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Chloroplast Division and DNA Synthesis in Light-grown Wheat Leaves[1]

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ABSTRACT

Light-grown 7-day-old wheat seedlings (*Triticum aestivum*, var. Maris Dove) showed an increase of 200% in plastids per cell between 1.7 and 4.5 centimeters from the leaf base. This increase was the result of divisions of young chloroplasts at various stages of development, and was well separated in distance, and therefore in time from the region of cell division in the basal meristem. [^{3}H]Thymidine was incorporated into plastid DNA throughout the zone of plastid division, but not above it.

During leaf development, proplastids differentiate into chloroplasts and their differentiation is accompanied by a large increase in the number of plastids per cell, caused by the division of both proplastids and young chloroplasts (11). In spinach leaves the division of young grana-containing, photosynthetic chloroplasts is the most important factor determining the number of chloroplasts per mature leaf cell: Possingham and Saurer (19) have shown that up to 90% of the final number of plastids can be accounted for by the division of young chloroplasts. Chloroplast division has also been observed to occur *in vitro* (8, 22) but no subsequent increase in size has been observed.

For ease of experimentation, the effects of exogenous factors on chloroplast division have been examined mainly by using discs excised from leaf tissue (4, 14, 16–18, 20); but in view of the large biochemical changes which occur rapidly after the excision of leaf tissue (15), the results of investigations of the control of plastid division in leaf discs should be interpreted with some caution. The division of young chloroplasts and its significance in the development and function of the leaf would be examined least equivocally in an intact, growing plant.

The aim of the present investigation was to use an intact plant in which a large increase in the number of plastids per cell occurred during leaf development and to examine the sequence of biochemical events associated with this increase. To follow the division process sequentially, we required plants from which large numbers of cells and plastids could be harvested at each stage of development. Dicotyledonous leaves are not an ideal tissue in which to study plastid division since the leaf is a mosaic of cells at different stages of differentiation. The advantage of many monocotyledonous plants is that in the leaves of their young seedlings all cell divisions occur in a basal meristem, resulting in a developmental sequence of cells from the base to the tip of each leaf. This has already been exploited in maize (2, 5, 6, 12), in maize and sorghum (25), in barley (23), and in oats (27) to study plastid and cell differentiation.

In the present paper we demonstrate how a similar sequential developmental system in wheat leaves can be used to investigate the replication process of young chloroplasts and the relationship between the timing of DNA synthesis and plastid division. Wheat was chosen because many varieties and special lines are available whose genetics are well understood, and the complication of chloroplast dimorphism, as found in maize, is avoided.

MATERIALS AND METHODS

Plant Material. Seeds of wheat, *Triticum aestivum*, var. Maris Dove, were soaked in running tap water at 20 C for 17 h, with surface sterilization in sodium hypochlorite solution (13% free chlorine) after the 1st h. The seeds were sown in Leavington Universal Compost (Fisons, U.K.) at a depth of 1 cm and were grown using a photoperiod of 16 h light at 20 C, 8 h darkness at 15 C, 70% RH. The light intensity at the level of the seedlings, measured with a solarimeter (Kipp & Zonen), was 4.0 mw cm^{-2}. Seedlings were harvested 7 days after sowing, 2 h after the start of the light period; the leaves were cut at their bases, and the coleoptiles gently pulled off.

Plastid Isolation and Measurement. Plastids were isolated by chopping 5-mm sections of leaf with a razor blade in an isolation medium containing 0.4 M sorbitol, 0.75 mM $MgCl_2$, 50 mM Hepes (pH 7.6). The slurry was filtered through eight layers of nylon bolting cloth (25-μm mesh) and was layered onto 1 ml of isolation medium, containing 0.4 M sucrose instead of sorbitol. After centrifugation for 5 min at $900g_{max}$, the pellet was resuspended in the medium containing sorbitol.

The percentage of dividing chloroplasts in each leaf section was determined by releasing the chloroplasts by chopping the sections in isolation medium on a microscope slide and photographing them. Plastids were counted and measured on the photographs only if their major axis was at least 2 μm long. Chloroplasts were regarded as dividing only if they had a distinct constriction halfway along the major axis. Duplicate photographs of each field were counted independently by two different people: the results of the two surveys were identical. To determine the Chl content of plastids at various stages of development, plastids were counted in a hemocytometer (improved Neubauer, depth 0.1 mm) and Chl was measured according to Arnon (1), using a Unicam SP 500 spectrophotometer.

Estimation of Numbers of Plastids per Cell in All Regions of the Leaf Above 2 cm. These were determined by counting the plastids in separated cells using a Zeiss photomicroscope fitted with Nomarski differential interference optics. The cells were separated by shaking leaf sections, fixed for 2 h in 3.5% glutaraldehyde, in 0.1 M EDTA at 60 C for 3 h (based on Possingham and Smith [20]). To ensure that the plastid counts were performed on a sample of cells representative of the whole population, each cell

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whose plastids were counted was photographed, and the projected cell area was measured using a 9864 A Hewlett-Packard digitizer linked to a model 30 Hewlett-Packard calculator. The average area of these cells was then compared with that of a very much larger sample of cells whose plastids had not been counted. Plastid counts were performed on at least 20 cells from each region, and their average area was never significantly different from the average of 100 other cells from the same region.

Estimation of Chloroplast Number for the Region Between 1.7 and 1.8 cm from the Leaf Base. Tissue pieces (1 × 2 mm) were cut from the first leaf. In this region chloroplasts but no proplastids are present in the mesophyll cells (electron microscope observations, R. M. Leech and W. W. T. Thomson, unpublished). After fixation in 2.5% (v/v) glutaraldehyde in phosphate buffer (pH 7.2) for 1 h at room temperature the tissue was postfixed in 2% (w/v) OsO_4 in buffer for 1 h at room temperature. After dehydration with acetone the specimens were embedded in Spurr's resin (26) using 10 g vinylcyclohexene dioxide, 7 g diglycidyl ether of polypropylene glycol, 26 g nonenylsuccinic anhydride, and 0.4 g dimethylaminoethanol. Polymerization was overnight at 70 C. Sections were cut on an LKB ultratome III at 1.0 μm using a glass knife, and examined using bright field illumination in the Zeiss photomicroscope after staining with 1% (w/v) toluidine blue in 1% (w/v) borax. Average chloroplast number per cell was calculated from the number of profiles observed in transverse cell sections (1.0 μm thick) and corrected for the length of cell and chloroplast length. Cell length was measured from longitudinal leaf sections. Chloroplast length was measured by counting the number of serial transverse sections (1.0 μm thick) required to section an individual chloroplast completely. The average value represents measurements of 37 separate chloroplasts.

Labeling and Isolation of DNA. To investigate the incorporation of [^{3}H]thymidine into DNA, 90 leaves with coleoptiles removed were placed with their cut ends in a solution of [methyl-^{3}H]-thymidine, 0.12 μCi/μl, 4.9 Ci/mmol (Radiochemical Centre, Amersham, U.K.). Transpiration was enhanced by artificial illumination from a 100-w tungsten bulb, 40 cm above the leaf bases, and by a flow of air. After 2 h the leaves were washed in distilled H_2O, and 80 of them were mixed with 240 nonradioactive leaves before being cut into 1-cm sections. Plastids were isolated from these sections as described above and were treated with DNase and phosphodiesterase according to Bennett and Radcliffe (3). The remaining 10 radioactive seedlings were cut into 1-cm sections, and DNA was extracted from these sections and from the plastid preparations according to Laulhere and Rozier (10). After precipitation of the whole-cell DNA, the pooled supernatants were sampled for estimation of the total free [^{3}H]thymidine in each section.

DNA was estimated from its *A* at 260 nm and by fluorescence with ethidium bromide (13). Radioactivity was determined by scintillation counting in a 1216 Rackbeta counter (LKB-Wallac), using 2,5-diphenyloxazole (PPO), 0.5% (w/v) in toluene:Triton X-100, 2:1 (v/v) as scintillant. Specific radioactivities were corrected for the levels of free [^{3}H]thymidine and for the dilution of radioactive plastids with nonradioactive ones. It was assumed that the endogenous pool sizes were the same in each section.

In order to assess any contribution to thymidine incorporation associated with bacteria contaminating the leaves, the following experiment was carried out. After incorporation, the leaf sections were vigorously agitated in a 0.1% (w/v) nonradioactive thymidine solution for 30 min. The solution was filtered through a bacterial filter (Millipore, U.K.) and washed twice with nonradioactive thymidine. The radioactivity left on the filters was 5 to 8% of the radioactivity incorporated into chloroplast-DNA.

Mitotic indices were determined according to Purvis *et al.* (21).

RESULTS

Plant Growth. Morphological studies can conveniently be performed on single seedlings, whereas most biochemical work requires more material than this. It is therefore important to use a system from which it is possible to obtain a large number of cells at the same stage of development. Figure 1 shows that wheat seedlings grown under appropriate conditions can meet this requirement. Between 5.5 and 7.5 days after germination the growth rate was almost constant at about 3.5 cm/day, and the variation in growth rates between seedlings was small. After 7 days, cells at any given height up to at least 8 cm were the same age and also at the same developmental stage. Seedlings which were shorter than 8 cm after 7 days growth were discarded.

Plastid Numbers. Having established that the system was suitable for the harvesting of large amounts of tissue at the same stage of development, it was important to discover if, on looking at sections progressively higher up the leaves, the number of chloroplasts per cell increased. Between 1.7 and 4.5 cm from the leaf base the number of plastids per cell increased from 45 to about 150, an increase of more than 200% (Fig. 2), but above 4.5 cm little change was observed. The increase of plastid number above 1.7 cm was well separated in distance, and therefore in time, from the zone of cell division: no cell division was seen above 1.0 cm from the leaf base, and mitotic figures were most abundant in the bottom 0.5 cm of the leaf. The Chl per plastid increased exponentially from 1 cm to 4 cm up the leaf (Fig. 2), indicating a progressive development of the plastids as they increase in number.

It is likely that the division of plastids is responsible for their increase in numbers per cell, and this idea is reinforced by the appearance of division profiles, such as those in Figure 3. Table I shows that the largest proportion of division profiles (13%) was found between 1.0 and 1.5 cm from the leaf base, but that as the cells became older the percentage of division profiles decreased; above 4.0 cm no such profiles were seen. It is also clear that the plastids increased in size as they developed, but, in those regions where most plastid division occurred, their average size increased most slowly. The ratio between the average sizes of the division profiles and the average sizes of all plastids remained almost constant. No measurements were made below 1.0 cm.

As wheat leaves contain a region in which a large increase in chloroplast number occurs in the absence of cell division, below a region in which chloroplast growth occurs without any increase

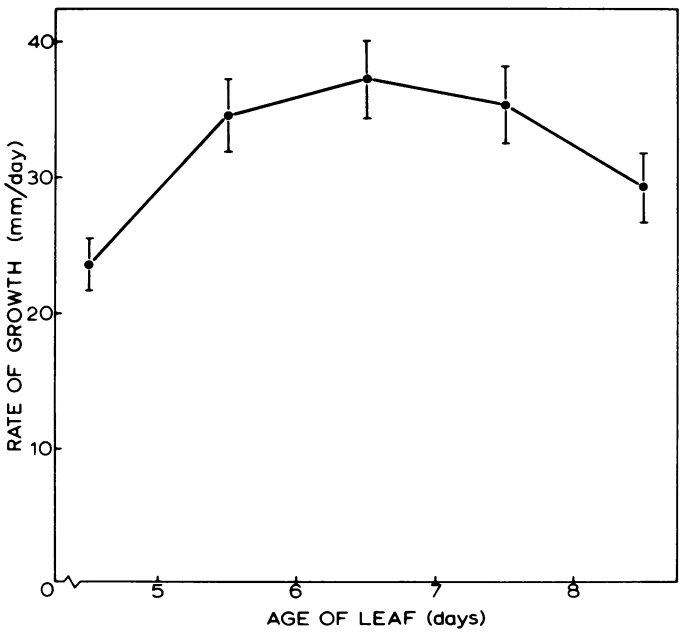

FIG. 1. Rates of growth of wheat seedlings. Bars show standard deviations in a sample of 20 seedlings.

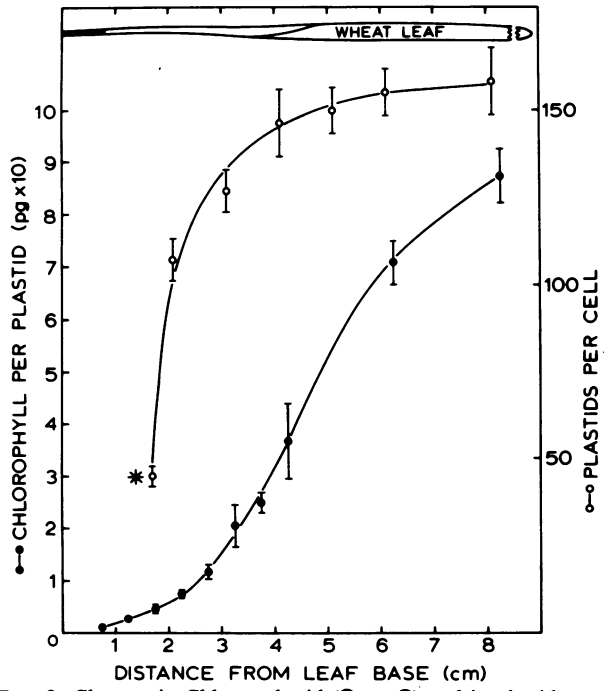

FIG. 2. Changes in Chl per plastid (●——●) and in plastids per cell (○——○) along the leaf. Bars show standard deviations of the means. Plastids were counted in cells using Nomarski optics, except at the lowest point (*) where sections of tissue were used as described under "Materials and Methods." Blacked-in section of leaf is the region of mitosis.

FIG. 3. Chloroplasts showing division profiles, in a cell separated from tissue between 1.5 and 2.0 cm above the leaf base, viewed by Nomarski microscopy. Bar represents 10 μm.

Table I. *Characteristics of Dividing and Nondividing Plastids in Wheat Leaves*

Plastids were isolated in a medium containing 0.5 M sucrose, 10 mM $MgCl_2$, 0.2% (w/v) bovine serum albumin, and 70 mM phosphate buffer (pH 8.0). The maximum dimension of each plastid was measured. The number of division profiles is expressed as a percentage of the total number of all chloroplast profiles in each section. At least 300 profiles in each section were measured.

Leaf Section	Chloroplast Division Profiles	Average Length of Chloroplasts (A)	Average Length of Division Profiles (B)	B/A
cm from leaf base	*% of all plastids*	*μm ± SE*	*μm ± SE*	
1.0–1.5	13	3.2 ± 0.1	4.6 ± 0.3	1.4
1.5–2.0	9	3.4 ± 0.1	5.0 ± 0.2	1.5
2.0–2.5	5	3.5 ± 0.1	5.2 ± 0.6	1.5
2.5–3.0	3	3.7 ± 0.1	5.1 ± 1.5	1.4
3.0–3.5	1	4.0 ± 0.1	6.3 ± 1.0	1.6
4.0–4.5	0	4.7 ± 0.1	—	—
8.0–8.5	0	5.2 ± 0.1	—	—

in numbers, the system appears to be very well suited to the biochemical investigation of chloroplast division. By using the incorporation of [^{3}H]thymidine into DNA as an indication of DNA synthesis in different sections of these leaves, it should be possible to discover which stages in chloroplast division and development require DNA synthesis.

Labeling of DNA. The specific radioactivity of the total DNA of each section, after leaves had been incubated with [^{3}H]thymidine, is presented in Figure 4. Incorporation of [^{3}H]thymidine into this DNA, at least 95% of it nuclear in origin (9), occurred almost entirely in the lowest cm of the leaf. By contrast, the specific radioactivity of DNA isolated from plastid preparations remained at a high level throughout the first 3 cm, falling dramatically in the 4th cm, and remaining at a negligible level above 4 cm. The significance of these changes is discussed below.

DISCUSSION

The 3-fold increase in plastids per cell we have observed in green wheat leaves is rather less than the increases seen by Possingham and Saurer (19) in spinach leaves; however, our measurements did not include those divisions which occurred below 1.7 cm from the leaf base. This lowest region was excluded from our measurements because its plastids were too small to identify inside cells by light microscopy, and because we wished to avoid including the zone of cell division.

At the base of the leaf all of the plastids are relatively undifferentiated, and so any increase of plastid number per cell must be a result of the division of proplastids. Further up the leaf this increase could be caused by the division of young chloroplasts at various stages of development, or alternatively by the replication of a subgroup of small plastids undergoing several cycles of division, as proposed by Honda *et al.* (7). On the basis of the results in Table I, this latter mechanism does not appear to operate in wheat. The incidence of division profiles at each level was seen to follow the same trend as the increase in chloroplast numbers per cell. Because the percentage of plastids with division profiles in each region was much lower than the percentage increase in plastids per cell across that region, it appears that not all of the plastids divided at once.

It is possible to make a rough estimate of the lifetime of a division profile. Between 1.8 and 2.1 cm from the leaf base the

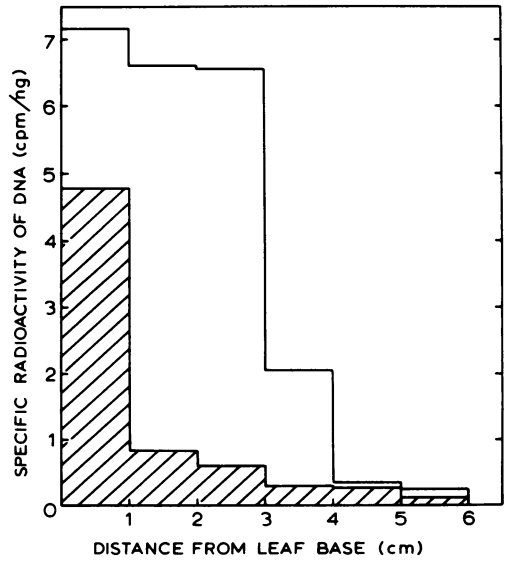

FIG. 4. Specific radioactivities of DNA prepared from whole cells (■) or from isolated plastids (□), in 1-cm regions along leaf. Values are corrected for levels of [^{3}H]thymidine in each section, and for the dilution of plastids with nonradioactive material. It was assumed that the pool size of thymidine was the same in each 1-cm section of the leaf.

chloroplast number per cell doubles, so, on average, each chloroplast divides once. It takes about 4 h for a cell to move from 1.8 to 2.1 cm above the leaf base, during which time 100% of the chloroplasts appear to divide. Only about 7% of all plastids in this region have division profiles at any time of sampling; hence, the average lifetime of a division profile is about 0.07×4 h, equal to about 20 min.

Immediately before separation, a dividing chloroplast consists of two attached daughter plastids. The shapes of the parent and daughter plastids are very similar and the volume of each daughter is approximately half that of the parent. It follows that the length of the major axis of each daughter will be 0.8 ($0.5^{\frac{1}{3}}$) that of the parent. Thus, the total length of the attached daughters will equal 1.6 times the length of the parent. Dividing chloroplasts at intermediate stages of division will be expected to have lengths between these two extremes. In our wheat leaves, the measured ratio of the average length of the division profiles to the average length of the other plastids remained constant at about 1.5 suggesting that not only small plastids, but all plastids in the population are capable of dividing. This view is further strengthened by the observation that the average size of the plastids rose only slowly throughout the region of most rapid increase in plastid numbers.

Throughout the region of plastid division, the content of Chl per plastid increased exponentially, indicating a very rapid differentiation, and hence an increase in internal membrane complexity, which obviously did not block division. This is consistent with the work of Possingham and Saurer (19) and Boasson *et al.* (4), who showed that an increase in plastid number per cell could occur in cells which contained no proplastids. We have never observed proplastids in electron micrographs of chloroplast-containing cells above 1.0 cm from the leaf base in wheat (Leech and Thomson, unpublished observations).

Because no cell division occurred above 1 cm from the leaf base, it was possible to examine the biochemistry of chloroplast division without the interference of mitosis. From the specific radioactivities of DNA in plastids isolated from seedlings incubated with [^{3}H]thymidine, it is evident that chloroplast DNA synthesis occurred in the same region as chloroplast division; above 4 cm from the leaf base both DNA synthesis and chloroplast division were negligible. The specific radioactivity of whole cell DNA was high in the bottom cm, where all mitosis occurs, but was very much less than that of the chloroplast DNA between 1 and 4 cm, showing that the observed high level of incorporation into chloroplast DNA was not caused by contamination with nuclear DNA. The results presented in Figure 4 are based on the assumption that the endogenous thymidine pool size is the same for the plastids in all sections of the leaf. An increase of 18-fold in this pool size between the 3rd and the 5th cm of the leaf would be necessary to account for the sudden decrease in the specific radioactivity of the chloroplast DNA. It seems reasonable to assume that the fall in incorporation of [^{3}H]thymidine into chloroplast DNA above 3 cm is largely the result of a decreasing rate of DNA synthesis, especially because we have evidence that the DNA per plastid remains constant above 4 cm from the leaf base (Boffey and Leech, unpublished).

Bennett and Radcliffe (3) and Rose *et al.* (24) have demonstrated an association between chloroplast DNA synthesis and chloroplast replication, although they have also shown that these two processes are not necessarily tightly coupled. It is evident from our results that synthesis of chloroplast DNA accompanies chloroplast division, but does not occur during the phase of chloroplast growth which follows the cessation of division. Because of the close match between the end of chloroplast division and the end of incorporation of [^{3}H]thymidine into chloroplast DNA, it seems likely that the synthesis of chloroplast DNA precedes the separation step of chloroplast division in wheat leaves.

Our results also show that in intact wheat seedlings concurrent synthesis of nuclear DNA is not necessary for chloroplast division or growth.

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