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DNA Content of *Beta vulgaris* Chloroplasts during Leaf Cell Expansion

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ABSTRACT

During the growth of beet leaves from 2 to 3 to 25 to 30 centimeters, the leaf cells increase in size, the average number of chloroplasts per cell increases from 11 to 65 and the amount of chloroplast DNA per cell increases from 1100 to 1900 plastome copies. The average number of copies of the plastome per chloroplast decreases from 104 in 2 to 3-centimeter leaves to 29 in 25 to 30-centimeter leaves during a period when the chloroplasts undergo two to three rounds of division and increase diameter from 1.5 to 4.9 micrometers. This result is at variance with previously published studies of beet chloroplasts but agrees with the conclusions reached in more recent studies of pea and spinach and wheat leaf cell expansion.

Using renaturation kinetics to measure the proportion of ctDNA[1] in total cellular DNA, Scott and Possingham (12) and Lamppa and Bendich (10) have shown that the amount of ctDNA per cell does not alter significantly in spinach and pea leaves during growth by cell expansion despite a large increase in the chloroplast number per cell. Similar results were recently obtained in wheat leaves (2) using different methodology. These studies showed that the amount of ctDNA per chloroplast decreases when chloroplasts divide and increase in size during the cell expansion phase of leaf growth.

Earlier autoradiographic work on beet of varying ploidy states (5, 8), measurement of DNA 'fibrillar' areas in chloroplasts from leaves of varying size (9), and measurement of DNA amounts in preparations of formaldehyde-fixed chloroplasts (7), were interpreted by Herrmann *et al.* (7) to show that the amount of DNA in chloroplasts was positively correlated with the size of the chloroplast. In the formaldehyde-fixed chloroplasts, after digestion with pronase and ribonuclease, estimates of the ctDNA content ranged from 10 plastome copies in chloroplasts with a diameter of 1.5 μm to 100 plastome copies in the larger chloroplasts with a diameter of 8.3 μm (7). These conclusions are at variance with the results quoted above for pea (10) and spinach (12). To resolve this question, we have used reassociation kinetics to measure the level of ctDNA in beet leaves and related the values to the change in size of chloroplasts, and the number of chloroplasts per cell in leaves of different ages.

MATERIALS AND METHODS

The methods used for the preparation of DNA, measurement of DNA in whole tissue, reassociation of ctDNA, and staining of ctDNA with DAPI have been described previously (12). Beet plants were grown in nutrient culture in growth cabinets as previously described for spinach (11) and the leaf laminae harvested after removal of the central midrib and the main veins. Leaf discs were taken randomly from the lamina for cell number and chloroplast number determinations (11) and for DNA measurement. DNA in discs was measured using a modification (12) of the method of Smillie and Krotkov (14). Up to four 1-h extractions at 70°C with 0.5 N $HClO_4$ were necessary for full hydrolysis of DNA from old beet leaves, whereas young leaves required only two extractions.

Beet or spinach ctDNA was prepared as described for spinach ctDNA and nick labeled (12) with [^{3}H]dCTP (19 Ci mmol^{-1}) to a specific activity of 8×10^6 dpm μg^{-1} DNA. A spinach ctDNA 1750-base pair restriction fragment (pSocE48), without the cloning vehicle, containing the gene for the large subunit of ribulose biphosphate carboxylase and an 11.5-kilobase pair fragment (pSocB149) which also contains the gene for the large subunit of ribulose bisphosphate carboxylase (16) were labeled with [^{32}P]dCTP (>400 Ci mmol^{-1}) to a specific activity of 2×10^8 dpm μg^{-1} DNA. Labeled dCTP was obtained from the Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

The eighth leaf of beet plants grown in liquid culture were sampled when it was 2 to 3 cm long and 25 to 30 cm long. Discs punched randomly from leaf laminae were used for the measurements shown in Table I. In the case of the 2 to 3 cm long leaves the entire lamina was used. The total amount of DNA per cell, calculated by dividing the DNA content per disc by the number of cells per disc, did not change significantly between leaves 2 to 3 cm long and leaves 25 to 30 cm long. These values of between 2.4 and 2.7 pg are close to the 2C nuclear DNA value of 2.5 pg reported by Bennett and Smith (1) which suggests that beet cells in this study do not become endopolyploid to any significant extent. In this study the average number of chloroplasts per cell increased 6-fold from 11 to 65 chloroplasts per cell and the average chloroplast diameters increased almost 3-fold from 1.8 to 4.9 μm (Table I).

With an appropriate probe for ctDNA in the presence of total DNA extracts from plants, estimates of the levels of ctDNA can be derived from the $C_0t_{\frac{1}{2}}$ values of the second order renaturation kinetics of the probe (12). In preliminary experiments, the same $C_0t_{\frac{1}{2}}$ values for beet and spinach ctDNA were obtained using beet or spinach [^{3}H]ctDNA or spinach [^{32}P]ctDNA fragments as probes. Figure 1A shows that the renaturation kinetics of the spinach ctDNA fragment ^{32}P-pSocE48, in the presence of either purified beet or spinach ctDNA, are almost identical. Consequently, in the experiments reported in Figure 1B and Table II, spinach ctDNA was used as a standard and either spinach [^{3}H]ctDNA or the spinach ctDNA fragment ^{32}P-pSocE48 as a probe. In another

[1] Abbreviations: ctDNA, chloroplast DNA; DAPI, 4, 6-diamidino-2-phenyl indale; $C_0t_{\frac{1}{2}}$, concentration (mol/L) × half-time.

Table I. *Measurements Made on the Laminae of Leaf 8 of Beta vulgaris (var. Fordhook giant) Plants Grown in Liquid Culture in Growth Cabinets*

Sample No.	Total DNA/Disc[a]	Cells/Disc[b]	Total DNA/Cell[c]	Chloroplast/ Cell[d]	Chloroplast Diameter[e]
	μg	$\times 10^{-6}$	*pg*		*μm*
A. 2–3-cm leaf (2-mm disc)					
1	0.430	0.137	3.14	10.8 ± 2.7	1.88 ± 0.39
2	0.268	0.108	2.48	10.9 ± 2.4	1.89 ± 0.41
3	0.430	0.189	2.28	9.2 ± 2.1	1.78 ± 0.25
4	0.408	0.173	2.36	10.9 ± 2.1	1.95 ± 0.50
5	0.381	0.170	2.24	12.2 ± 2.9	1.65 ± 0.31
6	0.313	0.150	2.09	10.7 ± 2.2	1.90 ± 0.30
Mean ± SE			2.41 ± 0.13	10.8 ± 0.39	1.84 ± 0.05
B. 25–30-cm leaf (4-mm disc)					
1	0.223	0.106	2.10	51 ± 23	5.28 ± 0.80
2	0.270	0.078	3.46	81 ± 33	4.83 ± 0.58
3	0.218	0.088	2.48	64 ± 34	4.91 ± 0.77
4	0.244	0.099	2.46	66 ± 24	4.65 ± 0.79
5	0.234	0.083	2.84	65 ± 24	4.80 ± 0.76
Mean ± SE			2.67 ± 0.23	65.4 ± 4.8	4.89 ± 0.24
LSD_5			0.60	9.8	0.24

[a] Means for five determinations, SE < 0.020.
[b] Means for eight determinations, SE < 0.016.
[c] Derived by dividing the total DNA values per disc by values in the second column.
[d] Mean ± SD for minimum of 20 measurements.
[e] Mean ± SD for a minimum of 100 measurements.

Table II. *Chloroplast DNA Content in Total DNA from Beet Leaf Lamina*

The $C_0t_{\frac{1}{2}}$ values for the renaturation of [^{3}H]- or [^{32}P]ctDNA probes in the presence of total DNA extracts from the laminae of 2 to 3-cm leaves and from the laminae of 25 to 30-cm leaves was followed. The $C_0t_{\frac{1}{2}}$ value for the spinach ctDNA standard was 0.10 mol/s L^{-1}. The proportion of ctDNA in beet leaf lamina DNA was calculated from the ratio of the $C_0t_{\frac{1}{2}}$ of the standard ctDNA to the $C_0t_{\frac{1}{2}}$ of the sample DNA (12).

Leaf Size	DNA $C_0t_{\frac{1}{2}}$	ctDNA
cm	*mol/s* L^{-1}	*% total*
2–3	1.34 ± 0.14	7.5 ± 0.8
25–28	0.875 ± 0.006	11.4 ± 0.08
	LSD_5 = 0.53	

Table III. *Chloroplast DNA Content of Cells of Beet Leaf Laminae*

The number of plastome copies was calculated using 0.97×10^8 as the mol wt of beet ctDNA (6). SE for plastome copy numbers were calculated from the SE of the values shown here (15).

Leaf Size	Chloroplasts/ Cell[a]	DNA/ Cell[a]	ctDNA	Plastome copies	
				Per cell	Per chloroplast
cm		*pg*	*% total*		
2–3	10.8	2.41	7.5	1118 ± 137	104 ± 13
25–30	65.4	2.67	11.4	1888 ± 211	29 ± 4

[a] From Tables I and II.

experiment, the renaturation of the probe ^{32}P-pSocE48, in the presence of spinach ctDNA, DNA from 2 to 3-cm beet leaves and 25 to 30-cm beet leaves is shown in Figure 1B for one measurement. The mean $C_0t_{\frac{1}{2}}$ values for all the measurements is shown in Table II. During the expansion of the beet leaf cells, ctDNA levels increased from 7.5% of the total DNA in 2 to 3-cm leaves to 11.4% in 25 to 30-cm leaves.

In Table III, the proportion of chloroplast DNA in total DNA (Table II) together with the amount of DNA per cell (Table I) are used to calculate the number of plastome copies per cell. The number of plastome copies per chloroplast was then calculated by dividing the number of plastome copies per cell by the number of chloroplasts per cell from Table I. The plastome copy number decreased almost 4-fold from 104 copies in chloroplasts from 2 to 3-cm leaves to only 29 copies in chloroplast from 25 to 30-cm leaves.

The plastome copy numbers and trends in copy numbers with cell expansion, on a per chloroplast basis, are similar to those recently reported for spinach (12) and pea (10). On a per cell basis, the plastome copy number is lower than that reported for pea and spinach but still comprises a vast excess over the nuclear genome copy number which we estimate as about 2C in these laminae.

The partitioning of nuclear and ctDNA amounts on a per cell and a per chloroplast basis described in Table III is an averaging process, and establishes trends rather than absolute amounts. While errors in this partitioning could be caused by cells with widely differing plastid numbers or ctDNA amounts, it seems unlikely that such factors would alter the trends we report. The changes that were measured were those associated with expanding mesophyll/palisade cells in the leaf laminae and the albino stems and main veins were excised.

Figure 2 shows epifluorescence micrographs of cells from 2 to 3 and 25 to 30-cm leaves stained with the fluorochrome DAPI (4). In the small chloroplasts of cells from 2 to 3-cm leaves, DAPI fluorescence is distributed throughout the chloroplast with a num-

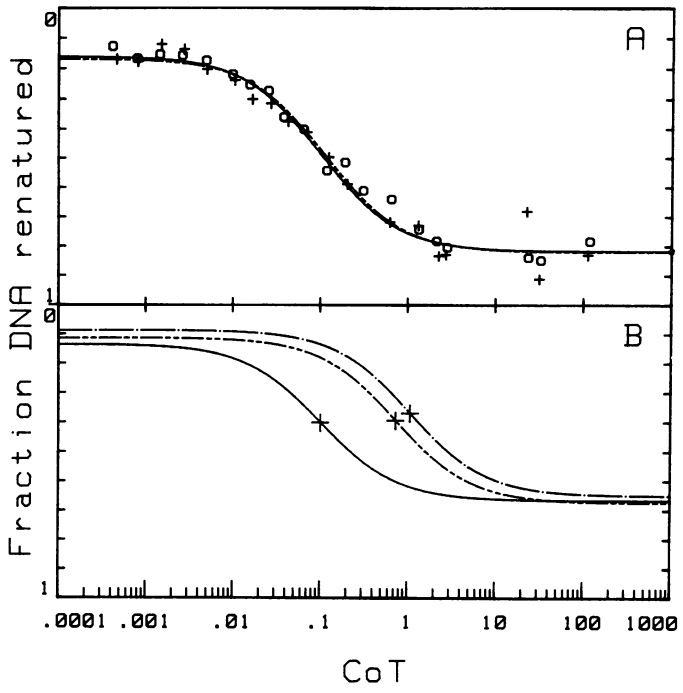

FIG. 1. The renaturation kinetics of the spinach ctDNA fragment ^{32}P-pSocE48 in the presence of spinach (——, +) or beet (– –, ○) ctDNA is shown in A. The $C_0t_{\frac{1}{2}}$ of the renaturation with spinach ctDNA is 0.100 mol/s L^{-1} and that for beet ctDNA 0.113, with SD of 0.015 and 0.010, respectively. In B, the $C_0t_{\frac{1}{2}}$ of ^{32}P-pSocE48 is shown in the presence of spinach ctDNA (——) and DNA from 2 to 3-cm beet leaf laminae (–·–) and 25 to 30-cm beet leaf laminae (— – —). In this experiment, the $C_0t_{\frac{1}{2}}$ for the DNAs, respectively, were 0.101, 1.06, and 0.725 with SD of 0.012, 0.079, and 0.116, corresponding to a ctDNA content in 2 to 3-cm laminae of 9.5% and in 25 to 30-cm laminae of 13.9%.

ber of intensely fluorescing regions present which could possibly be interconnected. In the larger chloroplasts from the cells from 25 to 30-cm leaves, DAPI fluorescence is present in fine discrete spots throughout the chloroplasts.

The decrease in the amount of ctDNA per chloroplast in beet leaves as the chloroplasts divide and increase in size, reported in this paper, is in contrast to the increase in ctDNA content with increased chloroplast size in beet claimed by Herrmann *et al.* (7). The increase in ctDNA content Herrmann *et al.* measured probably results from an overestimation of ctDNA in fixed chloroplast preparations which may have been contaminated by nuclear DNA. As well, Herrmann and Kowallik (8) identified one to five DNA-containing regions in young beet plastids following treatment of the preparations with trypsin to improve resolution, and found more such regions in larger plastids. Because of the difficulties involved in clearly identifying the 'DNA fibril-containing' areas, especially in mature plastids, and in the assumption that ctDNA packing is the same in young and old plastids, it seems unlikely that such observations are quantitative. Following autoradiography of tissue labeled with [^{3}H]thymidine, Herrmann and Kowallik (8) found up to 18 centers of labeling in large beet chloroplasts and equated these with increased DNA content of the chloroplasts. In the absence of information about ctDNA precursor pools and ctDNA repair, quantitative conclusions from these labeling experiments cannot be made. In these studies, beet plants of differing ploidy status were used to generate a wider than normal range of chloroplast sizes. We suggest that such a series may not be suitable for this purpose as chloroplast number per cell is known to vary and in some situations ctDNA content may vary also with the changes in ploidy level (3).

We consider that renaturation kinetics provide a reliable method for measuring changes in ctDNA levels in leaves. Using this method, we have found that during the expansion of leaf cells of beet as leaves grow from 2 to 3 to 25 to 30 cms in length, two to three rounds of chloroplast division take place and ctDNA is segregated to daughter chloroplasts. In beet, some additional ctDNA is synthesized during cell expansion because the decrease in plastome copies per chloroplast does not exactly parallel the increase in chloroplast number per cell that occurs in both spinach (12) and pea (10). The increase in the proportion of ctDNA in total DNA from 25 to 30-cm laminae compared to 2 to 3-cm laminae may be a reflection of the relatively early stage of development of the 2 to 3-cm beet leaf laminae. In the early stages of spinach leaf cell expansion, there is a stage when ctDNA is synthesized relative to nuclear DNA, the proportion of ctDNA in the tissue rises, and the plastome copy number per cell and per chloroplast rises also (13). This occurs before the stage of leaf cell expansion described previously (12) in which the major phase of formation and division of chloroplasts occurs. We consider the trend that chloroplast genome copy number falls as chloroplasts divide and increase in size may be a general phenomenon that

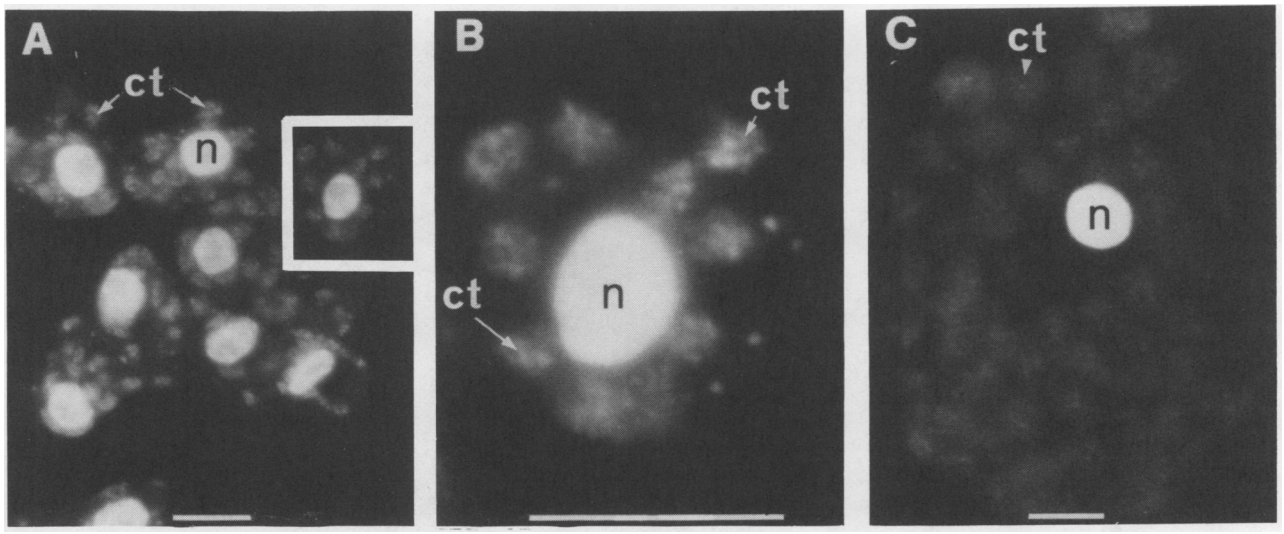

FIG. 2. Fluorescence photomicrographs of beet leaf cells showing DNA stained with the fluorochrome DAPI. A, Group of cells from 2.5-cm beet leaf. B, Higher magnification of cell shown in the inset in A. C, Cell from 25-cm beet leaf. Bar represents 10 μm. n, nucleus; ct, chloroplast.

takes place during the expansion of leaf cells of higher plants.

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