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Short Communication

Microspectrofluorometric Measurement of Chloroplast DNA in Dividing and Expanding Leaf Cells of *Spinacia oleracea*

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ABSTRACT

Absolute DNA amounts of individual chloroplasts from mesophyll and epidermal cells of developing spinach leaves were measured by microspectrofluorometry using the DNA-specific stain, 4,6-diamidino-2-phenyl indole, and the bacterium, *Pediococcus damnosus*, as an internal standard. Values obtained by this method showed that DNA amounts of individual chloroplasts from mesophyll cells fell within a normal distribution curve, although mean DNA amounts changed during leaf development and also differed from the levels in epidermal chloroplasts. There was no evidence in the data of plastids containing either the high or low levels of DNA which would be indicative of discontinuous polyploidy of plastids, or of division occurring in only a small subpopulation of chloroplasts. By contrast, the distribution of nuclear DNA amounts in the same leaf tissues in which cell division was known to be occurring showed a clear bimodal distribution. We consider that the distribution of chloroplast DNA in the plastid population shows that there is no S-phase of chloroplast DNA synthesis, all chloroplasts in the population in young leaf cells synthesize DNA, and all chloroplasts divide.

There are a number of papers in the literature reporting changes in chloroplast DNA amounts determined by methods involving DNA extraction and cell and chloroplast counting (3, 6, 16, 17). Problems with these methods include incomplete DNA extraction, difficulties in determining the ratio of nuclear to chloroplast DNA and inherent errors in averaging cell and chloroplast numbers from tissues with cellular heterogeneity. Significant errors also occur when chloroplast DNA levels are determined on isolated chloroplasts due to nuclear DNA contamination and leakage (2, 5, 15). These methods all necessitate the bulk averaging of millions of cells and/or chloroplasts and are not able to provide data on the differences either within or between the varying cell types of leaves. We recently established that absolute DNA amounts of individual chloroplasts can be measured by microspectrofluorometry using the DNA-specific stain DAPI,[1] and the bacterium, *Pediococcus damnosus*, as an internal standard (8). The DNA levels of individual chloroplasts measured in this way can provide information about the range and distribution of chloroplast DNA amounts within cells of the one type as well as indicating differences between cell types and between cells of different ages.

In this communication we report the results of experiments in which the DNA levels of individual chloroplasts in developing spinach leaves have been measured using microspectrofluorometry. The patterns of chloroplast DNA distribution in tissues with dividing cells was compared with the distribution of nuclear DNA amounts in the same tissue, determined by Feulgen microdensitometry.

MATERIALS AND METHODS

Spinach plants (*Spinacia oleracea* L.) (Hybrid 102, Yates and Co. Pty. Ltd., Australia) were grown in nutrient culture in growth cabinets as previously described (11). The culture of *Pediococcus damnosus* (*Cerevisiae*) ATCC 43013 cells, the preparation of standard bacterial slides, and the preparation and examination of leaf cells by microspectrofluorometry was described earlier (8). Briefly, this involved the simultaneous staining of leaf and bacterial cells on the same slides following fixation of all of the cells in 3:1 ethanol:acetic acid to extract Chl from the chloroplasts. Five positions were sampled along spinach leaves 2 cm long, as well as the distal 2 to 5 mm of leaves 2, 3, 4, 5, and 10 cm long. Four leaves 2 cm long, were collected and tissue strips 1 mm wide cut from the following regions: 0 to 1, 4 to 5, 9 to 10, 14 to 15, and 18 to 20 mm from the leaf base. The strips were processed independently for microspectrofluorometry, and all the strips from at least one leaf then measured during a single fluorometer sitting. Measurements of chloroplasts, backgrounds and *P. damnosus* cells were alternated throughout the measuring process until a total of 30 chloroplasts from each position on each of four leaves had been measured, giving a total of 120 chloroplasts per sampling position. Standard deviations were calculated from the pooled values. Samples from the distal 3 mm of leaves 2, 3, 4, 5, and 10 cm long were treated in a similar way. Epidermal cells were easily recognized and their chloroplasts were measured in the same leaf samples. A duplicate set of leaf samples was examined for chloroplast numbers per cell by the method of Possingham and Smith (12). Samples from the region 0 to 1 mm from the leaf base had earlier been collected from an additional six leaves 2 cm long, and their nuclear DNA amounts measured by the well established technique of Feulgen microdensitometry as described by Lawrence (7). Nuclei from the first 15 mesophyll cells encountered along a transect were measured on each side, as well as 15 nuclei of *Pisum sativum* root tip cells at prophase. The value of 19.46 pg DNA for *P. sativum* prophase cells was used to calibrate the spinach mesophyll cell nuclei values (1).

RESULTS AND DISCUSSION

The light microscope morphology of dividing mesophyll cells in the base of 2 cm spinach leaves is shown in Figure 1. The ultrastructure of the chloroplasts of these cells was described earlier and compared with those of mature leaves and with plastids of the stem apex (10). Mitotic figures were found to be associated with cells containing chloroplast numbers that ranged between 10 and 20 per cell. It was not possible to conclude that chloroplast numbers double before leaf cells can divide. Rather,

[1] Abbreviation: DAPI, 4'6-diamidino-2-phenyl indole.

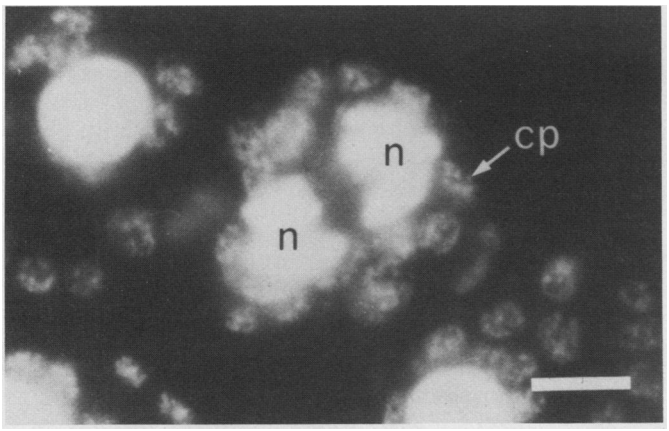

FIG. 1. Dividing cell from the base of a spinach leaf 2 cm long stained with DAPI. Chloroplasts (cp) are evident either side of the telophase nuclei (n). Bar = 10 μm.

it appears that chloroplasts of young leaves divide on a cycle time of between 24 to 30 h and their division is not tightly coupled to cell division (9, 11). Despite this apparent lack of coupling, close control over both events is exerted by the nucleus. In the case of cell division control is exercised via nuclear DNA synthesis and in the case of chloroplast division via the availability of chloroplast molecules that are nuclear coded.

The distribution of 120 measurements of chloroplast DNA amount measured in young dividing mesophyll cells from the base of a leaf 2 cm long is shown in Figure 2A. Values ranged from 2.9 to 17.6 fg with a mean of 8.5 fg and a standard deviation of 2.5 fg. The distribution of nuclear DNA amounts from the same tissue, measured by Feulgen microdensitometry, is shown in pg in Figure 3. In the region of cell division the nuclear DNA amounts fall into two populations with means of 2.65 and 5.30 pg. This distribution corresponds to nuclei having either a 2C or a 4C DNA amount; the latter being the nuclei that have completed their DNA synthesis phase prior to division. This is the pattern commonly observed in meristems, and results from nuclei having a short DNA synthesis time (19). The distribution of chloroplast DNA amounts in the same cells was not comparable and, instead, showed a normal distribution of values (Fig. 2A). These results show that chloroplasts do not have an identifiable short phase of DNA synthesis and suggest instead that DNA synthesis occurs continuously throughout the chloroplast division cycle. The results do not support the suggestion that only a subpopulation of chloroplasts within young cells divide (4). However, they support earlier proposals made by Rose *et al.* (13), based on pulse/chase experiments with tritiated thymidine, that all chloroplasts in a cell divide, and that chloroplast DNA segregates to all daughter chloroplasts during chloroplast replication.

Normal distributions of chloroplast DNA amounts were obtained for all of the leaf samples examined here (Fig. 2, A–E). Samples from the base and tip of leaves 2 cm long were examined using a QQ plot and no deviation from normality was found. The means and standard deviations of all leaf samples data are shown graphically in Figure 4, along with chloroplast numbers and cellular levels of chloroplast DNA calculated by multiplying chloroplast DNA amounts by the number of chloroplasts per cell. Chloroplast numbers in mesophyll cells from the basal 1 mm of leaves 2 cm long, were difficult to count by light microscopy due to the small chloroplast size, dense cytoplasm, and numerous vacuoles in these cells. A mean of eight chloroplasts per cell was recorded and we believe this number to be reasonably accurate, as no evidence has been found of large, cigar-shaped or irregular anastomosing chloroplasts in partial serial sections

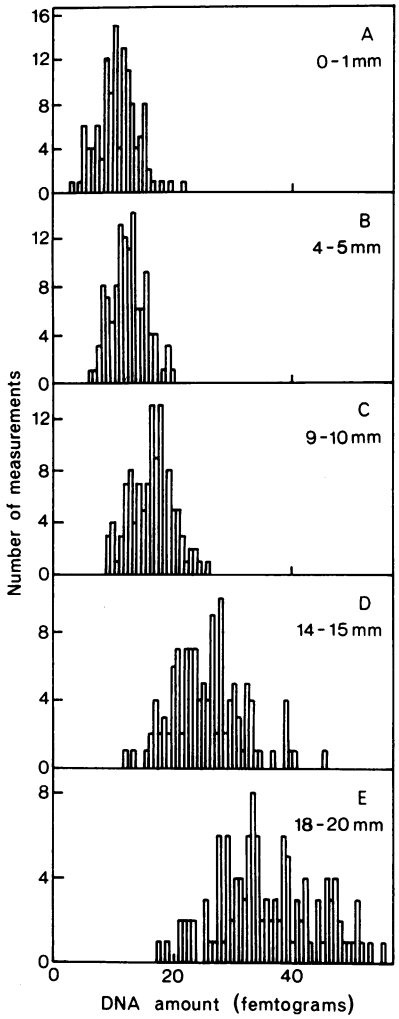

FIG. 2. Distributions of DNA amounts of 120 chloroplasts from mesophyll cells sampled at each of five positions along a spinach leaf 2 cm long. Relative DNA amounts were measured by microspectrofluorometry after DAPI staining, and were converted to absolute values using *P. damnosus* as an internal standard (8). The position of each sample, cut as 1 mm-wide strip, is indicated on the histograms.

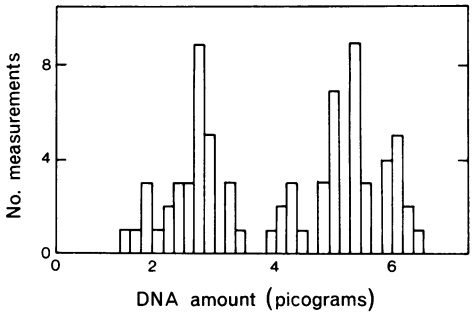

FIG. 3. Distributions of DNA amounts of 90 nuclei from mesophyll cells in the basal 1 mm of a spinach leaf 2 cm long. Relative DNA amounts were measured by Feulgen microdensitometry, and were converted to absolute values using *P. sativum* root tip nuclei as an internal standard (1, 7).

of these cells examined by transmission electron microscopy (ME Lawrence, JV Possingham, unpublished data).

The three phases of chloroplast division and chloroplast DNA synthesis proposed by Scott and Possingham (17) are evident in Figure 4. Our data, based on spectrofluorometric measurements

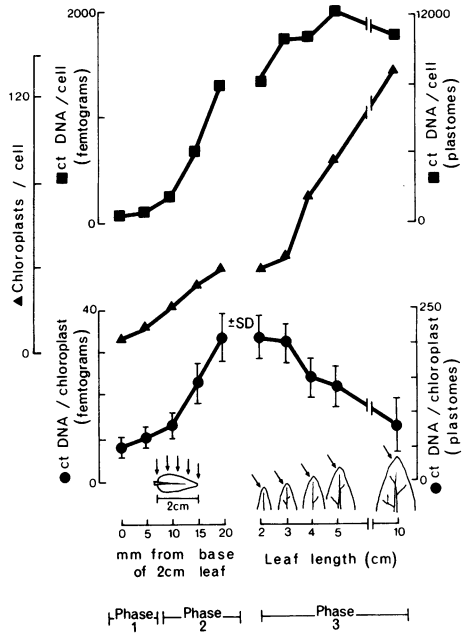

FIG. 4. Changes in DNA amount per chloroplast, chloroplasts per cell, and chloroplast DNA amounts per cell in mesophyll cells during spinach leaf development. DNA amounts per chloroplast are the mean of 120 measurements, and were converted to absolute terms using *P. damnosus* as a biological standard (8). Chloroplast numbers are the mean of 20 measurements. Plastome copy numbers were derived from DNA amounts assuming a plastome size of 1.6×10^{-16} g.

of mesophyll chloroplasts, shows more precisely the leaf regions in which the separate phases occur. Phase 1 corresponds to the basal 5 mm of leaves 2 cm long where growth is primarily by cell division. In this region chloroplast DNA synthesis and chloroplast division keep pace with cell division, so that chloroplast DNA amounts within cells and within chloroplasts remain relatively low at about 8 fg or 50 plastome copies per chloroplast. Chloroplast numbers per cell are maintained within the range of 10 to 15 per cell. The second phase corresponds to the remaining portion of leaves 2 cm long in which some cell division occurs, but growth by cell expansion commences. During this transition, the rate of chloroplast DNA synthesis exceeds the rate of chloroplast division, as there is a large increase in the amount of chloroplast DNA both per chloroplast and per cell. During the third phase there is very little, if any, chloroplast DNA synthesis but chloroplasts continue to divide and DNA amounts per chloroplast fall. This phase is evident in the distal regions of leaves greater than 2 cm in length (Fig. 4). This pattern for the overall trends in DNA per chloroplast and per cell is similar to that reported earlier in studies involving the averaging of whole tissues (16, 17). Not surprisingly, the absolute values we report are different. The DNA content per chloroplast measured here for mesophyll cells was lower in dividing tissue, underwent a greater change along leaves 2 cm long, and did not fall to such low levels in older leaves 10 cm long. Also, chloroplast DNA amounts per mesophyll cell reached 2,000 fg (12,500 plastome copies) which is significantly higher than the value reported earlier of approximately 5,000 plastomes per cell (16, 17). The differences may be due to previous averaging of the wide range of cell types found in samples of whole leaves.

A quite different pattern was found in epidermal cells from the same leaf samples when DNA amounts of individual chloroplasts were measured by microspectrofluorometry. In these cells, the DNA amount per chloroplast remained more or less unchanged, and at a level of about 8 fg or 50 plastome copies throughout leaf development (8; unpublished data). The value is the same as the DNA amount determined here for chloroplasts in the youngest mesophyll cells, and may therefore represent a basic level of chloroplast DNA for spinach leaves. Levels of nuclear endopolyploidy appear similar in both epidermal and mesophyll tissues of spinach leaves, with an equal representation of 2C and 4C states accounting for the majority of cells in both tissues (19; ME Lawrence, JV Possingham, unpublished data). Accordingly, the relative amounts of nuclear and chloroplast DNA per cell show a 25-fold change during leaf development. This large difference results from the combination of a 5-fold difference in the mean DNA content of individual chloroplasts between epidermal and mesophyll tissues and a 5-fold difference in mean chloroplast number per cell in these tissues.

We conclude from our observations of the amounts of DNA in individual spinach chloroplasts that (a) there is no identifiable S-phase of chloroplast DNA synthesis in young, dividing mesophyll cells and that chloroplast DNA is synthesized continuously throughout the chloroplast replication cycle (in contrast, nuclear DNA synthesis is known to occur at defined periods and to result in a bimodal distribution in nuclear DNA levels); (b) there is no evidence of discontinuous increments in DNA amounts per chloroplast, either within mesophyll cells of the same age or during the development of mesophyll cells as the leaves enlarge; (c) epidermal cells contain plastids with levels of DNA close to the minimum values found in mesophyll cells; and (d) within different tissues of spinach leaves there are significant differences in the relative amounts of nuclear and chloroplast DNA. These differences may either contribute to or merely be a consequence of cellular differentiation.

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