Planta (Berl.) 86, 186—194 (1969)

Changes in Chloroplast Number per Cell during Leaf Development in Spinach

J. V. Possingham and W. Saurer
CSIRO Division of Horticultural Research, Glen Osmond

Received November 22, 1968/January 17, 1969

Summary. The amounts of chlorophyll and nitrogen and the numbers of cells per unit area change as the green leaves of spinach plants grow and increase in size in the light. The changes in the numbers of chloroplasts per cell were measured by a new method. A 5-fold increase in the numbers of chloroplasts per cell took place in both palisade and mesophyll cells over a growing period of 10 days during which time the area of the leaves increased from 1 to 50 cm^2. Proplastids were not present in the young green leaves but electron-microscope and phase-contrast observations showed the presence of grana-containing chloroplasts, many of which appeared to be undergoing division by constriction. It is suggested that the large increase in chloroplast numbers as leaf cells grow and expand in the light is from the division of differentiated chloroplasts containing grana.

Introduction

The chloroplasts of plants are generally considered to be semi-autonomous organelles which increase in number by division (Granick, 1961; Frey-Wyssling and Mühlethaler, 1965; Kirk and Tilney Bassett, 1967). However, only limited data are available on changes in chloroplast number per cell as the leaves of higher plants mature and increase in size.

Granick (1938) observed that during the growth of tomato leaves from one-third of maximum size to full size the number of plastids per cell increased by about 30% but provided no quantitative data to support this important claim. Fasse-Franzisket (1955) found that during the differentiation of *Agapanthus umbellatus* leaves the numbers of plastids per cell increased from about 20 in meristematic cells to about 100 to 120 in mature palisade cells. Using phase-contrast microscopy she observed frequent division by constriction of the amoeboid granular plastids in the leaf cells of this plant, but less frequent division of fully differentiated and somatic chloroplasts. Division of the young, "amoeboid" proplastids of the meristematic cells of *Epilobium* has also been observed by Michaelis (1962). In this plant, most of the plastid division took place in the early stages of chloroplast development. By contrast, Wildman (1967), who has made extensive light-microscope observations on living leaf cells of *Spinacea oleracea*, has reported that he was unable to find any evidence of chloroplast divisions occurring in the mature cells of this plant.

This paper describes some of the changes that take place in the young, green leaves of spinach plants as they grow and increase in size in the light. In two experiments the changes that occurred in numbers of chloroplasts per cell as the leaves grew were measured by a new method, and as well the changes that took place in leaf area, cell number and in the important chloroplast constituents of chlorophyll and insoluble nitrogen.

Material and Methods

Plant Material. American Round-Seeded Spinach (*Spinacea oleracea* L.) plants were grown in a growth cabinet under fluorescent lights (12 hours, 3,000 ft-c) and under day and night temperatures of 25° and 22°, respectively. Seeds were germinated in vermiculite and the plants were transferred to nutrient-culture solutions after 10 days.

Sampling Technique. Leaf discs 0.5 cm in diameter were taken with a cork borer to provide leaf subsamples for the measurement of chlorophyll and insoluble nitrogen, and for determining numbers of cells and numbers of chloroplasts per cell. The discs were taken in rows, one either side of the midrib commencing at the leaf tip and extending to the leaf base, avoiding the veins as far as possible. In these experiments the leaves were numbered from the base of the plant with the oldest leaf being leaf 1.

Chlorophyll and Nitrogen Estimations. Chlorophyll was determined in 80% acetone extracts by the spectrophotometric method of ARNON (1949) and insoluble nitrogen in the acetone residues by the micro-Kjeldahl method as described by BROWN and POSSINGHAM (1957).

Cell-Number Measurements. These were made by the technique of BROWN and RICKLESS (1949) described for root tissue. The numbers of palisade and mesophyll cells per disc were counted separately and were added to provide the total cell number per disc. The number of epidermal cells per disc was not measured as these represent a small proportion of the total cells and contain few chloroplasts. Haemocytometer counts were usually made in quintuplicate on suspensions prepared from a single leaf disc.

Chloroplast Number per Cell. Leaf discs taken for estimations of both cell number and chloroplast number per cell were examined after being fixed in a solution of 3.5% glutaraldehyde in water for 2 hr. The technique for estimating chloroplast number per cell involved separating the leaf discs into single-cell suspensions by treating them with 1N HCl at 60° for approximately 10 min. A relatively dilute suspension of cells was placed on the microscope slide and the cover slip was pressed down sufficiently firmly to bring about cell rupture and release of the chloroplasts. The number of chloroplasts per cell was counted under phase contrast using an eyepiece graticule. Counts were made of the plastid numbers in at least 10 palisade or mesophyll cells in suspensions which were each derived from a single leaf disc.

Figs. 2 and 4 below show the appearance of separated single cells prepared from young and old leaves, respectively. Fig. 3 shows a ruptured single cell where the chloroplasts are all in the same focal plane and can be easily counted.

Leaf Area Measurements. Leaves to be measured were photocopied using a Xerox-750 copying machine. The leaf images were cut out and weighed and actual leaf areas read from a calibration line prepared by weighing known areas of Xerox paper.

Light Microscope Observations. Leaf strips or leaf discs were fixed in 3.5% glutaraldehyde for at least 12 hr, dehydrated in an alcohol series, wax-embedded, and sectioned with a rotary microtome. Unstained sections were examined under phase contrast.

Electron Microscope Observations. Leaf strips were fixed for 12 hr in 6.25% glutaraldehyde in phosphate buffer. After washing they were treated with 1% buffered OsO_4 for 2 hr, dehydrated through an ethanol series and embedded in araldite. A lead-citrate stain was applied before viewing the sections in an electron microscope (Siemens Elmiskop 1 A).

Results

Experiment I. Some of the changes that take place in leaves of spinach plants as they grow and develop were measured. In this experiment the changes in the leaves at positions 4 and 5 were followed over the period from day 10 to 24.

The plant material for this experiment was provided by 4 pots, each of 7 plants. Harvest 1 was made 10 days after transferring the seedlings to the nutrient cultures when 2 plants were taken from each pot to provide 2 replicate samples. At harvest the leaf area of the whole plant and of the leaves at positions 4 and 5 were determined separately. From each leaf at position 4, 5 leaf discs were taken. These were divided into 2 groups to provide 10 discs for chlorophyll and nitrogen estimations, and 10 discs for cell-number and chloroplast-number measurements. At harvest 1 the leaves at position 5 were approximately 2 cm long and only the 10 discs required for cell number and chloroplast number counts could be taken from a sample of 4 plants.

Harvest 2 was made on day 13 and again 2 plants per pot were taken and sampled as for harvest 1. By day 13 the leaves at position 5 were large enough to provide leaf discs as described above for position 4. On day 14 the nutrient solution of all pots was renewed. Harvests 3, 4 and 5 were made on days 16, 20 and 24, respectively. At these harvests only one plant per pot was taken as the increase in size of the leaves was such that it was possible to take 10, 0.5-cm diameter discs from each of the leaves 4 and 5.

Fig. 1 shows the principal data obtained in this experiment. Statistical analysis showed that significant increases in leaf area occurred between harvests for both leaves 4 and 5. For both these leaves there were significant increases in the amount of chlorophyll per disc between harvests, except between the 4th and 5th harvests. There were however no significant differences in the amounts of insoluble nitrogen per disc between harvests for either of the leaves.

There were significant and large increases in the number of chloroplasts per cell as the leaves grew. These changes occurred between all harvests except between the 4th and 5th. The same pattern of change occurred in both mesophyll and palisade cells and was similar for leaves 4 and 5. There was a concomitant decrease in the total number of cells (palisade plus mesophyll) per disc between day 10 and day 24 for both leaves 4 and 5.

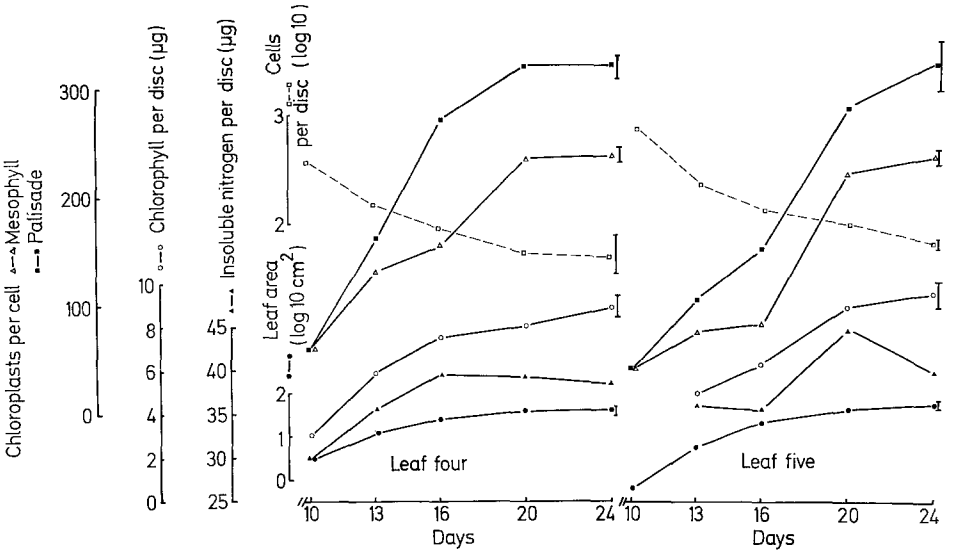


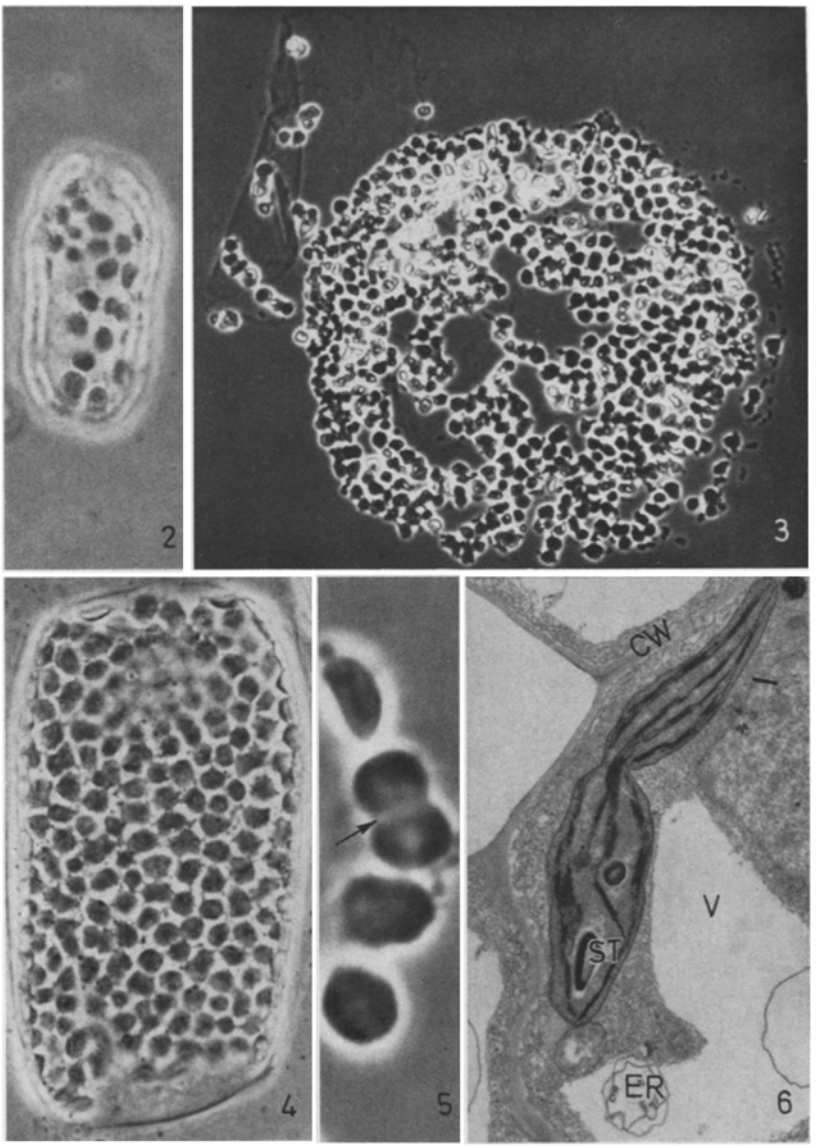
Fig. 1. Changes with time in leaf area and in amounts of chlorophyll, insoluble nitrogen and in number of cells per disc and in numbers of chloroplasts per palisade and mesophyll cell of leaves 4 and 5 of spinach plants. Vertical bars indicate least significant difference values ($P = 0.05$)

Experiment II. In this experiment the number of palisade cells per disc and the number of chloroplasts per palisade cell in 21-day-old, full-nutrient spinach plants were measured. The leaf area and the chlorophyll and nitrogen contents of leaf discs taken from leaves at positions 1 to 9 were measured. These data are given in the table.

Table. *Changes in leaf area and in chlorophyll, nitrogen and palisade cells per disc and in chloroplast number per palisade cell with leaf position in 21-day-old spinach plants*

Leaf position	Leaf area (cm^2)	Chlorophyll per disc (µg)	Insoluble N per disc (µg)	Number of palisade cells per disc ($\times 10^3$)	Number of chloroplasts per palisade cell
1 and 2	14.7	4.75	19.2	31.2	540
3 and 4	62.2	5.60	23.8	39.6	380
5 and 6	48.8	5.78	25.3	35.3	320
7 and 8	20.2	4.37	25.4	134.0	65
9	2.4	—	—	183.0	40

Statistical analysis showed that with the exception of the comparisons between leaves 1 and 2, and 7 and 8, and leaves 3 and 4 and 5 and 6, the leaf areas were significantly different one from the other. There were



Figs. 2—6

Fig. 2. Single palisade cell from leaf 10 of a 21-day-old spinach plant separated by treatment with hot 1 N HCl (×640)

Fig. 3. Pool of chloroplasts derived from a single mesophyll cell separated by treatment with hot 1 N HCl and subsequently ruptured by pressure on the cover slip (×250)

no significant differences between leaf positions in the amount of chlorophyll and of insoluble nitrogen per disc. The numbers of palisade cells per disc at leaf positions 7, 8 and 9 were significantly greater than those at the lower leaf positions. The numbers of chloroplasts per palisade cell in the leaves at positions 1 to 6 were significantly greater than those in leaves at positions 7 to 9.

Figs. 2 and 4 show the appearance of palisade cells in discs taken from leaves 9 and 1, respectively. It should be noted that the cells in Figs. 2 and 4 are shown at the same magnification (×640). It can be seen that the chloroplasts in cells of both the large leaf 1 and the small leaf 9 are similar in size.

Fig. 5 shows the appearance of chloroplasts in a mesophyll cell. Some of the chloroplasts have an appearance similar to yeast cells dividing by constriction.

Electron Microscopy. Leaf tissue from leaves at positions 1 to 10 were taken for electron microscopy from a 19-day old plant. The plant at this time had 10 green, partly-expanded leaves ranging in size from leaf 10, which was approximately 1 cm long, to leaf 4 which was about 10 cm long.

The general appearance and features of the cells of leaves 1 to 8 were similar to those described before for this plant (MURAKAMI, 1963; POSSINGHAM *et al.*, 1964). The plastids of these leaves had a well-defined limiting membrane and a well-developed lamellar system, and contained, in most cases, starch grains. The appearance of the immature cells of leaves 9 and 10 were generally similar to the young leaf cells of tobacco described by CRONSHAW and ESAU (1968). Many cells had differentiated grana-containing chloroplasts which appeared to be dividing by constriction (Fig. 6). These were similar in appearance to those described by LANCE (1958) for *Chrysanthemum segetum*, and by FREY-WYSSLING and MÜHLETHALER (1965) for *Solanum nigrum*.

Discussion

The data assembled in the first experiment of this series describe some of the changes that occur in spinach leaves as they grow and

Fig. 4. Single palisade cell from leaf 1 of a 21-day-old spinach plant separated by treatment with hot 1 N HCl (×640)

Fig. 5. Section of a mesophyll cell in leaf 10 of a 21-day-old spinach plant. Tissue fixed in glutaraldehyde, wax-embedded, viewed under phase contrast. Chloroplast marked with an arrow appears to be dividing by constriction (×3,000)

Fig. 6. Similar section to Fig. 5 viewed with the electron microscope, ×9,000. Chloroplast with central constriction. *CW* cell wall, *ER* endoplasmic reticulum, *St* starch, *V* vacuole

develop in the light. By far the most striking change was the large increase that occurred in the numbers of chloroplasts per cell. This took place in both mesophyll and palisade tissue and in the leaves at both positions 4 and 5. The increase in plastid number were approximately 5-fold over a 10 day period.

An essentially similar pattern of change occurred in the second experiment in which successive leaves of a 21-day-old plant were sampled. The differences that exist between leaves at differing stem positions may be considered as a time sequence modified by positional and competitive effects. We believe that the differences between the leaves at positions 7 to 9 and those at lower positions may be generally regarded as due to differences in leaf age. Figs. 2 and 4 illustrate that the predominant change as leaf cells grow is an increase in the number of chloroplasts. The changes that occur in chloroplast size are small.

This pattern of change contrasts sharply with those described by Gyldenholm (1968) for the regreening of dark-grown bean leaves. During regreening in this material there were large increases in the fresh and dry weight of the leaves and in chlorophyll and RNA content. During 45 hr of regreening, Gyldenholm found that there was no increase in cell number per leaf and in chloroplast number per cell, and also no increase in DNA per leaf and per plastid. In our experiments we found that there was an increase in the amounts of chlorophyll and nitrogen per unit of leaf tissue as the leaves grew and that this was associated with an increase in the number of chloroplasts per cell.

It should be noted that the smallest and youngest leaves sampled in the present series of experiments were green, as all the plants were grown in the light. Further, the American Round-Seeded spinach has a relatively open rosette habit of growth so that even the leaves smaller than those that were sampled here were green and contained differentiated chloroplasts. We consider it of importance that in our electron-microscope studies we found no evidence of proplastids or etioplasts in even the youngest leaves that were examined. No proplastid- or etioplast-division figures similar to those that have been described for maize and barley could be found (Vesk *et al.*, 1965; Sprey, 1968). Also, vesicles of the type described by Mühlethaler and Frey-Wyssling (1959) as proplastid initials were not a conspicuous feature in the young leaves we examined, but it is well known that objects of this size are difficult to locate in ultra-thin sections.

We consider that proplastids are not the source of the large number of chloroplasts that are formed as green leaves grow and develop. This conclusion is supported by the relatively high frequency with which we found chloroplasts of a shape suggestive of division by constriction. Every electron-microscope section of immature 1-cm-long leaves con-

tained at least one chloroplast-division figure. As well, under phase contrast chloroplasts with an appearance essentially the same as dividing yeast cells were found in a large proportion of the cells of immature leaves. In the case of the green alga *Nitella* this mechanism of chloroplast formation has been elegantly demonstrated by use of cinematography (GREEN, 1954). Unfortunately, because of their size it is not possible to view living cells of young spinach leaves cut free-hand, as has been done with the large, mature cells of this plant (POSSINGHAM *et al.*, 1964; WILDMAN, 1968). We suggest that constriction division of differentiated chloroplasts is the mechanism by which the bulk of the chloroplasts are formed in light-grown higher plants.

The authors are indebted to Mr. K. CELLIER, CSIRO Division of Mathematical Statistics, Glen Osmond, S.A. for statistical analysis of the quantitative data. They are also indebted to Mr. A. SOEFFKY for assistance with the electron microscope observations.

References

ARNON, D. J.: Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**, 1—15 (1949).

BROWN, R., and J. V. POSSINGHAM: Iron deficiency and the growth of pea roots. Proc. roy. Soc. B **147**, 145—166 (1957).

—, and P. RICKLESS: A new method for the study of cell division and cell extension with some preliminary observations on the effects of temperature and of nutrients. Proc. roy. Soc. B **136**, 110—115 (1949).

CRONSHAW, J., and K. ESAU: Cell division in leaves of *Nicotiana*. Protoplasma **65**, 1—24 (1968).

FASSE-FRANZISKET, U.: Die Teilung der Proplastiden und Chloroplasten bei *Agapanthus umbellatus* L'Hérit. Protoplasma **45**, 194—227 (1955).

GRANICK, S.: Chloroplast nitrogen of some higher plants. Amer. J. Bot. **25**, 561—567 (1938).

— The chloroplasts: Inheritance, structure and function. In: The cell (J. BRACHET and A. E. MIRSKY, eds.), vol. II, p. 489—602. New York: Acad. Press 1961.

GREEN, P. B.: Cinematic observations on the growth and division of chloroplasts in *Nitella*. Amer. J. Bot. **51**, 334—342 (1954).

GYLDENHOLM, A. O.: Macromolecular physiology of plastids. V. On the nucleic acid metabolism during chloroplast development. Hereditas **59**, 142—168 (1968).

FREY-WYSSLING, A., and K. MÜHLETHALER: Ultrastructural plant cytology. Amsterdam: Elsevier 1965.

KIRK, J. T. O., and R. A. E. TILNEY-BASSETT: The plastids: Their chemistry, structure, growth and inheritance. San Francisco: Freeman 1967.

LANCE, A.: Infrastructure des cellules du méristème apical et des jeunes ébauches foliaires de *Chrysanthemum segetum* L. (Composées). Ann. Sci. Nat., XI. Sér., Bot. **19**, 165—202 (1958).

MICHAELIS, P.: Über Zahlengesetzmäßigkeiten plasmatischer Erbträger, insbesondere der Plastiden. Protoplasma **55**, 177—231 (1962).

MÜHLETHALER, K., u. A. FREY-WYSSLING: Entwicklung und Struktur der Proplastiden. J. biophys. biochem. Cytol. **6**, 507—512 (1959).

MURAKAMI, S.: Ribosomes in spinach chloroplasts. Exp. Cell Res. **32**, 398—400 (1963).

Possingham, J. V., M. Vesk, and F. V. Mercer: The fine structure of leaf cells of manganese deficient spinach. J. Ultrastruct. Res. **11**, 68—83 (1964).

Sprey, B.: Zum Verhalten DNS-haltiger Areale des Plastidenstromas bei der Plastidenteilung. Planta (Berl.) **78**, 115—133 (1968).

Vesk, M., F. V. Mercer, and J. V. Possingham: Observations on the origin of chloroplasts and mitochondria in the leaf cells of higher plants. Aust. J. Bot. **13**, 161—169 (1965).

Wildman, S. G.: The organisations of grana-containing chloroplasts in relation to location of some enzymatic systems concerned with photosynthesis, protein synthesis and ribonucleic acid synthesis. In: Biochemistry of chloroplasts (T. W. Goodwin, ed.), vol. II, p. 295—319. New York: Acad. Press 1967.

Dr. J. V. Possingham
Division of Horticultural Research
CSIRO
Private Bag No. 1
Glen Osmond, S.A. 5064, Australia