Chloroplast replication and chloroplast DNA synthesis in spinach leaves

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(Communicated by R. Brown, F.R.S. – Received 17 October 1975)

Cultured leaf disks from young spinach leaves have been used to study chloroplast DNA synthesis in relation to chloroplast growth and replication. Autoradiographs prepared from thin sections show that labelling throughout disks is uniform. We consider that cultured leaf disks serve as a model system for studies of cell expansion and chloroplast replication during the development of spinach leaf cells.

Leaf disks from young spinach leaves were incubated continuously for 5 days in a medium containing \(^{3}\)H]thymidine. They were harvested daily and light microscope autoradiographs of separated cells prepared. In the expanding cells of disks all chloroplasts divide and the synthesis of chloroplast DNA as measured from grain counts was correlated with the rate of chloroplast formation. A doubling of chloroplast numbers was associated with a doubling of chloroplast silver grains, which is consistent with a duplication of chloroplast DNA during the chloroplast division cycle.

When disks were grown in green light, chloroplast replication but not chloroplast growth ceased in 3–4 days. The large chloroplasts of disks grown in green light appeared locked in a dumb-bell shaped configuration. Light microscope autoradiographs have established that these chloroplasts can synthesize DNA. These chloroplasts divided when the disks were exposed to high intensity white light and again it appears that all chloroplasts in the one cell divide. The chloroplasts of disks grown in green light for periods up to 9 days became uneven in size although all were dumb-bell shaped. We suggest that the size differences represent ploidy classes.

Chloroplast replication in dicotyledons has been discussed in relation to the division and growth of leaf cells.

Introduction

Studies employing light microscope autoradiography following the incorporation of \(^{3}\)H]thymidine (\(^{3}\)HTdR) have established that an active synthesis of both nuclear and chloroplast DNA (cDNA) occurs during the expansion of leaf cells of spinach (Rose, Cran & Possingham 1974, 1975). In these experiments it was...
found that in young cells, grown in white light, a 24 h pulse of thymidine labels all chloroplasts at the time of their division. Pulse-chase observations indicated that in expanding cells, virtually all chloroplasts divide rather than a small sub-population, as had been previously suggested (Honda, Hongladarom-Honda, Kwanyuen & Wildman 1971). The distribution of the label at the end of the chase in these experiments was consistent with semi-conservative replication of cDNA.

Other studies with spinach leaf disks have shown that there can be either replication of chloroplasts or alternatively chloroplast growth without an increase in chloroplast number per cell. The distinction between chloroplast division and enlargement depends on the combined effects of the intensity and quality of the light in which the disks are grown (Possingham & Smith 1972; Possingham 1973a, b; Possingham, Cran, Rose & Loveys 1975).

A number of other studies have been made of the relation between cDNA synthesis and chloroplast replication in higher plants, but in general have been limited by the absence of an experimental system where chloroplast replication is well defined and precursor incorporation into chloroplast DNA occurs readily. Wollgiehn & Mothes (1964) established, by using light microscope autoradiography, that young growing leaves, but not mature leaves, of *Nicotiana rustica* incorporate \(^{3}H\)TdR into plastid DNA. Mache, Rozier, Loiseaux & Vial (1973) have reported that when young, dark grown, maize leaves are transferred to white light, plastid number doubles in approximately 4 h, and DNA content measured by \(^{32}P\) incorporation per plastid increases sharply. They suggest that plastid DNA is synthesized just before and during plastid division. Somewhat similar results are available from experiments with cucumber hypocotyls which in contrast to leaves elongate in the dark. In this system, it has been reported that a wave of plastid replication and of \(^{3}H\)TdR incorporation into cDNA occurs some 12–16 h after hypocotyl excision (Kadouri & Atsmon 1974).

Evidence that the amounts of DNA per chloroplast can vary in the higher plant *Beta vulgaris*, comes from the work of Herrmann (1970), Herrmann & Kowallik (1970) and Kowallik & Herrmann (1972). Their studies indicate that the DNA of these chloroplasts is dispersed between a number of nucleoids and that the individual nucleoids can be polyploid to varying degrees. They have also shown that the amount of DNA per chloroplast is positively related to chloroplast size.

**Description of Plate 46**

*Figure 1.* Montage of three light microscope autoradiographs. Disks excised and incubated in the light for 24 h with \(^{3}H\)TdR. Tissue fixed in glutaraldehyde, post-fixed in osmium embedded in araldite; autoradiograph exposed for 55 days. (Magn. \(x\) 900.) Marking letters: thin arrow, labelled chloroplasts; black and white arrows, labelled and unlabelled nuclei respectively; p., palisade; s.m., spongy mesophyll.
Figure 1. For description see opposite.
Figure 2. For description see opposite.
Chloroplast replication and DNA synthesis

It is also of interest here that in Beta vulgaris there is a positive correlation between the number of plastids in a cell and the amount of DNA in the nucleus (Butterfass 1973). An explanation for this observation may come from the work of Honda et al. (1971) and from that of Boasson, Bonner & Laetsch (1972), who consider that plastid number per cell is closely dependent on cell size, which in turn is known to be influenced by the level of nuclear DNA per cell (Edwards & Endrizzi 1973; Machizuki & Sueoka 1955).

The purpose of the experiments reported here was to investigate the nature of the relation between cDNA synthesis and chloroplast growth and replication during the growth of spinach leaf cells. In these experiments cell and chloroplast development were manipulated by both light intensity and quality.

Experimental

(a) Uniformity of \([^3H]thymidine incorporation\)

In an earlier investigation on DNA synthesis during greening, nuclease treatments were used to establish the specificity of \([^3H]TdR\) incorporation into the DNA of separated cells (Rose et al. 1975). Initial experiments of this series were concerned with the uniformity with which \([^3H]TdR\) is incorporated into disk cells. Disks were excised and incubated for 24 h in \([^3H]TdR\) in the light prior to fixation in glutaraldehyde, post-fixation in osmium, and embedding in Araldite by previously described electron microscope methods (Cran & Possingham 1972a). Sections approximately 1 µm were cut from these blocks, attached to clean slides and autoradiographs prepared.

Figure 1, plate 46, is a montage made up from three autoradiographs. It shows a top to bottom cross section of a disk and indicates that virtually all chloroplasts in all cells become labelled but only about half the nuclei. These cross sections indicate that the palisade and spongy mesophyll cells of cultured disks are similar in size but differ in their orientation.

The variability in labelling across disks (side to side) was low. Labelling was not confined to the periphery of disks as would be expected if labelling was only in response to wounding.

(b) Continuous labelling of disks

Disks were taken from the base of young leaves of plants grown in nutrient culture for 14 days in a growth cabinet at a light intensity of 6.0 mW cm\(^{-2}\) s\(^{-1}\) (14 h day), and a temperature of 25–22 °C. They were cultured in the same

Description of Plate 47

Figures 2(a–e). Light microscope autoradiographs. On excision leaf disks placed in \([^3H]TdR\) in the light and harvests made on days 1, 2, 3, 4 and 5(a–e). Autoradiographs exposed for 5 days. (Magn. \(\times 800\).)
conditions for 5 days in 9 cm Petri dishes on a nutrient agar medium containing \([^3H]TdR\) 50 µCi ([\(6-^3H\)]thymidine, 27 Ci/mM, Radiochemical Centre, Amersham, U.K.) per 20 ml. At daily intervals three dishes from each of three dishes were taken for autoradiography.

Figure 2, plate 47, shows light microscope autoradiographs at the same magnification of cells from disks harvested on days 1–5. Figures 2a–e, plate 47, show

![Graphs](http://rspb.royalsocietypublishing.org/Downloaded from)

Figure 3. (a) Changes in chloroplast numbers per cell, with time, in disks cultured in the light (14 h day, 6.0 mW cm\(^{-2}\) s\(^{-1}\), 10 h night, temperature 25–22 °C). Each point is the mean of 10 replicate counts. (b) Change in total number of chloroplast grains per cell with time. Data from autoradiographs exposed for 5 days. (c) Change in average number of grains per chloroplast with time. (d) Relation between chloroplast grains per cell and chloroplast number per cell, in disks grown for 5 days in the light. The general pattern of cellular development with respect to changes in cell size and chloroplast number per cell. From this sequence of plates it is clear that at all harvests all chloroplasts become labelled and that chloroplast labelling parallels chloroplast formation.

Quantitative data from this experiment were assembled and are shown in figures 3a–d. For each harvest ten cells were counted for chloroplast number per cell, and ten chloroplasts from each of these latter cells were scored for the grain count. There was an exponential increase in chloroplast number per cell with time (figure 3a), while total number of chloroplast grains per cell showed a similar increase with time (figure 3b). Average grain numbers per chloroplast shown
in figure 3c were similar for all harvests with the exception of that for harvest 1, where a smaller proportion of the cDNA was labelled. Chloroplast grains per cell were directly related to chloroplast number per cell (figure 3d).

![Graphs showing cell area, chlorophyll per disk, chloroplast number per cell, and chloroplast area over days in culture.]

**Figure 4.** Effect of white light (●), 6.0 mW cm\(^{-2}\) s\(^{-1}\), green light (■), 0.6 mW cm\(^{-2}\) s\(^{-1}\) 525 nm, or darkness (▲), on (a) cell area (10\(^{-4}\) mm\(^2\)), (b) Chlorophyll per disk (μg), (c) chloroplast number per cell, (d) Chloroplast area (μm\(^2\)). Points for (a), (c) and (d), means of six replicates; and for (b), mean of 2.

(i) **Disks cultured in green light**

Previous work has shown that chloroplasts enlarge rather than divide when spinach leaf disks are grown for 14 days in low intensity (0.6 mW cm\(^{-2}\) s\(^{-1}\)) green light (Possingham 1973b; Possingham et al. 1975). In these experiments it was found that the large chloroplasts formed in green light will readily divide when exposed to high intensity white light.

Figures 4a–d shows the effect of low intensity green relative to high intensity white light and darkness on disk growth over a 9 day growing period. Values shown are averages of six replicate disks. Figures 4a, c indicate that both the chloroplast number per cell and the area of cells of disks grown in white light after three days begin to diverge from the corresponding values for disks grown in green light or darkness. By contrast, the chloroplasts of disks grown in low intensity green light are larger in area, relative to white light controls by day 3.
and are markedly so by day 4. The chlorophyll content of disks grown in green and white light were similar at all harvests, while that of disks grown in darkness did not increase above day 0 values.

(ii) Autoradiography
As the chloroplasts of disks grown in low intensity green light cease to replicate after 3–4 days, experiments were carried out to establish whether these chloroplasts can synthesize cDNA. Disks grown in continuous green light (0.6 mW cm⁻² s⁻¹) for 5 days were incubated with [³H]TdR for a further day in green light and light microscope autoradiographs prepared as before (Rose et al. 1975).

Figure 5a, plate 48, is an autoradiograph of a cell grown in green light showing there is incorporation of [³H]TdR into cDNA. Also many of the individual chloroplasts appear dumb-bell shaped, a configuration associated with chloroplasts undergoing binary fission.

(iii) Cytology
Sections 80 µm thick were cut from disks by using a sledge microtome. Living cells, as judged by the continuation of protoplasmic streaming, were viewed by interference contrast (Nomarski) microscopy. Figure 5b shows cells from a disk grown for 5 days in low intensity green light in which virtually all the chloroplasts have a dumb-bell shape. Figure 5c is of cells from a disk grown 5 days in green light and then illuminated for 24 h in high intensity white light. Over this period a marked increase in the area of the cells takes place and there is rapid formation of chloroplasts. When disks grown initially in green light are exposed for 12 h to white light, cells similar to that shown in figure 6a, plate 49, are found. In this cell all the chloroplasts are either dumb-bell shaped, or are grouped in pairs. It is suggested that the pairs of chloroplasts arise from the separation into two of dumb-bell shaped chloroplasts.

If disks are kept for longer periods (e.g. 9 days), in green light, little further chloroplast formation takes place. An example of this is shown in figure 6b, plate 49, and quantitative data is shown in figure 4c. However, the chloroplasts of disks grown for extended periods in green light maintain a dumb-bell shape.
Figure 5. For description see opposite.

(Facing p. 300)
Figure 6. For description see opposite.
but in many cases become uneven in size. Some chloroplasts are at least double the area of others. This is shown in figures 6b, c which are the same cell viewed by interference contrast and phase contrast microscopy respectively.

(d) The growth of spinach leaves and of cultured disks

The experiments reported here and those of a number of previous investigations have used cultured leaf disks to simulate developmental events in leaves (Possingham & Smith 1972). To justify this approach, comparisons have been made of the growth of 2 mm diameter disks cut from the base of 2 cm long leaves and cultured on agar, and of areas (2 mm in diameter on day 0) marked on the leaves with a felt tipped pen. At the end of a 7 day growing period, the cultured disks were compared with the areas marked on leaves. The relative positions of the marked areas on days 0 and 7 are shown in figure 7, plate 50, while table 1 provides data on the growth of cultured disks and of the marked areas of leaves. Individual cells in leaves and disks contain a similar total area of chloroplasts, as the leaf cells have fewer but larger chloroplasts than cultured disk cells (Cran & Possingham 1972a). The area of disk cells was almost twice that of leaf cells. The chlorophyll content of the areas marked on leaves was much higher than that of cultured disks and results from chlorophyll per chloroplast being lower in disks (Cran & Possingham 1974).

The data of table 1 indicates that between days 0 and 7 there is 10–20-fold increase in cell area, chloroplast number per cell and chlorophyll content per disk. The increase in chloroplast area over the same period is much less at 2–3-fold.

**Table 1. The Growth of Spinach Leaves and of Cultured Disks**

<table>
<thead>
<tr>
<th>day, treatment</th>
<th>fresh disk mass</th>
<th>chlorophyll</th>
<th>chloroplast number</th>
<th>cell area</th>
<th>chloroplast area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μg per disk</td>
<td>per cell</td>
<td>10⁻⁴ mm²</td>
<td>μm²</td>
</tr>
<tr>
<td>0 leaves†</td>
<td>0.4</td>
<td>1.1</td>
<td>18</td>
<td>4</td>
<td>8.5</td>
</tr>
<tr>
<td>7 leaves†</td>
<td>23</td>
<td>25.4</td>
<td>200</td>
<td>57</td>
<td>26.3</td>
</tr>
<tr>
<td>7 cultured disks‡</td>
<td>28</td>
<td>10.3</td>
<td>265</td>
<td>97</td>
<td>21.6</td>
</tr>
</tbody>
</table>

† Areas marked on leaves, 2 mm diam., day 0.
‡ Disks cultured on agar, initially 2 mm diam.

**Description of Plate 49**

Figure 6(a). Living section of a disk grown first for 5 days in green light and then for 12 h in white light. Section viewed by interference contrast microscopy. (Magn. x 1000.) Most chloroplasts in pairs or in various stages between dumb-bells and full separation.

(b) Some living cells from a disk grown for 9 days in (c) green light, viewed by interference contrast (b), or by phase contrast (c) microscopy. (Magn. x 1000.) Chloroplasts uneven in size but most in a dumb-bell configuration.
Discussion

Our earlier studies provided evidence of the specificity of \textsuperscript{3}H\textsuperscript{3}TdT incorporation into the cDNA of spinach leaf disks (Rose 	extit{et al.} 1975). The relatively uniform labelling within sections allowed us to use the cell separation technique for detailed studies of the developmental events occurring in expanding spinach leaf cells. These experiments also established that both wound and edge effects are small in spinach disks. It is also consistent with previous evidence that disk nuclei actively synthesize DNA in a situation where there is virtually no cell division (Rose 	extit{et al.} 1975).

The cells of cultured disks are similar in many respects to the cells of leaves, except that they are larger and their chloroplasts have a lower chlorophyll content. We consider that the qualitative changes associated with the expansion of disk cells are essentially similar to those which occur in leaves, and they can therefore serve as a model system for studies of the expansion phase of leaf development.

Data assembled in the continuous labelling experiment provide the clearest evidence so far that in developing leaf cells of spinach, the synthesis of cDNA parallels the rate of chloroplast formation. This is consistent with a doubling of cDNA during the chloroplast division cycle.

So far we have not been able to subdivide the sequence of chloroplast replication into specific cytological events. For example, we wondered whether there might be a period analogous to the S-phase of dividing cells during which cDNA synthesis takes place. We carried out shorter term labelling experiments (down to 3 h) on \textsuperscript{3}H\textsuperscript{3}TdT incorporation, and also did experiments on incorporation at different times of the day. In these experiments all chloroplasts were labelled when examined after a 6-7-fold increase in autoradiograph exposure time. Although it is distinctly probable that chloroplast replication is partially synchronous in disks cultured in regular light dark cycles, we doubt if all chloroplasts could have been labelled in our 3 h experiments if cDNA synthesis was confined to a discrete S-phase. Alternatively, it is possible that in immature, actively replicating chloroplasts, cDNA synthesis could be a continuous process keeping pace with membrane formation. This possibility receives support from recent work involving light and electron microscope autoradiography, which revealed an association between cDNA and the granal lamellar system; both appeared to be spirally arranged within the chloroplast (Rose & Possingham 1976a).

The possibility that cDNA could be continuously synthesized in immature replicating chloroplasts receives support from prokaryote bacterial systems whose nucleic acids have a number of features in common with chloroplasts and, dependant on growing conditions, bacteria such as \textit{Escherichia coli} can synthesize DNA for most of the cell cycle (Kirk 1972; Slater & Schaechter 1974).

There are a number of situations where cDNA synthesis is not obligatorily associated with chloroplast replication. In spinach disks grown in the dark for
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11–12 days, and in spinach epidermal cells where cDNA synthesis occurs, there is little chloroplast replication or growth (Rose et al. 1975). The reverse situation occurs, that of chloroplast replication in the absence of cDNA synthesis in expanding cells of tobacco treated with 5-fluorodeoxyuridine in the absence of cytokinin (Boasson & Laetsch 1969), and in *Acetabularia* (Woodcock & Bogorad 1970). Recently we have observed the same phenomena in γ-irradiated spinach cotyledons (Rose & Possingham 1976b).

In our experiments with green light, we have the situation where both cDNA synthesis and chloroplast growth occur in the virtual absence of chloroplast replication. However, the failure of chloroplasts grown in green light to divide is not due to their inability to synthesize cDNA. The dumb-bell shaped chloroplasts formed in green light will divide after 12–24 h of exposure to high intensity white light. Moreover the observations indicate that virtually all chloroplasts in the one cell can divide at the same time (figure 6a, plate 49). We consider this to be important independent evidence of the conclusion we came to from [3H]TdR pulse-chase experiments, where all chloroplasts in young cells were found to divide. The autoradiograph in figure 5a, plate 48, also provides additional evidence for cDNA segregation to daughter chloroplasts.

A striking feature of the chloroplasts formed in green light is their dumb-bell configuration. It seems that these chloroplasts complete some of the initial steps of division but become blocked partway through the process. Though few similarities exist between chloroplast division and cell division, both can block part-way through the process. For example, mitosis of cells is readily blocked by agents such as colchicine, high temperatures and irradiation which interfere with the formation of spindle fibres (Brown & Dyer 1972).

Ridley & Leech (1970) using both *Vicia faba* and spinach, and Kameya & Takahashi (1971) using *Nicotiana tabacum* have observed the division of isolated chloroplasts cultured in media containing Ficoll. Both groups report that chloroplast division figures were at a maximum some 70 h after isolation and that virtually no chloroplast growth took place *in vitro*. Preliminary experiments indicate that the dumb-bell shaped chloroplasts formed in green light divide *in vitro* when exposed to white light. These observations provide evidence that a single dividing chloroplast can separate into two daughter chloroplasts using mechanisms that exist within the plastid itself. The division of higher plant chloroplasts appears similar in some respects to cytokinesis in mammalian cells (Robbins & Gonatas 1964). Although chloroplasts have many features in common with prokaryotes, they mainly divide by constriction division and rarely by cross wall formation as occurs in bacteria (Cran & Possingham 1972b; Higgins & Shockman 1971).

Another important aspect of chloroplasts formed in green light is their unevenness in size. This is most marked in disks grown for longer periods (such as 9 days) in green light. Here chloroplasts many times larger than their neighbours can be found, and it is possible that these large chloroplasts are polyploid forms.
Our suggestions here are supported by the observations of Herrmann (1970) and Herrmann & Kowallik (1970), on Beta vulgaris chloroplasts.

In spinach leaves, as in many other dicotyledons, cell division and cell expansion proceed concomitantly rather than sequentially. Division continues in the expanding leaves of spinach until they are up to a third of their final area (Saurer & Possingham 1970). This phenomenon is shown in figure 7, plate 50, where a simple leaf marking technique has been used to demonstrate that extensive growth occurs at the basal end of partially expanded leaves. Cell division is associated with much of this growth and the chloroplasts for the newly formed cells arise by binary fission of existing chloroplasts. We have found that newly formed cells have between 10 and 15 chloroplasts (Possingham & Smith 1972). These subsequently increase in number to 200–300 per cell during cell expansion (Possingham & Saurer 1969).

The vast majority of the chloroplasts of spinach arise from the fission of grana containing chloroplasts, and our results establish that the continuity of genetic information is assured. In normal light grown leaves cDNA synthesis occurs in association with chloroplast replication in such a way that cDNA doubles during the chloroplast division cycle and at the time of binary fission segregates in similar amounts to each daughter chloroplast.

We thank Mrs J. Nikandrow and Mrs R. Luters for invaluable technical assistance, and Mr E. Lawton and Mr A. Soeffky for photographic assistance.

References


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Figure 7. Spinach leaves: l.h.s., 6th leaf from the base of a 14 day old plant showing position from which day 0 disks are taken for culture; r.h.s., a position 6 leaf, one week older, i.e. at day 21. The marks (2 mm diameter circles) were placed on this leaf 7 days before in the positions shown in the l.h.s. leaf. The marks on the l.h.s. of both leaves were retouched on the photograph.

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