Ribosomal bodies specific to both pollen and zygotic embryogenesis in *Datura*

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Summary. Induction of pollen embryos in vitro or zygotic embryos in situ is characterized by the formation of RNA-rich cytoplasmic bodies. These are observed in all pollen embryos irrespective of the different pathways of androgenesis followed in vitro.

Cultural conditions for the formation of a new sporophyte from the male gametophyte (microspore/pollen grain) have been intensely studied in *Datura*[2-7]. Deviation from the gametophytic to the sporophytic pathway is due to changes in the normal functioning of the pollen and to the acquisition of new morphogenic abilities. The structural modifications occurring in pollen grains at the time of embryogenesis could thus throw some light on the mechanism of androgenic induction. In this study we report the formation of ribosomal bodies (RB), specific to embryogenesis, whether androgenic or zygotic, and which occur in all the pathways of embryo formation, encountered in androgenesis.

Young diploid (2n = 24) zygotic plants (2-3 months old) of *Datura metel* L. were grown in a green house (Phytotron, Gif-sur-Yvette) at 24 °C (day) – 17 °C (night) and a 16-h light period. Flower buds (4 cm), containing pollen just before and just after the 1st haploid mitosis, were cold treated (48 h at 3 °C) and then surface sterilized. The anther culture technique used for obtaining optimum yields of embryos was very similar to that reported earlier[8,9]. The cultures were grown at 28 ± 1 °C and exposed to a continuous light intensity of 1000 lx.

The early evolution of the male gametophyte towards embryogenesis could be traced from Feulgen-squashes or sections of material fixed at regular intervals of 48 h for 16 days. Only 2.5% of the pollen formed embryos. In fact, after 2 days in culture, nearly half the pollen grains were dead, and their percentage increased regularly reaching 97.5 finally. Initiation of the embryos started after 2 days of culture and continued until the 16th day, as observed by the increase in the number of multicellular pollen[8]. Pollen embryo formation and development were not synchronous. Once induced, pollen embryos passed through successive stages of embryo development to give rise to young plantlets. In our culture medium, embryos were never accompanied by callus. Embryo formation was initiated either in the late uninucleate microspore or in the young bicellular pollen grains. 3 mains pathways of nuclear evolution during pollen embryo formation were observed in our cultures (table); this agrees with previously well-established schemes[3,8,10-12] particularly of Sunderland[4,6]. However, the majority of pollen embryos in *D. metel* were seen to originate from a single nuclear type (53%, type A or B) when 4-12 celled embryos were examined. In these, embryos arose either after the uninucleated pollen grains underwent a modified mitosis to give 2 equal nuclei or after the vegetative cell alone divided[8,9]. It is difficult to precisely distinguish these 2 types of nuclear morphogenesis from each other in the induced pollen embryos, because both show similar Feulgen staining. Though ultrastructural studies could be of some help[8], they do not permit a statistical evaluation of the proportion of the 2 kinds of morphogenesis. Hence, both are indicated here as of a single nuclear type. Pollen embryos were also formed, involving both the vegetative and generative cells (table, 30% type C). However, a small percentage of pollen embryos were of unknown origin (table, 15% type D) and we were unable to attribute any mode of formation to them.

When embryogenesis occurred 4-20 celled pollen embryos were observed after 4-8 days in anther cultures, irrespective of the segmentation pattern followed in the different pathways. The most important cytoplasmic feature of these early globular embryos was the formation of condensed pyroninophilic structures after UNNA staining and the Brachet test[13], for RNA. These cytoplasmic structures lasted only during the globular stage and disorganized progressively as pollen embryos entered the heart-shaped stage. They disappeared from globular embryos upon RNase treatment and their ultrastructure recalled that of ribosomal masses or bodies (highly rich in RNA) (fig., a-d), showing no cytoplasmic organelles within (e.g., plastids, mitochondria, lipid, etc.).

These bodies consisted mainly of dense masses of ribosomes grouped in polysomes, and a rough endoplasmic reticulum (RER) (fig. c and d). They were highly compact or condensed in the very young globular embryos (up to approximately 20 cells), and were observed as early as the 2-4 celled stage of the globular embryo. In older globular embryos, they were less compact and finally began to disorganize at the end of the globular stage. About 3-5 could be seen per cell section, situated around or very near the nucleus (fig., a and c). In the highly condensed state, the cisternae of the RER were long and occasionally arranged in more or less regular parallel arrays, while smooth endoplasmic reticulum was absent. In the less condensed state, we observed 1. a dilation and fragmentation of the cisternae of the RER, 2. the formation of a clear zone devoid of polysomes and 3. the migration of ribosomes/polysomes from RB to the surrounding cytoplasm.

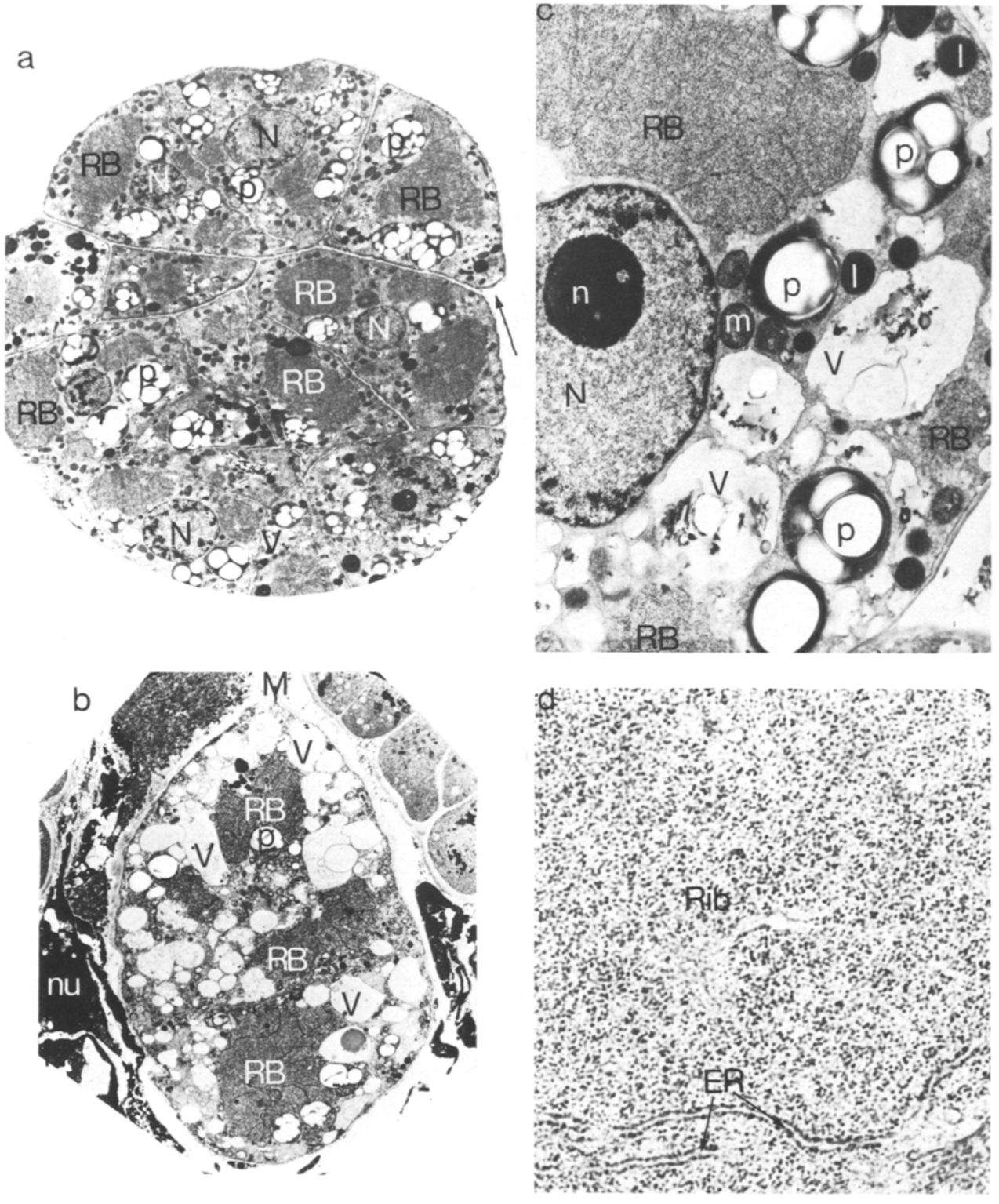
In the very early globular stage (4-20 cells), the cytoplasm contains very few ribosomes apart from those confined to the RB. As the late globular stage is attained, however, ribosomal density increases in the cytoplasm outside the RB as the latter's contents get dispersed.

Light and electron microscopical observations on young zygotic embryos of the same species (*D. metel* L.) confirmed the existence of such ribosomal bodies (fig. b), which were very similar in structure to those of young androgenic embryos, though occasionally enclosing plastids, mitochondria or lipids.

Percentage of pollen embryos arising through different pathways

Androgenic pathway	A or B	C	D
Number of cells per embryo	4-12	4-12	4-12
Percentage embryos	55	30	15

Feulgen-squashes from cultured anthers fixed for 3 h in ethanol (⅔) acetic acid (⅓). Means of 3 experiments done at different periods on a total of 160 embryos after 8 and 16 days of culture (the dead pollen grains and the embryos having more than 12 cells were not taken into consideration). A or B: 1 nuclear type; embryos formed either by the uninucleate microspore undergoing modified mitosis (A) or from the vegetative cell alone (B). C: Both vegetative and generative cells divide and form the embryos. D: Embryos originated through other than the above pathways i.e. of unknown origin.



Specimens were fixed for 3 h at room-temperature in cacodylate buffer (pH 7.4) and 2% glutaraldehyde, post-fixed for 2 h in 1% OSO_4, dehydrated through graded alcohols and embedded in araldite. Thin sections stained with uranyl acetate followed by lead citrate were examined in a Hitachi HU 11 E electron microscope (Sangwan[8]).

a A young androgenic embryo after 8 days of anther culture, characterized by the presence of numerous and voluminous ribosomal bodies (RB). Arrow indicates the location of the generative cell, which has degenerated. × 2200. *b* A young zygotic embryo after 8 days of pollination, showing dense RB traversed by cisternae of the endoplasmic reticulum. × 1800. *c* Detail of an androgenic embryonic cell with a large nucleus, 3 ribosomal bodies and other cytoplasmic organelles. Note the low density of ribosomes in the cytoplasm outside the RB. × 8500. *d* Detail of a RB, with dense polysomes and rough endoplasmic reticulum. × 33,000.

ER, endoplasmic reticulum; l, lipid; M, micropylar end; m, mitochondria; N, nucleus; n, nucleolus; nu, nucellus; p, plastid; RB, ribosomal bodies; Rib, ribosomes; V, vacuole.

These observations establish the prevalence of RNA-rich RB in both zygotic and androgenic embryogenesis of *Datura*. Most importantly, such bodies observed in all the pollen embryos, irrespective of the particular androgenic pathway, followed (table, pathways A, B and C, etc.) showing that their formation occurs with any of the different nuclear types. The phenomenon is therefore characteristic of early embryogenesis in whatever form. Moreover, it does not seem to be related to any stress resulting from prior cold treatment or centrifugation[8].

RB were not observed in pollen before embryogenesis in vitro nor during normal gametophytic development in vivo[8]. They appeared only when pollen advanced towards embryo formation. To our knowledge, this is the 1st report of ribosomal-body formation in plant embryology, whether androgenic or zygotic. In contrast, the origin, role and function of the RNA-rich ribosomal body are well described in animal embryology, particularly in that of the mollusc, *Lymnaea stagnalis*[14–16]. It has also been suggested that the RB are an important site of enzyme formation necessary for the induction and development of animal embryogenesis, and they are very active in the diffused state[14].

Consequently, the deviation from the gametophytic to the sporophytic pathway in higher plants like *Datura*, could be better understood by determining 1. the type of enzymes or proteins synthesized in the RB, and 2. whether their state is the condensed or diffused one.

1 We thank Drs Brigitte S. Sangwan-Norreel and A. Kovoor for discussions and Mr J. Schaeffer for technical assistance.
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Cholinergic nerves in the rat portal vein

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Summary. The innervation of the rat portal vein was studied using the cholinesterase histochemical method. 2 plexuses of cholinergic nerve fibers were found; an external plexus localized at the level of the outer adventitial layers and an inner plexus at the level of the adventitial-medial transitional zone and in the outer layers of the media.

A detailed knowledge of portal vein innervation is of particular interest since this vessel is widely used as a model for studies on the influence of various substances on vascular smooth muscle cells[3].

Histochemical studies demonstrate that the portal vein is provided with a rich adrenergic innervation[4–8] and in the rabbit with a purinergic innervation[8].

The available information about the cholinergic innervation of the portal vein is very poor, and limited to the results of Mootz[9] and Booz[5] who described the presence of few adventitial cholinergic nerve fibers (CNF) in the rat, and to the results of Ungvary et al.[6] and Burnstock et al.[8]. These authors[6,8] did not observe any appreciable cholinergic innervation of the portal vein in guinea-pig and rabbit.

The finding that there is a very poor cholinergic innervation in the rat portal vein (see Booz[5] and Mootz[9]) does not seem to be in agreement with the results of Reilly et al.[10] who observed a moderately rich cholinergic innervation of the intrahepatic branches of the portal vein in the same species.

With the purpose of clarifying the problem of the extent of the cholinergic innervation to extraparenchymal branches of the rat portal vein we have carried out the present experiments.

Methods. 15 young rats (1–3 months of age) were used in our study. 5 animals were chemically sympathectomized using an i.p. injection of 6-hydroxydopamine (6-OHDA, for references see Burnstock et al.[8]). The drug was injected in 2 doses of 200 mg/kg, the second was given 24 h after the first. The 6-OHDA (HCl salt, K.E.K., USA) was dissolved in 0,9% w/v saline containing 0.1 mg/ml ascorbic acid. 10 rats were used as a control and received 2 injections of a saline solution.

All animals were sacrificed under ether anaesthesia. The abdominal cavity was rapidly opened and the portal vein was dissected and washed in a Krebs solution at 4 °C. The pieces were divided into laminae and stretched flat on slides, or cut transversely on a cryotome (sections of 10–30 µm in thickness). The tissues were dried over P_2O_5 for 60–90 min and then processed for the histochemical detection of acetylcholinesterase activity as previously described[11]. The incubation was accomplished at room temperature for 2–6 h in a medium containing 10^{-5} M iso-OMPA to inhibit nonspecific cholinesterases[11].