Deoxyribonucleic Acid and Ribonucleic Acid Synthesis during the Cell Expansion Phase of Cotyledon Development in *Vicia faba* L.

ADELE MILLERD AND P. R. WHITFELD
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A.C.T. Australia

**ABSTRACT**

In *Vicia faba* L., the tissue specific proteins, legumin and vicilin, are synthesized during the cell expansion phase of cotyledon development. During this growth period, RNA and nuclear DNA increase 8- to 10-fold. $^3$H-Uridine and $^3$H-adenosine are incorporated into ribosomal RNA, both 25S and 18S, and into transfer RNA. DNA isolated from cotyledons in the cell division phase of growth has been compared with DNA isolated from cotyledons undergoing expansion growth. Results indicate that the DNA increase involves replication of the whole genome (endoreduplication).

After 1 week in a glasshouse at 24 C day, 19 C night temperature, plants were transferred to 18 C day, 13 C night temperature. Natural illumination was used, and the photoperiod was extended with supplementary low incandescent light. In summer, plants flowered 5 weeks from sowing. Cotyledons up to 13 mm length (about 25 days from flowering) correspond to phase I of Briarty et al. (3); cotyledons up to 28 mm, the initiation of dehydration, are equivalent to phases II and III (3). Cotyledons were 19 to 21 mm about 32 days from opening of the flower. Developing seeds were harvested, the testa and plant axis were removed, and the cotyledons were used for analysis. As described previously (19), the length of the long axis or the fresh weight of the cotyledons was used as a measure of development.

**Cell Number.** The number of cells per cotyledon was determined as described by Rijven and Wardlaw (24).

**Estimation of DNA and RNA.** Cotyledons were collected from developing seeds of all ages up to maximum fresh weight. Duplicate, pooled samples of each size were obtained. Fats and cold acid-soluble substances were extracted as described by Williams and Rijven (31) except that at step 5 four extractions (70% ethanol, 15 min) were employed. Nucleic acids were subsequently extracted with 0.5 N perchloric acid at 70 C for 15 min, and the UV absorption of the extracts was monitored. With older cotyledons (e.g., 25 mm in length) up to eight extractions were necessary for complete removal of nucleic acids. Total nucleic acid was estimated from absorbance at 260 nm of the combined perchloric acid extracts assuming that 1 mg nucleic acid/ml had E$_{260}$ of 30 units. An aliquot of the perchloric acid extract was used to determine DNA (6), and RNA was obtained by difference.

**Uptake and Incorporation of Labeled Nucleosides.** Detached cotyledons were incubated at 25 C, with gentle shaking in 5 ml of sterile nutrient (15), containing tritium-labeled nucleosides. Incorporation was measured following isolation of nucleic acids according to Solymosy et al. (27). Radioactive nucleosides were supplied by Amersham (thymidine, 27 c/mmole; uridine 4.6 c/mmole; adenosine, 8.43 c/mmole).

**Resolution of Nucleic Acids by Electrophoresis on Acrylamide Gels.** Isolated nucleic acids (30 mg) were resolved by electrophoresis on acrylamide gels as described by Loning (16), but modified by the addition of 0.5% agarose (9). The gels were scanned at 260 nm in a Gilford 240 spectrophotometer with a linear transport attachment, and then cut into 1-mm slices. Each slice was transferred to a scintillation vial, 0.5 ml of NCS solubilizer was added, and the vials were incubated for 2 hr at 60 C. Eight ml of scintillation fluid (6 g 2,5-diphenyl-oxazole/1 toluene) were added and after standing overnight in

**MATERIALS AND METHODS**

**Plant Material.** The data of Figure 1 were obtained using seeds of *Vicia faba* L. (cultivar unknown) as described previously (19). All other data were obtained using *V. faba*, cv. Early Long Pod. Plants were grown in the Controlled Environment Research Laboratory in Canberra. Seeds were planted in an equal mixture of perlite and vermiculite, and irrigated twice daily, once with half-strength Hoagland's nutrient solution.
the dark, samples were counted in a Beckman 100 liquid scintillation system.

**Autoradiography.** Following uptake and incorporation of 
$^3$H-thymidine, small pieces of cotyledon tissue were fixed in acetic acid-alcohol (1:3) and stained with Feulgen. Squashes of tissue were then prepared and dipped in Ilford K2 emulsion. Controls were prepared from tissue not exposed to $^3$H-thymidine. At the end of the exposure period, slides were developed with Dektol.

**Isolation of DNA.** Tissue was ground in a mortar with buffer (0.5 M NaCl, 0.05 M Na citrate, 0.1 M EDTA, pH 7.5) containing 2% sodium dodecyl sulphate, transferred to a tube and incubated 15 min at 50 C. Pronase was then added to a final concentration of 5 mg/ml and incubation at 50 C continued for another 3 to 5 hr. The sample was then extracted with buffer-saturated phenol in the usual way, and strings of DNA were spooled from the aqueous phase following addition of an equal volume of ethanol. Further purification of the DNA involved treatment with pancreatic and T1 ribonuclease, a second digestion with pronase and another phenol extraction. DNA was spooled from the final ethanol precipitation step, dissolved in 0.1 X SSC1 and dialyzed exhaustively against the same buffer.

**Shearing of DNA.** DNA was sheared to a molecular weight of approximately 200,000 as measured by analytical velocity sedimentation. Three to 5 ml of DNA (0.15 mg/ml) were sonicated with four bursts of 30 sec with the micro-tip of a Branson B12 sonifier at maximum allowable power output.

**Buoyant Density Determinations.** DNA samples were centrifuged to density equilibrium in CsCl according to the procedure of Mandel et al. (18). DNA from M. lysodeikticus (p 1.731 x 100 kg/ml) was included in each sample as a density marker.

**Reassociation Kinetics.** The reassociation kinetics of heat-denatured DNA were determined by monitoring (Gilford 2400 spectrophotometer) the hypochromicity during reassociation. Reassociation was carried out in 0.12 M phosphate buffer, an equimolar mixture of NaHPO4 and Na2HPO4, at 61 C or in 5 X SSC, 50% formamide at 37 C (these latter conditions are acceptable by the criteria of McConaughy et al. (20), since the Tm for V. faba DNA under these conditions is 61 ± 0.5 C). The rates of reassociation in 5 X SSC, 50% formamide have been corrected to those of 0.12 M sodium phosphate from the data of Britten and Smith (5). E. coli DNA (74 x 1000 kg/ml) was used as a standard to check for fluctuations in the reassociating conditions. Absorbance changes were measured at 260 nm in phosphate buffer, and at 270 nm in formamide. Rates of reassociation were generally monitored in 1-cm light path cuvettes at a DNA concentration of 75 to 80 µg/ml. For the very slowly reassociating component, 1-mm light path cuvettes and a DNA concentration of 900 to 925 µg/ml were used. This concentration of DNA was obtained by precipitating sonicated DNA with ethanol, dissolving the precipitated DNA in 5 X SSC, 50% formamide, and exhaustively dialyzing against 5 X SSC, 50% formamide. If necessary, the DNA solution was further concentrated in the dialysis sac with Ficoll (mol wt approximately 400,000; Pharmacia). The DNA solution was again dialyzed against 5 X SSC, 50% formamide. The hypochromicity observed on denaturation was 35 to 38% for all preparations of DNA.

**RESULTS**

**Pattern of Cotyledon Development.** The two phases of cotyledon growth have been defined by following changes in the number of cells present during development (19). In the material used for the experiments reported in this paper, the growing conditions were such that the full complement of cells was reached when the long axis of the cotyledons was 13 to 14 mm (about 25 days after flowering). An average measurement for the maximum number of cells per cotyledon was $2.8 \times 10^6$.

**DNA and RNA Content of Cotyledons during Development.** The changes in nucleic acids during cotyledon development are shown in Figure 1. DNA and RNA content increased throughout development, and the greatest increase occurred during growth by cell expansion. This continuing increase in DNA per cell is in agreement with the data of Wheeler and Boulter (30), who showed that the most rapid increase in DNA and RNA occurred between 36 and 45 days after flowering, and that cell division had ceased 25 days after flowering (3).

Using the data of Figure 1 and an average value of $2.8 \times 10^6$ cells per cotyledon, it can be calculated that the DNA content of a cell from a 13-mm cotyledon would be about $4.5 \times 10^{-12}$ g, but for a 29-mm cotyledon, it may be as high as $33.2 \times 10^{-12}$ g. McLeish (21) has reported 6.02 $\times 10^{-14}$ g DNA and Kung and Williams (14) 5.8 $\times 10^{-14}$ g per nucleus from root tips of V. faba. The increase in DNA content is reflected by an enlargement of the nuclei. Microscopic examination of Feulgen-stained squashes showed (Table I) a marked increase in size of nuclei and nucleoli of parenchymatous cells during growth by cell expansion. Increased size of nuclei has been correlated in Pisum with increased nuclear DNA content (25, 26).

**Nature of RNA Synthesized during Growth by Cell Expansion.** We tested the possibility that the rapid synthesis of storage proteins was correlated with changes in specific RNA species. Detached cotyledons (22 mm) were incubated at 25 C for 22 hr in a mixture of $^3$H-uridine (4 µCi/ml) and $^3$H-adenosine (4 µCi/ml). The nucleic acids were then extracted, the various species were resolved by acrylamide gel electrophoresis, and the distribution of the label determined. The absorbance and radioactivity patterns coincided, most of the label being incorporated into ribosomal RNA, both 25S and 18S, and into tRNA (Fig. 2). The incorporation in vivo of $^3$H-uridine into ribosomes of developing V. faba cotyledons has been reported by Payne and Boulter (23).

**Nature of DNA Synthesized during Growth by Cell Expansion.** Detached cotyledons (21 or 22 mm) were incubated for varying periods in medium containing 20 µCi/ml $^3$H-thymidine, and the fate of the label was studied by autoradiography of Feulgen-stained squashes. In all cases, the label was found to be restricted to the nucleus. After 22 hr incubation, all parenchymatous cells and cells of the vascular strands showed incorporation into the nuclei.

**Buoyant Density.** The increased DNA per nucleus may represent replication of part (selective amplification) or all of the genome (endoreduplication). As a criterion to assist in distinguishing between these alternatives, the buoyant density of DNA isolated from cotyledons in the cell division phase of growth was compared with that of DNA isolated from cotyledons growing by cell expansion. Both DNA preparations gave a single band of density 1.696 ± 0.001 g/cm3, a value in good agreement with those reported for nuclear DNA from roots (14) and leaves (29) of V. faba. It was concluded that the extra DNA synthesized during cell expansion had the same over-all base composition as the 2c DNA and, as there was no evidence of a satellite band in the DNA from the cell expansion phase, it was unlikely that there had been extensive amplification of a specific segment of the genome.

Additional support for the conclusion that the extra DNA synthesized in the cell expansion phase was similar to the pre-

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1 Abbreviation: SSC: 0.15 M NaCl, 0.015 M sodium citrate.
FIG. 1. Changes in content of nucleic acids during development of \textit{V. faba} cotyledons. Nucleic acids were extracted according to Williams and Rijven (31). Total nucleic acids were estimated at 260 nm assuming 1 mg nucleic acid/ml had E$_{260}^{	ext{cm}}$ of 30 units. DNA was determined (6) and RNA obtained by difference.

Table 1. Increase in Size of Nuclei and Nucleoli of Parenchymatous Cells during Expansion Growth in \textit{V. faba} Cotyledons

Measurements were made by microscopic examination of Feulgen-stained squashes.

<table>
<thead>
<tr>
<th>Length of Cotyledon (mm)</th>
<th>Nuclei Diameter (μm)</th>
<th>Nucleoli Diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>12.7 (se 0.6)</td>
<td>3.6 (se 0.4)</td>
</tr>
<tr>
<td>17</td>
<td>20.5 (se 0.9)</td>
<td>8.4 (se 0.4)</td>
</tr>
<tr>
<td>26</td>
<td>34.1 (se 1.5)</td>
<td>14.0 (se 0.8)</td>
</tr>
</tbody>
</table>

existing DNA came from experiments in which labeled DNA, extracted from young cotyledons (10 mm) which had been exposed to $^3$H-thymidine for 24 hr, was centrifuged to equilibrium in CsCl with unlabeled DNA isolated from cotyledons late in the cell expansion phase of growth. It can be seen (Fig. 3a), that the radioactivity pattern coincided with the absorbance pattern. A similar result (Fig. 3b) was obtained in the converse experiment, namely when labeled DNA from old (>22 mm) cotyledons exposed to $^3$H-thymidine for 24 hr was centrifuged with unlabeled DNA from young cotyledons in the cell division phase of growth.

Reassociation Kinetics. The time required for reassociation of denatured DNA reflects the number of different sequences represented and their relative concentrations (4). By plotting C$_t$ (the product of DNA concentration [moles/liter] and time [sec]) against per cent DNA reassociated, the relative proportion of these various renaturing species becomes apparent (4). A comparison was made of the reassociation kinetics of DNA isolated from the cell division phase of cotyledon growth with those of DNA from cotyledons in the cell expansion phase.

Reassociation in high salt and formamide at moderate temperature is a useful method for estimating moderately reiterated and unique sequences since the DNA must be incubated for long periods. However, with this method, the initial rates of reassociation of the highly reiterated sequences were so rapid, it was not possible to obtain accurate measurements. The kinetics of reassociation of the highly reiterated sequences...
were therefore measured at higher temperature (61°C) in 0.12 M phosphate, conditions which allow a more precise analysis of rapidly reassociating species.

As is shown in Figure 4, the kinetics of reassociation are similar for the two types of DNA. The small separation of the two curves at low Cₐt values was not reproducible and was, we believe, caused by experimental difficulties. When the kinetics of the rapidly reassociating components were measured in 0.12 M phosphate at 61°C, the curves were identical (Fig. 5) for DNA from both phases of cotyledon growth and from shoots of germinated seeds of *V. faba*.

For both types of DNA, the reassociation kinetics show (Fig. 4) approximately 30% unique sequences and 70% of families of sequences of varying degrees of reiterated. The patterns of reassociation, as indicated by the smooth form of the curves, show no distinct proportion of highly reiterated sequences. The evidence, then, indicates that in the large nuclei there has been replication of the whole genome (endoreduplication).

**Incorporation of ³H-Thymidine into DNA during Cell Expansion Growth in Cotyledons from Other Legumes.** To ascen-

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**Fig. 3.** Equilibrium density gradient centrifugation in CsCl of DNA from *V. faba* cotyledons in the cell division phase and in the cell expansion phase of growth. a: Three µg (40,000 cpm) ³H-thymidine labeled DNA from young cotyledons (<10 mm) and 47 µg unlabeled DNA from old cotyledons (23 mm) were centrifuged in CsCl (p 1.705 g/cm³) at 20°C for 66 hr at 33,000 rpm in the SW39 rotor of a Spinco centrifuge. Approximately 85 two-drop fractions were collected, diluted with 0.6 ml water, and the absorbance at 260 nm recorded. Radioactivity was measured by adding 3 ml of scintillation fluid (6 g PPO, 0.6 g POPP, 330 ml Triton X-100 per 670 ml toluene) to a 0.5-ml sample of the diluted fractions and counting in a Packard scintillation spectrometer. Absorbance at 260 nm (●); radioactivity (△). b: 2.5 µg (40,000 cpm) ³H-thymidine labeled DNA from old cotyledons (25 mm) and 48 µg unlabeled DNA from young cotyledons (<10 mm) were centrifuged in CsCl and analyzed as described for a).

**Fig. 4.** Comparison of reassociation kinetics of *V. faba* DNA from cotyledons in the cell division phase of growth (●) with that from cotyledons in the cell expansion phase of growth (○). *E. coli* DNA (●) was used as a standard to check for fluctuations in reassociation conditions. The rates of reassociation were measured at 37°C in 5 × SSC, 50% formamide by the method described previously. The rates of reassociation have been corrected to those in 0.12 M phosphate by the data of Britten and Smith (5). Hyperchromicity observed on denaturation was 35 to 38%.

**Fig. 5.** Reassociation profiles of the highly reiterated sequence of *V. faba* DNA from cotyledons in cell division phase of growth (●); from cotyledons in cell expansion phase of growth (○); from shoots of germinated seeds (■). *E. coli* DNA (●) was used as a standard to check for fluctuations in reassociation conditions. The rates of reassociation were measured at 61°C. Hyperchromicity observed on denaturation was 35 to 38%. © 1973 American Society of Plant Biologists. All rights reserved.
tain whether synthesis of DNA beyond the cell division phase was a general feature of legume cotyledons, the incorporation of $^3$H-thymidine during growth by cell expansion was checked in two other species. Cotyledons were removed from developing seeds of *P. sativum* var. Alaska and from fenugreek (*Trigonella foenum graecum* L.) and incubated in $^3$H-thymidine. In both types of seed, the developmental stage was well beyond completion of cell division and microscopic observation showed no mitotic figures in Feulgen-stained squashes. With both pea and fenugreek, it was found that $^3$H-thymidine was rapidly incorporated. Labeled nucleic acids were isolated and separated on acrylamide gels. The label was coincident with DNA, was removed by DNase, and was unaffected by RNase. Incorporation of $^3$H-thymidine does not necessarily imply extensive DNA synthesis, it may be due to repair. However, microscopic examination of expanding pea cotyledon cells showed a dramatic increase in nuclear size, comparable with that observed in *V. faba*, and a preliminary microspectrophotometric analysis of Feulgen-stained squashes has clearly demonstrated a net increase in nuclear DNA content. It seems likely then that DNA synthesis may be a normal feature of the cell expansion phase of cotyledon development in legumes.

**DISCUSSION**

Developing cotyledons of *Vicia* sp. and *Pisum* sp. accumulate large amounts of legumin and vicilin, and the bulk of this synthesis apparently takes place during the cell expansion phase of growth (1, 3, 10, 19). In this paper we have shown that, in the cotyledons of *V. faba* during this same phase of cell expansion, there is also a continuing increase in the amount of RNA and DNA. RNA content goes up 8- to 10-fold, and DNA content increases to a similar degree. Thus the quantity of DNA per cotyledon cell may reach an average value equivalent to 16c. The major portion, if not all, of this additional DNA appears to be confined to the nucleus.

The pattern of labeling, following incorporation of $^3$H-labeled uridine and adenosine into the cotyledon indicates that substantial synthesis of ribosomal and transfer RNAs is taking place. Synthesis of these RNA species is not unexpected because the cells are actively involved in the large scale production of protein, both storage and metabolic. The procedures used in the experiments do not allow us to comment on the question of messenger RNA synthesis. Obviously, the developing cotyledon system has considerable potential for studies on the synthesis of the specific messenger RNAs for legumin and vicilin, and experiments directed towards this problem are currently underway in our laboratory.

The continuing synthesis of DNA during the cell expansion phase of growth is not, however, so readily explained. There is considerable evidence that cell elongation (7, 13) and hormone-induced cell expansion (summarized in 17) may be dependent upon postmitotic DNA synthesis. The parenchymatous cells of legume cotyledons are very large and more DNA may be necessary simply to allow for an increased supply of messenger RNA molecules to support the greater activity of the enlarged cells. Alternatively, accumulation of DNA in the cotyledon cells may be related to the future role of this tissue in the germinating seed rather than to current needs. Supplying nutrients necessary for the growth of the young seedlings is a major function of the cotyledons of legumes. The additional DNA synthesized during seed development may merely serve as a ready source of deoxynucleosides and phosphate for the next generation.

Another possible role for the extra DNA might be in connection with the specialized process of storage protein synthesis. For instance, amplification of genes concerned in the production of legumin and vicilin might conceivably account for some of the increased synthesis of DNA. However, this does not appear to be so. The proportions of reiterated (70%) and unique sequences (30%) are the same (Figs. 4 and 5) for DNA isolated from cotyledons in the cell division phase of growth and from cotyledons undergoing growth by cell expansion. Likewise, the kinetics of reassocation are identical (Figs. 4 and 5) for DNA isolated from both growth phases. Thus the increased DNA would appear to represent replication of the whole genome (endoreduplication) and this view is supported by the CsCl density equilibrium patterns which are identical for the DNAs from the two stages of growth (Fig. 3). On the basis of our data, the possibility of selective amplification of the structural genes for legumin and vicilin cannot be eliminated. It is interesting to note, however, that in the case of the specialized silk glands of *Bombyx mori* specific amplification of the gene for fibroin does not occur (28).

Many other examples are known of cells in which differentiation to a specialized function is accompanied by a large increase in the DNA content of the cells. In some cases endoreduplication seems to be involved, and this is so in the silk gland of *B. mori* (11). On the other hand, there are cases known where differentiation to specialized function does not involve uniform replication of the whole genome. In *Drosophila* during polytenization of the salivary-gland cells much of the reiterated DNA is excluded from replication (8, 12). Although polytenic chromosomes have been observed in the suspensor cells of *Phaseolus vulgaris* (22), we have not found any indications of polytenic chromosomes during cotyledon development in *V. faba*. As mentioned above, the evidence favors the view that the extra rounds of DNA synthesis in *V. faba* cotyledons involve endoreduplication and are thus analogous to the situation in the silk gland of *Bombyx mori*.

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**LITERATURE CITED**