the relative frequency (%) of the configurations and the generation time. Values are the means from three experiments.

Culture Condition	Generation Time	Chloroplast Configuration			
		S type	O type	D type	Dn type
	h				
A. Continuous white light (1 d)	19.4	13.4 h (69.3 ± 1.8%)	2.8 h (14.4 ± 2.2%)	3.1 h (16.2 ± 2.8%)	0.3 h (1.8 ± 0.8%)
B. Continuous dark (3 d)	51.5	22.8 h (44.2 ± 5.4%)	8.4 h (16.4 ± 3.3%)	20.2 h (39.3 ± 3.5%)	1.9 h (3.6 ± 0.6%)
Ratio of (A) to (B)		1.70	3.00	6.52	6.33

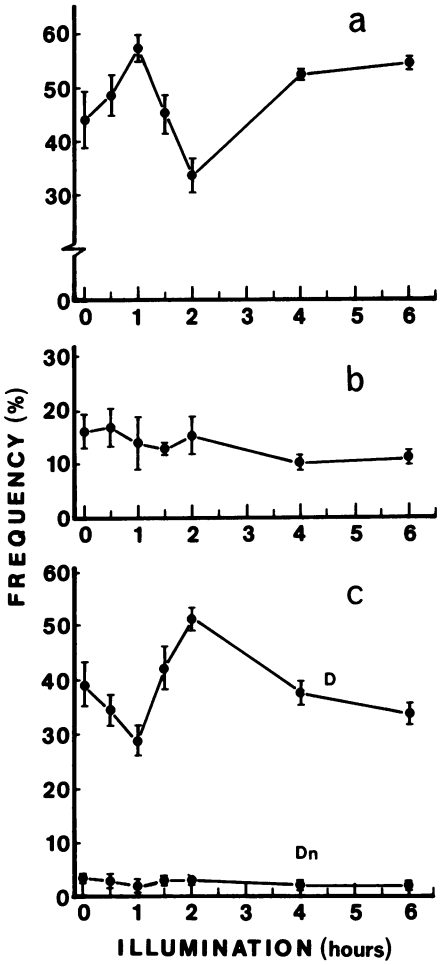

Figure 3. Changes in relative proportion of (a) S-type, (b) O-type, and (c) D-type chloroplasts and deeply constricted chloroplasts with a narrow neck (Dn type) when the discs cultured for 3 d in the dark were transferred to white light. Values are means of three experiments. Vertical bars are standard errors.

small increases in chloroplast number per cell as DNA level per chloroplast remained virtually constant.

When discs were transferred from darkness to white light the DNA level of chloroplasts increased markedly. Here the rate of synthesis of chloroplast DNA was higher than the rate of chloroplast division (Fig. 1h). In these discs the cellular level of chloroplast DNA increased approximately threefold within 1 d after transfer (Fig. 1i).

Both the rate of chloroplast division and chloroplast DNA synthesis may be influenced by changes in environmental conditions from intact leaves of whole plants to cultured leaf discs and by culture conditions of the discs. In cultured leaf discs both processes do not always keep pace with each other as observed in intact leaves (9).

Figure 4, a to c, shows the distribution of DNA amount in S-, O-, and D-type chloroplasts, respectively, in discs cultured for 2 d under continuous white light. The amount of DNA per chloroplast rose as chloroplast division proceeded from S to O, then to the D stage. From these patterns of DNA distribution no evidence could be obtained for discrete periods of chloroplast DNA synthesis. It is likely that chloroplast DNA is continuously synthesized throughout the division cycle.

The pattern of increase in chloroplast DNA per cell was similar to those for cell enlargement and for chloroplast number per cell (Fig. 1, c, d, and i).

DISCUSSION

We describe here the nature of the chloroplast division cycle in cultured spinach leaf discs. In this study we used a gently rotating liquid medium for the culture of leaf discs taken from the distal end of 2-cm leaves. With this culture system the fresh weight of leaf discs increased at a more rapid rate than in discs cultured on solid nutrient agar used previously by Possingham (11) and Tymms *et al.* (17). The faster growth rates are thought to be due to the better penetration of nutrients. Gentle rotation was an essential part of this culture system and maintained the discs at the surface of the liquid medium. Disc growth ceased when they became submerged.

Cells in discs taken from the distal end of 2-mm leaves expanded over 10-fold in culture and the chloroplasts of these cells divided without concommitant cell division occurring in the disc. The absence of cell division made it possible to use the disc culture system for the determination of chloroplast generation time.

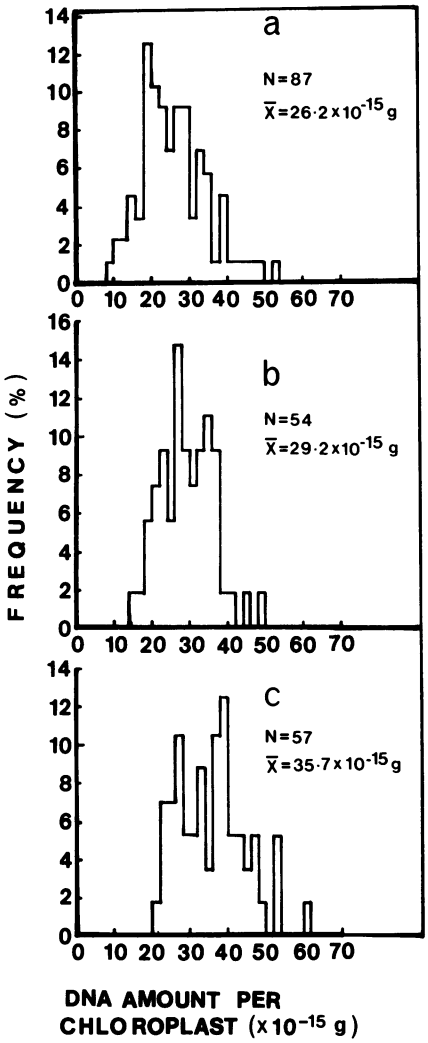

Figure 4. Distribution of DNA in chloroplasts in different configurations in discs cultured for 2 d under continuous white light. a, S-type; b, O-type; c, D-type chloroplasts.

The shortest generation time for chloroplasts that was obtained in this study was 19.4 hours under continuous white light (60 μmol $m^{-2}s^{-1}$ PAR 400–700 nm) during the first day of culture (Table I). However, the generation time of chloroplasts was markedly influenced by the environmental conditions and by the age of the cultures. In darkness chloroplast division was slowed down as a generation time of 51.5 h was recorded during the initial days of culture. This is in marked contrast to the situation in the primary leaves of monocotyledons such as *Avena sativa* (6) where plastid division rates were similar in either light or darkness. Although proplastids and etioplasts in both roots and shoot apices of dicotyledons can divide in the dark (3) in cultured leaf discs light markedly affects the rate of plastid division (11).

In spinach leaf discs light does not uniformly shorten the duration of the individual stages of the chloroplast division cycle. Furthermore when discs are transferred from darkness to light the separation of dumbbell-shaped chloroplasts takes place more rapidly than the formation of plastids with this configuration (Fig. 3, a–c). No satisfactory explanation is available for the stimulating effect of light on chloroplast division in spinach, except that light tends to promote cell enlargement and chloroplast numbers per cell and cell volumes are known to be closely correlated (13). It is possible that photosynthetically produced high energy compounds such as ATP, glyceraldehyde phosphates, etc., may function as a control mechanism for the division of chloroplasts in spinach leaves. In *Avena* seedlings where there is a similar rate of plastid division both in darkness or in light, it has been suggested that reserve starch in the endosperm may provide the high energy compounds for plastid division (6). In spinach it is possible that high energy compounds may be required for the actual separation of dumbbell-shaped chloroplasts rather than for the formation of chloroplasts with this configuration.

In spinach leaves, chloroplast DNA synthesis was enhanced by transfer of discs from dark to light, but as in the case of chloroplast division light was not essential for chloroplast DNA synthesis. Tymms *et al.* (17) measured chloroplast DNA level per cell in discs from the base of 2-cm spinach leaves using Cot curve analysis. They reported a 7.2-fold increase in chloroplast DNA level per cell over 6 d of culture on solid nutrient agar in white light. In the present study the cellular level of chloroplast DNA in discs from the distal end of 2-cm leaves increased 10-fold from 1.1×10^{-12} g to 11×10^{-12} g over 4 d of culture in continuous white light in gently rotating liquid media (Fig. 1i). Assuming a plastome size of 1.6×10^{-16} g, plastome copy number was calculated to be the high value of 69,000 copies per cell at the 4th d of the culture.

In the cells of expanding spinach leaves of whole plants, the maximum value of chloroplast DNA per cell was recorded in the distal region of 5 cm leaves and was approximately 2×10^{-12} g (9). This value is lower than the level of chloroplast DNA per cell in cultured discs. It is suggested that these variations in chloroplast DNA may be due to differences in the levels of nuclear DNA in the cells of whole plants and cultured discs as the cellular levels of nuclear and chloroplast DNA tend to be correlated (17).

Microspectrofluorometry was used to measure chloroplast DNA amount in individual chloroplasts at the different stages of the division cycle. The mean chloroplast DNA content reached higher values as the cells enlarged and chloroplast division proceeded. This result is consistent with the conclusion of Lawrence and Possingham (9) for intact spinach leaves that there is no specific S phase of chloroplast DNA synthesis. The DNA content of individual chloroplasts does not double immediately prior to chloroplast division but rather chloroplast DNA synthesis occurs throughout the chloroplast division cycle. In the light-grown discs of this study the average DNA amount per chloroplast actually decreased during the 4 d of culture since the rate of chloroplast DNA synthesis was lower than the rate of the chloroplast division (Fig. 1h). There is an equal partitioning of chloroplast nucleoids to daughter chloroplast during division probably resulting from chloroplast DNA being attached to the internal plastid membranes (16). Evidence was obtained which indicates that final separation of nucleoids occurs as late as the Dn phase of chloroplast division (Fig. 2h). There was no indication that the

separation of chloroplast DNA to daughter chloroplasts precedes plastid division.

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LITERATURE CITED

1. **Bisalputra T, Bisalputra AA** (1970) The ultrastructure of a brown alga *Sphacelaria* sp. III. The replication and segregation of chloroplast genophore. J Ultrastruct Res **32:** 417–429
2. **Boffey SA, Ellis JR, Sellden G, Leech RM** (1979) Chloroplast division and DNA synthesis in light-grown wheat leaves. Plant Physiol **64:** 502–505
3. **Chaly N, Possingham JV, Thomson WW** (1980) Chloroplast division in spinach leaves examined by scanning electron microscopy and freeze-etching. J Cell Sci **46:** 87–96
4. **Green PB** (1964) Cinematic observations on the growth and division of chloroplasts in *Nitella*. Am J Bot **51:** 334–342
5. **Hashimoto H** (1986) Double ring structure around the constricting neck of dividing plastids of *Avena sativa*. Protoplasma **135:** 166–172
6. **Hashimoto H, Possingham JV** (1989) DNA levels in dividing and developing plastids in expanding leaves of *Avena sativa*. J Exp Bot (in press)
7. **Honda S, Hoglandarom T, Laties GG** (1966) A new isolation medium for plant organelles. J Exp Bot **17:** 460–472
8. **Lawrence ME, Possingham JV** (1986) Direct measurement of femtogram amounts of DNA in cells and chloroplasts by quantitative microspectrofluorometry. J Histochem Cytochem **34:** 761–768
9. **Lawrence ME, Possingham JV** (1986) Microspectrofluorometric measurement of chloroplast DNA in dividing and expanding leaf cells of *Spinacia oleracea*. Plant Physiol **81:** 708–710
10. **Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–479
11. **Possingham JV** (1973) Effect of light quality on chloroplast replication in spinach. J Exp Bot **24:** 1247–1260
12. **Possingham JV** (1976) Controls to chloroplast division in higher plants. J Microsc Biol Cell **25:** 283–288
13. **Possingham JV, Lawrence ME** (1983) Controls to plastid division. Int Rev Cytol **84:** 1–56
14. **Possingham JV, Saurer W** (1969) Changes in chloroplast number per cell during leaf development in spinach. Planta **86:** 186–194
15. **Possingham JV, Smith JW** (1972) Factors affecting chloroplast replication in spinach. J Exp Bot **23:** 1050–1059
16. **Rose RJ, Cran DG, Possingham JV** (1975) Changes in DNA synthesis during cell growth and chloroplast replication in greening spinach leaf disks. J Cell Sci **17:** 27–41
17. **Tymms MJ, Scott NS, Possingham JV** (1982) Chloroplast and nuclear DNA content of cultured spinach leaf discs. J Exp Bot **33:** 831–837