Plant Science, 70 (1990) 255–265
Elsevier Scientific Publishers Ireland Ltd.

NUCLEAR DNA CONTENT AND SEPARATION OF *NICOTIANA SYLVESTRIS* VEGETATIVE AND GENERATIVE NUCLEI AT VARIOUS STAGES OF MALE GAMETOGENESIS

R. DE PAEPE[a,*], A. KOULOU[a,**], J.L. PHAM[a] and S.C. BROWN[b]

[a]*Génétique et Physiologie du Développement des Plantes, and* [b]*Institut des Sciences Végétales. CNRS, 91198 Gif-sur-Yvette (France)*

(Received October 19th, 1989)
(Revision received April 17th, 1990)
(Accepted April 17th, 1990)

At all stages of male gametogenesis, generative and vegetative pollen nuclei of *Nicotiana sylvestris* can be distinguished without ambiguity after Feulgen or ethidium bromide staining. They differ by their morphology and their apparent DNA content, always lower in vegetative nuclei. These differences provide a basis for their separation by sedimentation and fluorometry. After elimination of the anther somatic cells and after crushing the pollen, vegetative and generative nuclei are separated by two successive Percoll gradients (purity 80–90%). Analysis of the gradient fractions and final purification can be done with a cell sorter. DNAs of both types are isolated by a cetyltrimethylammonium method, followed by a RNase treatment. Yields are lower for vegetative than for generative nuclei, and decrease with the age of pollen. Molecular weights and digestibility by restriction enzymes are compatible with molecular analyses.

Key words: pollen nuclei and DNA; androgenesis; cytophotometry; flow cytometry; *Nicotiana sylvestris*

Introduction

In higher plants, the mature male gametophyte consists of one generative cell (G) or two sperm cells, according to the species [1], and a single vegetative cell (V) that assumes most of the pollen metabolism and does not participate in the reproductive pathway in vivo [2]. However, in vitro pollen culture gives the opportunity to obtain plants from the V nucleus. Indeed, in species where androgenesis is carried out with binucleated pollen, regenerated plants generally derive from the V cell, the G nucleus dividing only once [3]. This is the case in the diploid species *N. sylvestris*, where binucleated pollen, taken 1 or 2 days after the first pollen mitosis, develop into plantlets by direct embryogenesis without callus formation [4]. Regenerated diploid plants (Doubled-Haploids, DHs) were all different from their parent pure line for the same type of characteristics — slow growth, reduced dimensions, morphological abnormalities ("crumpling") [5,6] — that were inherited in a nuclear mode. Moreover, nuclear genome organization of the DH was found to be changed, possibly by amplification of some classes of repeated sequences [7]. Similar phenomena have been described in the related species *N. tabacum* [8]. The question is: do these DNA changes occur during pollen culture, or do they preexist in the nucleus from which the androgenetic plants originate, i.e. the V nucleus of immature pollen? To try to answer this question, we developed a method to separate G and V nuclei at all stages of male gametogenesis, and to obtain sufficient amounts of DNA from each of them to compare their nuclear organization. To our knowledge, such protocols

*Present address: Génétique Moléculaire des Plantes, URA 115 CNRS, Université Paris-Sud, 91405 Orsay Cedex, France.
**Present address: Faculté des Sciences, Université d'Abidjan, Cote-D'Ivoire.

Abbreviations: CTAB, cetyltrimethylammonium bromide; DH, Doubled-Haploid; EtBr, Ethidium bromide; G, generative; V, vegetative.

0168-9452/90/$03.50
Printed and Published in Ireland

have not yet been designed for immature gametophytes. Previous reports concern isolation of either nuclei from pollen tubes in *Trandescantia* [9], or sperm cells of mature pollen in *Plumbago zeylanica* [10], maize [11] and *Vicia faba* [12]. *N. sylvestris* pollen has been stained with the fluorochrome ethidium bromide (EtBr); this DNA intercalary dye [13] is compatible with further DNA isolation and analysis and inhibits DNAse activity [14]. As observed in other plant species [15], EtBr fluorescence is very different in *N. sylvestris* V and G nuclei. Fractions enriched in the two different types of nuclei were obtained by two centrifugation steps on Percoll gradients. Analysis of the gradient fractions and complete separation were performed by flow cytometry ("cell sorting").

Material and Methods

Plant material and culture

The *N. sylvestris* line used was a botanical pure line provided by the SEITA (Bergerac, France) and maintained in Gif-sur-Yvette Phytotron greenhouses by self-pollination. The flowering plants, source of anthers, were grown under natural light supplemented with 16 h fluorescent lighting (inductive conditions). Temperature was 25 °C day and 17 °C night. They were fed with Nitsch nutrient solution [16].

Feulgen cytophotometry

Anthers were fixed with ethanol/acetic acid solution (3:1) and their pollen (approx. 350 000 grains per flower) stained with Schiff reagent. Nuclear DNA content was estimated by the absorbance method using a Leitz MPV microphotometer (560 nm) as described previously for leaf cells [7]. Aproximately 40–50 pollen grains were analysed per stage. The reference 1C value was given by the telophasic nucleus of first pollen mitosis.

Fluorometry

Pollen were stained by the addition of 0.1 mg/ml of EtBr to the isolation solution. After crushing of the pollen and during the isolation procedure, free nuclei were examined either by light microscopy after acetocarmine coloration, or under violet lighting (400 nm) with a Reichert microscope, enabling simultaneous use of interference contrast optics and epifluorescence (IV2 filter). Nuclear DNA content was estimated on a Leitz MPV microfluorometer from more than 50 free nuclei, either immediately after pollen crushing, or after they had been stored on ice for 3–5 h, with or without RNAse A treatment (50 μg/ml). The reference 1C value was estimated from young tetrad nuclei.

Separation of pollen nuclei

Anthers of 200–300 flowers of the same development stage were collected (they can be stored at −20 °C but subsequent DNA yields may thus decrease) and opened in a Potter tube containing 10 ml of Honda buffer [17], all the operations being performed close to 0 °C. The solution was filtered under low vacuum through a 100 μm nylon net to eliminate anther somatic cells. Pollen was centrifuged at 3000 × *g* for 5 min in a fixed angle rotor to obtain a compact pellet. After eliminating as much isolation buffer as possible, pollen was crushed with a mortar and pestle. As checked by light microscopy (acetocarmine coloration), approximately 80–90% of the grains must be broken. They were resuspended in 10 ml Honda buffer, centrifuged at 400 × *g* for 5 min and mixed with 15 ml of a 78% Percoll solution (0.4 M sucrose, 0.02 M $CaCl_2$, 0.1 mg/ml EtBr, Tris–HCl 25 mM pH 7.5), and centrifuged at 4500 × *g* for 30 min in a fixed angle rotor. The gradient was recovered as 2-ml fractions with a peristaltic pump via a needle reaching the bottom of the tube but carefully avoiding the pollen pellet stuck to the side. Each fraction was diluted 5-fold with the isolation buffer and centrifuged at 3000 × *g* for 20 min. The pellets of the lower fractions were stored for DNA extraction. The pellets of the upper fractions were resuspended in 2 ml EtBr isolation buffer, loaded on a preformed 50% Percoll gradient (run at 30 000 × *g* in a 65 Ti rotor or equivalent for 50 min, without brake) and run at 800 × *g* for 20 min. The gradient fractions were recovered and their contents pelleted as previously described for the 78% gradient.

Isolation of pollen DNA

Pollen DNA was extracted by a CTAB treatment, modified from Murray and Thompson [18], all operations being performed in Eppendorf tubes. Freshly crushed pollen, or pellets from the 78% and 50% gradients, were suspended in 0.7 ml of 1% CTAB, 0.7 M NaCl, 60 min at 60 °C., and deproteinised by adding one volume of a chloroform/octanol (10:1) mixture. The chloroform treatments were repeated until the interface was clear. The CTAB-DNA complex was precipitated by adding 1/5 volume of 5% CTAB, 0.7 M NaCl (15 min at 60 °C), followed by one volume of 1% CTAB, without NaCl, and centrifuged at 5000 $\times$ *g* for 20 min. The pellet was resuspended in Tris—HCl 50 mM (pH 7.5), EDTA 1 mM, NaCl 1 M, and nucleic acids precipitated with two volumes of cold alcohol. This step was repeated to fully eliminate CTAB, the pellet was then resuspended in 50 mM Tris—HCl (pH 7.5), 1 mM EDTA, and treated with a mixture of RNAse A (0.1 mg/ml) and T1 (1000 U/ml) (37 °C 30 min), followed by cold ethanol precipitation. If the amount of DNA was greater than 10 μg, it was purified by CsCl ultracentrifugation.

Cell sorter analysis

The consecutive Percoll gradient fractions were analysed by cytometry using either one (fluorescence intensity) or two parameters (light scatter and fluorescence), on an EPICS V machine (Coulter, Florida), with a 0.1 mm nozzle. The light source was a Spectra-Physics 2025-05 Argon laser at 488 nm, 400 mW; the emission was collected above 590 nm. Using the principle of measuring "fluorescence pulse area" against "fluorescence pulse height" [19], we specifically eliminated aggregated nuclei. The objects sorted out from the chosen windows were identified using the Reichert microscope, in the same manner as freshly crushed pollen.

Results

Male gametogenesis and principles of separation of V and G nuclei

In *N. sylvestris*, male gametogenesis is highly synchronous in all five anthers of one flower, each containing approximately 70 000 pollen grains [20], and correlates to well defined sizes of other floral parts [4]. This allows the collection of gametophytes at a precise developmental stage. First pollen mitosis takes place 2 days after meiosis and the following day practically all pollen are binucleated (Table IA). V and G nuclei differentiate immediately after their formation. As observed in aceto-carmine and Feulgen preparations (Fig. 1A), the G nuclei are small and ovoid with highly condensed chromatin, while the V nuclei are larger, round and more diffuse. V and G differences were also clearly observed after pollen staining by EtBr, either in situ, or after crushing in the isolation medium (Fig. 1B,C). In both cases, V nuclei show little fluorescence as compared to the G, but the difference in size is not yet apparent in free nuclei. Aspects of V and G remain essentially the same up to the mature stage, although the G become more crescent-shaped.

Relative DNA content per nucleus was estimated by three methods: Feulgen cytophotometry of fixed microscopes and immature pollen; EtBr fluorescence of free nuclei of mature pollen at several intervals after crushing; and cell sorter analysis of each Percoll gradient fraction (see the separation protocol), for all binucleated stages up to anthesis. Results obtained by the different methods are given in Table IB. Either Feulgen or EtBr stained G nuclei reached a maximum close to the 2 C value in 2 days after pollen mitosis. Feulgen values decreased slightly in older stages (mature pollen were not studied with this method), but this effect was not observed by EtBr fluorescence. The intensity of staining was similar in freshly crushed pollen and after the separation procedure; furthermore, it was not affected by the RNase treatment (not shown).

At all gametophytic stages, V nuclei appeared to be much more heterogeneous than G nuclei, and their apparent DNA content differed markedly according to the staining method. Indeed, by Feulgen, the highest mean of 1.4 C was reached in 2 days, while EtBr mean

Table I. Microgametophytic development and pollen DNA content.

Days from meiosis		1	2		3–4		6		10	
(A) Development parameters										
Flower (mm)		8	12		20		50		90	
Anther (mm)		3.2	3.5		4.0		4.5		4.5	
Gametophyte (μm)		15	19		22		22		22	
Binucleated pollen (%)		0	10		98		99		99	
Days from	1	2			3–4		6		10	
	M	M	G	V	G	V	G	V	G	V
(B) Nuclear parameters										
Diameter (μm)	6	8.1	1.7	5.1	2.5	8.2	4.2	8.7	5.7	9.0
DNA content										
Feulgen m	1.9	1.9	1.0	1.0	1.8	1.4	1.6	1.4	—	—
Feulgen s (10^2)	16	6	12	28	22	28	24	34	—	—
EtBr										
fluor. (a) m	—	—	—	—	1.8	0.6	1.8	0.5	1.8	0.5
(b) m	—	—	—	—	—	—	—	—	2.0	0.4
(b) s	—	—	—	—	—	—	—	—	0.1	0.1

M, microspore; G, generative; V, vegetable. m, mean, s, standard error (although the distribution was obviously not gaussian); —, not measured. Feulgen: nuclear DNA content of fixed pollen nuclei, relative to the telophasic nucleus of first pollen mitosis (1 C value); EtBr fluorometry: nuclear DNA content relative to the tetrad nucleus (1 C value); (a): cell sorter analysis of Percoll gradient fractions; (b): microfluorometric measurements (Leitz MPV microscope) of free nuclei after pollen crushing.

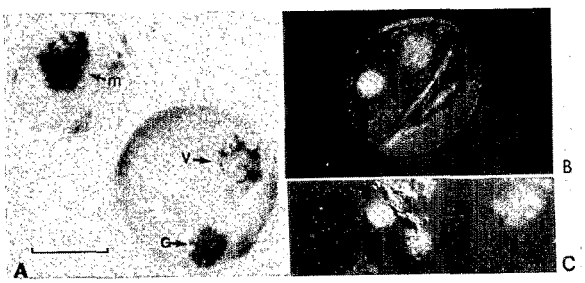


Fig. 1. *N. sylvestris* microgametophytic nuclei in situ and after pollen crushing. (In all figures, Bar = 10 μm). (A) Feulgen coloration of a microspore and a young binucleated pollen immediately after first pollen mitosis: m, microspore nucleus; V, vegetative nucleus; G, generative nucleus. (B) Differential interference contrast (DIC) and epifluorimetry of EtBr stained pollen, 2 days after first pollen mitosis. The G nucleus (left) is smaller and more fluorescent than the V. (C) Free V and G nuclei (DIC) immediately after crushing pollen in the isolation medium. Note that the V is now smaller than in situ.

values never exceeded 0.5 C. As measured with the Leitz cytophotometer, approximately 50% of mature pollen V nuclei had a EtBr fluorescence of only 0.25 C, the highest values ranging between 0.4 C and 0.7 C. As for G nuclei, V fluorescence did not significantly decrease after RNase treatment and during the separation procedure.

Separation of V and G nuclei

Observation of the Percoll gradient fractions. The pellets were examined after acetocarmine staining or by fluorometry with the Reichert microscope. As for native pollen, V and G nuclei could be distinguished without ambiguity at each step. G nuclei were ovoid, densely stained by aceto-carmine, and fluoresced intensively orange-yellow with EtBr. From immature pol-

len, they had often lost their membrane integrity, being no longer visible by transmission microscopy without differential interference contrast. Mature pollen G nuclei were routinely in better state. They contained no visible nucleole. In contrast, V nuclei were round, faintly stained by aceto-carmine, and fluoresced weakly yellow-green with EtBr. Contrasting with the in situ state, isolated V nuclei were smaller than G nulcei. They were not obviously damaged and contained one or several nucleoli.

In the 78% Percoll gradient, the lower fractions consisted mainly of V nuclei, still contaminated with wall and cytoplasmic fragments, and of approximatey 10% of either intact or broken G nuclei (Fig. 2A). Upper fractions contained a mixture of V and G nuclei, and unbroken pollen.

In the 50% Percoll gradient, G nuclei banded in the middle (apparent buoyant density = 1.355 g/ml) (Fig. 2B). They were often aggregated, being associated with plasma and wall fragments, and represented 80—90% of the nuclei found in the corresponding fractions (Fig. 2C). Bottom of the gradient consisted of V nuclei, pollen and wall fragments.

Analysis and purification by cell sorter. Cell sorter analyses of 78% and 50% gradient fractions are shown in Fig. 3. They confirm the microscope observations, V and G nuclei having fluorescence values similar to those of freshly crushed pollen (Table IB).

With one parameter analysis, V and G subpopulations could be identified on the basis of fluorescence intensity (Fig. 3A,B). G nuclei were grouped around the 1.8 C value, while V nuclei showed a multimodal distribution: the more visible peaks correspond to 1/5 (V1) and 1/3 (V2) of the G values (respectively 0.4 C and 0.7 C). V1 and V2 positions remained constant throughout pollen maturation, but the proportion of less fluorescent nuclei increased with age. A third peak of more fluorescent nuclei (V3), faintly visible in middle-aged pollen and more apparent in young stage pollen (not shown), corresponded to the 1 C value. This 1 C peak was absent in mature pollen. The less fluorescent nuclei (0.25 C) observed with the Leitz microscope, were not resolved from the background

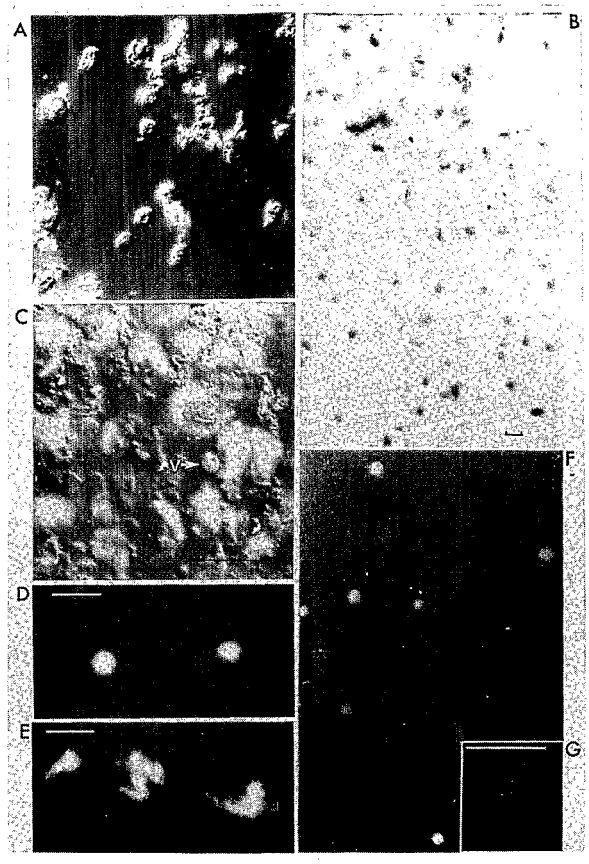


Fig. 2. V and G nuclei after separation. All figures, except B are DIC/epifluorescence mix. (In all figures, Bar = 10 μm). (A) Vegetative nuclei in bottom fraction of the 78% Percoll gradient. Note the nuclei heterogeneity in size and morphology. Fluorescent stretches are G or wall fragments. (B) G nuclei in the middle fraction of the 50% gradient (aceto-carmine staining). (C) EtBr fluorescence of G nuclei in the middle of the 50% gradient. A contaminating vegetative nuclei (→ V) is clearly distinguishable. (D,E) Intact and damaged G nuclei sorted out of window B of Fig. 3C. Intact G represented about 50% of the recovered nuclei, and were preferentially found in the bottom of B area. (F) Intact vegetative nuclei sorted out of window A of Fig. 3C. Note the size heterogeneity and the presence of a more densely stained nucleus near the bottom edge, probably belonging to the V3 peak. (G) Higher magnification of a nucleus sorted from window A, having the typical V morphology, with several nucleoli.

in cytograms, and therefore the mean DNA values of V nuclei from mature pollen were higher as estimated by flow cytometry than by microscopy (Table IB).

Two parameter analysis, using forward-

angle light scatter and integral fluorescence intensity on a linear scale, resolved G nuclei from all other components. In contrast, the population of V nuclei was contiguous with other objects in the preparation (Fig. 3C).

Both types of analyses confirmed the virtual absence of G nuclei from the lower fractions of the 78% gradient and their concentration in the middle fractions of the 50% gradient. The physical sorting out of objects gave 50% of generally intact V nuclei and 50% debris from window A (bottom fraction of the 78% gradient), and 100% more or less damaged G nuclei from window B (middle fraction of the 50% gradient) (Fig. 2, D—G). Intact G nuclei were preferentially recovered from the lower part of the B area.

At all gametophytic stages, numerical yield of G nuclei at the end of the separation procedure was lower than that of V nuclei (Table

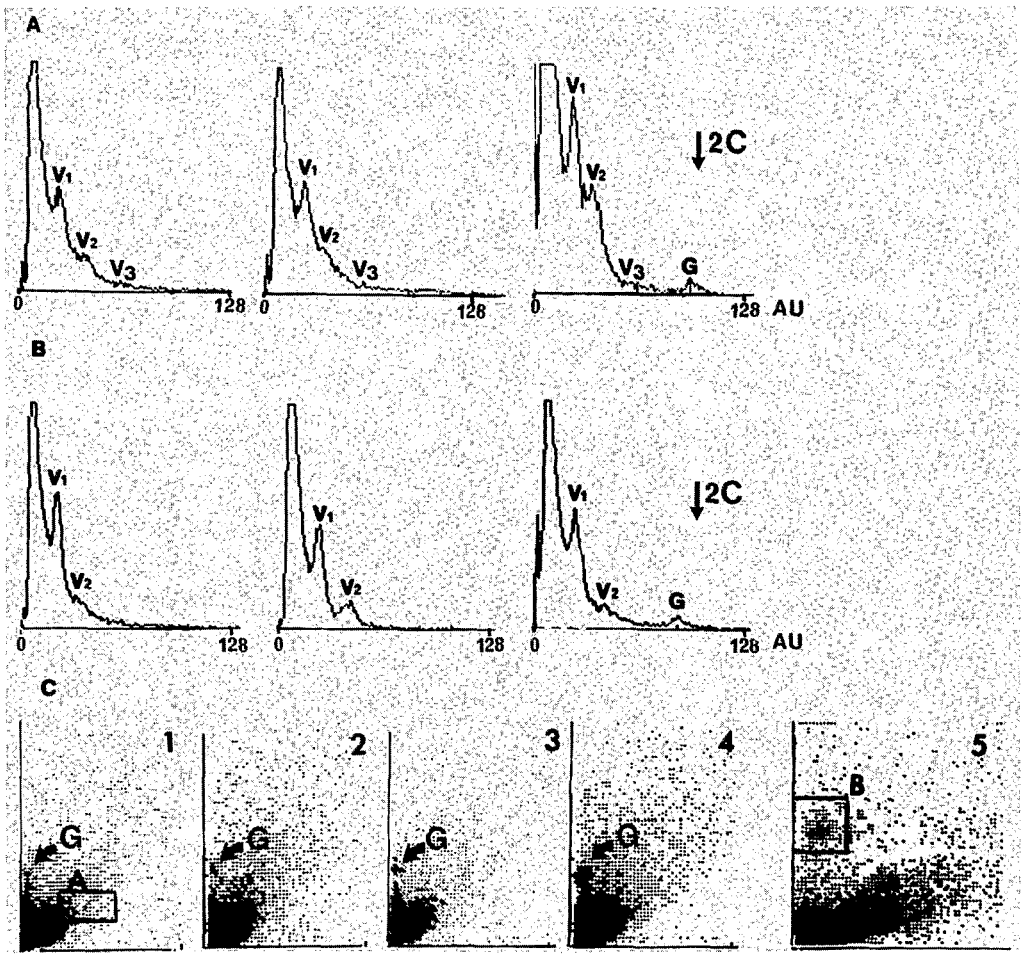
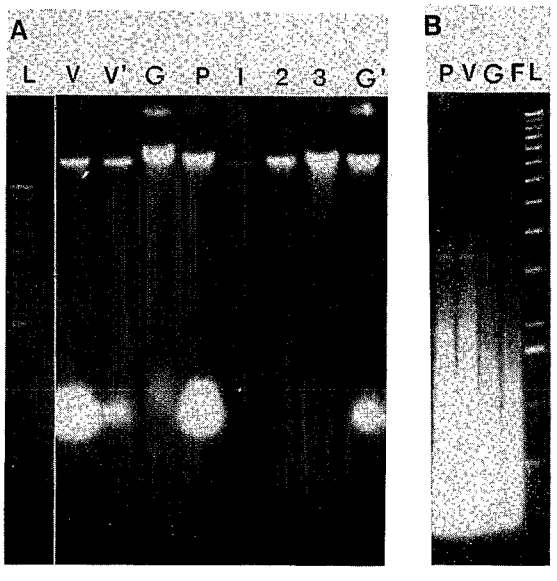


Fig. 3. Cell sorter analyses of Percoll gradient fractions. (A) One parameter histogams (integral fluorescence intensity, arbitrary units AU) of the 78% gradient of mid-aged pollen. From left to right: bottom, middle and top fractions; G (1.8 C): generative nuclei; V1 (0.4 C), V2 (0.7 C), V3 (1 C): sub-populations of V nuclei. The 2 C value was given by microspore nuclei, taken just before first pollen mitosis, and checked to possess the 2 C content by comparison with tetrad nuclei (not shown). (B) As A, for mature pollen. (C) Two parameters cytograms (X: forward-angle light scatter; Y: integral fluorescence intensity) of mid-aged pollen. Relative frequencies are represented by grey levels. From left to right: four successive 78% gradient fractions (1—4) and middle 50% gradient fraction (5) (amplifier gains were increased for the 50% gradient). Only the G population is resolved. Objects sorted out from windows A (mean fluorescence 0.8 C) and B (mean fluorescence 1.8 C) are shown in Fig. 2 D—G.

Table II. Nuclei and DNA yields from Percoll gradient fractions.

	Pollen and DNA yields (/100 flowers)						
Gametophytic stage	Microscope	Binucleated pollen				Anthesis	
		Young		Intermediate			
	—						
	M	V	G	V	G	V	G
In situ							
Ploidy	2 C	1.4 C	1.8 C	1.6 C	1.8 C	1.4 C[a]	1.6 C[a]
Number of nuclei ($\times 10^{-6}$)	35	35	35	35	35	35	35
DNA amounts (μg)	300	210	270	240	270	210[a]	240[a]
After separation							
Number of nuclei ($\times 10^{-6}$)	—	11	4	11	4	11	4
Expected DNA (μg)	300	63	27	72	27	63[a]	24[a]
Obtained DNA (μg)	45	5	20	5	15	1	5

Pollen stages: young, 1—2 days, intermediate, 3—5 days after first pollen mitosis
In vivo: M, microspores taken just before the first pollen mitosis; V, vegetative nucleus; G, generative nucleus. Ploidy: on the basis of Feulgen estimations; [a]probably underestimated (see discussion). Nuclei and DNA amounts: on the basis of 350 000 grains/flower [20] at 4.4 pg for the C value in *N. sylvestris* [21].
After separation: Number of nuclei: estimated from cell sorter cytograms: V, pooled bottom fractions of the two Percoll gradients (vegetative nuclear purity: 90%); G, pooled middle fractions of the 50% Percoll gradient (generative nuclear purity: 90%); Theoretical DNA/fraction: on the basis of 30% of the nuclei initially present recovered in the pooled V fractions, and 10% in the G fractions; M: nuclear DNA was extracted from crushed microspores, with the same protocol as for the Percoll fractions.



IIA). Values pooled from the different fractions indicate a final yield of approximately 10% for G (most of them in the middle fractions of the 50% gradient), and approximately 30% for V nuclei (half of them in the lower fractions of the 78% gradient, most of the others staying in the bottom fractions of the 50% Percoll gradient).

Fig. 4. Electrophoresis of pollen DNAs. (A) Native DNAs obtained from non-separated pollen (P), two different extractions of vegetative (V, V') and generative (G, G') fractions, without RNase treatment (1% agarose gels run at 4 V/cm for 6 h); L: BRL 1 kb ladder; 1, 2, 3: calf thymus DNA standards of quantitation (respectively 0.25, 0.5 and 1 μg). By visual comparison with standards, the DNA amounts were estimated respectively: 0.4 (V, V'), 0.7 (P), 1.5 and 0.7 μg (G, G'). The low molecular weight band was essentially RNA, as it was sensitive to RNAse, as shown in B. (B) MboI restriction of RNase treated DNAs (0.8% agarose gels run at 3 V/cm for 15 h); P, V, G, L: as in A; F: *N. sylvestris* foliar DNA.

Pollen DNA quality and yield

Pooled pellets of the lower fractions of both gradients (called V), and those of the middle fractions were significantly lower at all stages, extracted by a CTAB treatment as described in Material and Methods. Agarose gel electrophoresis showed that DNAs of high molecular weight (30—40 kb) were obtained from both V and G fractions, and that they could be efficiently digested by restriction enzymes (Fig. 4). As preparations still contained some RNA, especially from V fractions, DNA yields were estimated by visual comparison of the native forms with λ and calf thymus standards. Table II shows DNA yields from V and G fractions of mature and immature pollen, relative to theoretical amounts in vivo on the basis of 350 000 grains per flower [20] and 4.4 pg of DNA per haploid genome in *N. sylvestris* [21]; moreover, the amounts of DNA expected from the number of nuclei in each fraction are also shown. DNA yields from G fractions were close to expected amounts for immature pollen, and show a 5-fold decrease at mature stage. DNA yields from V fractions were significantly lower at all stages, and decreased dramatically with pollen age.

Discussion

As deduced from Feulgen measurements, vegetable and generative pollen nuclei of *N. sylvestris* seem to undertake a single round of DNA synthesis. Similar results have already been obtained for a few other plant species [22—25], namely the related species *N. tabacum* [22]. However, Aruga et al. [26] were unable to detect any [^{3}H]thymidine uptake in *N. tabacum* V nuclei, so the situation is not at all clear for these nuclei. From our measurements, DNA synthesis in G nuclei takes place soon after first pollen mitosis and seems to be completed within 2 days. G Feulgen absorption then decreases with pollen age, probably by interference with the starch that accumulates during gametophytic maturation. A similar decrease has been observed in *Datura* pollen [25]. Whether the 2 C level is truly reached in V nuclei remains unclear, their maximum absorption value being 1.4 C. Differences between V and G apparent DNA levels were even more obvious by Ethidium Bromide fluorometry. Indeed, 2 days after their formation, G show EtBr fluorescence values close to the 2 C level, that remain unchanged up to anthesis. In contrast, V values are heterogeneous, being always lower than 1 C. Thus EtBr fluorometry seems valid for DNA assessment in G nuclei up to mature pollen stage, but probably gives wrong estimations of DNA content for the V nuclei. The reliability of fluorochromes for estimating DNA values in microspores and mitotic pollen cells has been studied by Coleman and Goff [27], but these authors did not analyse differentiated G and V nuclei. The low V values we obtained by EtBr fluorescence must be due mainly to a differential reactivity of V chromatin with this intercalating dye [28]. Whatever the basis of the phenomenon. EtBr stained V and G nuclei differ at all stages of male gametogenesis, allowing their identification by fluorometry and their separation with a flow cytometer.

We found that the best way of disrupting immature pollen walls was to crush with mortar and pestle, after elimination of as much isolation buffer as possible. In our hands, alternative methods, such as bursting in hypotonic buffer [9—12], sonication, enzymatic digestion, dissolution by 4-methyl *N*-oxide morpholine monohydrate [29], were either ineffective or gave damaged nuclei. After pollen crushing, nuclei were stained with EtBr and fractions respectively enriched in G and V nuclei were recovered by two successive Percoll gradients (purity 80—90%). Dense fractions of the first gradient (78% Percoll) contained essentially free V nuclei and pollen fragments. G nuclei, often aggregated with attached membrane fragments, were found in the middle of the second gradient (50% Percoll) at a specific density of 1.355. V nuclei mostly remained intact through the separation procedure, while G nuclei, especially from immature pollen, were fragile.

Complete separation of the two types of nuclei can be achieved with a flow cytometer.

This technique has recently been used in plant cell biology (see Refs. 19 and 30 for reviews), including to quantify DNA content in pollen tetrads [31]. Here, in addition to their preparative function, cell sorter analyses give interesting information on nuclear cycling in binucleated pollen and on protocol efficiency. They confirm the virtual absence of G nuclei from the lower fractions of the 78% gradient and their accumulation in the middle fractions of the 50% gradient. In all fractions, EtBr fluorescence of unbroken V and G nuclei is close to that of the corresponding nuclei of freshly crushed pollen preparations, showing that no substantial loss of DNA has occurred during the separation procedure. G nuclei are grouped around the 1.8 C values. Heterogeneity of the V nuclei is clearly evident, major peaks occurring at the 0.4 C and 0.7 C levels. As several peak values were also observed by fluorescence microscopy, they are not likely to be due to an orientation artefact and more probably represent distinct subpopulations. A similar heterogeneity has been found in tetrad nuclei of several plant species [31]. The increase in proportion of less fluorescent V nuclei with ageing indicates either chromatin structural changes or DNA loss in situ, due to nuclease activity. High levels of nucleases are indeed known to exist in mature pollen of tobacco [32,33]. As decrease of EtBr fluorescence with age is not apparent in G nuclei, it must be assumed that either nucleases are not present in the generative cell, or that G nuclei are protected by their condensed chromatin structure.

The proportion of nuclei present in each fraction can be estimated from the cytograms. Approximately 30% of the initial V nuclei were recovered, half of them in the lower fractions of the 78% gradient. On the other hand, only 10% of the initial G nuclei were recovered in the middle of the 50% gradient. These differences between V and G yields are consistent with the microscopic observations, showing that immature G nuclei are more fragile than the V. This could be the consequence of the plasticity of G in situ, that elongate during pollen maturation and tube growth, presenting in some cases filament-like extensions towards the V nuclei [34]. Another cause of nuclear breakage may be that, as observed in all species analysed so far, the wall of the young *N. sylvestris* G cell is attached to the pollen intine [35]. This physical link may be an obstacle to the recovering of intact nuclei. During maturation, the *Nicotiana* G cell becomes free and completely embedded in the V cell [36,37], that could explain why mature G nuclei are more resistant. However, their recovery did not increase.

Yields of high molecular weight DNA, as estimated by agarose gel electrophoresis, are always lower for V fractions than for the G, and decrease dramatically with age: from mature pollen, DNA yields were only 20% of those expected for G nuclei, and 3% for V nuclei (on the basis of 4.4 μg per haploid genome in *N. sylvestris* [21], 1.8 C value for G and 1.4 C value for V, Feulgen estimations). Pollen nucleases could be activated after nuclear disruption in the EDTA containing medium. EDTA-dependent nucleases have been found in wheat seedlings [38]. Large amounts of low weight DNA were not specifically detected on the electrophoretic patterns of mature pollen, but the degraded DNA could have been lost, either by poor precipitation with CTAB, or by polysaccharide trapping. Such an effect has been suggested by Rogers and Bendich [39] to explain the wide differences they observed in recovering DNA from various tissue types, namely pollen. The accumulation of starch during pollen maturation is consistent with this latter hypothesis, and could be an additional cause of poor DNA yields from mature pollen.

In conclusion, V and G enriched fractions can be obtained from Percoll gradients at all stages of pollen development, namely those stages induceable for androgenesis, i.e. 1–2 days after first pollen mitosis. As indicated by Feulgen cytophotometry, both types of nuclei have undertaken a phase of DNA synthesis at this stage. However, EtBr fluorescence indicates that V and G are in a very different state of chromatin structure. V and G DNA molecular weights and digestibilities by restriction enzymes are compatible with molecular ana-

lyses. However, as DNA yields from V nuclei are much lower than those from G nuclei, low contamination of V fractions by G nuclei — even as little as 5—10% — may introduce strong bias in the comparisons. The possibility to completely separate V and G nuclei by flow cytometry is an attractive alternative. In addition, cell sorter analyses could help to understand cell cycle events in pollen nuclei, using several dyes (e.g. EtBr, mithramycin, DAPI: see Refs. 15 and 27) to distinguish changes in DNA content and DNA structure.

Acknowledgements

We thank Mme J. Knight for intensive technical assistance (collecting the anthers of several tens of thousand flowers), as also Prof. P. Manigault (CNRS, Gif-sur-Yvette) and Dr. Essad (CNRA, Versailles) for kindly helping us to use their cytophotometers.

References

1 J.L. Brewbaker, The distribution and phylogenetic significance of binucleate and trinucleate pollen grains in the angiosperms. Am. J. Bot., 54 (1967) 1069—1083.

2 M.R. Vuayaraghavan and Karuna Bhatia, Cellular changes during microsporogenesis, vegetative and generative cell formation: a review based on ultrastructure and histochemistry. Int. Rev. Cytol., 96 (1985) 262—295.

3 N. Sunderland, Pollen and anther culture, in: H.E. Street (Ed.), Plant Tissue and Cell Culture, Oxford, Blackwell, 1973, pp.205—239.

4 R. De Paepe, C. Nitsch, M. Godard and J. Pernes, Potential from haploid and possible use in agriculture, in: W. Barz, E. Reinhard and M.H. Zenk (Eds.), Plant Tissue Culture and its Biotechnological Application, Springer-Verlag, 1977, pp. 341—352.

5 R. De Paepe and J. Pernes, Exemples de variations à hérédité mendélienne induites au cours du développement des plantes. Physiol. Vég., 16 (1978) 195—204.

6 R. De Paepe, D. Bleton and F. Gnangbe, Basis and extent of genetic variability among doubled-haploid plants obtained by pollen culture in N. sylvestris. Theor. Appl. Genet., 59 (1981) 177—184.

7 R. De Paepe, D. Prat and T. Huguet, Heritable nuclear DNA changes in doubled-haploid plants obtained by pollen culture of *Nicotiana sylvestris*. Plant Sci. Lett., 28 (1982) 11—28.

8 S.K. Dhillon, E.A. Wernsman and J.P. Mikshe, Evaluation of nuclear DNA content and heterochromatin changes in anther-derived dihaploid of tobacco (*Nicotiana tabacum*) cv Cooker 139. Can J. Genet. Cytol., 25 (1983) 169—173.

9 K.L. La Fountain and J.P. Mascarenhas, Isolation of vegetative and generative nuclei from pollen tubes. Exp. Cell Res., 73 (1972) 233—236.

10 S.D. Russell, Isolation of sperm cells from the pollen of *Plumbago zeylanica*. Plant Physiol., 81 (1986) 317—319.

11 I. Dupuis, P. Roeckel, E. Matthys-Rochon and C. Dumas, Procedure to isolate viable sperm cells from corn (*Zea mays* L.) pollen grains. Plant Physiol., 85 (1987) 876—878.

12 C. Zhou, Isolation and purification of generative cells from fresh pollen. Plant Cell Rep., 7 (1988) 107—110.

13 J.B. Le Pecq and C. Paoletti, A fluorescent complex between ethidium bromide and nucleic acids. J. Mol. Biol., 27 (1967) 179—189.

14 N. Kislev and I. Rubenstein, Utility of ethidium bromide in the extraction from whole plants of high molecular weight maize DNA. Plant Physiol., 66 (1980) 1140—1143.

15 T. Hough, P. Berhardt, R.B. Knox and E.G. Williams, Application of fluorochromes to pollen biolgy. II. The DNA probes ethidium bromide and Hoechst 33258 in conjunction with the callose-specific aniline blue fluorochrome. Stain Technol., 60 (1985) 155—162.

16 J.P. Nitsch, Les phytotrons et la bioclimatologie expérimentale, in: G. Gallimard (Ed.) Encyclopédie La Pléiade, 1969, pp. 1602—1617.

17 S.I. Honda, T. Honglaradom and G.G. Laties, A new isolation medium for plant organelles. J. Exp. Bot., 17 (1966) 460—465.

18 H.G. Murray and W.F. Thompson, Rapid isolation of high molecular weight DNA. Nucleic Acids Res., 8 (1980) 4321—4325.

19 S.C. Brown and C. Bergounioux, Plant flow cytometry, in: A. Yen (Ed.), Flow cytometry: Advanced Clinical and Research Applications, Vol. II, CRC Press Inc, Boca Raton, Florida, 1989, pp. 195—220.

20 R. De Paepe, D. Prat and J. Knight, Effects of consecutive androgeneses on morphology and fertility in *N. sylvestris*. Can. J. Bot. (1983) 2038—2046.

21 J. Ingle, J.N. Timmis and J. Sinclair, The relationship between satellite deoxyribonucleic acid, ribosomal nucleic acid, gene redundance and genome size in plants. Plant Physiol., 55 (1975) 496—501.

22 F. D'Amato, M. Devreux and G.T. Scariasca Mugnozza, The DNA content of the nuclei of the pollen grain in tobacco and barley. Caryologia, 18 (1965) 377—382.

23 R. Jalousot, Différenciation nucléaire et cytoplasmique du grain de pollen de *Lilium candidum*, Exp. Cell Res., 55 (1969) 1—8.

24 C.U. Hesemann, Untersushungen zur pollenenwicklung and pollenschauchbildung bei hoheren pflanzen III. DNS-replication bei vegetative und sperma-kernen

in reifen pollenkorne von gerste. Theor. Appl. Genet., 43 (1973) 232–241.

25 B.S. Sangwann-Norreel, Evolution du contenu en DNA nucléaire dans les gamétophytes males du *Datura innoxia* au cours de la période favorable ă l'androgenèse. Can. J. Bot., 57 (1979) 451–457.

26 K. Aruga, K. Yamamoto and T. Nakajima, Nucleic acid synthesis required for embryogenic induction in pollen grains of *Nicotiana tabacum* L., in: A. Fuwara (Ed.), Plant Tissue Culture, Maruzen, Tokyo, 1982, pp. 537–538.

27 A.W. Coleman and L.J. Goff, Application of fluorochromes to pollen biology. Mithramycin and 4′, 6-diamino-2-phenylindole (DAPI) as vital stains and quantitation of nuclear DNA. Stain Technol., 60 (1985) 145–154.

28 M. Lefort-Tran, M.H. Bre, M. Pouphile and P. Manigault, DNA flow cytometry of control *Euglena* and cell cycle blockade of vitamin B12-starved cells. Cytometry, 8 (1987) 46–54.

29 F.A. Loewus, B.G. Baldi, V.R. Francheschi, L.D. Meinert and J.J. Mac Collum, Plant sporoplasts: dissolution of pollen walls. Plant Physiol., 78 (1985) 652–654.

30 D.W. Galbraith, Analysis of higher plants by flow cytometry and cell sorting. Int. Rev. Cytol., 116 (1989) 164–228.

31 L.K. Anderson, S.M. Stack, M.H. Fox and Z. Chuanshan, The relationship between genome size and synaptonemal complex length in higher plants. Exp. Cell Res., 156 (1985) 367–378.

32 J. Matousek and J. Tupy, The release of nucleases from tobacco pollen. Plant Sci. Lett., 30 (1983) 83–89.

33 J. Matousek and J. Tupy, The release and some properties of nucleases from various pollen species. J. Plant Physiol., 119 (1985) 169–178.

34 B.A. Palevitzt and M. Cresti, Cytoskeletal changes during generative cell division and sperm formation in *Trandescantia virginia.* Protoplasma, 150 (1989) 54–71.

35 J. Heslop-Harrison, Synchronous pollen mitosis and the formation of the generative cell in massulate orchids. J. Cell Sci., 3 (1968) 457–466.

36 M. Cresti, F. Campiolini, D.L.M. Mulcahy and G. Mulcahy, Ultrastructure of *Nicotiana alata* pollen, its germination and early tube formation. Am. J. Bot., 72 (1985) 719–727.

37 Y. Hong-Shi, H. Shi-Yi and C. Zhu, Ultrastructure of sperm cells and the male germ unit in pollen tubes of *Nicotiana tabacum.* Protoplasma, 152 (1989) 29–36.

38 M.C. Jones and S.A. Boffey, Deoxyribonuclease activities of wheat seedlings, FEBS Lett., 174 (1984) 215–218.

39 S.O. Rogers and A.J. Bendich, Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol., 5 (1985) 69–76.