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Chloroplast Growth and Replication in Germinating Spinach Cotyledons following Massive γ-Irradiation of the Seed

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ABSTRACT

Spinach seeds (*Spinacia oleracea L.*) given massive doses of γ-irradiation (500 krad) germinate and form a seedling with two green cotyledons and a radicle, but develop no further. Irradiated cotyledons show no increase in cell number or total DNA over a 7-day period in the light, while in control cotyledons there is a small increase in cell number and large increases in total DNA and chloroplast number. The chloroplasts of irradiated cotyledons are delayed in their division, become greatly enlarged and contain large amounts of starch. The whole population of chloroplasts subsequently undergoes a wave of division. The daughter chloroplasts show normal thylakoid development, but have some abnormal structural features caused by the radiation stress. Information on the effect of X-irradiation, ultraviolet irradiation, and 5-fluorodeoxyuridine on chloroplast replication and on chloroplast and nuclear DNA synthesis was obtained from cultured spinach leaf discs. It appears that chloroplast replication is more resistant to ionizing radiation than cell division and can proceed in the absence of nuclear DNA synthesis and greatly reduced chloroplast DNA synthesis.

When immature spinach leaf discs are cultured in the light, there is normally a close relationship between chloroplast DNA synthesis and chloroplast replication during cell growth (21, 23, 24). The two processes are not irrevocably coupled. Rates of chloroplast DNA synthesis in dark-grown spinach discs are comparable to those in light-grown discs, though the division rate is greatly reduced (24). Boasson and Laetsch (1) found that light-stimulated chloroplast replication could occur in the presence of the DNA inhibitor, FdUrd,[1] in tobacco leaf discs. They suggested that chloroplasts contained multiple copies of chloroplast DNA and could divide a number of times without additional DNA synthesis. It is now established that there are many DNA copies in chloroplasts (16). More recently, it has been shown that in *Euglena* plastids can replicate using a reduced number of genomes (17).

In higher plants, relationships between chloroplast replication and cell growth (2, 14, 22), and chloroplast replication and nuclear DNA have been found (6, 24). Recently gibberellic acid has been shown to stimulate cell elongation, chloroplast numbers, and the rate of synthesis of both mainband DNA and organelle-rich satellite DNA in cucumber hypocotyls (15).

It was the purpose of this investigation to examine the regulatory relationships between cell growth, chloroplast DNA synthesis, nuclear DNA synthesis and chloroplast replication and development. In the first instance, we used cultured spinach discs, but this report is primarily concerned with the growth and development of chloroplasts in spinach cotyledons that have developed from heavily γ-irradiated seed. These are "γ-plantlets" similar to those studied by Haber (11).

MATERIALS AND METHODS

Growth of Leaf Discs and Cotyledons. Methods used in growing spinach plants (*Spinacia oleracea L*), for culturing leaf discs on sterile nutrient agar where growth is largely by cell expansion, have been previously described (22).

Cotyledons were obtained from normal or γ-irradiated spinach seed. The seeds were germinated in vermiculite in the dark at 16 C for 7 days. The seedlings were then moved to growth cabinets where they were kept in vermiculite for 4 days and then transferred to nutrient solutions for the final 5 days. This is essentially the same procedure routinely used for the growth of spinach plants for leaf disc culture. Growth cabinet conditions refer to a 14-hr day with a light intensity of 6 mw cm^{-2} sec^{-1}, a day temperature of 25 C and a night temperature of 22 C. Growth of discs in continuous darkness was at 25 C.

Radiation Treatments. γ-Irradiation of seeds was carried out at the Australian Atomic Energy Commission, Lucas Heights, N.S.W., at a rate of approximately 380 krad/hr, to give a final dose of 500 krad. The seeds used were not more than 3 years old.

X-irradiation of spinach leaf discs was carried out using a Philips PW1009 x-ray generator with a cobalt target. The machine was adjusted to 40 kv and 15 mamp, and the dose rate delivered to the discs was 19 krad/hr.

UV-irradiation (254 nm) was delivered to the leaf discs at a fluence rate of 2 joules m^{-2} sec^{-1}, using a germicidal lamp.

Chloroplast Number, Chloroplast Area, Cell Area, and Cell Number Measurements. Chloroplast number per cell, chloroplast area, and cell area were measured in squashes of glutaraldehyde-fixed cells using light microscopy (22). Cell numbers were measured using the Brown and Rickless (4) method.

^{3}H-Thymidine Incorporation. Leaf discs were incubated in 50 μCi (6-^{3}H) thymidine (27 Ci/mM, Radiochemical Centre, Amersham, U.K.), added in 1 ml under sterile conditions to the 20 ml of nutrient agar medium in 9-cm Petri dishes (containing six discs). Half of the discs from each of two plates were used to obtain duplicate values of incorporation into trichloroacetic acid-insoluble material, as previously described (24). The remaining discs from the two plates were used to measure incorporation into chloroplasts using grain counts from light microscope autoradiography of EDTA-separated cells (24).

DNA Determination. DNA determinations were carried out in duplicate using the diphenylamine reaction (5), as we have previously described (24).

Electron Microscopy. Tissue was fixed in 6.25% glutaralde-

[1] Abbreviation: FdUrd: 5-fluorodeoxyuridine.

hyde, postfixed in osmium and embedded in araldite (7). Sections were mounted on uncoated copper grids and stained in uranyl acetate in 50% ethyl alcohol, followed by lead citrate.

RESULTS

The effect of X-irradiation on cell growth and chloroplast replication in cultured spinach leaf discs is shown in Figure 1. Both processes are inhibited by the radiation treatment to a similar extent. From studies we have done at 50 krad (unpublished data), the effects of X-irradiation and γ-irradiation are essentially the same. There are initially about 12 chloroplasts per cell when the radiation treatment is commenced, so that at doses as high as 300 krad, there are at least two cycles of chloroplast replication.

X-irradiation inhibits ^{3}H-thymidine incorporation into total cellular DNA by 88% at 300 krad (Fig. 2A), but in this system, total incorporation appears to be largely a reflection of nuclear DNA synthesis (24). Incorporation of ^{3}H-thymidine into chloroplast DNA is also inhibited by the radiation treatment (Fig. 2B). It is recognized that these incorporation measurements may reflect repair synthesis rather than the formation of new molecules. In the control situation the incorporation probably does not represent repair as total DNA increases in the discs (24) and the kinetics of long term labeling of chloroplasts (21) are more consistent with replication. It appears from Figure 2, A and B, that the incorporation into chloroplast DNA is less sensitive to radiation than nuclear DNA synthesis. If the reduction in chloroplast numbers is taken into account, the total incorporation into chloroplast DNA per cell shows the same inhibition as the total incorporation.

We obtained essentially similar results to X-irradiation with the DNA synthesis inhibitors UV-irradiation and FdUrd (Table I). In all these cases, effects on cell growth and chloroplast replication could not be clearly separated. Although DNA synthesis is not an absolute requirement for cell elongation (13), inhibitors of DNA synthesis can reduce cell size in certain cases where DNA endoreduplication is closely associated with cell growth (18).

As an alternative way of looking at the relationships between cell growth, chloroplast replication, and DNA synthesis, cotyledons of plants grown from seeds given 500 krad of γ-irradiation were used. In the dormant seed, the plastids are undifferentiated and during germination form amyloplasts or etioplasts before forming chloroplasts in the green cotyledon (7).

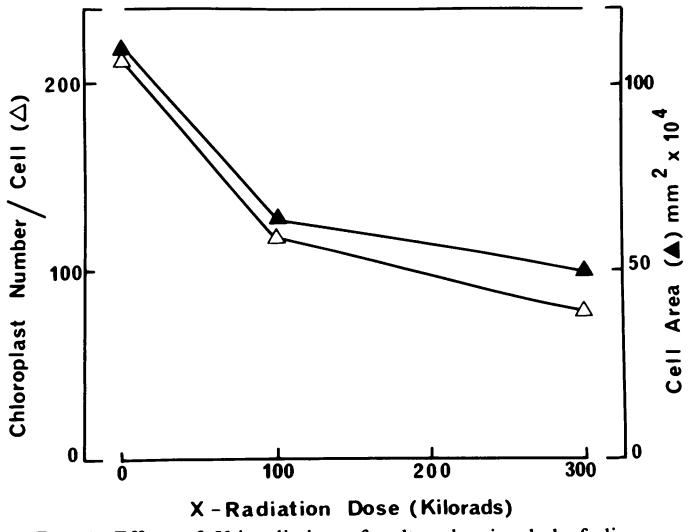

FIG. 1. Effect of X-irradiation of cultured spinach leaf discs on chloroplast number per cell and cell size. Spinach leaf discs were excised from the leaf and subjected to the different radiation doses. The discs were then cultured on fresh nutrient agar for 5 days in darkness, then 7 days in the light, before being harvested for chloroplast number and cell size determination.

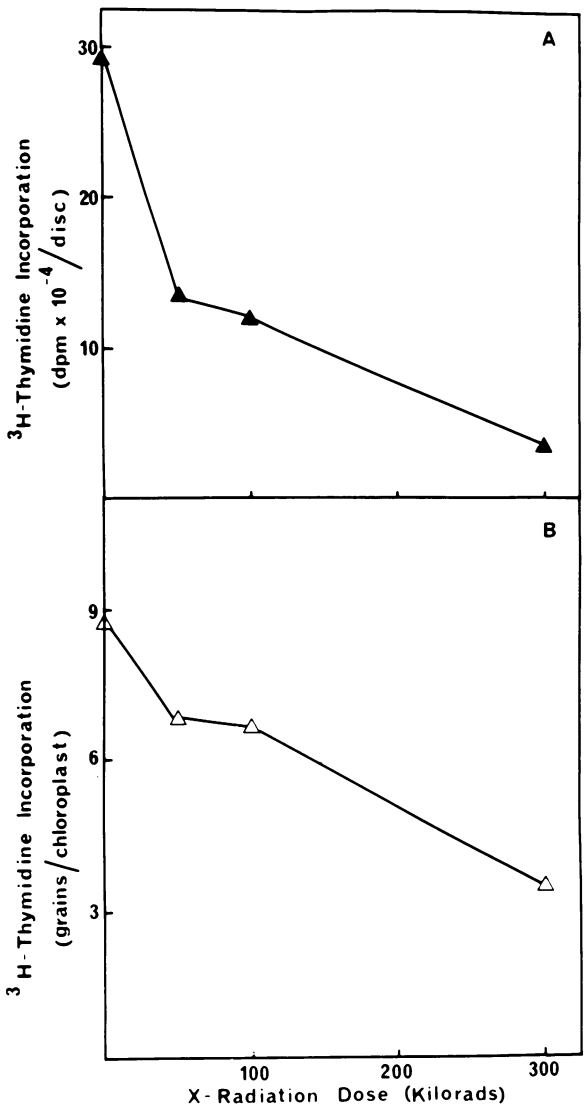

FIG. 2. Effect of X-irradiation of cultured spinach leaf discs on ^{3}H-thymidine incorporation into trichloroacetic acid insoluble material (A) and on ^{3}H-thymidine incorporation into chloroplasts, using light microscope autoradiography (B). The discs were treated as in Fig. 1, and isotope incorporation measured for 24 hr, 1 day after transfer to the light.

Table I. *Effect of UV-Radiation and FdUrd on Leaf Discs*

After excision, leaf discs were UV-irradiated or FdUrd applied and incubated in the dark for 7 days, then in the light for 7 days before measurements were made. Each value was obtained from six replicate discs.

Treatment	Cell Area	Chloroplast per Cell
	$mm^2 \times 10^4$	*No.*
UV Control	93	251
UV 1,000 joules m^{-2}	88	154
UV 2,000 joules m^{-2}	62	76
FdUrd Control	127	236
FdUrd 0.001 μg/ml	59	119
FdUrd 0.01 μg/ml	18	32

The control and irradiated seedlings are shown in Figure 3. It should be noted that the apical meristems develop in the controls, which will grow into mature plants. The irradiated seedlings do not develop meristems.

Over the period 2 to 9 days after transfer to the light, control cotyledons show an increase in cell size, from 35 to 92 $mm^2 \times 10^{-4}$, and a small, but reproducible, increase in cell number, 20 to 25 $\times 10^4$. The irradiated cotyledons show a much smaller increase in cell size, 25 to 40 $mm^2 \times 10^{-4}$, and a small change in cell number, 20 to 18 $\times 10^4$. Over the same period, there is a doubling of the amount of DNA in control cotyledons, from 2.33 to 4.67 μg per cotyledon, while there is virtually no increase in DNA in the irradiated cotyledons (1.75 compared with 1.98).

Under the growth situation described in Figure 3, we find the changes in chloroplast number and chloroplast size shown in Figure 4. Chloroplast numbers increase in the control but the mean chloroplast size remains constant. The chloroplast size and number changes in the irradiated cotyledons are quite different. The chloroplasts from the irradiated cotyledons become extremely large (Figs. 4B and 5), and then return to a size similar to the controls (Fig. 6), following a wave of partially synchronous division.

We examined the chloroplasts of the cotyledons with the electron microscope (Figs. 7–13). The large chloroplasts of irradiated cotyledons (Fig. 5) are packed with starch (Fig. 7). Though grana stacks are present in the large chloroplasts of the irradiated cotyledons, prolamellar bodies are still apparent 3 days after transfer to the light (Fig. 7). Little starch and no prolamellar bodies were seen in the control sections (Fig. 8). The large chloroplasts in the γ-irradiated cotyledons divide while in this starch-packed condition (Figs. 4, 9, and 10). Some days after this division cycle, these chloroplasts (Figs. 11 and 12) resume a structure more similar to the controls (Fig. 13). Some differences remain as their stroma is dense and osmiophilic bodies are often present in large numbers (Fig. 11), while peripheral vesicles are commonly found (Fig. 12).

DISCUSSION

The observations we have made of the effects of irradiation on cultured leaf discs and of the cotyledons of heavily irradiated seeds are of relevance in understanding the complex interrelationships between chloroplast replication and development,

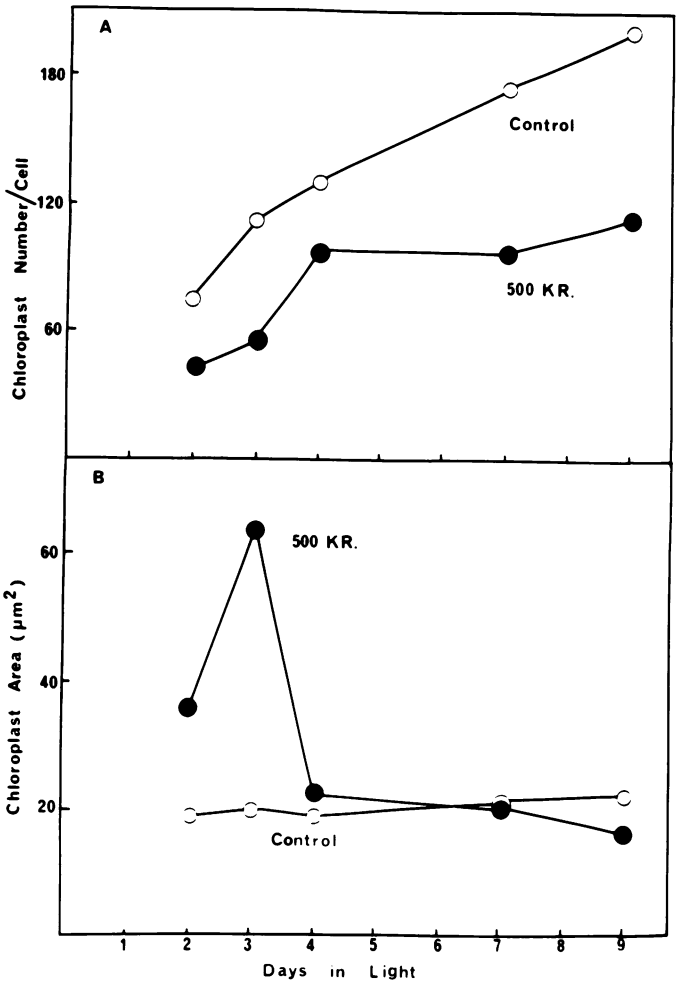

FIG. 4. Time course of chloroplast number per cell (A) and chloroplast size (B) in control and "γ-plantlet" cotyledons. The seeds were germinated in the dark for 1 week before being transferred to the light.

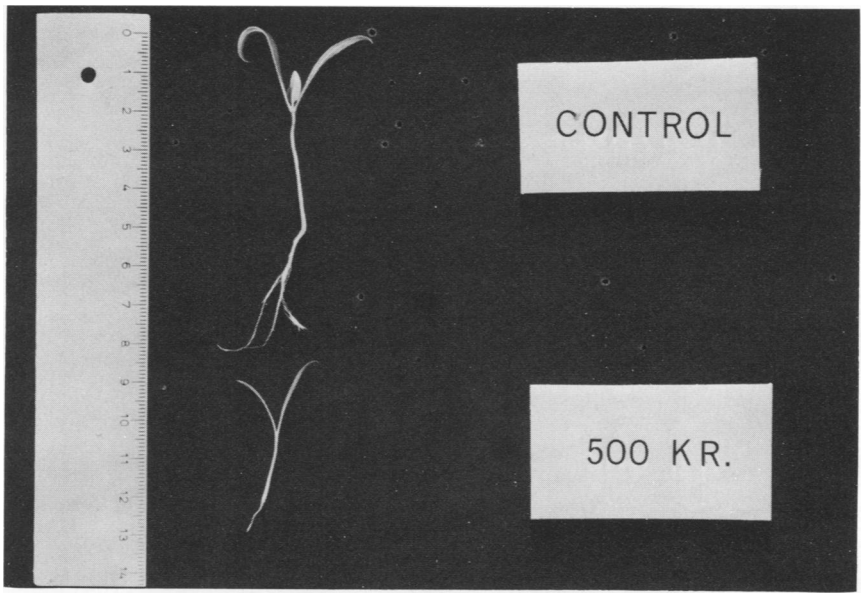

FIG. 3. Spinach seedlings that have developed from unirradiated and massively irradiated seed (500 krad). The seeds were germinated in the dark for 1 week and photographed 6 days after transfer to the light.

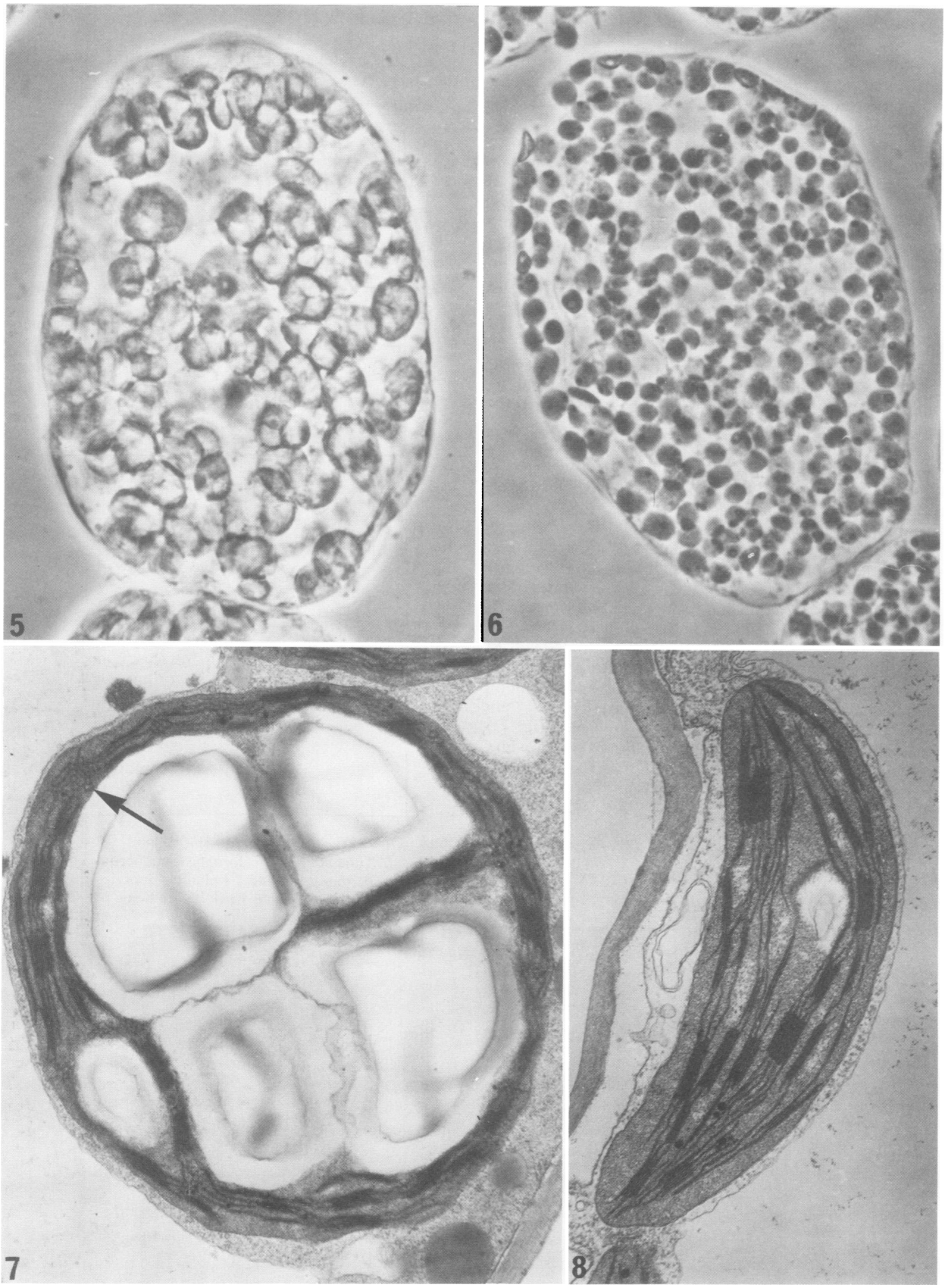

FIG. 5. Light micrograph of cell from "γ-plantlet" spinach cotyledon, 3 days after transfer to the light. Note the greatly enlarged chloroplasts. × 900.

FIG. 6. Light micrograph of cell from normal spinach cotyledon, 3 days after transfer to the light. × 900.

FIG. 7. Electron micrograph of chloroplast from "γ-plantlet" spinach cotyledon, 3 days after transfer to the light. Note the large amount of starch and remnants of prolamellar bodies (→). × 16,000.

FIG. 8. Electron micrograph of chloroplast from normal spinach cotyledon, 3 days after transfer to the light. × 19,000.

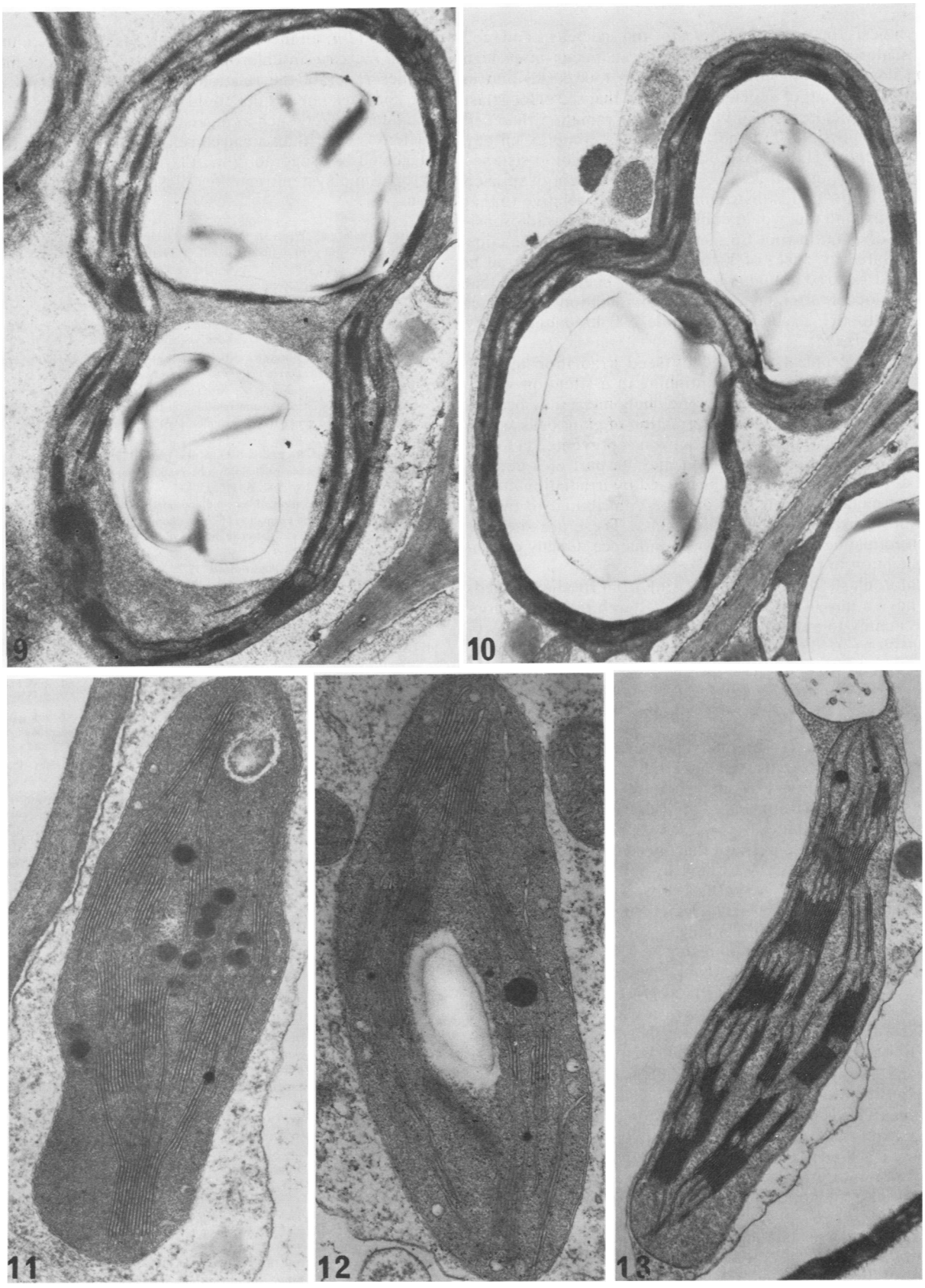

FIG. 9. Partially constricted chloroplast from "γ-plantlet" spinach cotyledon, 3 days after transfer to the light. × 27,000.

FIG. 10. Similar to Fig. 9, but more advanced constriction. × 12,000.

FIG. 11. Chloroplast from "γ-plantlet" cotyledon, 9 days after transfer to the light. Thylakoids are normally stacked, a number of osmiophilic bodies are present. × 27,000.

FIG. 12. As for Fig. 11, with peripheral vesicles more common. × 33,000.

FIG. 13. Chloroplast from normal cotyledon, 9 days after transfer to the light. × 20,000.

DNA synthesis in both chloroplasts and nucleus, and cell growth. Chloroplast replication clearly can occur after high radiation doses in both cultured leaf discs (300 krad) and in cotyledons (500 krad) of spinach, indicating that the chloroplast division process is less sensitive to ionizing radiation than cell division. It is possible that the absence of a complex mitotic apparatus in chloroplasts may contribute to the radioresistance of chloroplast replication. As well, the DNA content of spinach chloroplasts is low and is distributed differently relative to that of the nucleus, and this would confer a difference in radiosensitivity. Cell division in root tip meristems is known to be completely inhibited by doses of X-radiation as low as 3.2 krad in onion (8) and 1.6 krad in *Vicia faba* (9). It has been reported that mitosis can occur after higher doses of radiation (120 krad) when given to dry maize seeds, while 500 krad completely prevents mitosis (25).

The giant chloroplasts induced by seed irradiation may be caused by chloroplast growth continuing in a situation where chloroplast replication is delayed. The phenomena may bear an analogy to the radiation-induced formation of giant cells which usually occurs in plants when cell division is prevented (11, 27). The delay of chloroplast replication may be part of a general delay of developmental sequences caused by irradiation affecting cellular metabolism. It may also be contributed to by the absence of meristems in irradiated plants. These are sites for cytokinin formation which is known to influence directly chloroplast replication in tobacco (2).

The chloroplasts of cotyledons arising from irradiated seeds initially have a massive accumulation of starch relative to controls due mainly to a delay in the normal amyloplast to chloroplast transition (7). Subsequently, these chloroplasts have structural features developed in response to radiation such as dense stroma, osmiophilic bodies, and peripheral vesicles but normal thylakoids; high x-ray doses reduce thylakoid formation in etiolated barley leaves (26).

Our experiments establish that chloroplast replication can occur in the spinach leaf discs at 300 krad, although chloroplast DNA synthesis is greatly reduced. At 500 krad chloroplast DNA synthesis would be expected to be low in the cotyledons if radiosensitivity was similar to the discs. Approximately 50% of discs survive 500 krad, and those that survive show at least one chloroplast division cycle. We think it unlikely that the small amount of chloroplast DNA synthesis observed after these high levels of radiation represents intact molecules. Little information is available on the repair of single strand or double stranded breaks after ionizing radiation in plants (19). It seems that some spinach chloroplast division like that in tobacco (1) and *Euglena* (17) can occur in the absence of chloroplast DNA replication. The 20 to 30 chloroplast DNA copies present in higher plant chloroplasts (16) confer on the chloroplast the flexibility to divide without synthesizing chloroplast DNA.

Nuclear DNA synthesis, though usually associated with chloroplast division in spinach (24), can be separated from this relationship. This is shown in the cotyledon experiments where no increase in total DNA occurs. The ratio between the level of chloroplast DNA per cell and the nuclear DNA may possibly be the factor of importance. It is of interest here that in yeast cells mitochondrial DNA appears to be a constant proportion of total cellular DNA (10).

In both the leaf discs and cotyledons, the final chloroplast number was closely related to the final cell size. As indicated by the work of Boasson *et al.* (2), Honda *et al.* (12), and Possingham (20), an increase in cell size is probably a prerequisite for an increase in chloroplast numbers. The present radiation studies reiterate the relationship between the two processes. We consider our results could arise because of some common regulatory effect, such as a generalized effect of radiation on the nucleus, rather than a causal relationship between the two. As suggested by Bogorad *et al.* (3), it is probable that a nuclear-cytoplasmic system regulates plastid development and replication.

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