EVIDENCE THAT THE AMOUNT OF CHLOROPLAST DNA EXCEEDS THAT OF NUCLEAR DNA IN MATURE LEAVES

CHARLENE A. JOPE, ATSUSHI HIRAI, and S. G. WILDMAN

From the Department of Biology, Molecular Biology Institute, University of California, Los Angeles, California 90024. Dr. Hirai's permanent address is Institute for Biochemical Regulation, Faculty of Agriculture, Nagoya University, Nagoya, Japan.

ABSTRACT

Cell-free homogenates containing intact chloroplasts and nuclei were allowed to settle for up to 1 h before the top 2 ml of the 5-ml homogenate was withdrawn. Whereas <18% of the chloroplasts moved from the top to the bottom portions, the ratio of nuclei to chloroplasts in the top portion changed from ~1/200 to 1/900. The total numbers of chloroplasts and nuclei were counted in the homogenate before settling and in the top 2 ml and bottom 3 ml after settling. The total DNA content of the homogenate and the top and bottom portions after settling was determined by the diphenylamine colorimetric assay. By simultaneous equations, the absolute amount of DNA in chloroplasts and nuclei was determined. The results are consistent with previous observations of chloroplast DNA by fluorescence microscopy which indicated that the amount of chloroplast DNA per chloroplast is a function of chloroplast size. In addition, the results show that the amount of chloroplast DNA per average chloroplast in large leaves is 0.14×10^{-12} g, a magnitude higher than previous reports in the literature, and that large leaves contain about twice as much chloroplast DNA as nuclear DNA.

KEY WORDS chloroplast DNA nuclear DNA diphenylamine total leaf DNA

It is more or less an article of faith that chloroplast DNA constitutes ~10% and nuclear DNA ~90% of the total DNA in leaves. Mitochondrial DNA amounts to <1% of the total (6). James and Jope (5) described the visualization of the chloroplast DNA genome in isolated chloroplasts. After *Nicotiana excelsior* chloroplasts were stained with 4'6 diamidino-2-phenylindole (DAPI), a dye which binds specifically to DNA, fluorescence microscopy gave evidence that the amount of chloroplast DNA is proportional to the area of a chloroplast. The observations also left the impression that the amount of DNA per chloroplast appeared to be more than previously reported (6). We were therefore prompted to make an analysis of the DNA contained in nuclei and chloroplasts of these same plants under conditions where the nuclear/chloroplast ratio could be significantly changed and the absolute numbers of nuclei and chloroplasts correlated with change in the total DNA content of the extracts. Such a measurement allowed quantitation of the amount of DNA per organelle. The results are the subject of this communication.

MATERIALS AND METHODS

Plant Material

Nicotiana excelsior plants were grown under greenhouse conditions. When the plants were 2–3 mo old and before floral primordia were evident, they were topped

by removing the apical meristem. As lateral buds developed, they were also removed. This procedure inhibited growth in height and forced expansion of the leaves remaining on the plant in length, width, and thickness.

Thin sections of laminar tissue were made as described previously (8). Rough estimates of the number of chloroplasts present in living leaf cells could be obtained by counting the number of chloroplasts present on a single face of a palisade cell, which represents approximately one-third of the surface area of the cell.

Enhanced separation of isolated chloroplasts from nuclei was achieved by depleting the chloroplasts of starch to reduce the density of chloroplasts. Removing the plants from the greenhouse in the early morning and placing them in the dark for 24 h resulted in the disappearance of starch grains from the chloroplasts of parenchymous cells.

Isolation of Chloroplasts and Nuclei

Leaves of predetermined length were removed from the plants just before use. The midrib and all major veins were excised and the remaining strips of laminar tissue weighed. Individual strips precooled on ice were immersed upside down in a homogenization medium consisting of 0.5 M sucrose and 5 mM magnesium acetate contained in a shallow polyethylene dish glued to an aluminum plate. By standing the metal plate on ice, the medium was cooled to ~4°C. With a new single-edged razor blade, a succession of parallel cuts into the mesophyll was made as close as possible. The cuts were made in one direction only and were deep enough to open up the spongy and palisade parenchyma cells and release their protoplasmic contents into the medium. However, the cuts did not completely sever the strip of tissue. Each gram of tissue was sliced beneath 2 ml of homogenization medium. The object of this gentle chopping procedure was to cut through the cell walls of lower epidermal and mesophyll cells so that their organelles and other protoplasmic contents could be released into the homogenization medium with the least possible damage to the chloroplasts and nuclei. The sliced tissue was gently squeezed against the side of the chopping vessel to maximize release of the cell-free homogenate which was filtered through one layer of Miracloth (Chicopee Mills, Inc., Milltown, N. J.).

Comparison of organelles produced by this means with their *in vivo* counterparts by phase and fluorescence microscopy showed that the organelles were intact. The microscopic observations permitted the easy identification of fragmented nuclei. Separate analyses of breakage were carried out to obtain an estimate of the percentage of nuclei that showed fragmentation. Chloroplast damage is extremely rare and the few free nuclear DNA particles which are visible by fluorescence techniques do not adsorb to chloroplast surfaces. Care was also taken to determine the error introduced by the inclusion of the estimated percentage of broken nuclei in the chloroplast fraction. Details of this are given in the Discussion.

Extraction and Assay of DNA

DNA was measured by the diphenylamine reaction (2). For this colorimetric procedure to be reproducible, it was necessary to first remove interfering pigments by extraction with 80% acetone which simultaneously precipitated the DNA. The precipitate containing DNA was resuspended in 80% acetone, allowed to stand for 1 h in the dark, then centrifuged and washed with this reagent in this manner until the precipitate was completely free of green color. After the final wash with 80% acetone, the procedure used for extracting DNA from the precipitate was similar to that of Ogur and Rosen (7). After draining off the few drops of remaining 80% acetone, the pellet was thoroughly resuspended in 5 ml of ice-cold 0.2 N perchloric acid (PCA) using a glass rod. The suspension was spun at 7,700 *g* in the cold for 5 min. The supernate was discarded. The pellet was resuspended and washed two more times in 5 ml of cold PCA. The final pellet from these washings was resuspended in 0.3 ml of 0.5 N PCA and heated for 20 min at 70°C. After cooling, 0.6 ml of diphenylamine reagent (2) was added to the suspension which remained at room temperature for 18 h. The suspension was filtered and the absorption spectrum of the filtrate between 400 and 800 nm was recorded by a Cary Model 15 Spectrophotometer (Cary Instruments, Applied Physics Corp., Monrovia, Calif.). Standard curves for DNA concentration were constructed for each experiment by using known amounts of highly polymerized calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) which were also heated to 70°C in 0.3 ml of 0.5 N PCA for 20 min and cooled before addition of the diphenylamine reagent. Although the absorption maximum was consistently found to be between 590 and 596 nm, the convention was followed of making comparisons at 600 nm. With pure DNA, absorption minima were found at 515 nm (OD 594/515 = 1.6–1.7). All values reported in this paper for leaf DNA quantities were obtained from solutions whose absorption spectra matched those of the color of diphenylamine reagent produced by reaction with pure DNA. Analysis of our standard curve showed that in the range of 2 μg the standard error of the estimate was ± 0.14 μg.

Chloroplasts and Nuclei Counts

To a 50-μl aliquot of sample was added 25 μl of a 0.1 mg/ml acridine orange solution and 25 μl of homogenization medium. 10 μl of the mixture was placed in each of the two chambers of a Spencer Bright-Line haemocytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). With a 25× Neofluar phase objective, chloroplasts contained in 80 out of 4,000 0.5-mm squares were counted in duplicate with reproducibility within 10% as in erythrocyte counting. The absolute number of chloroplasts in the aliquot was calculated according to the equation: number chloroplasts/μl = chloroplasts counted × dilution × 4,000/80. There were

many fewer nuclei per aliquot, and they were counted in duplicate in 9 out of 10 1-mm squares, as in the case of counting leukocytes. The nuclei were sometimes concealed by being covered by chloroplasts. Greater accuracy was achieved by making the counts with a combination of low intensity tungsten light (to allow visualization of the lines demarking the squares of the chambers) and mercury light, which caused the nuclei, stained with acridine orange, to fluoresce brilliantly. The absolute number of nuclei in the aliquot was calculated by the formula: number of nuclei/μl = nuclei counted $\times$ dilution $\times$ 10/9.

Microscopy

Microscopic examinations were made with the same equipment described by James and Jope (5).

RESULTS AND DISCUSSION

Altering the Ratio of Chloroplasts to Nuclei in Leaf Homogenates

Attempts were made without success to significantly alter the ratio of nuclei to chloroplasts in cell-free homogenates by very low speed centrifugation. However, it was found that the ratio could be changed by allowing the organelles to settle under the influence of gravity. 2 ml of 0.5% agar made a flat bottom surface in 5-cm diameter petri dishes. The dishes were then positioned on a level piece of aluminum placed on ice. 5 ml of cell-free homogenate, which was gently but thoroughly mixed, was layered over the solidified agar surface of each petri dish. This created a vertical path length of 0.6 cm, from the top of the fluid to the surface of the agar, for the organelles to traverse during settling. After allowing time for settling, the petri dishes were very gradually tilted by a mechanical device to 45°. Then, with the aid of a cathetometer, a pipette was slowly lowered into the fluid to a position where the top 2 ml (hereafter referred to as the upper phase) could be slowly withdrawn without mixing with the lower 3 ml. After returning the petri dish to the horizontal, the small green layer which had collected on the agar surface was gently stirred until it became completely mixed with the lower 3 ml of fluid (hereafter referred to as the lower phase) which was then withdrawn from the petri dish.

An experiment was performed to test the rate at which chloroplasts and nuclei were removed by 1 *g* from the upper phase of cell-free homogenates. 25 ml of cell-free homogenate was prepared and a 2-ml aliquot reserved as a control. Three 5-ml aliquots were layered on agar in petri dishes which were allowed to stand on a level surface for 15, 30, and 60 min before the upper phase was withdrawn as described. Duplicate 50-μl aliquots were removed from the control and the three other samples and used to count the absolute numbers of nuclei and chloroplasts. The remainder of the samples were made to 80% acetone to extract the chlorophyll which was measured by the method of Arnon (1). The results for counting together with chlorophyll determinations are shown in Fig. 1. It can be seen that a significant change in the ratio of nuclei to chloroplasts (1/214 compared with 1/900) occurred within 1 h without a great change in chlorophyll. The greatest change in chlorophyll concentration of the homogenate occurred in the first 15-min interval. Evidently, this change was associated with some chloroplasts settling at a rate at which nuclei were settling. The chlorophyll concentration curve thereafter approached an equilibrium as time of settling was extended. Even after 1 h, only 18% of the chloroplasts contained in the original cell-free homogenate had settled out of the upper phase. But, in the meantime, an appreciable change in the nuclei/chloroplast ratio of the upper phase had occurred after 30 min of settling (1/400) and the ratio changed even more (1/900) after 60 min. Thus, it was evident that this simple technique was producing the desired result of altering the nuclei/chloroplast ratio without greatly changing the number of chloroplasts remaining in the homogenate. Additional advantages of this technique were: (*a*) the absence of clumping which occurs when mixtures of organelles are subjected to even low-speed preparative spins; (*b*) elimination of damage to organelles which occurs during resuspension of pellets.

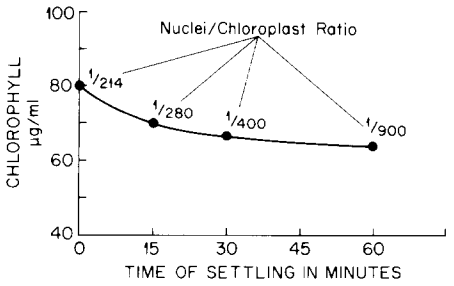

FIGURE 1 Comparison of rate of removal of chloroplasts from the upper portion of a cell-free leaf homogenate vs. change in ratio of nuclei to chloroplasts as a function of time of settling of the homogenate.

Reliability of the Diphenylamine Assay for Measuring Total DNA in Leaf Homogenates

Preliminary analyses indicated that a homogenate from mature *N. excelsior* leaves would contain about 5 μg DNA/ml. This meant that it would be desirable for accuracy to use at least 2 ml of homogenate per analysis. But it was found that >1 ml of homogenate produced spurious colors. Instead of a peak near 600 nm, there was frequently observed a continuous increase in absorbance from 600 down to 500 nm. However, this difficulty was eliminated by spinning the homogenate at 7,700 *g* for 10 min to pellet all chlorophyll-containing objects and discarding the yellow supernate. After resuspending the green pellet in 80% acetone and removing the chlorophyll as described in Materials and Methods, the extracted DNA produced the proper absorption spectrum shown in Fig. 2, compared with the continuous spectrum obtained when the 80% acetone-precipitable material in the supernate was analyzed in the same way.

As a further test of the reliability of the assay for DNA, an internal standard was employed. 3–20 μg of highly-polymerized calf thymus DNA dissolved in standard saline citrate were added to the 7,700-*g* pellet of cell-free homogenate, the pellet having first been resuspended in a volume of homogenizing medium equal to the original volume of homogenate. The resuspended organelles plus added DNA were made to 80% acetone, and the acetone-precipitable material was analyzed for DNA as described. Typical results are shown in Fig. 3. In this experiment, 6 μg of DNA was added to 1 ml of the resuspended organelle fraction from 1 ml of cell-free homogenate. The results show that 96% of the calf-thymus DNA was recovered. Therefore, we were confident that the analysis for total DNA in chloroplasts plus nuclei was reliable.

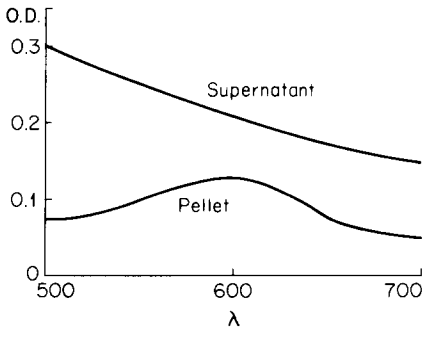

FIGURE 2 Colorimetric reactions produced by diphenylamine reacting with constituents contained in a cell-free leaf homogenate.

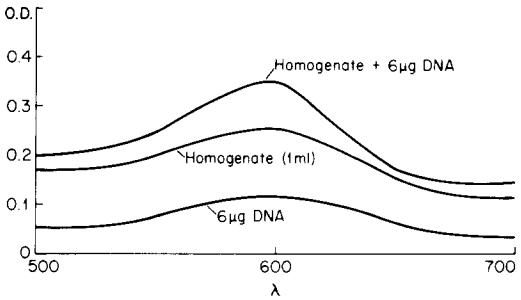

FIGURE 3 Recovery of calf-thymus DNA added to a cell-free leaf homogenate as measured by colorimetry after reaction with diphenylamine.

The possibility was also considered that DNA might in some curious way preferentially leak out of nuclei into a supernatant solution at a faster rate than out of chloroplasts. An experiment was performed using 0.5-ml aliquots of cell-free homogenates. One aliquot was precipitated directly after being made to 80% acetone. After removing the precipitate by centrifugation and washing it several times with 80% acetone, the final precipitate was analyzed for its DNA content. The amount of homogenate had been kept low enough to prevent spurious colors from interfering with the diphenylamine reagent. The second aliquot was centrifuged, and the pellet of organelles was resuspended in 80% acetone, washed, and the precipitate was analyzed for DNA. There was no difference in the DNA content of the two aliquots, permitting the conclusion that no significant amount of DNA remained in the supernate after centrifuging out the chlorophyll-containing organelles of the cell-free homogenate.

Total DNA Content of Cell-free Homogenates in Relation to Nuclei/Chloroplast Ratio and Size of Leaves

We have found that the average number of chloroplasts as well as the average diameter of the chloroplasts increases as the volume of the palisade cell increases in *N. excelsior* leaves. This result conforms to those found for other species of plants by Honda et al. (4). According to James

and Jope (5), the fluorescence produced in chloroplasts as a consequence of the binding of the dye DAPI to DNA gave the impression that the amount of chloroplast DNA was proportional to the area of the chloroplasts. Accordingly, experiments were performed to determine the DNA content of chloroplasts and nuclei in cell-free homogenates obtained from three sizes of *N. excelsior* leaves whose average chloroplast areas were different. In each case, 30 ml of cell-free homogenate was prepared from 15 g of leaf laminal tissue. Four 5-ml aliquots of each cell-free homogenate were allowed to settle for 1 h; the top phases were removed and combined, as was also done for the bottom phases. The absolute numbers of nuclei and chloroplasts in aliquots of the homogenate, top, and bottom phases were counted and the total DNA content of the three fractions was determined. The results are compiled in Table I. By knowing the absolute numbers of chloroplasts and nuclei present and the total amount of DNA in each sample, it was possible to set up simultaneous equations to solve for the amount of nuclear DNA/nucleus and chloroplast DNA/chloroplast. For example, in the case of the 30 × 15-cm leaves, let x = nuclear DNA/nucleus and y = chloroplast DNA/chloroplast. Using the numbers italicized in Table I, the DNA in the top phase is distributed between nuclei and chloroplasts according to the equation:

$$0.2 \times 10^{6}x + 1.1 \times 10^{8}y = 18\ \mu g\ DNA, \text{ or}$$
$$y = (16.4 \times 10^{-8} - 0.18 \times 10^{-2}x).$$

The distribution of DNA in the bottom phase is:

$$1.5 \times 10^{6}x + 2.7 \times 10^{8}y = 59\ \mu g\ DNA, \text{ or}$$
$$1.5 \times 10^{6}x + [2.7 \times 10^{8}\ (16.4 \times 10^{-8} - 0.18 \times 10^{-2}x)]$$
$$= 59\ \mu g\ DNA \text{ and } x = 14.68 \times 10^{-6}\ \mu g\ DNA/nucleus.$$

Knowing x, y calculates to be 0.14×10^{-6} μg DNA/chloroplasts.

Since the unfractionated cell-free homogenate contained 221 chloroplasts for each nucleus, it is evident that these large leaves contained ~2.0 times more chloroplast DNA than nuclear DNA. With medium size leaves, similar calculations using the data in Table I showed the chloroplast DNA to be about equal in amount to the nuclear DNA. In the smaller leaves, one-third of the total leaf DNA was contained in chloroplasts. By extrapolation of the data in Table I, to obtain a ratio of 90 parts nuclear DNA to 10 parts chloroplast DNA, as tradition would have it, would probably necessitate extracting the organelles from leaves of about a centimeter or less in length.

James and Jope (5), utilizing the fluorescent dye DAPI, presented data suggesting that chloroplasts contain much greater amounts of DNA per chloroplast than previously reported (6). Thus, the results in this paper support that finding. In regard to the DNA content per nucleus, our values of 15 × 10^{-12} g per nucleus for large leaves and 5 × 10^{-12} g per medium and 10 × 10^{-12} g for small leaves are in the same range as values for DNA content per nucleus reported by Hamilton et al. (3). However, our values for chloroplast DNA per chloroplast are a magnitude higher than what had been previously reported. Earlier values ranged from 0.2 to 1.0 × 10^{-14} g per chloroplast. Our values were 0.08 × 10^{-12} g for chloroplasts in small leaves and up to 0.14 × 10^{-12} g for chloroplasts isolated from large leaves.

Since these values differ from those previously reported by such a large margin, it is necessary to consider the error that might be introduced from various sources. As stated in Materials and Methods, separate experiments were carried out to evaluate the contribution of broken and distorted nuclei to the value for DNA per chloroplast. This was done by counting the number of broken nuclei per total number of nuclei in any one homogenate. For example, in an extreme sample there were 45

TABLE I

Number of Nuclei and Chloroplasts and Total Amount of DNA in Cell-Free Homogenates as a Function of Leaf Size Before and After Fractionation of Organelles

	μg DNA			Nuclei × 10^{6}			No. Chloroplasts × 10^{8}		
Leaf size	Homogenate	Top	Bottom	H	T	B	H	T	B
14 × 6 cm	145 (1/99)	28 (1/37)	99 (1/90)	6.9	1.0	5.3	6.9	2.4	4.7
21 × 11 cm	65 (1/159)	11 (1/471)	47 (1/161)	2.6	0.3	2.2	4.1	1.3	3.6
30 × 15 cm	80 (*1/221*)	*18* (1/509)	*59* (1/188)	2.1	*0.2*	*1.5*	4.6	*1.1*	*2.7*

Quantities in parentheses indicate ratio of nuclei to chloroplasts. Italicized numbers have been used for a calculation in the text.

broken nuclei per 249 nuclei (i.e., 18%). If the DNA in each nucleus is the equivalent of the DNA in 100 chloroplasts, then in a sample starting with 100 nuclei there will be enough broken nuclear DNA to equal 1,800 chloroplasts. Since the ratio of nuclei to chloroplasts was 1:427 in the homogenate, the error introduced by broken nuclei would be 1,800/42,700 or 4.2% of the chloroplast value. This is a maximum estimate since a majority of these nuclear fragments are very large and would sediment with the nuclei. The possibility that the error is this large can be argued against on the grounds that the determination of soluble DNA which does not come down with the organelles proved to be exceedingly low.

The values given for the DNA per nucleus are, of course, average values. The amount of DNA per nucleus can vary as a result of many factors. For example, it can vary through the cell cycle (cells in G_2 having twice the amount of DNA as cells in G_1), or it can vary as a result of polyploidy. Careful observations and counts of cells and chloroplasts in expanding leaf tissues have led us to the conclusion that the increase in leaf area is the result of increased cell size and not continuous division of a population of cells initially present. This excludes variability related to cell cycle. Within the cells, the chloroplasts are continuously increasing in size and number.

One event which could distort our estimates of the amount of DNA per organelle would be the presence of a subpopulation of highly polyploid nuclei within our sample, especially if, for some reason, these nuclei sedimented differently than normal (2N) nuclei. Samples from each homogenate were stained with the DNA-specific dye DAPI and examined by fluorescence microscopy. There was no detectable difference within the nuclear population, of any homogenate, in terms of the amount of DNA fluorescence per nucleus. The uniform intensity of the nuclear fluorescence suggests the absence of polyploidy.

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