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Relations between the plastid gene dosage and the levels of 16S rRNA and rbcL gene transcripts during amyloplast to chloroplast change in mixotrophic spinach cell suspensions

Pierre Aguettaz, Patrick Seyer, Hélène Pesey & Anne-Marie Lescure
Laboratoire de Biologie Moléculaire Végétale, CNRS UA 1178, Université de Grenoble 1, BP 68, F-38402 Saint Martin d'Hères Cédex, France

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Summary

Spinach cell suspension cultures maintained in photomixotrophic conditions exhibit plastids which undergo cyclic morphological transformations along a growth cycle. Ultrastructural studies show that the green chloroplasts present at the initial stage differentiate into amyloplasts during the subsequent log phase and then return to chloroplasts in stationary phase. The changes of the levels of plastid DNA (pt DNA) per cell have been determined along the growth cycle, as a percentage of total DNA by hybridization of definite amounts of total DNA to a radioactive probe of cloned pt DNA. The number of pt DNA copies have been estimated to 1125 per cell at the maximum of amyloplast development and to 5940 copies per cell at the maximum of chloroplast differentiation. Hybridizations of defined amounts of total cellular RNA to labelled probes of the 16S rDNA and of the rbcL gene allowed estimations of the variations of the corresponding cellular RNA pools. These variations are well correlated with the changes of the ptDNA cellular levels. These results show that the ptDNA gene dosage plays a central role in the regulation of the plastid transcript levels in this system.

Introduction

Plastids in higher plants are submitted to various processes of differentiation upon environmental changes such as light or nutrient supplies (14, 22). These morphological changes imply noteworthy qualitative and quantitative alterations of the plastid protein contents. Such changes, which are quite obviously tightly controlled by the cell, raise the question of the molecular level at which the regulation occurs. Most of the data reported on that field deal with the light-induced transformation of etioplasts to chloroplasts (for review see 24).

In heterotrophic tobacco cell suspension cultures, it has been shown that plastids undergo definite morphological changes along a growth cycle, under continuous light (19). Ultrastructural studies have shown that the chloroplasts present in the cells at ,the transfer time, are rapidly transformed into amyloplasts during the cell growth period and then return to green plastids at the end of the growth cycle. Nothing is known about the plastome replication and the related gene transcription during these morphological changes.

In the present work we use a spinach cell suspension which presents similar plastid alterations during a cell growth cycle. Our goal was first to measure the contents of plastid DNA (ptDNA) per cell along the cycle. Then, we were interested to know whether these plastid structural changes were correlated to alterations of the plastome transcription, especially whether the greening and chloroplast differentiation observed in stationary phase were correlated with a stimulation of the transcription of chloroplast genes. To respond to this question the variations of the sizes of plastid transcript pools were measured for two genes: the 16S rDNA and the rbcL gene (encoding for the large subunit of ribulose bisphosphate carboxylase, LSU).

Materials and methods

Cell culture conditions

The spinach strain G20 used in this work has been provided by Dr C. C. Dalton (B.P. Research Center, U.K.). The cell cultures were performed in erlenmeyer flasks as previously described (8): the gas exchanges with the atmosphere were limited by a tight closure of the flasks with a polythene membrane (2) and a constant CO_2 concentration of 5% was maintained by addition of a carbonate-bicarbonate buffer in a central tubule. For the maintenance, cells were transferred every 14 days into fresh medium containing 5 g · l^{-1} glucose.

For the experiments described here the initial glucose concentration in the medium was increased from 5 to 10 g · l^{-1}.

Growth and chlorophyll measurements

Growth was measured by numeration of cell number (2). Chlorophyll contents were determined according to (21), using the equations of Vernon (26).

Purification of spinach cell nucleic acids

For the total cellular DNA, 2 g of drained cells were ground to a powder in liquid nitrogen and thawed into five volumes of lysis buffer containing 0.15 M NaCl, 2% (w/v) SDS, 2% (w/v) sodium-N-lauroyl sarcosine, 10 mM EDTA and 50 mM Tris HCl pH 8.0. The homogenate was then extracted with phenol, twice with chloroform and the nucleic acids concentrated by precipitation with ethanol. After centrifugation, the pellet was washed twice with 70% ethanol, slightly dried, and resuspended in TE buffer (12). After 1 h incubation at 37 °C, with 100 μg · ml^{-1} RNAse I, followed by a further incubation with 100 μg · ml^{-1} proteinase K, the solution was extracted with phenol and chloroform as described above and nucleic acids were ethanol precipitated. The pellet was washed twice with 70% ethanol, dried and dissolved in 2.5 ml TE buffer.

Spinach leaves ptDNA purification was performed according to (17). Total cellular RNA was extracted from frozen cells as described in (10). DNA and soluble RNA were eliminated by two successive differential solubilisations in the presence of 2 M LiCl. Electrophoretic analyses have shown that DNA is completely eliminated by this procedure.

Concentrations of DNA and RNA were determined spectrophotometrically assuming 1 OD_{260} equal to 50 μg · ml^{-1} for DNA and to 40 μg · ml^{-1} for RNA.

Isolation and labelling of cloned ptDNA fragments

The recombinant pBR325 plasmid pWH4031 E2 contains a 1750 bp EcoR 1 insert. It comprises 1350 bp of the coding region for the LSU and 400 bp upstream (27). This clone was a gift of Professor R. G. Herrmann (Botanisches Institute der Universität München). The recombinant pBR322 plasmid, psoc B10 containing the structural gene for the 16S rRNA was prepared in our laboratory (1).

Plasmid DNA was hydrolysed with appropriate enzymes and the inserted fragments separated from the vector by electrophoresis on agarose gels. Selected fragments were electroeluted and the DNA purified from the gel according to (12). DNA fragments were radiolabelled with [^{32}P]dCTP using the nick translation kit of Amersham, following the supplier recommendations. Specific activities ranging from 10^6 to 10^7 cpm per μg DNA were obtained.

Hybridization experiments

Spinach cellular DNA or RNA were blotted onto nitrocellulose filters BA 85 (Schleicher and Schull), according to the technical procedures of the supplier. Baked filters were introduced into a 20 ml vial and prehybridized for 20 h at 42 °C in a buffer containing 5 × SSC (1 × SSC consists of 0.15 M NaCl, 0.015 M Na citrate), 50% (v/v) deionised formamide, 200 μg · ml^{-1} sonicated heat denatured thymus DNA and 5 × Denhardt solution (1 × Denhardt consists of 0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% bovine serum albumin).

The prehybridization medium was removed and replaced by hybridization buffer identical to the foregoing except that Denhart solution and thymus DNA concentration were reduced five times. The appropriate nick translated probes were denatured at 100 °C for 10 min, cooled rapidly to 0 °C and ad-

ded to the hybridization buffer. Hybridization was allowed to proceed for at least 48 h at 42 °C. Filters were washed twice in 2 × SSC at room temperature for 30 min followed by two 30 min washes in 2 × SSC, 1% SDS (w/v) at 60 °C and finally two washes in 0.1 × SSC at room temperature. Filters were dried and counted in 10 ml of scintillation fluid.

Electronmicroscopy

Electronmicroscopy was performed according to (7).

Results

Plastid differentiation during a cell growth cycle

A typical growth curve of a spinach cell suspension is presented in Fig. 1. Cells were growing rapidly during six days with a generation time of about three days. A residual growth, proceeding at a very slow rate, was observed from day 6 to day 14, which corresponded to a pseudo-stationary phase.

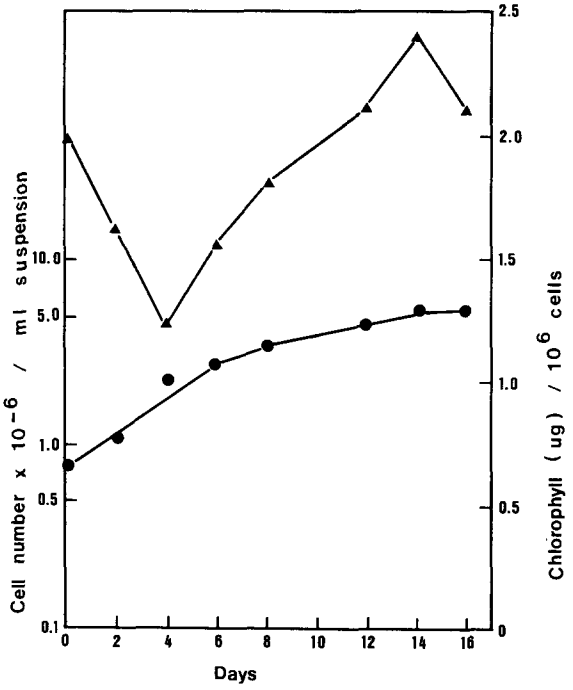

Fig. 1. Growth and chlorophyll contents of spinach cells during a culture cycle. ●——● cell number per ml suspension. ▲——▲ chlorophyll (μg per 10^6 cells).

The chlorophyll contents per cell were found to decrease during the first four days of growth and then to increase during the end of the exponential phase and the pseudo-stationary phase (Fig. 1).

After 14 days the number of cells and the chlorophyll contents fell in these culture conditions which did not allow a total autotrophic growth (8).

Examination of ultrastructures (Fig. 2) shows that plastids underwent a series of morphological changes during the cell growth cycle. The initial stage (Fig. 2A) was characterized by chloroplasts with slightly stacked lamellae. During the cell growth period, thylakoids disintegrated and plastids stored large starch grains (Fig. 2B and 2C). During the pseudo-stationary phase (Fig. 2D and 2E) starch disappeared and the lamellar system differentiated. The residual growth observed from day 8 to day 14 probably results from the metabolism of starch and from a low photosynthesis which has been shown to be efficient in these mixotrophic conditions (8).

Variations of plastid DNA contents per cell during a growth cycle

The ptDNA contents per cell were determined by hybridization of constant amounts of total cellular DNA (20 μg) extracted from the cells at different periods of the growth cycle to a radioactive DNA fragment coding for the LSU. The use of this probe supposes that in spinach the rbcL gene is contained only in the chloroplast genome. It has been shown by Lonsdale *et al.* (11) that in maize the mitochondrial DNA contains a sequence homologous to the rbcL gene. However, Stern and Palmer (20) have extended these experiments to other species of angiosperms. Their results clearly show that only a very weak hybridization is observed between spinach mitochondrial DNA and a ptDNA fragment containing the psbA and rbcL genes. According to Lonsdale (personal communication) the spinach mitochondrial DNA does not contain the rbcL gene.

The results of the hybridizations are reported in Table 1. The hybridized radioactivity was found to decrease during the first six days, which corresponded to the cell growth period, where amyloplasts became abundant. Then the hybri-

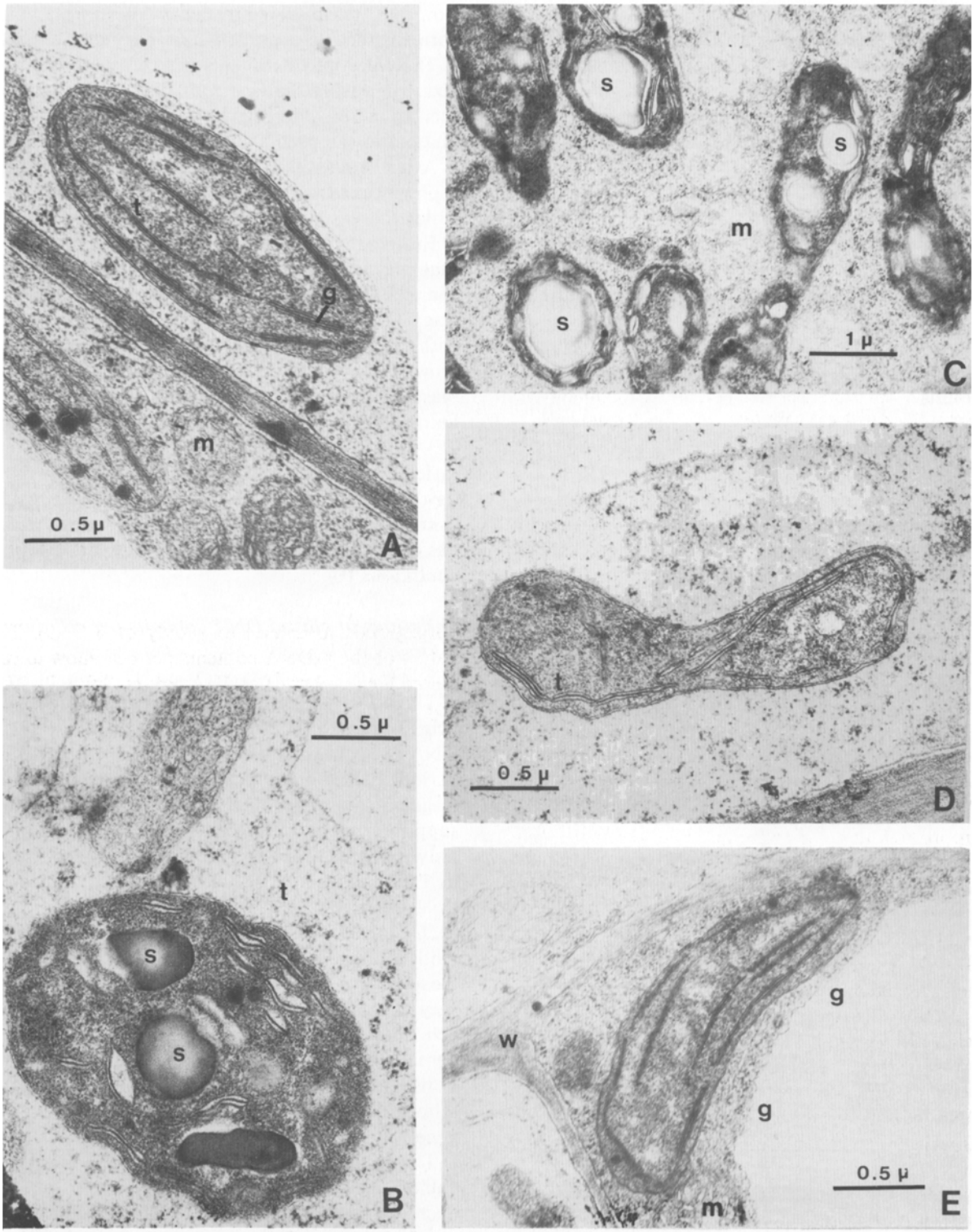

Fig. 2. **Ultrastructural changes** of plastids during a culture cycle A: Inoculum; **B: day 4; C: day 6; D: day 8; E: day 14.** g = grana; t = thylakoids; m = mitochondria; s = starch; w = cell wall.

Table 1. Evolution of the percentage of ptDNA versus total cellular DNA during the growth cycle.

Culture day	cpm × 10^{-3}/ 20 μg total DNA[a]	ptDNA μg/20 μg total DNA[b]	pt DNA % total DNA
0	16	1.74	8.7
2	14	1.43	7.1
4	11	1.20	6.0
6	6	0.68	3.4
8	17	1.78	8.9
12	25	2.60	13.0
14	33	3.40	17.0
16	32	3.24	16.2

[a] Each value corresponds to the average of triplicate hybridization measurements with 20 μg of DNA extracted at the different times of culture. For each point, no experimental value deviated more than 12.5% from the average value.

[b] The amount of ptDNA contained in 20 μg total DNA has been calculated with the reference curve (Figure 3). The reference curve and the hybridization experiments have been performed at the same time, with the same nick translated LSU probe: the radioactivity was the same for all the measurements.

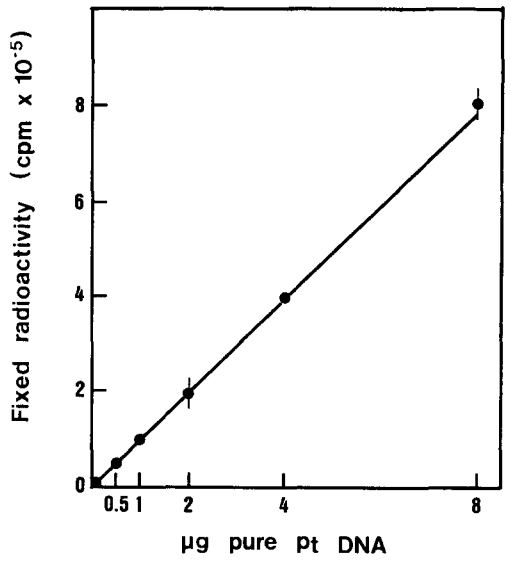

Fig. 3. Hybridization of the rbcL gene radioactive probe with definite amounts of highly purified ptDNA. Purified ptDNA was extracted from leaf chloroplasts (15). Each value is the average of triplicate measurements. Error bars have been reported for each point.

dized radioactivity increased during the stationary phase (day 6 to day 14) when chloroplasts differentiated.

In order to establish a definite relation between the fixed radioactivity and the amounts of ptDNA, increasing amounts of highly purified plastid DNA were blotted onto nitrocellulose filters and hybridized to the labelled probe. Figure 3 shows that the proportionality was respected from 0.5 to 8 μg of ptDNA. Using this reference curve, it is possible to correlate the radioactive counts with the actual ptDNA contents into the 20 μg of cellular DNA and to estimate the percentage of ptDNA versus total cellular DNA for each time (Table 1).

To estimate the ptDNA contents per cell, it was necessary to know the absolute DNA amounts per cell at each step of the growth cycle. Total DNA was purified from a determined number of cells as described in Materials and Methods. The total DNA contents per cell measured at the different times are reported in Table 2. Using the percentages of ptDNA versus total DNA (Table 1), it is possible to calculate the nuclear and ptDNA contents per cell for each point. The nuclear DNA cellular content does not change significantly during the growth cycle. The average value of 5,3 pg per cell corresponds to a level of ploidy of 4 C (9). The values of the ptDNA contents per cell allow to calculate the number of ptDNA copies (Table 2). This number decreases from 3190 to 1125 from day 0 to day 6 and it raises up to 5940 copies at day 14.

This experiment has been repeated twice: similar

Table 2. Evaluation of the ptDNA copies per cell during a growth cycle.

Culture day	Total DNA pg/cell[a]	Nuclear DNA pg/cell[b]	ptDNA pg/cell	ptDNA copies/cell[c]
0	5.58	5.37	0.51	3190
2	6.70	6.23	0.47	2940
4	5.98	5.63	0.35	2190
6	5.43	5-25	0.18	1125
8	5.80	5.29	0.51	3190
12	6.38	5.55	0.83	5190
14	5.60	4.65	0.95	5940
16	5.60	4.69	0.91	5690

[a] Each value corresponds to the average of triplicate extractions: no experimental value deviated more than 10% from the average value.

[b] The average of these values corresponds to 5.3 pg nuclear DNA per cell.

[c] Calculated assuming that the size of the spinach plastome is 1.6×10^{-16} g (5).

results were obtained, that is a decrease of ptDNA content per cell during the growth phase and an increase during the pseudo-stationary phase. Only the absolute values varied from one experiment to the other according to the initial value of the ptDNA contents at the time of the transfer.

Variation of the levels of 16S rRNA and of the mRNA for the LSU during a cell growth cycle

To determine the variations of the cellular level of plastid 16S rRNA, total cellular RNA extracted from cells along the growth cycle was hybridized to the radioactive 16S rDNA fragment. Two preliminary controls were performed first, the total cellular RNA was electrophoresed on a denaturing agarose gel in the presence of methyl mercuric hydroxyde (3) and transferred onto nitrocellulose (Northern). The 16S rDNA radioactive probe was found to hybridize only with the U.V. band corresponding to the 16S rRNA on the gel (result not shown). Secondly, increased amounts of a preparation of total cellular RNA were hybridized with the probe: a good proportionality between the hybridized radioactivity and the amounts of RNA was observed between 0.6 μg RNA (Fig. 4). Taking into account these results, total cellular RNA was extracted from the cells at the different times of the growth cycle and in each case, hybridization was performed with 5 μg RNA. The amounts of radioactivity hybridized at the different times are reported in Table 3.

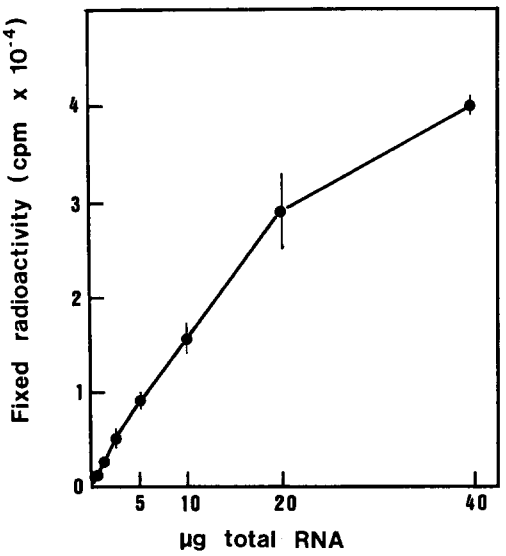

Fig. 4. Hybridization of the radioactive probe of the 16S rDNA with increased amounts of total cellular RNA. Each value is the average of triplicate measurements. Error bars have been reported for each point.

We have shown in a previous work with tobacco cell cultures (18) that the cellular RNA contents are not constant during a cell growth cycle, especially there is a burst of the RNA contents per cell just after the cell transfer. In order to correlate the amount of the hybridized radioactivity to the number of cells in our present experiments, the contents of the total RNA per cell have been established at the different times of the growth cycle. These contents are reported in Table 3: as in tobacco cells, a burst of the total cellular RNA contents was observed at day 2. These values allowed a direct relation to be established between the hybridized radioactivity and a definite number of cells at each time. The curve representing the alterations of the 16S rRNA per cell is given in Fig. 5 (curve II). The variations of the number of ptDNA copies per cell have been reported on the same figure (curve I). During the period of amyloplast formation (day 0 to day 6), the number of ptDNA copies per cell is divided by 2.8. The cellular level of 16S rRNA is also reduced but only by a factor 1.5. Then during the chloroplast differentiation (day 6 to day 14) the two levels increase, with an excess of the ptDNA contents versus the 16S rRNA.

The same procedure was used to determine the

Table 3. Variations of the 16S rRNA content per cell along the growth cycle.

Culture day	Labelled 16S rDNA (cpm × 10^{-3}) fixed tot 5 μg total RNA[a]	Total RNA (pg)/cell[b]	Labelled 16S rRNA cpm/10^2 cells
0	617	9.5	118
2	324	16.6	108
4	348	13.9	97
6	320	11.3	72
8	469	11.7	110
12	667	10.7	143
14	780	10.6	166
16	580	10.9	126

[a] Each value is the average of triplicate measurements. For each point, no experimental value deviated more than 15% from the average value.

[b] The statistical variations of the amount of total RNA extracted from 6 samples is ± 16% (with $P = 0.05$).

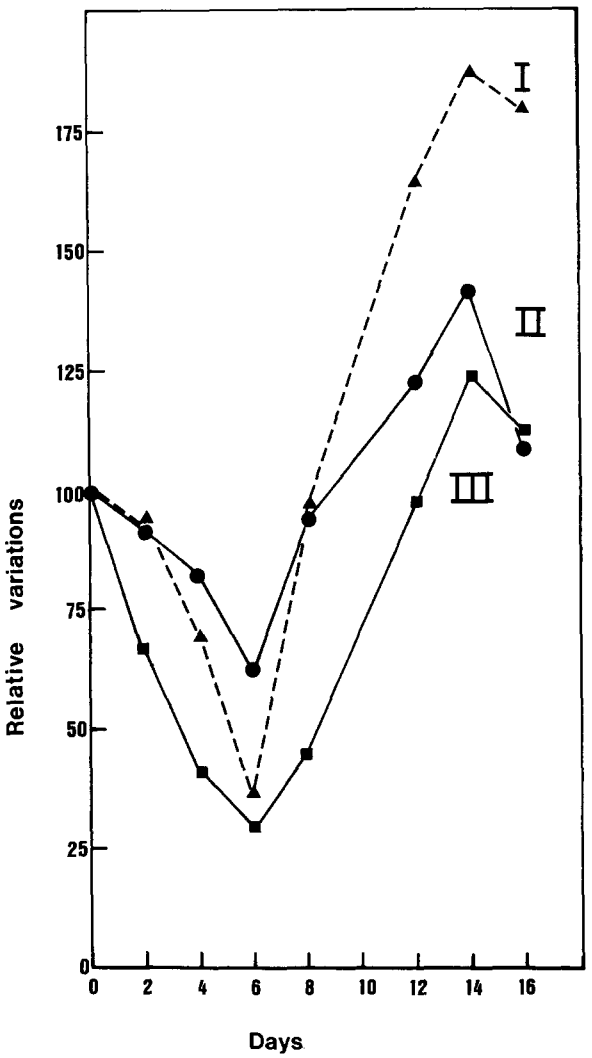

Fig. 5. Relative variations of the ptDNA contents per cell and of the cellular levels of transcripts from the rDNA and from the rbcL gene. ▲——▲ ptDNA; ●——● rRNA; ■——■ LSU mRNA. In order to allow a direct comparison, all the initial values at day 0 have been standardized by giving an arbitrary value of 100.

changes of the rbcL gene transcript contents along the growth cycle. In this case, the hybridizations were performed with the radioactive DNA fragment coding for the LSU. The curve representing these variations is reported in Fig. 5 (curve III). As the ptDNA and the 16S rRNA levels, the cellular level of the mRNA for the LSU decreases during the amyloplast formation (day 0 to day 6). In this case the decrease is slightly higher than that of the ptDNA. Then the mRNA for the LSU contents rise during the chloroplast formation (day 6 to day 14): an excess of the ptDNA levels versus the mRNA for the LSU is observed, as for the 16S rRNA.

Discussion

In a previous work (8), we have shown that spinach cell suspensions were able to develop chloroplasts in the presence of glucose only when exogenous supply of oxygen was suppressed: in these conditions, the consumption of glucose becomes dependent of the chloroplast development and of the resulting production of photosynthetic oxygen. In the present experiments, at the beginning of the cell growth cycle, the composition of the gas phase inside the flasks was that of the atmosphere and the culture medium contained 10 g · l^{-1} glucose. In these conditions, the chlorophyll contents of the cells decreased and chloroplasts were transformed into amyloplasts as shown by ultrastructural studies. Then, as the exchanges with the atmosphere were limited by a tight closure of the flasks, the amount of oxygen inside the flasks decreased during the growth cycle and as expected, chloroplasts differentiated again at the end of the culture. However, it is noteworthy that the thylakoid development in these spinach cell suspensions remains poor as well as the cellular chlorophyll content which is about 10-fold lower than that reported for spinach leaves (15).

Hybridizations of equal amounts of total cellular DNA with the radioactive DNA fragment containing the rbcL gene allowed the percentage of ptDNA to be estimated at the various steps of these plastid changes. This percentage decreased from 8.7% to 3.4% (3190 to 1125 DNA copies per cell) during the amyloplast formation (day 0 to day 6). Then at the end of the culture, the percentage of ptDNA rose to 17% (5940 ptDNA copies per cell) during chloroplast differentiation.

These data require some comments. First, it is noteworthy that the number of ptDNA copies is reduced by a factor 2.8 during the exponential cell growth, whereas the number of cells is multiplied by 4. On the contrary, the plastome copy number is multiplied by 5,3 at the end of the cycle when cell divisions are almost stopped. These results, which indicate that nuclear DNA and ptDNA replications

are not synchronized in these cultures, are in agreement with other reports: Heinhorst *et al.* (4), using different inhibitors of DNA synthesis, have found that ptDNA and nuclear DNA in tobacco cells were not necessarily coupled to one another. In a previous work, Tymms *et al.* (25) had also suggested that ptDNA replication could be independent of that of nuclear DNA in cultured spinach leaf discs.

In our experiments, the number of ptDNA copies in the green cells at the end of the growth cycle has been estimated to about 6000. This number is similar to that reported for the spinach leaves (15). Therefore, the low development of the thylakoid system in our spinach cell suspensions, when compared with that of leaves, does not appear to result from a reduction of the plastid genome contents in the cell suspension cultures.

Amyloplast ptDNA has been measured in mature potato tuber cells (16). Expressed as a percentage of the total DNA, the level of ptDNA found in tubers was 5.2%, while that found in leaves was 7.6%. Surprisingly, when the values were reported to plastids, tuber amyloplasts were found to contain ten times as much ptDNA as leaf chloroplasts. In our experiments with spinach cell suspensions, the number of plastids per cell is difficult to measure and has not been determined; therefore we cannot say if the decrease of ptDNA copies per cell observed during the amyloplast formation corresponds to a diminution of the number of plastids or to a decrease of the ptDNA contents inside the amyloplasts.

The hybridizations of defined amounts of total cellular RNA with labelled probes containing the 16S rDNA or the rbcL gene allowed the variations of the corresponding RNA pools to be estimated as the different steps of plastid differentiation along the culture cycle. In the case of the 16S rRNA, during the active growth phase (day 0 to day 6), the cellular level decreases with the ptDNA copy number. However, at day 6, the ratio of the two levels shows an excess of the 16S rRNA level versus the ptDNA. This excess might be due to a stimulation of 16S rRNA gene transcription at the end of the active growth phase. Then, during the stationary phase, the two levels increase with a relative excess of ptDNA versus 16S rRNA: therefore, during amyloplast to chloroplast differentiation (day 6 to day 16) no stimulation of the 16S rRNA gene transcription appears to occur and only the gene dosage would be responsible for the rRNA accumulation.

In the case of the mRNA for the LSU, the diminution of the cellular pool during the first 6 days is higher than that of the ptDNA. This higher decrease may result from a negative regulation of the transcription of this gene in amyloplasts or from an increased turnover of the mRNA for the LSU inside these organelles. Then, during the chloroplast differentiation, the accumulation of the mRNA for the LSU appeared to be correlated to the number of ptDNA copies. Such a direct relation between the gene dosage and the increased level of LSU mRNA has been reported in the case of the etioplast to chloroplast differentiation in pea (6, 13, 23).

Our present data show that the number of ptDNA copies per cell is able to vary considerably with the plastid differentiation steps along a cell growth cycle. In this system, the gene dosage appears to play a central role, although not exclusive, in the control of the plastid transcript levels, during the transformation of amyloplasts to chloroplasts.

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