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Genome Expression during Normal Leaf Development[1]

I. CELLULAR AND CHLOROPLAST NUMBERS AND DNA, RNA, AND PROTEIN LEVELS IN TISSUES OF DIFFERENT AGES WITHIN A SEVEN-DAY-OLD WHEAT LEAF

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ABSTRACT

Changes in genome expression during normal cellular and plastid development in the first leaf of young (7-day-old) wheat (*Triticum aestivum* var. Maris Dove) were investigated by examining homogeneous populations of leaf cells and plastids of several developmental ages present in the same leaf. The cells were characterized over a period immediately following the last cell division. All of the leaf cells had cytoplasmic contents and nuclei, and between 44% (young tissue) and 54% (older tissue) of the leaf cells were mesophyll cells. Chloroplast development was complete 36 hours after the chloroplasts had ceased dividing. Extremely large changes occurred in cellular constituents over a very short period of leaf development. Maximum rates of accumulation of ribulose bisphosphate carboxylase per mesophyll cell (80 picograms/hour), chlorophyll per mesophyll cell (9 picograms/hour), and 70S ribosomes per mesophyll cell (19×10^5/hour) were recorded.

Total cellular DNA varied from 40 to 60 picograms/cell, reflecting the changes in nuclear and chloroplast DNA synthesis during different phases of cellular and chloroplast division. The period of maximum accumulation of protein, total RNA, and both 80S and 70S ribosomes occurred between 36 and 48 hours after the last cell division. Between 48 and 60 hours, 70S rRNA per cell and protein content per cell continued to increase as 80S rRNA per cell declined. Ribulose bisphosphate carboxylase per cell increased 20-fold between 15 and 60 hours.

Chloroplast development depends on the continuing biochemical interaction of chloroplast and cytoplasmic components and specific examples of the collaboration of nuclear and chloroplast genetic systems, as in the formation of RuBPCase[2] (14, 15) and the ATP synthase complex (28), are well documented. The investigation of normal plastid differentiation in leaves has been hampered because of the lack of synchrony in tissue development in many leaves and the complication of continuing cell division during plastid differentiation.

It seemed that young developing graminaceous leaves might provide an excellent system in which to study the characteristics and control of gene expression during normal leaf development. All cell division occurs in the basal meristem, and the tissues between this region and the leaf tip provide a gradient of cellular and plastid development within a single leaf (24, 34). Above the meristem, developmental changes can be studied in the absence of the cell cycle, and sections of tissue cut at similar distances from the leaf base provide cells and plastids of uniform age and uniform developmental stage. This allows the precise investigation of developmental parameters as the cells and plastids mature in a way which has not been previously possible using leaf tissue composed of a mosaic of developmental stages (39) such as whole dicotyledonous leaves (18, 36) or whole monocotyledonous leaves (7, 8, 30, 33). The young leaves of several graminaceous species have already been used to study changes in composition and structure associated with plastid development (1, 4–6, 11, 19, 24, 25, 29, 31, 32, 35, 37, 38).

For the present investigation, we chose young hexaploid wheat since its development is particularly rapid and uniform, chloroplast development is completed within 36 h (4) and is associated with a concomitant 4-fold increase in cell size and a 5-fold increase in cell protein. In addition, protoplasts and chloroplasts showing high levels of photosynthetic CO_2 assimilation can be isolated with ease from young wheat leaves (13). For studies of gene expression, wheat has an additional advantage since many wild and cultivated varieties are available whose genetic make-up is understood in considerable detail.

In the first stage of the investigation reported in this paper, the organization of the wheat leaf tissue is described, and the changes in DNA, RNA, and protein as the plastid differentiates are quantified. The changing pattern of RuBPCase accumulation during leaf development is also described. Counts of numbers of cells and numbers of chloroplasts enabled the quantitative changes to be expressed as values per cell and per plastid. The significance of the very large quantitative and qualitative changes is discussed.

MATERIALS AND METHODS

Plant Material. Wheat seedlings, *Triticum aestivum* var. Maris Dove (National Seed Development Organisation Ltd., Newton Hall, Cambridge), were grown according to Boffey *et al.* (4). Seedlings were harvested 2 h after the start of the light period. The leaves were cut at their bases and washed, and the coleoptiles and second leaves were removed. Twenty, 50, or 100 seedlings (according to the assay) were aligned at their bases on a cooled glass plate, and 5-mm sections were cut progressively from the base to the tip of the leaf with a razor blade.

Estimation of Mesophyll Cell Number and Total Cell Number. Mesophyll cell number per leaf section was determined by a modification of the method of Brown and Rickless (9). Ten 5-mm sections in 5% (w/v) chromium trioxide were kept at 4°C for 7 d. The cells were separated by flushing the sections through a Pasteur pipette. Mesophyll cells were counted in a 0.2 mm depth hemocytometer. After chromate treatment, there was no evidence that mesophyll cell breakage had occurred, and the suspensions were free from fragments of broken cells. This is, therefore, a satisfactory, quantitative method for counting wheat mesophyll cells as previously shown for other tissues (2, 9). The chromate method was not suitable for counting epidermal and vascular cells since epidermal cells folded or collapsed because of their considerable

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[2] Abbreviation: RuBPCase, ribulose 1,5-bisphosphate carboxylase.

length and could not be counted accurately; single vascular cells which were very small and fibrous could also not be identified. The proportion of mesophyll cells in the total cell population was determined in 1-mm-thick leaf sections after fixation and embedding in Spurr's resin as in Boffey *et al.* (4). Transverse sections, 2.5 μm thick, were cut on a Reichert ultramicrotome III, stained in 1% (w/v) toluidine blue in 1% (w/v) disodium tetraborate, and examined microscopically to determine the proportion of mesophyll cell profiles in leaf sections from different parts of the first leaf. The lengths of the epidermal, mesophyll, and vascular cells were measured in 2.5-μm-thick longitudinal sections, and corrections were made to take account of the differing cell lengths of the three cell types in the different parts of the leaf. From the cell length measurements and the counts of the number of profiles of a given cell type in transverse section, the percentage of mesophyll cells in the total cell population and the total cell number per leaf section were calculated. The pyronine-methyl green stain used to visualize cytoplasm and nuclei in hand-cut leaf sections was 0.5% (w/v) methyl green and 0.5% (w/v) pyronine Y in 0.1 M acetate buffer (pH 4.1).

Computation of Cell Age. Cell age was calculated using a modification of the computer program devised by Boffey *et al.* (6). The program was extended to cover the whole length of the leaf up to 10 cm from the leaf base by inserting the growth rates of the seedlings at earlier stages of development into the program. The computer program relies on two features of the young monocotyledonous leaves. (a) All cell division occurs in a basal meristem. Cells are formed by repeated divisions of the meristematic cells, and for the purposes of this analysis, the age of a cell is measured from the time it is cut off from the meristem by its final cell division. Cells do not divide again once they have been displaced from the meristem. (b) There is a zone of constant length in the lower part of the leaf in which all cells are elongating, extending from the basal meristem to 3 cm above the leaf base. Above this zone of elongation, no further increase in cell length occurs. The details of this structure are described in Boffey *et al.* (6).

Estimation of Chloroplast Number per Cell. Plastid number per cell was determined by counting chloroplasts in fixed, separated cells using a Zeiss photomicroscope fitted with Nomarski differential interference optics as described in Boffey *et al.* (4).

Extraction of Total RNA and Total DNA. Fifty 5-mm leaf sections were ground in K-phosphate buffer (pH 7.5). Quantitative RNA estimations were made according to the method of Becker *et al.* (2). This method was chosen since it provides a very reliable quantitative estimation for the simultaneous estimation of DNA and RNA in the same tissue. It has previously been validated for a developing cotyledon system (2). Nucleic acids were precipitated with an equal volume of cold 10% (w/v) $HClO_4$ and allowed to stand on ice for 1 h. After centrifugation at $3{,}000g_{max}$ for 10 min, the pellet was washed twice with 5 ml of 5% (w/v) $HClO_4$ and twice with 5 ml of ethanol:chloroform:ether (2:1:1, v/v/v). The pellet was allowed to drain and then was dissolved in 1 ml of 0.3 N NaOH, and the RNA was hydrolyzed for 18 h at 37°C. After incubation, the tube was cooled on ice and the sample was acidified with cold 70% (w/v) $HClO_4$. To check that the hydrolysis conditions chosen to ensure complete hydrolysis of the RNA had not caused any protein degradation (20, 21), 260:280 nm absorption ratios of the hydrolysates after 1 h and after 18 h were compared. These were 1.78 and 1.79, respectively, indicating no protein degradation had occurred.

Estimation of Total RNA and Total DNA. The resulting precipitate (A, see below) was removed by centrifugation at $3{,}000g_{max}$ for 10 min, and the supernatant whose pH was adjusted to 8.0 was assayed for RNA content by determination of A_{260}, assuming an A of 1.0 to correspond to an RNA concentration of 31.7 μg/ml (2). The 260:280 and 260:230 ratios were never less than 1.8 and 1.85, respectively. The spectral ratios were similar for RNA samples from all the leaf sections of different ages which were analyzed, and addition of a hydrolysate of pure yeast RNA increases the ratios additively. Thus, there is no indication of under or over estimation of RNA in the samples which are being analyzed.

DNA amounts were estimated using the diphenylamine assay of Burton (10) which for wheat leaf DNA gives more consistent results than fluorometric methods. Precipitate A, removed from the RNA estimation above, was hydrolyzed in 5% (w/v) $HClO_4$ for 1 h at 70°C. One-ml aliquots were then taken through the diphenylamine assay using freshly prepared calf thymus DNA (Boehringer; mol wt, $1.0–1.2 \times 10^7$) in sodium saline citrate (150 mM NaCl, 15 mM Na_3 citrate) /10 as a standard. The diphenylamine assay method had been previously validated in our laboratory (R. J. Ellis, A. J. Jellings, and R. M. Leech, unpublished results) using wheat leaf nuclei. The DNA from isolated wheat leaf nuclei was extracted and assayed as described in this paper. In addition, the DNA per leaf nucleus was estimated by Feulgen microdensitometry (3). The values of DNA (pg per nucleus) determined by the two different assay methods differed by less than 5%, showing that both the diphenylamine assay method and the DNA extraction procedure are quantitatively reliable for young wheat leaf tissue.

Estimation of Ribosomal RNA. Ribosomal RNA measurements were performed on nucleic acids extracted by the method of Leaver and Ingle (23). One hundred 5-mm leaf sections were frozen in liquid N_2 and quickly ground in a mortar in a grinding medium consisting of 6% (w/v) para-aminosalicyclic acid, 1% (w/v) tri-iso-propylnaphthalene sulfonic acid, sodium salt, 0.1 M Tris-HCl (pH 8.5). Proteins were extracted from the homogenate by shaking with an equal volume of phenol solution saturated with 0.1 M Tris-HCl (pH 8.5) containing 10% (v/v) *m*-cresol and 0.1% (w/v) hydroxyquinoline. After centrifugation at $1000g_{max}$ for 10 min, the aqueous layer was removed, and the proteins were re-extracted two more times. The aqueous layer was made to 0.3 M K-acetate (pH 5.5), and two volumes of cold absolute alcohol were added. After 12 h at −20°C, the samples were centrifuged at $1000g_{max}$ for 15 min and the pellets were washed three times in cold 80% (v/v) ethanol, dried down, and taken up in sterile water to a concentration of approximately 1 mg/ml. Forty μg RNA/ml was assumed to give an A of 1.0 at 260 nm. All steps were performed at 4°C to avoid breakdown of the 23S chloroplast rRNA (22).

Fractionation of the nucleic acids was achieved by electrophoresis on 2.4% (w/v) polyacrylamide tube gels (30 μg RNA/gel) as described by Loening (26) but at 4°C to maintain the integrity of the 23S rRNA. Gels were run for 3 h with a current of 4 mamp/gel. The 7.5 cm gels were washed in distilled H_2O for 2 h to reduce background absorbance and scanned at 260 nm using a Joyce-Loebl UV scanner. To quantitate the 25S and 18S cytoplasmic rRNA, the 23S and 16S chloroplast rRNA, and the low-mol-wt RNA, the areas under the individual peaks were measured using a Hewlett-Packard digitizer. The areas were expressed as a function of the total area under the curve for a particular scan. Each fraction was then multiplied by the total RNA content (μg/leaf section) determined as described for the total RNA assay.

Estimation of Protein. Twenty frozen leaf sections were ground in 0.5 ml buffer containing 25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonylfluoride in a mortar. The homogenate was decanted into tubes, and the mortar was washed with further grinding buffer. The wash was added back to the original homogenate. Protein was precipitated from the total homogenate by the addition of 10% (w/v) TCA and incubated on ice for 1 h. The samples were boiled for 15 min, and the pellets were washed with 80% (v/v) acetone. Protein was determined according to Lowry *et al.* (27) using BSA (Cohn fraction V) as a standard.

Electrophoretic Assay for RuBPCase. RuBPCase was determined by fractionation of the protein homogenate on non-denaturing 6% (w/v) polyacrylamide tube gels. The appropriate gel region was excised, and the protein was recovered and quantified. The gel constituents, 6% (w/v) acrylamide, 0.12% (w/v) bisacrylamide, 0.375 M Tris-HCl (pH 8.5), were polymerized with 0.06% (w/v) *N,N,N',N'*-tetramethylethylenediamine and 0.07% (w/v) ammonium persulphate, and pre-run for 1 h at 1 mamp/tube. Each gel was loaded with 100 to 300 μg protein in 2.5 mM Tris-HCl (pH 8.5), 19.2 mM glycine, 10 mM 2-mercaptoethanol, 5% (w/v) sucrose with bromophenol blue as tracker dye. Gels were run at 1 mamp/tube for 6 h and stained overnight in 0.5% (w/v) Amido Black 10B in 7% (v/v) acetic acid. Destaining was carried out electrophoretically at 1 amp/8 gels for 1 h in 7% (v/v) acetic acid. The RuBPCase band, identified by coelectrophoresis of the purified enzyme (see "Acknowledgments"), was excised from the gels and incubated at 37°C for 16 h in 2 ml 1 N NaOH. The protein content of the NaOH extracts was quantitated from *A* at 615 nm using a standard curve for 25 to 200 μg of purified wheat RuBPCase. The standard was electrophoresed and extracted in NaOH using the same procedure as for the samples.

RESULTS

The seedlings were first examined to establish the growth rate of the leaves and the rate of chloroplast development.

Plant Growth. The growth rate of the wheat seedlings increased during the first 5 d after sowing and then remained constant at 3.53 cm/d until 7 d after sowing. The variation in the growth rates of different plants was less than 8%. After 7 d, the growth rate of the first leaf began to decline. Seven-d wheat leaves were chosen since this stage of development provided the widest range of cells at different ages in leaves still growing at their maximal rate.

The first part of the analytical investigation was aimed at establishing the details of the pattern of the cellular development occurring in cells of a 7-d wheat leaf to characterize the system before further biochemical analysis. The characterization included the determination of the number and age of mesophyll cells in each leaf section.

Cell Number per Leaf Section. Mesophyll cell number/leaf section dropped rapidly in the first 3 cm above the leaf base (Fig. 1) because of rapid cell elongation. In older tissue, the number of cells per leaf section remained constant indicating that no ontogenetic variation (29) in cell number occurs in the first leaf of wheat. The cells in successive stages of development form a linear series from base to tip of the first leaf.

The mesophyll cell profiles, including both arm palisade and thin-walled bundle sheath cells (16), constituted about 40% of the total cell population in the transverse sections taken from the embedded leaf sections. The rest of the cell population was made up of about 37% vascular cells and 23% epidermal cells. These proportions were constant in transverse sections of all the leaf tissue of the first leaf. A comparison of the cell length of mesophyll cells with the cell lengths of epidermal and of vascular cells is shown in Table I. The changes in relative cell lengths occurring as the leaf tissue matures are clearly revealed. The largest change in ratio, seen as an increase in vascular cell length relative to mesophyll cell length occurs, as the xylem vessel end walls breakdown (40–60 mm). The insert in Figure 1, shows the proportion of mesophyll cells in leaf tissue of different ages calculated by taking into account the changes in relative cell length. Throughout the leaf, only 44 to 54% of the leaf cells are mesophyll cells. This is a remarkably low proportion and has important consequences in a consideration of the distribution of the biochemical function between the different cell types in a green leaf.

Using the values of mesophyll cells per leaf section (Fig. 1; curve A) and the proportion of mesophyll cells to total cells (Fig. 1; curve B), the total cell number per leaf section was calculated

Table I. *Ratios of Mesophyll Cell Lengths to Epidermal and Vascular Cell Lengths in 7-Day-Old Leaves of T. aestivum var. Maris Dove*

Longitudinal paradermal serial sections were cut from one leaf epidermis to the other, and the cells in every fifth section were photographed. Cell lengths were measured from the photographs, and each value was an average of the measurements from at least 15 sections.

Leaf Section (distance from leaf base)	Ratios of Cell Lengths	
	Mesophyll:epidermal	Mesophyll:vascular
mm		
2–3	0.83	0.70
19–20	0.42	1.12
40–41	0.28	1.11
60–61	0.39	0.72
80–81	0.27	0.78

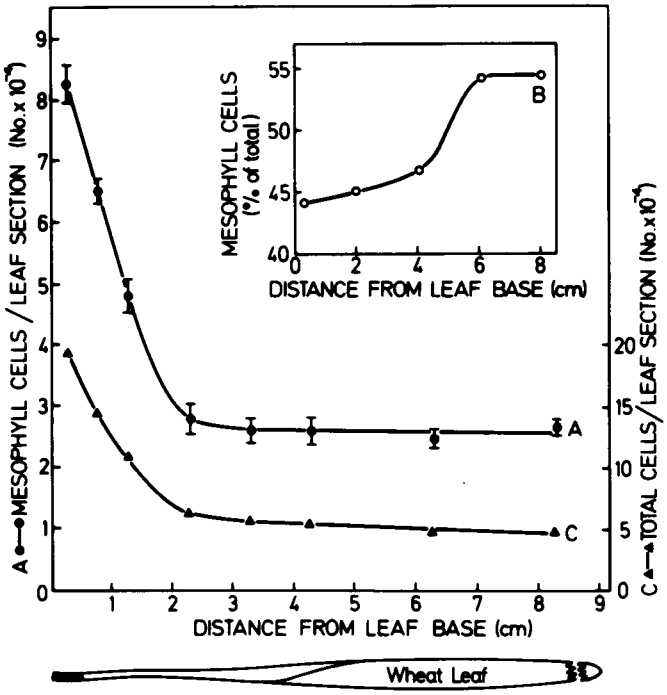

FIG. 1. Changes in cell number in leaf sections taken along the first leaf of *T. aestivum* var. Maris Dove. Mesophyll cell number per section (A) and the proportion of mesophyll cells in the total cell population (B) were measured as described in the text. Points are means ± SE for at least three determinations on different batches of leaves. Total cell number per leaf section (C) was calculated using A/B. The black section on the drawing of the first leaf of wheat represents the basal meristem.

(Fig. 1; curve C). Because of the high proportion of vascular tissue, it is also important to establish the proportion of cells in this category which are still alive, particularly in the present studies when nucleic acid values per cell are being determined. Unexpectedly, all cells of the vascular tissue examined under bright field microscopy were found to have cytoplasmic contents and also nuclei, even in the more mature region of the leaf. The nuclei were seen in toluidine blue-stained sections, and the cytoplasmic contents and nuclei were differentially stained using pyronine-methyl green (see "Materials and Methods"). Nuclei and cytoplasm were also found in thin sections of vascular tissue cells of all ages examined in the electron microscope by Dr. K. Platt-Aloia. It is therefore assumed that all cells in a 7-d-old wheat leaf (vascular, epidermal, and mesophyll) will contain nucleic acids, and all the subsequent calculations in this paper are based on this

assumption. The details of the microscopical examinations will be published elsewhere.

Determination of Leaf Cell Age. The relationship between the position of a cell in the leaf and its age is shown in Figure 2. At the base of the leaf, extension growth is small and mainly reflects successive cell divisions pushing cells away from the meristem. Rapid elongation of the cells occurs just above the meristem and thus accounts for the faster leaf extension higher up the leaf. In cells between 1.5 and 7.0 cm from the leaf base, the relationship between cell age and distance from leaf base is linear. The distal 3 cm of the first leaf contains the cells produced in the first 4 d after sowing when the growth rate of the leaf had not reached its maximum. This is a reflection of the incomplete development of the elongation zone which, in turn, is reflected in the nonlinear relationship between cell age and distance from leaf base in cells above about 7 cm from the leaf base, shown in Figure 2.

Determination of Plastid Number per Leaf Section. The next phase of the investigation was to relate plastid development to cell development. Chloroplast number per cell increases from the youngest cells up to cells 8 cm from the leaf base (Table II). Using the values for mesophyll cell number per leaf section (Table II, line 2), the total number of chloroplasts in each section can be calculated (Table II, line 3). There is a 75% increase in chloroplasts per leaf section contrasting with the doubling of chloroplasts per cell. This is a reflection of the decrease in cell number per leaf section with increasing age.

Total DNA, RNA, and Protein Levels per Leaf Cell. Having established the details of cell number and cell age in leaf tissue of different ages, the next phase of the work was a biochemical characterization of the leaf. The DNA, RNA, and protein content per leaf cell for leaf tissue of different cell age is shown in Figure 3. Since both green and non-green cells of all ages are living, the total cell number was used to calculate nucleic acids per cell. The cellular content of DNA decreased from the base of the leaf to 1 cm above the base. The meristematic zone of the first leaf is found in this region, and the high levels of DNA per cell reflect the presence of cells in which nuclear DNA replication had taken place, but cell division had not yet occurred. The absolute levels of DNA in cells of all ages are high compared with the 2C level for wheat quoted as 34.6 pg/nucleus (3).

The total amount of RNA increased at first slowly, then linearly for the first 2 d of development (Fig. 3), and then dropped until it reached the level comparable with that found 1.75 d after cellular development was first measured. The levels of RNA were always much greater than the amount of DNA, and the ratio of RNA to DNA reached a maximum of about 3.

The rate of protein accumulation increased progressively as the cells matured, with a 4-fold increase in 36 h, the most rapid increase following the initiation of the rapid increase in RNA (Fig. 3). The patterns of RNA and protein per cell show general increases as the cells mature.

The next stage of the work was to establish the contributions of the individual RNA and protein components to the overall changes.

Chloroplast and Cytoplasmic Ribosomal RNA. Figure 4 shows representative scans of the polyacrylamide gels from which the quantitative information was obtained. As might be expected, the two cytoplasmic subunits (25S and 18S) change in parallel as do the two chloroplast subunits (23S and 16S). In the youngest leaf sections, the contribution from the chloroplast ribosomal subunits is negligible and increases rapidly as the chloroplasts develop until, in the more mature sections of the leaf, the contributions from chloroplast and cytoplasmic ribosomal RNAs are approximately the same. However, although all the cells of the leaf possess cytoplasmic ribosomes (80S), only the mesophyll cells (44–54% of the total) contain chloroplast (70S) ribosomes. When this distribution is taken into account and the values of RNA per cell are adjusted to accommodate the changes in the proportion of meso-

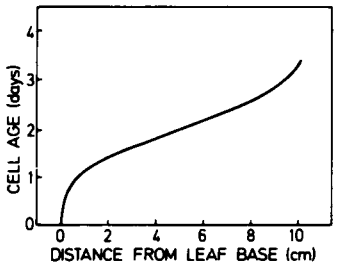

FIG. 2. Relationship between age of a leaf cell and its distance from the leaf base in a 7-d-old wheat leaf. The curve was computed using measured growth rates at leaf tips and in sections at different heights from the leaf bases (details in Ref. 8 and in the text).

Table II. *Calculations of Chloroplast Number per Leaf Section in Leaf Sections along the First Leaf of T. aestivum var. Maris Dove*

Chloroplast number per leaf section is derived from the product of mesophyll cell number per leaf section and the chloroplast number per cell (values are means ± SE for at least 13 estimations). Each leaf section taken was 5 mm above the height from the leaf base. The calculation assumes that only mesophyll cells contain chloroplasts. Guard cells are also chloroplast-containing cells, but since the guard cells constitute only about 3% of the total cell number and contain only about 5 very immature plastids in wheat, the contribution of these to the chloroplast population was considered to be negligible.

Distance from Leaf Base	Chloroplast No./ Mesophyll Cell	Mesophyll Cell No./Leaf Section × 10^{-4}	Chloroplast No./Leaf Section × 10^{-6}
cm			
2	69 ± 4	2.85	1.97
4	90 ± 5	2.62	2.36
6	109 ± 7	2.62	2.86
8	133 ± 6	2.62	3.49
10	135 ± 7	2.62	3.54

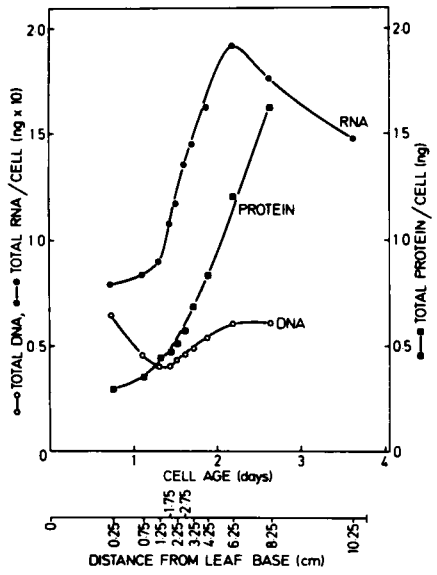

FIG. 3. Changes in total RNA, DNA, and protein per cell during cell development in the leaf cells of *T. aestivum* var. Maris Dove. Maximum SE for RNA and DNA estimations was ±6% and for protein estimations was ±8%. All measurements were estimated using 5-mm leaf sections. All leaf cells are assumed to be alive (see text). The total number of cells per section was used to give the values calculated on a per cell basis.

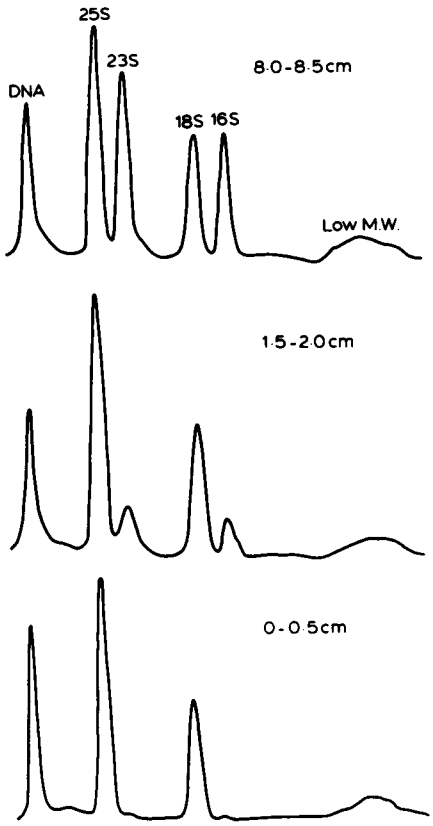

FIG. 4. Acrylamide gel profiles for RNA extracted from leaf sections 0 to 0.5, 1.5 to 2, and 8 to 8.5 cm from the leaf base of 7-d-old *T. aestivum* var. Maris Dove. Thirty μg phenol-extracted total nucleic acid was loaded onto 2.4% polyacrylamide gels. The gels were subjected to electrophoresis at 4°C for 3 h with a current of 4 mamp/gel, washed in distilled H_2O, and scanned at 260 nm. The between gel variation for one sample was ±3%.

Table III. *Changes in the Ribosomal Subunits per Cell during the Development of a 7-Day-Old Leaf of T. aestivum var. Maris Dove*

Ribosomal RNA values per leaf section were expressed on a per leaf cell basis using the total cell number per leaf section for cytoplasmic ribosomal subunits and the mesophyll cell number per section for chloroplast ribosomal subunits. The values are means ± SE for two measurements.

Distance from Leaf Base	Cytoplasmic rRNA Subunits/Cell		Chloroplast rRNA Subunits/Mesophyll Cell	
	25S	18S	23S	16S
mm	*pg*			
0–5	41 ± 0.5	22 ± 0.1	2.5 ± 1.5	1.8 ± 0.7
5–10	39 ± 1	24 ± 1	11 ± 3	5.5 ± 1.5
10–15	45 ± 0.5	25 ± 1.5	12 ± 3	8.7 ± 1.3
15–20	49 ± 0.5	27 ± 1.5	19 ± 5	12 ± 1
20–25	51 ± 0.5	28 ± 0.5	24 ± 6	18 ± 3
25–30	61 ± 0.5	33 ± 1	35 ± 0.5	21 ± 0.1
30–35	59 ± 0.5	33 ± 2	41 ± 4	28 ± 0.5
40–45	62 ± 1.5	36 ± 0.1	54 ± 0.5	36 ± 1
60–65	64 ± 3	36 ± 0.5	69 ± 3	45 ± 1.5
80–85	55 ± 4	31 ± 0.5	79 ± 0.5	48 ± 1

phyll cells during leaf development, then the values shown in Table III are obtained. There is a 30-fold increase in chloroplast ribosomal RNA per cell in contrast to the cytoplasmic RNA which only increases by 60%. The figures shown in the legend to Figure 5. were used to convert pg RNA to give the number of ribosomes.

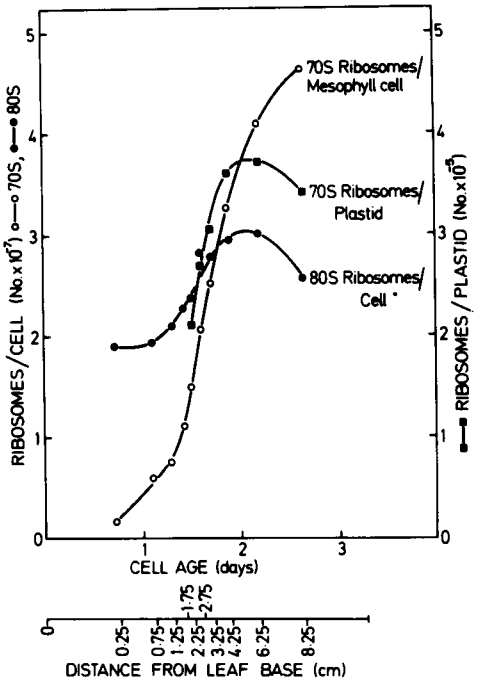

FIG. 5. Changes in ribosome number during cell and chloroplast development in leaf cells of *T. aestivum* var Maris Dove. Conversion of pg RNA per cell (from Table III) to 70S ribosome number was calculated assuming one molecule rRNA to weigh $\frac{(0.56 + 1.06)}{6.023 \times 10^{-23}} \times 10^6$ g = 2.76×10^{-6} pg RNA/70S ribosome. Conversion of pg RNA per cell (from Table III) to 80S ribosome number was calculated assuming one molecule rRNA to weigh $\frac{(1.3 + 0.7)}{6.023 \times 10^{-23}} \times 10^6$ g = 3.32×10^{-6} pg RNA/80S ribosome. (○), 70S ribosomes per mesophyll cell; (■), 70S ribosomes per plastid; (●), 80S ribosomes per cell.

Table IV. *Changes in the Level of RuBPCase in Cells and Chloroplasts of a 7-Day-Old Leaf of T. aestivum var. Maris Dove*

RuBPCase content per leaf section was determined electrophoretically as described in the text. The values are means for three separate experiments. Similar measurements in different experiments differed by only 10 to 15%. Mesophyll cell number per leaf section and chloroplast number per leaf section were used to determine RuBPCase per mesophyll cell and RuBPCase per plastid, respectively.

Distance from Leaf Base	RuBPCase		
	% of total leaf protein	/Mesophyll cell	/Plastid
mm		*pg*	
0–5	12	82	—[a]
5–10	16	125	—
10–15	11	107	—
20–25	13	146	2.1
30–35	17	242	3.0
40–45	19	324	3.6
60–65	37	821	7.5
80–85	56	1,691[b]	12.7

[a] Values for RuBPCase per plastid below 2 cm could not be calculated as the plastids were too small to be counted in the cells under light microscopy.

[b] The higher figures for RuBPCase per mesophyll cell than protein per cell reflect the higher total protein content of mesophyll cells compared with epidermal and vascular cells.

The rapid increase in 70S ribosomes per cell is shown in Figure 5; the 7-fold increase which occurs in the 18-h period between 30 and 48 h after development begins is particularly dramatic. The increase in 70S ribosomes per cell is due in part to the doubling in chloroplast number per cell; the number of 70S ribosomes per plastid actually decreases in the older cells. The patterns of development for 70S ribosomes per plastid and 80S ribosomes in all the cells changed in parallel, both increasing for the first 2 d of development and then decreasing.

Quantification of the Major Leaf Protein RuBPCase. The increases in RuBPCase during leaf and plastid development were measured as a concentration per cell and per plastid. Using these methods of analysis, the extremely dramatic changes taking place can be quantified. The results are shown in Table IV. The absolute quantities of RuBPCase per mesophyll cell increase 20-fold from young cells containing proplastids to those containing fully mature chloroplasts. This change is particularly impressive inasmuch as it occurs in a period of only 2 d. Even in this young leaf tissue, 56% of the total protein in the older cells is RuBPCase. The most rapid phase of increase expressed on a per plastid basis occurs after the phase of chloroplast division has ceased.

DISCUSSION

This paper describes the biochemical and structural development of the tissue of the first leaf of *T. aestivum* var. Maris Dove. Three significant conclusions can be drawn from this work.

(a) Extremely large changes occur in many of the cell constituents over a very short period of leaf development. The maximum rates of accumulation are: RuBPCase per mesophyll cell, 80 pg/h; Chl per mesophyll cell, 9 pg/h; and 70S ribosomes per mesophyll cell, 19×10^5/h. These values compare with similar parameters measured in cucumber cotyledons (2) where maximum rates of accumulation of RuBPCase per cell, Chl per cell, and 70S ribosomes per cell were found to be 8 pg/h, 4 pg/h, and 4×10^5/h, respectively.

(b) An unexpectedly small number of chloroplast-containing cells is present in the total cell population of the young wheat leaf; the maximum contribution from mesophyll cells reaches only 54% in the mature tissue. This has important consequences when parameters such as nucleic acids are expressed on a per cell and per chloroplast basis. It has generally been assumed by plant physiologists that the contribution of non-green tissue in the leaf is negligible and can be ignored in biochemical analyses. This is clearly incorrect at least in the case of wheat, spinach, and barley (A. J. Jellings and R. M. Leech, manuscript in preparation).

(c) All the cells in a 7-d-old wheat leaf, including the vascular tissue, have cytoplasmic contents and nuclei.

The DNA levels per cell fall between the leaf base and 1 cm above it because of the decreasing proportion of cells undergoing DNA replication and then cell division. After 1.25 d of cell development, the DNA per cell value reaches a minimum of 40 pg. This compares with the published 2C value for wheat nuclei of 34.6 pg (3) and indicates that these young cells contain about 5 pg organellar DNA. The 25% increase in DNA per cell between 1.4 and 1.75 d of development can be accounted for by increased chloroplast DNA synthesis in this region of leaf tissue as shown previously (4). The increase in DNA per cell between 1.75 and 2.2 d of development almost certainly reflects the breakdown of the end walls in the xylem, which can be observed after 1.8 d, causing the xylem vessels to become highly polyploid. Since the vascular tissue contributes about 40% of the leaf cells in mature tissue (Table I; Fig. 1), their increase in polyploidy will considerably alter the value for total DNA per cell calculated for the whole leaf.

The drop in total RNA parallels the decrease in 70S ribosomes per plastid and 80S ribosomes per cell and occurs without a concomitant drop in DNA per cell.

Although nuclei and cytoplasmic contents are seen in all cells of the vascular tissue in all positions in the leaf, the decrease in RNA per cell may reflect the beginning of the degradation of cytoplasmic contents known to occur during xylem vessel formation. Dyer and Osborne (12) found that DNA was degraded much later than RNA in senescing tissue and suggested that, as DNA is turned over less than RNA, it is more resistant to cytoplasmic degradation.

The pattern of development of 70S ribosomes per plastid and 80S ribosomes per cell is very similar; both increase for the first 1.75 d of development and then decrease. Using quantitative electron microscopy as an alternative to the biochemical estimations, we have recently been able to demonstrate a 3-fold increase in 70S ribosomes per plastid during the development of young corn leaves (H. M. Nice and R. M. Leech, unpublished results). The calculation of the number of 80S ribosomes per cell assumes all living cells in the leaf have the same complement of ribosomes. We plan to investigate the validity of this assumption in wheat leaves. The ribosome numbers decrease at the stage of development where Chl per plastid, chloroplast number per cell, and chloroplast area (C. Dean and R. M. Leech, unpublished results) are still increasing and the increase in RuBPCase per plastid is at its maximum. The period of most rapid protein synthesis clearly occurs before the completion of the development of the photosynthetic apparatus.

In mature wheat leaf cells, 50% of the total protein is RuBPCase, a high value compared with 20 to 50% quoted previously for dicotyledons (15, 17). These lower values may reflect a sampling error which occurs in all developmental work on dicotyledonous plants. The developing leaves of dicotyledons are made up of a mosaic of cells of different ages and also contain chloroplasts at different stages of development so an estimate of chloroplast proteins such as RuBPCase will be an average for plastids of many different developmental ages.

The changes in RuBPCase level per cell found in the present work on wheat is very large when compared to previous levels found in *Lolium multiflorum* (31) and in *Cucumis sativus* (2).

In all cell development studies, since many characteristics of the tissue are changing simultaneously, the parameter used for expressing the developmental changes is critical. We cannot emphasize too strongly the importance of using as many different parameters as possible as a basis for comparisons. By expressing protein values on a per cell and per chloroplast basis, the changing characteristics of the cell and of the plastid can be considered independently. This is illustrated by the values of RuBPCase shown in Table IV. The RuBPCase content per mesophyll cell increases rapidly and steadily as the cells develop. However, the greatest increase per plastid does not occur until after chloroplast division has ceased, *i.e.* after 1.75 d of development, in leaf tissue 4 cm from the leaf base. At this point, the dilution effect of chloroplast division is no longer a contributing factor to the levels of RuBPCase per plastid.

The wheat leaf therefore provides a good developmental system in which to study both qualitative and quantitative changes in gene expression and to relate these changes to cell and chloroplast differentiation. We are at present engaged in the identification and study of the control of synthesis of the polypeptides which undergo major changes during the development of the wheat leaf.

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