

Changes in Chloroplast DNA Levels during Development of Pea (*Pisum sativum*)¹

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ABSTRACT

Determinations were made of the percentage of chloroplast DNA (ct DNA) in total cell DNA isolated from shoots of pea at different stages of development. Labeled pea ct DNA was reassociated with a high concentration of total DNA; the percentage of ct DNA was estimated by comparing the rate of reassociation of this reaction with that of a model reaction containing a known concentration of unlabeled ct DNA. The maximum change in ct DNA content was from 1.3% of total DNA in young shoots to 7.3% in fully greened shoots. Analyses were also performed on DNA from embryos, etiolated tissue, roots, and leaves. The first leaf set to develop in pea was excised over a growth period of 8 days during which leaf length increased from 4 to 12 millimeters. Young leaves contained about 8% ct DNA; in fully greened leaves the level of ct DNA approached 12%, equivalent to as many as 9,575 copies of ct DNA per cell. Root tissue contained only 0.4% ct DNA.

Chloroplast development is essentially a light-induced phenomenon. The process includes reorganization of the prolamellar bodies to form the thylakoids and grana, reduction of Pchl to Chl, and plastid enlargement (11). In higher plants the number of chloroplasts per cell can increase by as much as a factor of 20 during greening and leaf expansion (19).

There is some evidence that chloroplast development is accompanied by ct DNA² synthesis (6, 19, 20). However, the contribution of ct DNA to the total cellular DNA complement at any stage of higher plant development has not been accurately determined. A major difficulty is the similar base composition of chloroplast and nuclear DNAs (10), which has prevented resolution of these components by CsCl density gradient analysis of total cell DNA. In the algae the buoyant densities of the two DNAs may differ considerably. It has been found that ct DNA comprises about 5% of total DNA in the primitive alga *Olisthodiscus* throughout the growth cycle (2), and as much as 11 to 15% in *Chlamydomonas* (22, 26). Although 9% ct DNA has been estimated for tobacco leaves by the density method (23), reassociation kinetics analysis has indicated that tobacco leaves contain 4% ct DNA and roots less than 1% (21).

The purpose of this study was to determine the amount of ct DNA in total DNA extracted from shoots throughout development of peas using reassociation kinetics analysis. In an earlier report we noted that ct DNA could increase by a factor of 2.5

during shoot development (13). Here we show that the increase can actually be as great as 5.6. DNA from embryos, etiolated tissue, roots, and leaves has also been analyzed. From the rates of reassociation we have estimated the number of chloroplast genomes in leaf cells.

MATERIALS AND METHODS

Growth of Tissue. Pea seeds (*Pisum sativum* L., cv. Alaskan wilt-resistant) were planted in plastic trays on 4 cm of Vermiculite and covered with an additional 2 cm. Illumination (18 h light/6 h dark) was from cool-white fluorescent bulbs, 600 ft-c at tray height. Germination of all seeds occurred within 24 h at 28 C. Days in shoot development were assigned relative to the day of planting: after 24 h plants were labeled day 1, after 48 h day 2, and so on. Since emergence from the substrate varied from one developmental series to another, the onset of greening did not correspond closely to the age of the plant. Chl content of the series presented in Figure 1 increased most rapidly between day 6 and day 10. Root tissue harvested from day 5 seedlings was washed with 1 M NaCl, 0.1 M EDTA, and 1% Sarkosyl before DNA extraction, as described below. Embryos were excised from the seed after 8- to 12-h water imbibition in the dark at room temperature. Etiolated tissue was grown in the dark and harvested in green light. Day 4 etiolated shoots were 3 to 4 cm high; day 6 were 7 to 11 cm. Etiolated tips were taken from day 6 shoots and included only the leaf primordia.

Isolation and Labeling of Chloroplast DNA. Covalently closed circular ct DNA was isolated from pea seedlings at day 10 in development following the method of Kolodner and Tewari (12). UV absorbance ratios were as follows: $A_{260/280}$, 1.75; $A_{260/230}$, 2.5. Chloroplast DNA was labeled *in vitro* with deoxythymidine 5'-triphosphate, tetrasodium salt, methyl-³H using the procedure of Maniatis (15). Specific radioactivities were approximately 1 to 2 × 10⁶ cpm/μg DNA.

Isolation of Total Cell DNA from Pea Tissues. Two methods of extraction were employed. Method I included freezing and pulverization of the tissue at the temperature of solid CO₂, 5 to 10 min of grinding with a mortar and pestle on ice in 1 M NaCl, 0.1 M EDTA, 1% Sarkosyl followed by repeated chloroform-isopentyl-OH (96:4, v/v) extraction of the aqueous and interphases, centrifugation at 8,000 rpm in the Sorval SS34 rotor for 15 min, and overnight ethanol precipitation. The pellet was resuspended in 0.015 M NaCl, 0.01 M Tris, 1 mM EDTA (pH 8) and digested with RNase (66 μg/ml pancreatic, 33 units/ml T1, 30 min) and pronase (500 μg/ml, 3 to 4 h) at 37 C before an additional chloroform extraction and ethanol precipitation. All ethanol precipitates were collected by centrifugation for 20 min at 8,000 rpm in the SS34 rotor. Method II was essentially the same, except that homogenization was carried out in 0.1 M Tris, 0.1 M EDTA (pH 8), 2% Sarkosyl, and 100 μg/ml pronase. The slurry was incubated at 60 C for 30 min and extracted with chloroform and then phenol. The aqueous phase was raised to 0.3 M NaCl before each ethanol

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² Abbreviations: ct DNA: chloroplast DNA; P₀: probe concentration (moles/liter); P₀t: probe concentration × time (seconds); P₀t_{1/2}: point of half-reassociation of probe DNA; DABA: diamino benzoic acid.

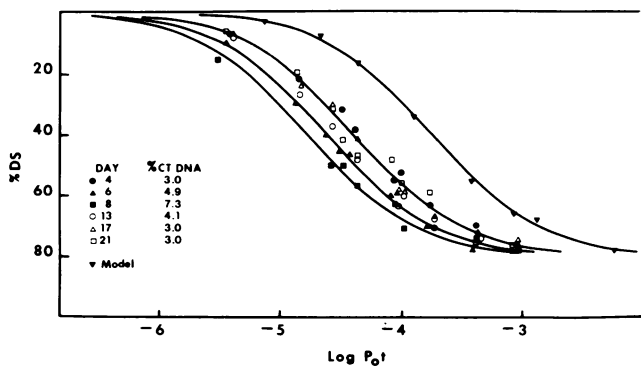


FIG. 1. Reassociation of labeled pea chloroplast DNA with total DNAs from different stages of shoot development. Tritiated ct DNA (probe I) at 7.9×10^{-8} mol/l was reassociated with unlabeled total DNA isolated by method I. P_0t equals the probe concentration \times time in seconds. Reactions were at $150 \mu\text{g/ml}$ unlabeled driver DNA in 1 M NaClO_4 buffer at 60 C . Eighty per cent component curves are drawn through the data points after subtraction of zero time binding equal to 8%. Linear least squares regression analysis of Scatchard plots (16) of the data gave correlation coefficients greater than 0.90. Shown are curves for days 4, 6, and 8; points for the other days cluster between those for day 4 and day 6. The $P_{0t_{1/2}}$ values are $4.1, 2.5, 1.6, 2.9, 4.0,$ and 3.0×10^{-5} for days 4, 6, 8, 13, 17, and 21, respectively. In the model reaction the probe was reassociated with unlabeled pea ct DNA at $1.03 \mu\text{g/ml}$; the $P_{0t_{1/2}}$ is 1.75×10^{-4} . Inset shows symbols for reaction and the percentage of ct DNA estimated from the model reaction. Vertical axis is per cent double-stranded probe DNA that bound to hydroxylapatite.

precipitation step. The DNAs were sonicated (weight average length = 375 bases) as described (13), dialyzed against 1 mM Tris , 1 mM EDTA (pH 8), and stored at -20 C .

Reassociation of Labeled Chloroplast DNA with Unlabeled Total Cell DNA. The reassociation procedure has been described (13). Reactions were carried out in 1 M NaClO_4 , 0.03 M Tris , 1 mM EDTA (pH 8), 0.1% Sarkosyl at 60 C . Double strands were separated from single strands on hydroxylapatite. The concentrations of unlabeled DNA are given in the figure legends. Three different labeled ct DNA probes were used, all prepared from the same isolate of covalently closed circular DNA. To compare $P_{0t_{1/2}}$ (mol/l \times s of incubation) values for reactions with different probes, root DNA was reassociated with each as described in the text, and in several cases other total DNAs were also included.

The concentration of the total DNA was determined by both UV absorbance and the DABA assay for microquantities of DNA (3). DNA was applied directly to the filters in 1 mM Tris , 1 mM EDTA (pH 8). Although the DABA assay reproducibly gave lower DNA concentrations, the ratio of the DABA to A values was almost the same for all of the DNA samples in the developmental series analyzed, *i.e.* 0.8 to 0.85. For roots this value was 0.99 and for etiolated tissue, 0.85. DNAs from leaves and embryos were not analyzed by the DABA assay. Where possible curves have been adjusted to the concentration of DNA determined by the DABA assay.

The size of the single component kinetic curves drawn for the different sets of reactions ranges from 70 to 80% due to decreases in the length of the probe with time (from a weight average length of 1,660 to 700 bases) (13) leading to less total DNA binding to hydroxylapatite at the termination of reassociation (unpublished observations). This should have no effect on the comparisons of reassociation rates obtained simultaneously.

Construction of Model Reaction with Unlabeled Chloroplast DNA. The percentages of ct DNA presented rely completely on an accurate determination of the concentration of unlabeled ct DNA in the model reaction. In preparation of the ct DNA ($1.03 \mu\text{g/ml}$) for the model reaction shown in Figure 1, calf thymus DNA was included as a carrier during the sonication and dialysis

steps to permit evaluation of ct DNA recovery. Eighty-nine per cent was recovered as estimated by A .

RESULTS

Changes in Percentage of Chloroplast DNA in Shoots during Development. Figure 1 shows single component kinetic curves for the reassociation of labeled pea ct DNA with unlabeled total cell DNAs extracted at day 4 through day 21 of pea shoot development. Days in development have been defined under "Materials and Methods." The reassociation data are presented following the method of Chilton *et al.* (4), where P_0 equals the concentration of labeled chloroplast "probe" DNA. A leftward shift in the curve indicates an increase in the fraction of unlabeled "driver" DNA that is homologous to the probe DNA. Figure 1 also presents the reassociation of labeled ct DNA with unlabeled ct DNA at $1.03 \mu\text{g/ml}$. Calf DNA, which does not accelerate ct DNA reassociation (13), was added to compensate for viscosity differences between this reaction and those using total pea DNA. From this model reaction the μg of ct DNA in the total DNAs were estimated. The model reaction has a $P_{0t_{1/2}}$ of 1.75×10^{-4} . Since the reassociation curve for DNA from day 4 in development has a $P_{0t_{1/2}}$ of 4.06×10^{-5} , that DNA must have contained 4.3-fold ($17.5/4.06$) more ct DNA than the model reaction, or $4.3 \times 1.03 = 4.4 \mu\text{g/ml}$. Since the total driver DNA concentration for this reaction was $150 \mu\text{g/ml}$, 3% of the DNA from day 4 constitutes chloroplast sequences. Similar computations for the other curves show that the percentage of ct DNA increased to 5% by day 6, over 7% by day 8, and then decreased gradually to less than 3% by day 21. An earlier report (13) described ct DNA levels as increasing from 2.4 to 6% during shoot development. The values given here have been corrected to DNA concentrations determined by the DABA assay (see under "Materials and Methods") rather than A .

Plant DNA may contain polysaccharide contaminants that artificially accelerate reassociation reactions (17, 18). The rate of reassociation of *Bacillus subtilis* DNA in the presence of DNA from day 4 and day 8 was the same as that for *B. subtilis* reassociated alone or in the presence of calf DNA. It was concluded that the differences in the percentage of ct DNA shown in Figure 1 are due to changes in the ratio of chloroplast to nuclear DNAs.

DNA obtained from another developmental series and isolated by the same procedure (method I) was analyzed (Fig. 2). In this instance embryos excised within 12 h of water imbibition were used to assess the basal level of ct DNA. Both shoot primordia

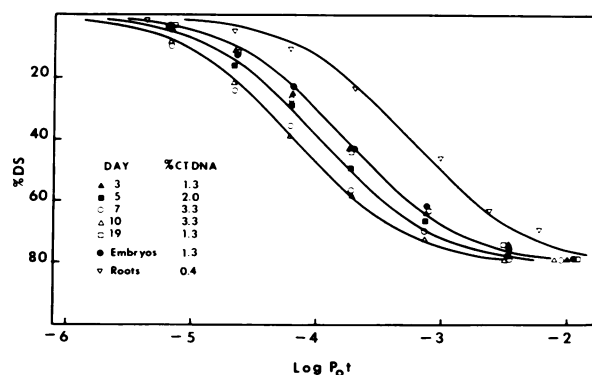


FIG. 2. Reassociation of labeled pea chloroplast DNA with total DNAs from a second developmental series. Tritiated ct DNA (probe II) at 7×10^{-8} mol/l was reassociated with total DNAs at $143 \mu\text{g/ml}$ isolated by method I. Eighty per cent component curves are drawn through the data points for the embryo, day 5 and day 7. $P_{0t_{1/2}}$ values are $1.7, 1.7, 1.0, 0.66, 0.66, 1.6,$ and 5.5×10^{-5} for embryos, days 3, 5, 7, 10, 19, and roots, respectively. Root DNA contained about 0.4% ct DNA as estimated in Figure 4. See Figure 1 for other details.

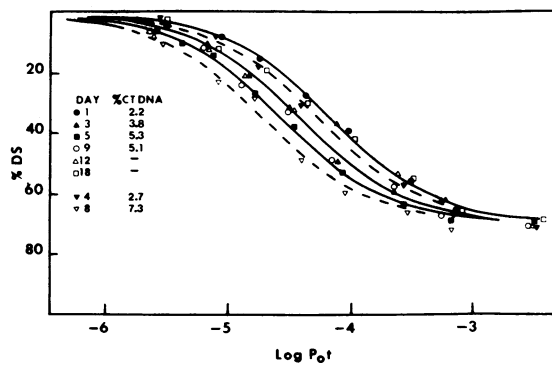


FIG. 3. Reassociation of labeled chloroplast DNA with total DNAs extracted by an alternate method. Unlabeled DNAs isolated by method II were at 100 $\mu\text{g}/\text{ml}$ in reassociation reactions with probe I. DNAs isolated by method I, days 4 and 8, were included as standards. Chloroplast DNA percentages were estimated relative to day 8 shown to contain 7.3% in Figure 1. Seventy per cent component curves (—) are drawn through the data points for days 1 (an embryo after 24-h water imbibition), 3, and 5, and (---) through days 4 and 8. $P_{0t_{1/2}}$ values are 6.4, 3.7, 2.6, 2.7, 5.2, and 1.9×10^{-5} for embryos, days 3, 5, and 9 DNAs of method II (no estimates for days 12 and 18, see text), and day 4 and day 8 DNAs of method I, respectively. See Figure 1 for other details.

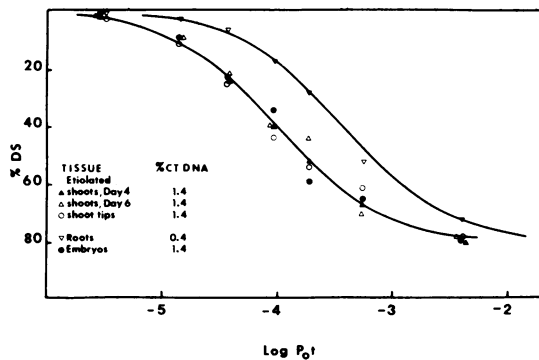


FIG. 4. Reassociation of labeled chloroplast DNA with DNAs from etiolated tissue, roots, and embryos. Conditions are described in Figure 1. Percentages of ct DNA were determined relative to the $P_{0t_{1/2}}$ values of the model reaction, Figure 1, since the reactions were done at the same time. Eighty per cent component curves are drawn through the data points for roots and etiolated tissue, day 4.

and the radicle were included. The percentage of ct DNA increased from 1.3% in the embryo to 3.3% in day 7 shoots; it remained at this level until at least day 10 and by day 19 had returned to the embryonic level. These percentages were estimated with respect to the rate of reassociation of labeled ct DNA with root DNA; root DNA was used to compare rates found with different chloroplast probe DNAs. Morphological changes in the pea shoots were also followed in this series. There was no greening in the shoots until after day 3 when they emerged from the substrate. The first leaf set was fully greened by day 5 and the second by day 7. One other leaf set appeared before senescence. By day 19 the first two leaf sets had senesced and flowers and tendrils were on all plants.

To investigate whether the apparent changes in ct DNA content could be attributed to the extraction procedure with nuclei or chloroplasts selectively lysed at various stages in development, another procedure (method II), was employed to isolate total DNA. The results of the reassociation reactions between these DNAs and labeled pea ct DNA are shown in Figure 3. An increase in the ratio of chloroplast to nuclear DNAs was observed from early development through day 5. In this series the shoots were fully greened by day 3, although the first leaf set was not entirely

opened until day 4. DNA from the next day analyzed, day 9, contained the same amount of ct DNA as day 5. A single component curve will not fit the data points obtained for days 12 and 18.

DNAs isolated from days 4 and 8 using method I were included with the series presented in Figure 3 to control for probe length decreases that occur as a probe "ages" (13) which result in reduced rates of reassociation (7). If the $P_{0t_{1/2}}$, 1.9×10^{-5} , for day 8 DNA, method I, represents 7.3% ct DNA, then in this group the ct DNA level increased from about 2% in a day 1 embryo to 5% at days 5 and 9. By day 18 the percentage approached that found for day 1. This range of ct DNA contents is in good agreement with that shown in Figures 1 and 2, indicating that the changes are not artifacts of the extraction procedure. Over-all, there seems to be a continuous change in ct DNA content of the shoot during development. The amount of ct DNA increases and then gradually decreases.

Percentage of Chloroplast DNA in Different Tissues. Labeled ct DNA was reassociated with total DNA from embryos, etiolated shoots at day 4 and day 6 in development, etiolated shoot tips, and root tissue. Embryos (12 h of water imbibition) and etiolated tissue, whether from the entire shoot or the tips exclusively, contained the same level of ct DNA, 1.4% (Fig. 4), as indicated by their overlapping reassociation curves. The lowest level of ct DNA was found for the roots, 0.4%. These reactions were performed at the same time as the model reaction in Figure 1 so the percentages were derived directly from the concentration of ct DNA in that reaction, showing a $P_{0t_{1/2}}$ of 1.75×10^{-4} .

The same root DNA was analyzed with the developmental series presented in Figure 2 and the leaf series in Figure 6 to estimate the relative percentages of ct DNA in those cases.

Reassociation of labeled ct DNA with total leaf DNAs is shown in Figure 5. In a separate study the average increase in the length of the first leaf set was found to increase from 4 mm at the start of greening to 12 mm at full greening (manuscript in preparation). Here leaves showing that pattern of development were selected for analysis. Figure 5 lists both the length of the first leaf set and the day in development of leaf excision. There was not a dramatic change in the level of ct DNA in the leaf during development. Between a length of 4 and 7 mm the increase was 1.4-fold; there was no further change as the leaves expanded to 12 mm. A random selection of senescing leaves at day 20 showed a drop in ct DNA below that found for young leaves.

The percentage of ct DNA in leaf DNA was estimated (Fig. 6)

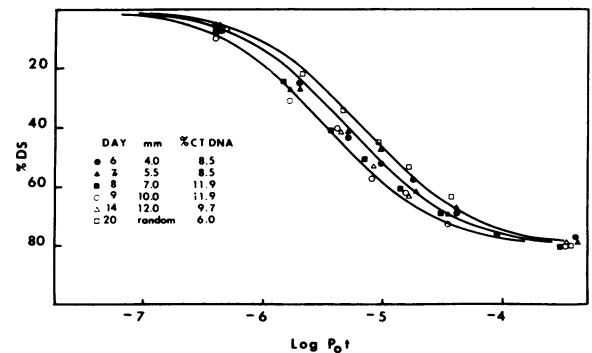


FIG. 5. Reassociation of labeled pea chloroplast DNA with DNA from leaves at different stages of greening and growth. Total DNA from leaves (first leaf set) 4 to 12 mm in length was reassociated with probe III, 1.2×10^{-8} mol/l. Inset shows both leaf length and day in development of leaf excision. Eighty per cent curves are drawn through the data for 4 mm, 10 mm, and a random selection of leaves from day 20 plants. $P_{0t_{1/2}}$ values are 5.0, 5.0, 3.5, 3.5, 4.0, and 7.0×10^{-5} for leaves with the following respective lengths (mm): 4.0, 5.5, 7.0, 10.0, 12.0, and random. Other details are given in Figure 1. Percentages are relative to that found for 5.5-mm leaves in Figure 6.

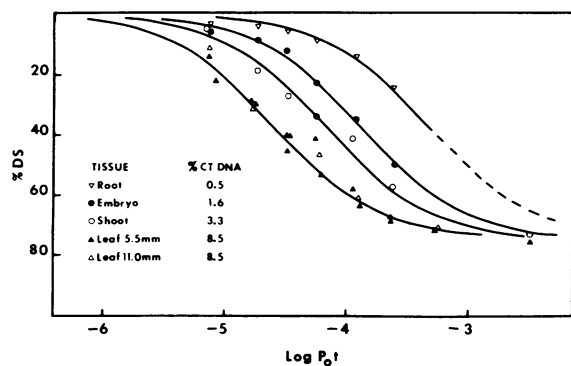


FIG. 6. Determination of percentage of ct DNA in leaves. Reassociation was with probe III at 6.7×10^{-8} mol/l and total DNAs from 5.5-mm (day 7) and 11.0-mm (day 11) leaves. Seventy-five per cent component curves are drawn through the data. DNAs from root tissue, embryos, and day 10 shoots (see text for description of DNAs) were simultaneously analyzed to estimate the percentage of leaf ct DNA. Percentages presented are relative to day 10 shoots; $P_{01/2}$ represents 3.3% ct DNA. $P_{01/2}$ values are 4.5, 1.3, 0.65, 0.25, and 0.25 for roots, embryos, day 10 shoots, 5.5-mm leaves, and 11-mm leaves, respectively.

by reassociating DNA from day 7 leaves, 5.5 mm, in a set of reactions with root DNA (same sample used for Figs. 2 and 4) and DNAs from 12-h embryos and day 10 shoots analyzed in Figure 2. If the reassociation curve for the root DNA is used as a standard, young leaves contained about 7% ct DNA in total DNA. Alternatively, if ct DNA from day 10 comprised 3.3% of total DNA, then young leaves contained about 8.5%. Since the ct DNA in the leaves increased by a factor of 1.4 (Fig. 5) during greening and growth, the final level of ct DNA may have reached 9.8 to 11.9%.

DISCUSSION

The percentage of chloroplast DNA in total cell DNA changes during development and varies from tissue to tissue. Fully greened leaves showed the highest level of ct DNA, from 9.8 to 11.9%, while roots had the lowest, 0.4%. Embryos and etiolated tissue both contained about 1.4% ct DNA which may be the basal level in a pea diploid cell since both the shoot primordia and radicle of the embryo are considered to be 2 C (8, 25), and roots are at least 4 C (14). During shoot development the percentage of ct DNA increased from 1.3% to an average of 5.3% at full greening; maxima of 3.3, 5.3, and 7.3% were observed in different developmental series. The variation in the maximum amount of ct DNA in total DNA does not seem surprising considering that in one group more leaves may have developed fully, raising the ct DNA in the entire shoot. The continuous change in ct DNA levels probably reflects the heterogeneity of cell type and age that contribute to each shoot total DNA preparation.

The percentage of ct DNA represents the ratio of chloroplast to nuclear DNAs (plus an unknown contribution from mitochondria) which will change if one of the DNAs is replicated or degraded without cell division. Endoreduplication of nuclear DNA is well documented in pea tissue. In light-grown epicotyls there is an increase from 2 to 4 C within 4 days, with 10 to 20% of the nuclei reaching an 8 C DNA level (24). If the tissue used in this study followed such a pattern, the ct DNA would have had to replicate once to maintain 1.4%, twice to attain 2.8%, and three times to reach 5.6%, which would be equal to an 8-fold amplification of ct DNA beyond the embryonic level. In this report it was shown that etiolated tissues (both the shoot tips and entire shoots) contained the same level of ct DNA as the embryo. Since dark-grown epicotyls increase their nuclear ploidy levels by at least a factor of 4 (24), the synthesis of ct DNA appears to keep pace with nuclear DNA endoreduplication in dark-grown shoots, but not exceed it.

TABLE I. Copy Number of Chloroplast DNA in Leaf Cells

Ct DNA CONTENT ¹	COPIES/DIPLOID CELL ²	COPIES/Ct ³
8% young	6106	244
12% fully greened	9575	174

1 Percentages estimated from Figures 5 and 6 for leaves.

2 Determined as follows:

- 6.32×10^{12} daltons + (fraction ct DNA) (x) = x,
- (fraction ct DNA) (x) = daltons ct DNA/cell,
- (daltons ct DNA/cell) $(1/90 \times 10^6 \text{ daltons})$ = copies ct DNA/cell, where x is the total daltons of DNA in a pea cell; the fraction of ct DNA is obtained from the percentages in column 1; pea diploid DNA content (6.32×10^{12} d) came from reference 1, and approximate pea chloroplast genome size from 12.

3 Copies/cell divided by the average number of chloroplasts/cell estimated to be 25 and 55 for the first leaf set at 4 mm and 10 mm, respectively (manuscript in preparation).

In contrast, the low levels of ct DNA in root tissue, 0.4%, could be explained by nuclear DNA increases (14) unaccompanied by ct DNA replication.

Young leaves contained about 8% (7–8.5%) ct DNA. Calculations have been made to estimate the number of copies of the 90×10^6 dalton (12) chloroplast genome in total DNA. Assuming a diploid genome size of 6.32×10^{12} daltons, equivalent to 10.5 pg (1), Table I shows that a diploid cell from young pea leaves would contain at least 6,106 copies of ct DNA, or 244 circles per chloroplast based on an average number of 25 chloroplasts per cell (manuscript in preparation). Similar calculations are given for 12% ct DNA per leaf cell. Twenty-six hundred copies of ct DNA have been calculated for a tobacco leaf cell by reassociation kinetics analysis (21). In diploid *Chlamydomonas* cells 80 to 140 copies per chloroplast have been estimated (26).

A haploid cell of pea would contain about 3,000 molecules of chloroplast DNA with a complexity of 90×10^6 daltons, or 1.4×10^5 base pairs. A component repeated 2,800-fold per haploid genome with a complexity only slightly higher has been found in total DNA isolated from soybean leaves (5). The percentage of total DNA comprising this component was 28 to 40%. Perhaps a plant with a smaller genome (1.95 pg/haploid) (5) than pea has a similar number of ct DNA molecules per leaf cell and this results in a larger repetitive component in total DNA. However, such a component has been detected in *Petroselinum sativum* (parsley) nuclear DNA preparations thought to be chloroplast-free (9). The present finding that leaves may contain 20 to 30 times more ct DNA than root tissue should prove useful in designing a simple test for the origin of a repetitive component identified in total cell DNA.

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