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# Induction and Regulation of Chloroplast Replication in Mature Tobacco Leaf Tissue[1]

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## ABSTRACT

**Chloroplast replication was induced in mature tobacco leaf tissue (*Nicotiana tabacum* L.) by culturing leaf discs on a sterile medium composed of salts and sucrose. Chloroplast replicaton is greatly enhanced by the addition of kinetin to this medium. Kinetin also enhances cell enlargement, but cell division does not occur. Chloroplast replication is nonsynchronous and proceeds most rapidly when the cell enlargement rate decreases. Chloroplast replication is light-dependent, but cell enlargement occurs in both light and dark. Chloroplast replication resumes when discs cultured in the dark are returned to the light. It appears that chloroplast replication is related to cell expansion. The possibility of inducing synchronous replication of chloroplasts in tobacco cells is discussed.**

It is well known that proplastids and young chloroplasts divide (1, 2, 12–14, 16, 17), but very little is known about the ability of mature chloroplasts to replicate.

We observed that cytokinins enhanced replication of developing chloroplasts in etiolated leaf tissue exposed to light (1), and this led to an investigation of the effects of such compounds on the replication of mature chloroplasts. This paper presents evidence that mature tobacco chloroplasts in fully expanded tobacco leaves can be induced to divide, and that a variety of factors regulate this process.

## MATERIALS AND METHODS

Mature leaves were obtained from greenhouse-grown *Nicotiana tabacum* L. cv. Maryland Mammoth. Leaf discs were placed on sterile culture media as described earlier (1). The standard medium contained mineral salts as described by Murashige and Skoog (1), as well as 5 g/liter sucrose and 8 g/liter agar (S + S medium[3]). Kinetin (6-furfurylaminopurine) at a concentration of 0.5 mg/liter was added for the cytokinin medium. Chloroplasts were counted and cells measured in the following manner: tissue was fixed in 3.5% glutaraldehyde in $H_2O$ (w/v) (this fixation prevents damage to the cells by pectinase, and also preserves the tissue so that chloroplast counts can be made at convenient times). The cells were separated with pectinase (Nutritional Biochemical Co.) according to Humphries and Wheeler (5), except that the incubation period was reduced to 1 day. Plastid numbers and cell lengths were determined in the separated cells. The techniques for counting chloroplasts in the cells as well as for counting cells and for determining chlorophyll have been described (1). Palisade cells were used for all results described below, as these cells enlarge primarily along their longitudinal axes. Spongy mesophyll cells enlarge in all directions, and their large size and irregular shape make it very difficult to count chloroplasts in them. However, mesophyll cells were counted in some experiments, and, though these cells generally have more plastids per cell, results were similar for both cell types.

## RESULTS

There is great variability between leaves in size of mature cells and in cell chloroplast numbers, and the magnitude of responses to experimental conditions is also variable. Replicate experiments, however, always show the same pattern, and representative experiments are illustrated.

Figure 1 shows the time course of chloroplast replication and cell enlargement in discs cultured in the light on the S + S + K medium. The cells enlarge considerably, and this is accompanied by a large increase in chloroplast number per cell. Plastid number increases steadily for about 6 days and subsequently reaches a plateau. There is no indication of any synchronization of chloroplast division. Figure 1 also shows the end points for chloroplast number, cell size, and plastid number/cell size (PN/CS) ratio in tissue kept on S + S medium. Similar experiments, where samples were taken at 2-day intervals, show that the S + S medium does not induce chloroplast replication until the tissue has been cultured for about 4 days.

The PN/CS ratio mentioned above seems instructive, as plastid replication does not occur without cell expansion. In general, this ratio is higher in the kinetin-treated tissue than in the S + S control; the difference between the two means is significant (12 experiments, paired sample $t$ test, $P < 0.01$). When the ratio in the starting material is arbitrarily defined as 1.00, the various replicate experiments can be directly compared. The average of the final ratios in kinetin-treated tissue is slightly above 1.00, and is below 1.00 for tissue grown on S + S. There is a dip in the middle of the PN/CS curve (Fig. 1), which implies that cell expansion goes faster than chloroplast replication and also reaches a plateau sooner. There is no detectable cell division in kinetin-treated tissue or in tissue on S + S medium during the course of these experiments.

Because the tissue increases greatly in fresh weight, the

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[3] Abbreviations: S + S medium: medium containing Murashige and Skoog salts, 5 g/liter sucrose and 8 g/liter agar; S + S + K medium: same as above with 0.5 mg/liter kinetin added; PN/CS ratio: ratio between chloroplast number and cell size.

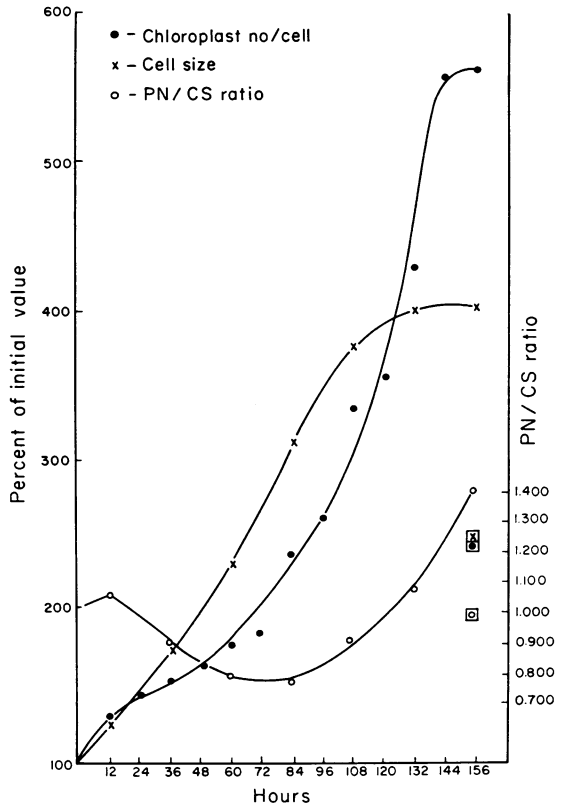


FIG. 1. Changes in chloroplast number per cell, cell size, and the ratio between them (PN/CS) over time in mature tobacco leaf discs cultured in the light on S + S + K medium. The points in the squares give the final values for the same parameters in tissue on S + S medium.

chlorophyll content per gram fresh weight decreases drastically. The final chlorophyll content is again variable, but on the average there is no net loss of chlorophyll in kinetin-treated tissue. Material on S + S does lose chlorophyll during the experiment. Chloroplast size is not significantly changed by either medium.

The effect of kinetin concentration on replication is shown in Figure 2. The response is independent of concentration within the range used. The figure also shows that the kinetin effect can be duplicated with the cytokinin, benzyladenine.

Some chloroplast replication occurs when leaf tissue is cultured on a medium containing only salts and sucrose, and the effect of sucrose concentration on replication is shown in Figure 3. Sucrose seems to be somewhat limiting at 0.5 g/liter, but not at the concentration of 5 g/liter which is regularly used in these experiments. Higher concentrations may even be slightly inhibitory and seem to inhibit chloroplast replication more than cell expansion, since the PN/CS ratio drops. Experiments with various sucrose concentrations in media containing kinetin showed no evidence for synergism, but in these experiments high sucrose concentrations were not inhibitory and the PN/CS ratio did not drop. The inhibitory effect of high concentrations of sucrose, and a general paucity of knowledge concerning the relationship between cell expansion and plastid replication, suggested the use of mannitol in the medium as an osmoticum. We thought that high osmotic tension in the medium might reduce cell expansion without directly influencing chloroplast replication, thus making it possible to separate the two events. Chloroplast replication, however, seems to be more sensitive to increasing mannitol concentrations than in cell expansion (Fig. 4), suggesting that mannitol has other, more specific effects on cell metabolism besides its action as an osmoticum.

Two other growth substances, IAA and GA were tested for their effects on chloroplast replication (Table I). They neither stimulate plastid replication nor cell expansion as much as kinetin.

Chloroplast replication, both with and without kinetin, is strictly light dependent (Fig. 5). The cells expand considerably in the dark, but the expansion on both types of media is less pronounced in the dark than in the light. Chloroplast numbers remain essentially at the initial value in the dark, so that PN/CS ratios drop drastically. The dark-induced inhibition

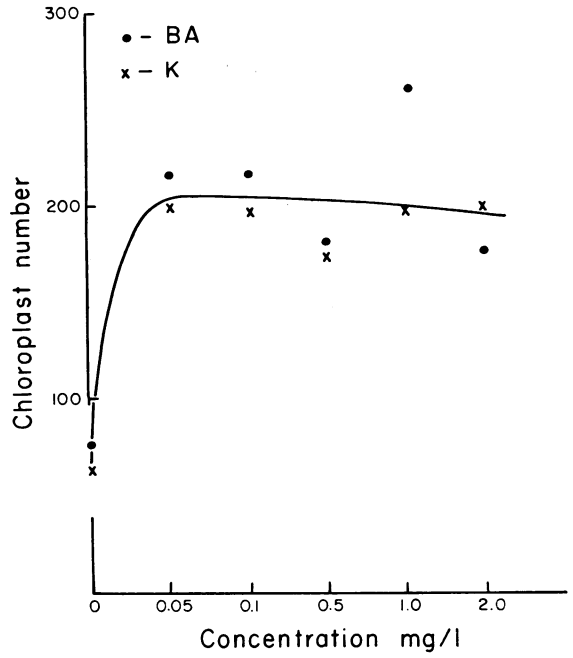


FIG. 2. Effect of different concentrations of kinetin (K) and benzyladenine (BA) on chloroplast number per cell in mature tobacco leaf discs cultured in the light for 7 days.

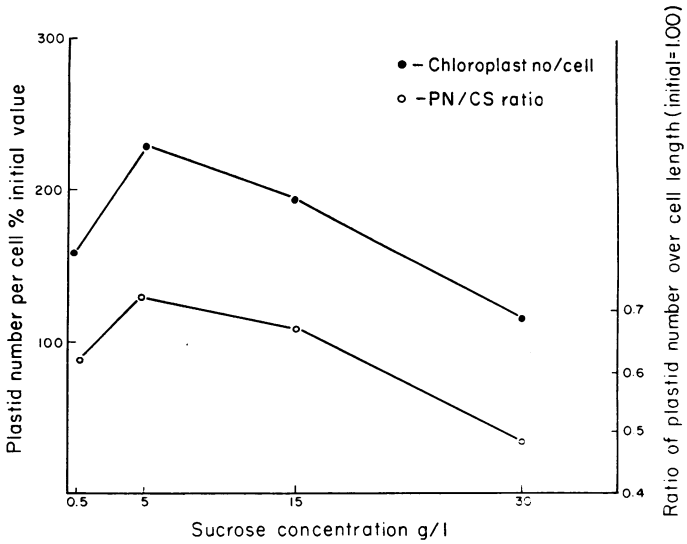


FIG. 3. Effect of different sucrose concentrations in a medium without kinetin on chloroplast number per cell and on the PN/CS ratio in mature tobacco leaf discs cultured in the light for 7 days.

can be released at any time. If leaf discs are kept in the dark for 7 days and then exposed to light, the number of chloroplasts increases (Fig. 6) just as in tissue that has not been pretreated in the dark. Cell size continues to increase, but at a slower rate than chloroplast number, and the PN/CS ratio rises.

The light dependency of chloroplast replication raises the question of the role of light in this phenomenon. The most obvious interpretation is photosynthesis, but these discs are provided with sucrose in the medium so gross energy requirements are met. It is conceivable, however, that photosynthesis could provide energy that is more readily available or provide some limiting substances for chloroplast division. The involvement of photosynthesis in replication was tested with DCMU. When 10 $\mu$M DCMU was added to a medium containing kinetin, chloroplast replication was almost completely inhibited, but cell expansion also tended to be inhibited. Experiments with lower concentrations showed that DCMU indeed inhibits both processes but that plastid replication is more sensitive (Fig. 7).

## DISCUSSION

Chloroplasts in palisade cells of mature tobacco leaves cover most of the available wall space, and they often appear to squeeze each other out of shape. This suggests that cell enlargement might be a precondition for chloroplast replication. Granick (3) also suggested that the number of chloroplasts per cell may be related to the available cell surface, and recently Honda *et al.* (4) showed a strong correlation between cell size and number of chloroplasts.

Our data indicate that cell enlargement in the light and in the presence of certain minimal nutrients does induce chloroplast replication and that specific factors are not required for this process. Under these conditions, the cells in the leaf discs expand to two or three times their original size, and the number of chloroplasts increases. The curves for the processes are very similar to those for cell expansion and mitochondrial replication obtained by Juniper and Clowes (7). The addition of kinetin to the medium greatly enhances chloroplast replica-

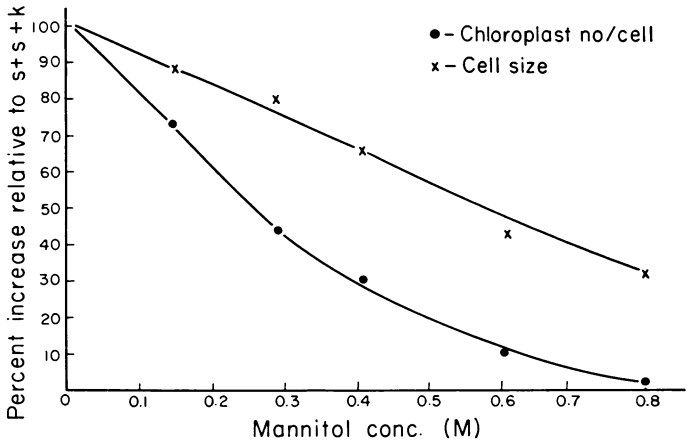


FIG. 4. Effect of mannitol added to S + S + K medium on chloroplast number per cell and on cell size in mature tobacco leaf discs in the light for 7 days.

Table I. *Effect of Various Growth Substances on Chloroplast Replication and Cell Expansion in Mature Tobacco Leaf Discs Cultured on Sterile Media for 1 Week in the Light*

| Treatment in the Light | Percentage of Initial Value | | |
|---|---|---|---|
| | Chloroplast No./cell | Cell size | PN/CS ratio |
| S + S | 148 | 287 | 52 |
| S + S + kinetin (0.5 mg/liter) | 279 | 339 | 82 |
| S + S + IAA (0.5 mg/liter) | 197 | 253 | 78 |
| S + S + GA (0.5 mg/liter) | 172 | 161 | 107 |

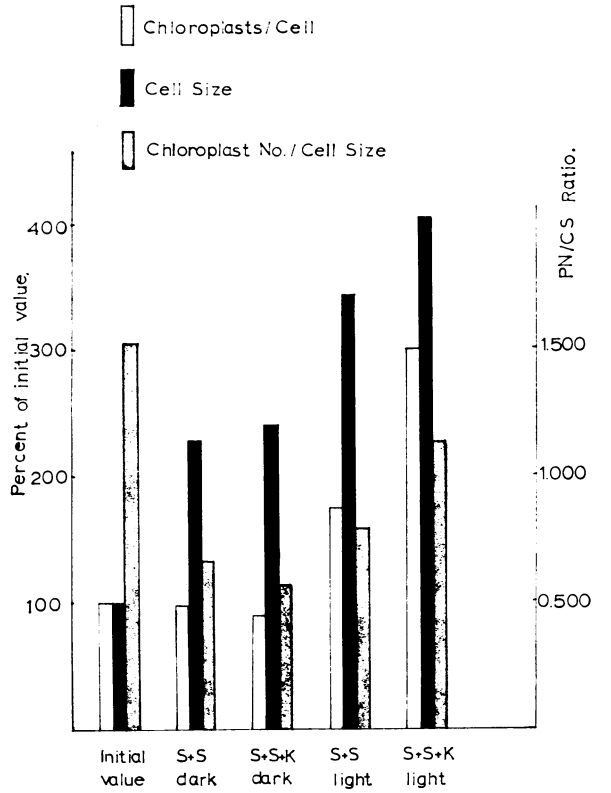


FIG. 5. Changes in chloroplast number per cell, cell size, and PN/CS ratio in mature tobacco leaf discs cultured for 7 days in light and dark on both S + S and S + S + K media.

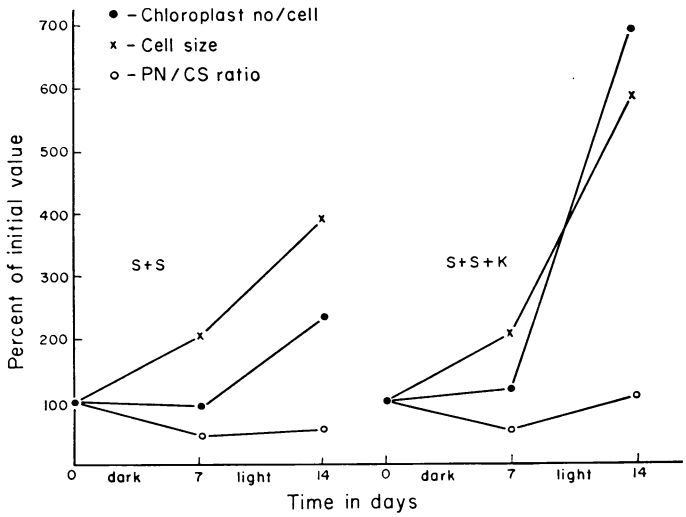


FIG. 6. Release of dark inhibition of chloroplast replication in mature tobacco leaf discs. Discs were kept in the dark for 7 days on medium with or without kinetin and then exposed to light for 7 days on the same medium.

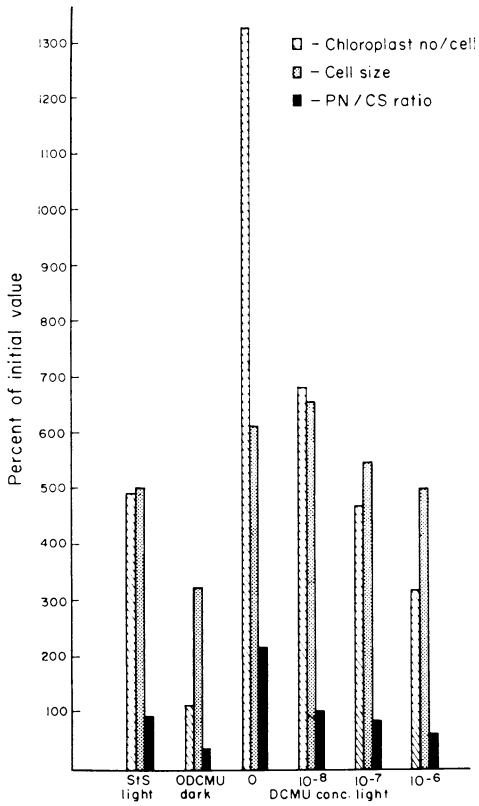


FIG. 7. Comparison of chloroplast number per cell, cell size, and PN/CS ratio in discs cultured in the light both with and without DCMU.

tion (Fig. 1). It also increases cell expansion, and this may partly explain the increase in plastid replication. The significant difference, however, in the final values of the PN/CS ratios between the tissue on S + S and the kinetin-treated tissue indicates that kinetin enhances chloroplast replication more than cell enlargement. The most likely explanation seems to be that kinetin, which has known senescence-retarding properties (9), maintains the cells in a better condition. The possibility that kinetin exerts a more specific influence cannot be ruled out. However, it does not seem likely because treatment with IAA and GA (Table I) also appears to result in higher values for the PN/CS ratio, even though these growth substances do not enhance cell expansion as much as the S + S medium.

The few reports in the literature dealing with division of mature chloroplasts seem to fit this pattern. Mothes (10) found in his experiments with rooted tobacco leaves that leaf tissue increased greatly in fresh weight because of cell expansion and that chloroplast numbers per cell increased. Reinhard (12) found active division of chloroplasts during cell enlargement in certain galls. We find no evidence for a synchronous division of chloroplasts, even though there is a definite starting point for the stimulus. This may be a general phenomenon in plastid division in tobacco, since etioplasts exposed to light do not show synchronization (1). Nonsynchronous replication is probably explained by the fact that cell expansion occurs continuously and at a faster rate than chloroplast replication (Fig. 1). It appears that chloroplasts are constantly being induced to divide by the ever enlarging cell. When cell expansion rate decreases, the replication rate is the highest. Replication ceases very abruptly at a point soon after cell expansion has stopped.

The most intriguing aspect of the replication process is the strict light dependency. The inhibitory effect of DCMU suggests that photosynthesis is involved, although it is disturbing that DCMU also inhibits cell expansion. In some cases there is more inhibition with DCMU than occurs in darkness. If photosynthesis is required for chloroplast replication, this may also explain the observation that high concentrations of sucrose and mannitol inhibit chloroplast replication more than cell expansion (Figs. 3, 4). It has been shown (6) that even at relatively low soil moisture tension photosynthesis may be inhibited. Osmotic stress would have the same effect. It is known, however, that high sugar concentrations have a bleaching effect on chloroplasts (15). High sucrose concentrations inhibit chlorophyll synthesis in etiolated tobacco leaf discs exposed to light (8), and the higher concentrations of sucrose and mannitol caused a considerably greater loss of chlorophyll from the tissue in the present study. Although the mechanism of this sugar effect is not understood, it is likely that anything which has an adverse effect on chlorophyll metabolism will influence chloroplast behavior as well.

If chlorophyll is the pigment involved in the light effect, then etioplasts react differently than chloroplasts. Etioplasts in tissue exposed to light begin to divide immediately, whereas there is no detectable amount of chlorophyll, and consequently no photosynthesis, until between 16 and 24 hr after exposure to light (1). Recent experiments have shown this to be a low energy response, so it is possible that the light-dependent response in mature leaves is a phytochrome-mediated phenomenon. It is expected that work in progress will solve this problem.

The light effect also complicates the relationship between cell size and chloroplast replication, because cell enlargement occurs in the dark while replication does not (Fig. 5). The kinetin stimulation of cell enlargement in the dark is almost eliminated, and cell enlargement on both types of media is less than in the light. This experiment demonstrates that cell enlargement does not always induce chloroplast replication. It remains true, however, that replication does not occur in the absence of cell enlargement.

When the cells are exposed to light after they have doubled in size in the dark, the stimulus for chloroplast replication is not a gradual increase in available space, but the sudden removal of a block in a situation where space is not limiting. This relationship between cell enlargement and chloroplast replication suggests it might be possible to induce synchronous replication of chloroplasts, and the precise regulation would be a definite advantage in experiments concerned with chloroplast autonomy.

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