growth weighed. This weight was corrected for material cut away by the saw, generally 10 percent of the weight.

23. Because grunt schools consistently associate with the same coral heads and because recruitment from the plankton was low over this period, < 30 grunts were found on this head both 4 and 8 months after the original school of 304 was removed.
24. Over the 8-month period, mean growth rate of the head with fish (W) was higher than the control head (WO), but the difference was not statistically significant. A decreased number of grunts on head W and environmental factors other than the presence of grunts (weather-related factors or simply more squirrelfish on head WO over this period) were obscuring the impact of grunts.
25. Families include acanthurids, apogonids, carangids, chaetodontids, holocentrids, lutjanids, mullids, pempherids, pomacentrids, priacanthids, scarids, sciaenids, serranids, and siganids.
26. We thank the staff of the West Indies Laboratory for their assistance in all phases of this research, and L. R. Pomeroy, G. J. Smith, A. Szmant-Froelich, and an anonymous reviewer for useful comments on the manuscript. This research was supported by NSF grant OCE 79-19406. This is contribution No. 91 of the West Indies Laboratory, Fairleigh Dickinson University.

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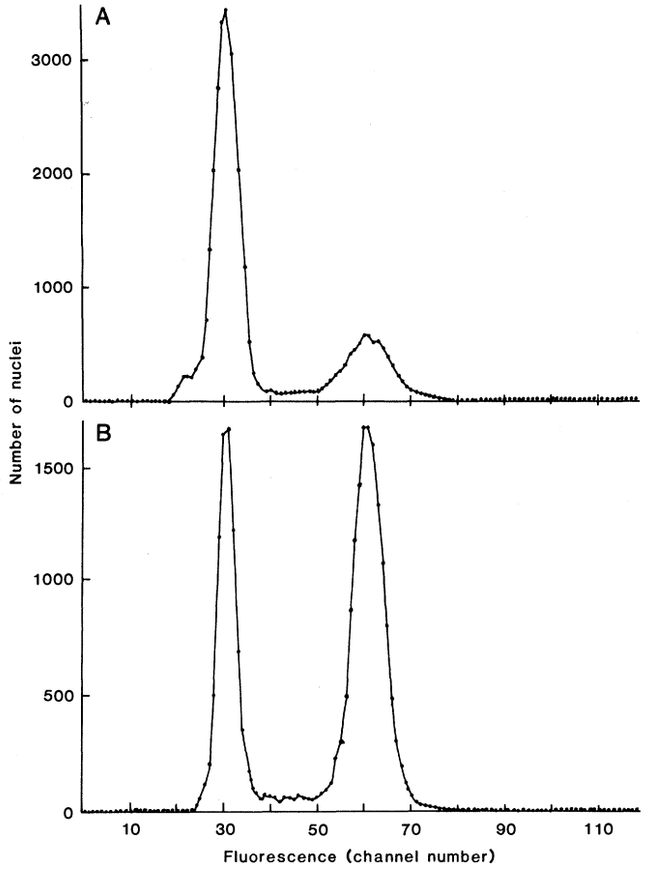
## Rapid Flow Cytometric Analysis of the Cell Cycle in Intact Plant Tissues

Abstract. *Mechanical chopping of plant tissues in the presence of mithramycin released intact nuclei representative of the cells within the tissues. The amount of nuclear DNA in the homogenates of monocotyledonous and dicotyledonous plants was accurately and rapidly determined by flow microfluorometry, and the distribution of nuclei involved in the cell cycle was charted for tissues selected from different physical locations or developmental stages.*

Flow cytometric methods have several advantages over conventional microscopic procedures for determining the cell cycle status of eukaryotic cells. In particular, they are exceptionally rapid, accurate, convenient, and sensitive (*1*). A disadvantage is the requirement for single-cell suspensions. Although some animal cell types exist as single cells in vivo, and many other animal tissues can be converted into single-cell suspensions by proteolytic digestion, higher plant cells commonly exist as complex three-dimensional tissue structures. Furthermore, these structures often display a gradation in cellular differentiation as a function of position, particularly in terms of distance from a meristematic region.

It is clear that cell cycle activity is intimately connected with differentiation and that an understanding of plant cell cycle control is central to an understanding of the establishment of plant form during growth. Less clear is the means for establishing a method to accurately and conveniently determine the status of the cell cycle in higher plant tissues, as reflected in the cellular nuclear DNA contents. Investigators have been using quantitative microphotometry with Feulgen staining or DNA-specific dyes such as Hoechst 33258 (*2, 3*). The slowness and inaccuracy of such techniques have hindered the satisfactory resolution of questions on the cell cycle in higher plant tissues. Although the preparation of protoplasts by the use of cell wall–digesting enzymes can be an appropriate first step for the analysis of the plant cell cycle (*4*), in that protoplasts represent single-cell populations, it is difficult to determine the precise prior location of the cells in the tissue from which the protoplasts are obtained. Furthermore, many plant tissues do not readily yield satisfactory preparations of protoplasts. Finally, it cannot be determined whether a proto-



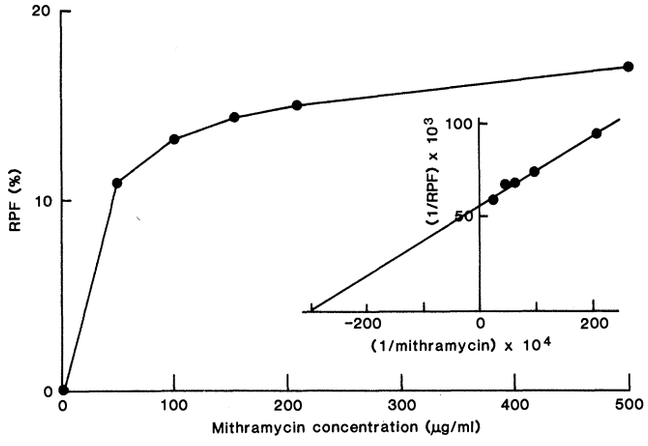




Fig. 1 (left). Flow cytometric analysis of nuclei released by chopping of leaf (A) or terminal root (B) tissue of *N. tabacum*. The nuclei were stained with mithramycin before analysis. The coefficients of variation for the $G_1$ peaks were 8.1 and 6.0 percent, respectively, and the profiles represent (A) phase $G_1$, 71.1 percent; S, 6.6 percent; and $G_2$, 22.3 percent; and (B) $G_1$, 30.5 percent; S, 13.6 percent; and $G_2$, 55.9 percent. Fig. 2 (right). Binding characteristics of the mithramycin-DNA interaction in nuclei released by chopping *N. tabacum* leaf tissue. The degree of fluorescence of the $G_1$ peak is expressed as the relative peak fluorescence (*RPF*), a value obtained by dividing the channel number of the $G_1$ peak by the channel number of the peak fluorescence of Fullbright fluorescent microspheres, which were included in each flow cytometric determination as internal standards.

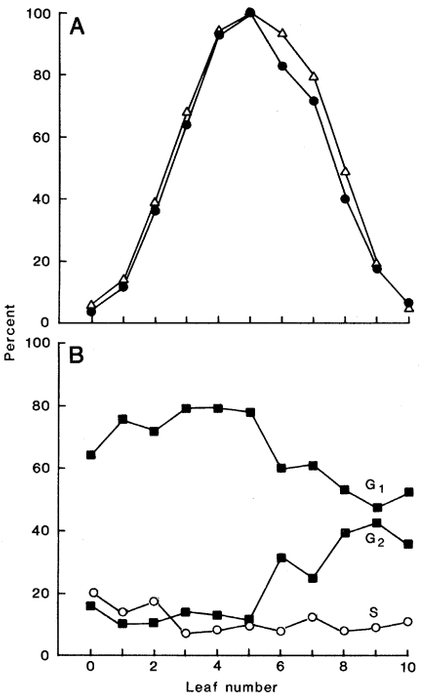



Fig. 3. Sequential cell cycle status of leaves of *N. tabacum*, as determined by flow cytometric analysis of nuclei produced by separate chopping of leaves numbered from the apex to the bottom of the plant. (A) Surface areas (△) and fresh weights (●) of the leaves, expressed as percentages of the values of the largest leaf (leaf 5). (B) Proportions of nuclei characteristic of the various phases of the cell cycle, calculated from the appropriate DNA histograms by use of the Extended Analysis System. An increase in the proportion of $G_2$ nuclei is apparent in leaves located below leaf 5.

plast population is truly representative of the starting tissue. In this report we outline a rapid, simple method for accurately determining the distribution of plant cell nuclear DNA in which positional information can be retained without significant loss of accuracy. The method enables determination of the nuclear DNA status of a tissue, a plant, or an entire plant population.

Since eukaryotic animal nuclei retain both structure and DNA under the mechanical and chemical stress of hypotonic lysis (*5–7*), in which the plasma membrane is essentially solubilized, we wondered whether plant nuclei can be mechanically released from intact plant tissues. We reasoned that gentle chopping procedures, such as those developed for the isolation of intact, enzymatically active dictyosomes (*8*), would be unlikely to stress the nuclei significantly more than hypotonic lysis. Since the physical locations of such tissues could be accurately charted, positional information related to the cell cycle could be obtained. Finally, chopping would provide an impartial, random selection of nuclei in the tissue zone to be analyzed.

Plants were grown from seed under standard greenhouse conditions (Table 1). Haploid plantlets were produced by anther culture with conventional techniques (*9*). Selected tissues were excised, immediately chilled on ice, and chopped in a cold room with a single-edged razor blade in a glass petri dish (diameter, 5 cm) containing a "chopping buffer" (*p*H 7.0) of the following composition: 45 m*M* magnesium chloride, 30 m*M* sodium citrate, 20 m*M* 4-morpholinepropane sulfonate, and Triton X-100 (1 mg/ml) (*10*). After 2 minutes the cellular debris, consisting of finely minced tissue fragments, was passed through two nylon filters (pore size, 60 and 15 μm). The nuclei in the filtrate were stained with a ninefold excess of a solution containing mithramycin (100 μg/ml) dissolved in chopping buffer. After 5 to 60 minutes the stained nuclei were analyzed by flow microfluorometry (rate, 50 nuclei per second) with a Coulter EPICS V flow cytometer–cell sorter operating at a laser wavelength of 457 nm and a power output of 200 mW. Barrier filters LP510 and BG38 permitted optimum detection of the mithramycin-DNA complex. Fluorescent microspheres (Coulter Fullbright) were included as internal standards for experiments in which stain binding characteristics were determined. For measurements of absolute DNA values, chicken red blood cells containing 2.33 ± 0.22 pg of DNA per nucleus (means ± standard deviation, $N = 7$) were included as an internal standard (*11*). Cell cycle parameters were calculated with fitting routines provided by the Coulter Electronic Analysis System.

Figure 1A shows the DNA histogram obtained when a fully expanded *Nicotiana tabacum* cv. Xanthi leaf (fresh weight, 2.3 g; surface area, 90.7 $cm^2$) was subjected to chopping and staining with mithramycin. Most of the nuclei have a single DNA peak, which, by comparison with the chicken red blood cell standard, represents a mass of 9.67 pg (*11*). Figure 1B shows the DNA histogram obtained when *N. tabacum* root tips were subjected to the same chopping and staining procedure. The major peaks of fluorescence are coincident with those observed for the leaf tissue. Because normal root tip cells contain 2C nuclei that are active in the cell cycle (*12*), it follows that the lowest peak in the DNA histogram represents those nuclei in phases $G_0$ and $G_1$, and therefore that the

Table 1. Nuclear DNA contents of leaf tissues, as measured by flow cytometry.

| Species | Nuclear DNA content (pg) | Coefficient of variation of $G_1$ peak (%) | Reported 2C value (*15*) (pg) |
|---|---|---|---|
| *Agropyron smithii* Rydb. | 0.81 | 7.3 | |
| *Antirrhinum majus* L. | 1.03 | 7.1 | 3.7 |
| *Bouteloua gracilis* (HBK) Lab. ex Steud. | 39.38 | 6.4 | |
| *Capsicum annuum* L. | 5.52 | 4.7 | |
| *Catharanthus roseus* L. | 4.84 | 9.7 | |
| *Coleus blumei* Benth. | 3.43 | 6.3 | |
| *Elymus canadensis* L. | 21.60 | 4.4 | |
| *Euphorbia pulcherrima* Willd. | 2.62 | 10.8 | |
| *Helianthus annuus* L. | 3.57 | 6.2 | 6.3 |
| *Ipomoea purpurea* L. | 1.08 | 8.1 | |
| *Lycopersicon esculentum* Mill. | 1.48 | 6.7 | 2.0 to 5.1 |
| *Nicotiana glauca* Grah. | 6.91 | 5.7 | |
| *Nicotiana glutinosa* L. | 4.08 | 5.9 | |
| *Nicotiana knightiana* Goodsp. | 6.04 | 6.4 | |
| *Nicotiana nesophila* Johnst. | 10.15 | 6.9 | 9.7 |
| *Nicotiana paniculata* L.* | 5.53 | 6.4 | |
| *Nicotiana paniculata* (haploid, from anther culture)† | 2.74 | 7.1 | |
| *Nicotiana sylvestris* Speg. & Comes‡ | 5.43 | 4.8 | |
| *Nicotiana sylvestris* (haploid, from anther culture)§ | 2.74 | 6.9 | |
| *Nicotiana tabacum* L. cv. Xanthi | 9.67 | 3.8 | |
| *Nicotiana stocktonii* Brand. | 9.45 | 6.3 | |
| *Panicum thermale* Boland. | 2.12 | 10.8 | |
| *Pisum sativum* L. | 7.72 | 5.2 | 9.8 to 10.5 |
| *Solidago canadensis* L. | 3.13 | 7.0 | |
| *Solanum melongena* L. var. *esculentum* Nees. | 2.33 | 4.7 | |
| *Zea mays* L. | 5.99 | 5.2 | 4.7 to 11.0 |

*Standard deviation, 4.86 percent ($N = 23$). †Standard deviation, 4.86 percent ($N = 14$). ‡Standard deviation, 3.97 percent ($N = 6$). §Standard deviation, 5.82 percent ($N = 7$).

second, upper peak represents those nuclei in $G_2$ and M, with a spectrum of S-phase nuclei between these two peaks.

The nuclear DNA contents of a variety of monocotyledonous and dicotyledonous plants are presented in Table 1. These data are in broad agreement with the results of studies in which Feulgen staining was used. Exact correspondence would not be expected, since considerable variability can be observed in the nuclear DNA values obtained with different lines or cultivars of the same species by identical Feulgen staining. In contrast, the data obtained for haploid and diploid *Nicotiana* species with flow cytometry show the predicted doubling of the nuclear DNA content in $G_1$. Furthermore, in a single species the $G_2$ peak always appears at exactly twice the DNA content of the appropriate $G_1$ peak, and the coefficients of variation for the 2C DNA peaks of the various species (Table 1) compare favorably with those obtained with animal cell lines (*13*).

These observations, coupled with a complete absence of artifactual nuclear adhesion (peaks corresponding to the adhesion of three or more nuclei were never observed), lead us to conclude that mechanical chopping does not result in significant DNA hydrolysis or in nuclear degradation or adhesion of intact nuclei liberated by this procedure. The reproducibility of measurements for different individuals of a single species was extensively investigated in *Nicotiana*. The standard deviations of these determinations fall in the range of 4 to 6 percent. This compares to a range of 6.2 to 39.8 percent attainable by microspectrophotometry with Feulgen staining (*2*). Figure 2 shows the binding characteristics of the mithramycin-DNA complex and their analysis by double-reciprocal plotting. The data indicate a single class of binding sites, half-saturated at a mithramycin concentration of 32 μg/ml. Fluorescence was abolished by treatment with deoxyribonuclease (1 mg/ml) for 30 minutes at 37°C, but was unaffected by ribonuclease under the same conditions.

The power of the technique is further illustrated in Fig. 3. The nuclear DNA status of 11 sequential leaves (counting from the apex) of a single *Nicotiana tabacum* was analyzed. The complete analysis, from greenhouse to histograms, required less than 2 hours. The results clearly show the occurrence of a prolonged period of DNA synthesis during leaf maturation after leaf expansion has become complete. Since neither mitotic figures nor binucleate cells were observed in leaf sections that were fixed and observed by conventional microscopy (*2*), we conclude that the process of leaf maturation includes the development of a significant population of cells arrested in $G_2$.

This method is directly applicable to (i) measurements of nuclear DNA degradation during leaf senescence as a function of leaf age, (ii) measurements of DNA synthesis in tissues in which infiltration with [$^3$H]thymidine is inappropriate or impossible, (iii) measurements of possible positions of cell cycle arrest in response to micronutrient or phytohormone deprivation, (iv) measurements of endopolyploidy in storage tissues, (v) correlations of ploidy level with cell cycle status, photosynthetic efficiency, and plant yield in isogeneic polyploid series, (vi) measurements of the distribution of evolutionary polyploid series in ecology and taxonomy (*7, 14*), and (vii) a systematic, accurate determination of 2C nuclear DNA levels in many, if not all, mono- and dicotyledonous plant species.

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10. The minimal convenient sample size was 12 mg chopped in a volume of 0.2 ml of buffer. The coefficients of variation for the $G_1$ peaks of nuclei prepared in this manner were similar to those obtained with larger tissue samples.
11. For measurement of nuclear DNA contents, chicken red blood cells were used as internal standards. Normally, the amplification and high voltages of the flow cytometer photomultipliers were adjusted for each species such that the $G_0/G_1$ peaks were located wherever possible, toward the upper ends of the 256-channel histograms. This maximizes the accuracy of measurement of the position of the fluorescence peaks. The absolute DNA contents of the chicken red blood cells were determined by the method of K. Burton [*Biochem. J.* **62**, 315 (1956)] in which calf thymus DNA is used as the standard.
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## Desuppression of Leaf Primordia of *Plagiochila arctica* (Hepaticae) by Ethylene Antagonists

Abstract. *Silver nitrate, α-aminooxyacetic acid, and aminoethoxyvinylglycine, three potent inhibitors of ethylene synthesis and action, induced the same kind of phenovariation in the liverwort* Plagiochila arctica *Bryhn and Kaal (Hepaticae) as do antagonists of the synthesis of hydroxyproline-containing protein. This finding (i) supports the hypothesis that hydroxyproline-protein has a role in ethylene-mediated suppression, (ii) provides evidence that the role of ethylene in the correlative development of leafy liverwort gametophytes may be similar to its role in flowering plants, and (iii) contributes to the characterization of a morphoregulatory system that mediates cellular suppression in leafy liverworts and possibly all land plants.*

A series of experiments with leafy liverworts (*1–10*) led to a new hypothesis concerning the morphoregulatory role of a cell-surface protein sensitive to antagonists of the synthesis of hydroxyproline-containing protein (hyp-protein). According to this hypothesis, the cell-surface protein plays a pivotal role in suppressing cellular development in local populations of cells such as leaf and branch primordia. Furthermore, changes in the time or location (or both) of the synthesis of this protein during ontogeny (heterochronous changes) could account for some of the phylogenetically significant differences in leaf and branch morphology of leafy liverworts. Some idea of the different positions of the cell populations (primordia) that may be influenced in liverworts can be seen in Fig. 1. We refer to the hypothesis concerning both morphogenetic action and phylogenetic implications as the suppression hypothesis (*7–10*).

Suppression of cellular development is commonly associated with the phytohormone ethylene. Ridge and Osborne (*11, 12*) suggested that the inhibitory action of ethylene involved hyp-protein. They had found that ethylene-induced sup-