Plant embryology during and after Panchanan Maheshwari's time – Changing face of research in the embryology of flowering plants

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Much of the past research in the embryology of flowering plants, pioneered by the late Panchanan Maheshwari has been in the areas of descriptive, comparative, and experimental embryology concerned with the control of fertilization, embryogenesis, and endosperm development. In recent years, there has been a shift of emphasis in research in flowering plant embryology from these areas to genetic and molecular aspects of embryogenesis and endosperm development, using Arabidopsis thaliana as a model system.

THE year 2004 marks the centenary of the birth of Panchanan Maheshwari, the famous plant embryologist, who was Professor and Head of the Department of Botany at the University of Delhi from 1949 until his death in 1966. Maheswari's work in plant embryology may be said to have followed the momentum created by the discovery of 'double fertilization' in 1898, which is now recognized as a defining feature of the reproductive biology of flowering plants. To commemorate the centenary of Maheshwari's birth, this article attempts to embed the work done by him and his students in the context of double fertilization and to show how research on descriptive, comparative, and experimental embryology pioneered by Maheshwari continues to influence a significant fraction of modern-day research in the embryology of flowering plants.

The conventional wisdom is to trace the history of sex in living organisms to the ancient Greek philosophers; but for the purpose of this article, the study of the reproductive biology of flowering plants is considered to have had a shorter history dating back to the early nineteenth century, beginning with the discovery of the pollen tube[1]. However, sustained investigations on this topic began with the discovery of the actual fusion of the male and female gametes during fertilization in *Monotropa hypopitys* by Strasburger[2]. Although this work identified the embryo as the resulting product of fertilization, understanding of the fate of the second male gamete discharged by the pollen tube and the origin of the endosperm remained as major hurdles in gaining a complete insight into the dynamics of fertilization and seed formation in flowering plants. The legendary discovery by Nawaschin[3] showed that in ovules of *Lilium martagon* and *Fritillaria tenella*, both male gametes from the pollen tube penetrated the embryo sac; whereas one of them fused with the nucleus of the egg cell, the other fused with the polar fusion nucleus floating in the central cell, initiating a second fertilization event. This process later dubbed as double fertilization, results in the development of the diploid zygote and the triploid primary endosperm nucleus[4]. Whereas the zygote gives rise to the embryo – the progenitor of the future plant – the primary endosperm nucleus forms a tissue known as the endosperm that plays an important role in regulating maternal nutrient fluxes to the embryo. Confirmation of the occurrence of double fertilization in many flowering plants within slightly over a year after its discovery spawned a flurry of research into the development of the male and female gametophytes, embryo and endosperm in a large number of plants. The results obtained served to connect the dots in the life cycle of flowering plants into a stunningly simple model of an alternation of generations between a gametophytic phase and a sporophytic phase. The model led to the comforting dogma that embryological processes lie at the root of the pathway that initiates phase changes in the life cycle of plants culminating in the development of the seed[5].

Descriptive, comparative and experimental embryology

When descriptive accounts of embryo development in flowering plants began to appear in the 1870s, a fairly clear picture of the structural organization of the male and female gametophytes was already available. The choice of *Capsella bursa-pastoris* for an important part of the first account of embryo development in flowering plants received wide acceptance in later years as a paradigm species to follow cleavage patterns of early-stage embryos and trace the ancestry of cells in a typical dicot embryo[6]. For nearly 90 years following the work on *C. bursa-pastoris*, the field

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of descriptive embryology involving the events of micro- and megasporogenesis, gametogenesis, and embryo and endosperm development in plants belonging to widely scattered families emerged as a preeminent field of study[7]. These investigations provided ample evidence of the diversity in the patterns of cell division during the development and organization of embryos of dicots and monocots, to suggest that cell lineages during embryo development are programmed by a blueprint characteristic of each species. They also led to an appreciation of the role of the tapetum in the nutrition of microspore mother cells in the developing anther, of the suspensor and its bizarre haustorial outgrowths in anchoring the embryo and positioning it in relation to the endosperm and seed tissues, and of apomixis in short-circuiting the sexual pathway of reproduction in the formation of viable seeds[1,8]. Embryological data were used in later investigations to identify realignment of doubtful genera and species delimited solely on the basis of vegetative characters and floral morphology[1,9]. By providing a new level of information, these investigations opened up the field of comparative embryology for solving disputed taxonomic assignments of flowering plants. Maheshwari and his students were major players in the field of descriptive and comparative embryology. Beginning with a study of the embryology of *Boerhaavia diffusa*[10], Maheshwari's bibliography from 1929 to until his death included about 150 publications, many of them in the field of descriptive and comparative embryology; in addition, more than 300 publications have been credited to his student[11].

The use of transmission electron microscope to monitor ultrastructural changes that occur during transformation of the egg into an embryo profoundly influenced the study of flowering plant embryology beginning in the 1960s. This examination resulted in the accumulation of a body of knowledge on the subcellular organization of embryos of different ages. The ultrastructural studies provided new insights into the metabolic status of the egg before and after fertilization, the basis for polarity of the egg and zygote, and the functional differentiation of cells formed from the first division of the zygote. Besides establishing the cellular nature of the sperm of flowering plants, the electron microscope also served as a powerful tool for providing the first glimpses of double fertilization and for understanding the function of cells of the female gametophyte and the subtending suspensor[5,12]. Electron microscopic examination of sperm cells of *Plumbago zeylanica* provided evidence for a physical association between the two sperm cells and the vegetative cell nucleus in the pollen grain or in the pollen tube as a package and for possible gamete-level recognition during double fertilization[13,14].

Beginning in the 1930s, advances made in the fields of plant physiology, biochemistry, and genetics, and refinements in the culture of plant organs, tissues, cells and protoplasts under aseptic conditions seemed to offer unique advantages to examine reproductive processes in flowering plants from a different perspective. This led to the development of the field of experimental embryology, involving the control of pollination and fertilization and manipulations of the anther, pollen grain, ovary, ovule and embryo by excision and culture, and by chemical, hormonal and surgical treatments. A focus area of research in experimental embryology was culture of embryos of different ages in defined media, first introduced by Hannig[15]. These studies showed that early-stage embryos, bombarded as they are in the natural habitat of the embryo sac with nutrient substances present in the endosperm, require complex, exogenously supplied metabolites to maximize their chances for survival and growth in culture, whereas late-stage embryos, especially seed embryos can be nurtured to the stage of seedlings in relatively simple media consisting of mineral salts and a carbon energy source such as sucrose. This confirmed what was suspected from other studies that early-stage embryos are heterotrophic and depend on the nutrient materials present in the endosperm, whereas late-stage embryos are autotrophic and are able to synthesize the array of metabolites necessary for their growth[16].

A cherished objective of experimental plant embryologists is to promote *in vitro* fertilization with isolated, single gametes under controlled conditions, on a level comparable to that feasible in most animals and in some brown algae. The technical difficulty of isolating sperm from the confines of the pollen grain or the pollen tube, and of the egg from the embryo sac has been a major handicap in developing an *in vitro* fertilization system in flowering plants. A beginning toward controlled encounter of the egg and sperm was achieved by Maheshwari's group by culturing unpollinated receptive ovules of *Papaver somniferum* and dusting them with viable pollen grains. In this technique known as test-tube fertilization, pollen germination, pollen-tube entry into ovules, and double fertilization proceeded normally, as attested by the transformation of cultured ovules into seeds enclosing embryo and endosperm[17]. In important extensions of this work, the technique was refined to overcome self-incompatibility[18] and to obtain seeds from a large number of flowering plants by overcoming other types of crossability barriers[19]. After long frustrations, intensive efforts in several laboratories around the world beginning in the 1970s, in isolating viable egg cells and sperm and in standardizing conditions for *in vitro* fertilization were finally rewarded by the success obtained in fusing isolated egg and sperm of maize (*Zea mays*) *in vitro* and generating fertile plants[20], and in fusing sperm and polar fusion nucleus to form the endosperm[21] to demonstrate *in vitro* double fertilization for the first time in maize. Although successful *in vitro* fertilization in a flowering plant is a remarkable technical feat, its significance does not end there. It is clear that genetic transformation of plants via the egg, sperm or zygote is on the horizon as a crucial methodology for crop improvement.

An idea attributed to a prophesy by Haberlandt[22] that it is possible to grow embryos from vegetative cells of plants, was realized when tissue culture approaches showed that

single somatic cells originating from a callus produced from the secondary phloem of carrot (*Daucus carota*) grown in a suspension culture give rise to fertile plants simulating stages strongly reminiscent of normal embryogenesis by a process known as somatic embryogenesis[23,24]. The enormous advantages inherent in the clonal multiplication of plants by somatic embryogenesis are increasingly being exploited in horticultural and agricultural practices. Two crucial pieces of information that added weight to the unfolding story of somatic embryogenesis in carrot in the 1960s came from the work done by Maheshwari and his students on plants totally unrelated to carrot, before the latter would become the most popular model for studying many aspects of somatic embryogenesis. One was the demonstration of the single-celled origin of adventive embryos (somatic embryos) from the superficial cells of cultured embryos of *Cuscuta reflexa*[25]. The other was the surprising finding of somatic embryogenesis directly on cultured explants of *Ranunculus sceleratus* without the intervention of a callus phase[26,27].

Research using tissue-culture approaches to study the embryology of flowering plants was culminated by the seminal discovery of pollen embryogenesis by Guha and Maheshwari[28]. It was found that when excised anthers of *Datura innoxia* at the pollen-grain stage were cultured in a complex medium, embryo-like structures appeared from the sides of the anther. Although it was suspected that these outgrowths might have originated from the somatic tissues of the anther, their origin from pollen grains and consequently their haploid nature was confirmed late[29]. Much of the interest in this discovery lies in the fact that it opened up the way to produce haploid plants reproducibly and in quantity for genetic and breeding experiments by the simple expedient of culturing anthers enclosing pollen grains at an appropriate stage of development in a suitable medium.

The advent of molecular embryology

Although robust embryological investigations of many additional species of plants will be necessary for enlarging the database of wild-type relatives of our cultivated crop plants, the need for this work was overshadowed by developments in molecular biology and genetics to study flowering plant embryology. This heralded the advent of molecular embryology beginning in the 1970s, which sadly, Maheshwari did not live to see establish its roots and flourish. The goal of this area of research was to obtain answers to questions about the genetic and molecular mechanisms that modulate the development of the complex embryo of flowering plants from a single-celled zygote and of the endosperm from the primary endosperm nucleus. It appears that the buzzwords that will constitute a common thread binding these mechanisms will be signal transduction molecules.

A new level of understanding of the genetic and molecular biology of development of the embryo and endosperm of flowering plants has become possible by drawing largely on the advantages of *Arabidopsis thaliana* as a useful experimental system. These investigations have been aided in a large part by the isolation of mutations that affect in an informative way virtually every aspect of embryo development from the morphology of the mature embryo down to the early-stage embryo generated by the first few rounds of division of the zygote combined with cloning of the mutated genes, identification of their protein products, and transgenic approaches. From these and other studies which have thrust *Arabidopsis* as an excellent hunting ground for embryo developmental mutants, evidence has emerged that meristem initiation, pattern formation, morphogenesis and cytodifferentiation of the embryo portrayed in exquisite detail by descriptive embryology are regulated independently by different sets of genes. Some of these studies, which really picked up from where descriptive embryology signed-off, are highlighted below to provide a glimpse of future research in plant embryology.

In flowering plants, the lineage of the shoot apical meristem is traced to cells in the apical half of the globular-stage embryo, although the meristem itself becomes first visible later in the torpedo-shaped-stage embryo. Central to the functioning of this meristem in the embryo is the maintenance of a reservoir of stem cells that are available for ongoing organogenesis throughout the life of the plant. Work done on *Arabidopsis* to trace the progression of the shoot apical meristem from a set of undistinguished cells in the globular-stage embryo has identified more than a dozen mutations, most importantly, *wuschel* (*wus*), *shoot meristemless* (*stm*), and *clavata* (*clv*) that affect in subtle ways the organization of the embryonic shoot apical meristem. Based on the analysis of expression of wild-type genes as molecular markers, the prevalent view is that an organizing centre specified by the *WUS* gene in four inner cells of the 16-celled embryo followed by a step-wise appearance of characteristic transcriptional domains signalled by other genes, collectively defines the initiation and subsequent maintenance of the shoot apical meristem[30–32].

Descriptive embryology had established long ago that in plants related to *Arabidopsis*, the root apical meristem is derived from cells cut-off by both the terminal and basal cells of the two-celled embryo[1]. In addition, a group of cells in the root apical meristem known as the quiescent centre which divide rather infrequently, or not at all, has been found to endow this meristem with unusual cytological features[33]. Histological techniques combined with clonal analysis of the embryonic or seedling root apical meristem have shown that in *Arabidopsis* the quiescent centre is surrounded by the stem cells; these observations impart the quiescent centre the function of an organizing centre for stem-cell maintenance in the root apical meristem, probably by contact-dependent, short-range signal transduction[34,35]. Histochemical localization of free auxin in individual cells

of the root apical meristem by the use of a reporter gene under the control of auxin-responsive regulatory sequences has suggested that cellular organization of the embryonic root meristem of *Arabidopsis* is modulated by a localized concentration of auxin, which apparently functions as a signalling molecule[36,37]. Investigations of mutants such as *monopteros* (*mp*) impaired in the formation of the root apical meristem[38,39], *hobbit* (*hbt*) impaired in the development of the quiescent centre[40], and *plethora* (*plt*) impaired in the development of the quiescent centre and root cap columella[41], have provided strong indications that the mutated genes are involved in auxin signalling by encoding transcription factors required for stem-cell specification and maintenance in the root apical meristem. Collectively, these investigations have also underscored the fact that determination of cell fate in the root apical meristem is under the control of genes that are activated during early stages of embryogenesis and is less lineage-dependent than previously thought.

The rudiments of the body plan of a flowering plant are carved out during early embryogenesis by two distinct and largely independent processes – one that defines the apicobasal pattern and the other the radial pattern – and elaborated during later embryogenesis. Numerous studies, utilizing experimental, genetic and molecular approaches have been employed in recent years to investigate the mechanisms that initiate the establishment of the apicobasal and radial patterning in embryos. These studies have shown that the genome of *Arabidopsis* harbours an amazingly large and diverse set of genes whose mutations can cause havoc in the patterning of embryos. Underscoring the significance of specific cell-division patterns in the crafting of an embryo, most of the mutations have been traced back to defects in the early stages of embryogenic divisions. Evidence that specification of cotyledons is directed mostly, if not exclusively, by a single gene has come from an analysis of the *gurke* (*gk)* mutant in which the cotyledons are missing due to failure of organized cell divisions in the globular/heart-shaped embryos which initiate cotyledons[42]. The gene which has the spatially restricted task of hypocotyl specification is *FACKEL* (*FK*), as mutations in this gene give rise to seedlings in which the hypocotyl is missing and hence the cotyledons are attached directly to a short root. The mutant phenotype has been traced to cytokinetic defects such as enlarged cells, random orientation of cell divisions, and incomplete cell walls beginning with globular-stage embryos[43]. The finding that the *fk* mutation causes a lesion in the pathway of C-14 sterol reductase synthesis has given some insight into how a biochemical defect is translated into a phenotypic abnormality[43,44]. Mutants identified with impaired capacities for the production of both hypocotyl and root are *mp*[38] and *bodenlos* (*bdl*)[45]. Features that characteristically distinguish mutant embryos from their wild-type relatives are confined to a narrow developmental window between the two- or four-celled stage and the heart-shaped stage.

The gene, which when mutated, causes defects in both the shoot and root of *Arabidopsis* seedling is *GNOM* (*GN*); mutant phenotypes appear mostly cone-shaped with reduced root and cotyledons, or in strong alleles, as an undifferentiated mass of tissue with no apparent apicobasal axis. At the physiological level, developmental abnormalities have been attributed to defects in the polarity of auxin flow in cells along the apicobasal axis of the embryo. Cytologically, defects in the mutant lines have been precisely traced to the zygote, whose first division is deflected to produce two nearly equal cells rather than two asymmetrical cells. These observations support the view that the cellular target of the *GN* gene is the usual asymmetric division of the zygote[46,47]. The GN protein shows partial sequence homology to yeast proteins YEC2, of unknown function, and SEC7 (a member of a family of ADP-ribosylation factor [ARF] nucleotide exchange factors) that facilitates intracellular transport mediated by Golgi bodies[48,49]. This raises the possibility that stabilization of the apicobasal axis may in part depend upon targetted vesicle trafficking.

After the apicobasal axis of the embryo is established, the shoot and root apical meristems delimit the three sets of primary meristems, namely the protoderm, procambium and ground meristem, which subsequently differentiate into the main tissues of the embryo axis to provide the radial pattern of the embryo. In *Arabidopsis*, radial patterning is initiated as early as the eight-celled stage of the embryo, when these cells divide periclinally to give rise to eight inner and eight outer daughter cells. The outer cells which form the epidermis, differ from the inner cells in the expression of a specific gene named *Arabidopsis thaliana Meristem Layer 1* (*AtML1*), which is not expressed in the inner cells[50]. Genetic screens have identified mutations in *Arabidopsis* that specifically cause disturbances in tissue differentiation or deletion of specific cell layers in the embryonic organs. Two of the most informative and thoroughly investigated mutants in which imperfections in cell differentiation can be traced to early-stage embryos are *knolle* (*kn*) and *keule* (*keu*). The severity of the mutations varies in the seedling phenotypes which appear mostly as round or tuber-shaped structures with a rough epidermis and lacking functional meristems in *kn* alleles and as elongate axis topped by reduced cotyledons in *keu* alleles. The cellular effects of the mutations are vividly illustrated by the generally anarchic divisions in the early-stage embryos, resulting in the formation of large multinucleate cells with gapped or incomplete crosswalls. Pattern defects inflicted by these mutants have been attributed indirectly to defects in cytokinesis, because vesicles transported to the equator of the dividing cell do not fuse to form the cell plate[51–54]. Suggestive of a possible link between molecular functions of the *KN* gene and cytokinetic defects during embryogenesis resulting in the accumulation of unfused vesicles at the site of the cell plate, the predicted protein product of this gene is found to have similarity to syntaxins, members of a protein family known as SNARE[53,55]. The SNARE

complexes have been assigned important roles in membrane-fusion events and in diverse vesicle-trafficking pathways in eukaroytic cells, although the extent to which they contribute to the specificity of the processes is not determined.

After many years of latency, some fundamental questions about the endosperm have been revitalized in recent years. Two early debated hypotheses, one that proposed that the endosperm is a modified second embryo and the other that considered the endosperm as evolutionarily homologous to the female gametophyte, were entwined with the shifting views on angiosperm phylogeny and consequently, it was difficult to distinguish empirically between them[56]. New evidence has shown that in contrast to the triploid endosperm found in the overwhelming majority of flowering plants, a diploid endosperm predominates in some of their ancient lineages. From this observation it has been inferred that over evolutionary time, addition of a male nucleus by a second fertilization event would have provided the specific genetic and developmental basis to transform a diploid biparental endosperm into a triploid one[57]. This is a simple idea, but considering the past vicissitudes of the current hypotheses, there are likely to be surprises ahead in this field before a conclusion is reached.

Several features of the endosperm have made it a useful model for cell biological, genetic, and molecular studies as a snapshot of events in a single tissue consisting of one or two uniform cell types. In many plants, cytokinesis is uncoupled from nuclear division cycle as the endosperm goes through a stage of a multinucleate mass of protoplasm or syncytium. Although eventual wall formation takes place to form a cellular tissue, in many respects the mechanism of placement and growth of walls in the syncytium has turned out to be unusual. An important observation made in barley (*Hordeum vulgare*) endosperm using immunolocalization techniques is that in preparation for cellularization, radial arrays of microtubules that proliferate from the nuclear surface organize the free nuclei into nuclear–cytoplasmic domains. Shortly thereafter, wall materials in the form phragmoplast configurations (cytoplasmic phragmoplasts) are deposited at the interstices of the nuclear–cytoplasmic domains to establish the initial pattern of cellularization[58]. The early process of cellularization of the endosperm of *Arabidopsis* has been resolved in great detail to show that small groups of overlapping microtubules that radiate from neighbouring nuclei initially assemble into mini-phragmoplasts. These phragmoplasts are put together in a patchwork to generate a novel kind of cell plate, the syncytial-type cell plate to divide the free nuclear domains into cells[59]. A three-dimensional reconstruction aided by high-voltage electron microscopy has indicated the involvement of Golgi-derived vesicles transported along the phragmoplast microtubules, probably mediated by kinesin-like motor proteins in the formation of syncytial-type cell plate[60].

The obvious connection between the ploidy level of the primary endosperm nucleus ranging from diploid in the Oenothera type of embryo sac to $9n$ in the Peperomia type, has engendered the notion that except in a small number of plants with the Oenothera type of embryo sac, in majority of the flowering plants, presence of nuclei with more than the diploid number of chromosomes is a way of life for the endosperm generated by double fertilization[1]. It is now known that besides this natural diversity in chromosome numbers, endosperm nuclei exhibit a capacity to increase their DNA content by endoreduplication. Research on the mechanism of endoreduplication has been dominated by the work done on maize in which DNA content of endosperm cells of some genotypes reaches a maximum level of 690C (ref. 61). The direct involvement of a cyclin-dependent kinase (CDK) in the regulation of endoreduplication was recently demonstrated by reducing the level of endoreduplication in endosperm cells of transgenic maize by ectopically expressing a gene encoding a dominant negative form of the CDC. This work showed that whereas overexpression of a wild-type *CDK* gene did not affect endoreduplication, the gene for the defective enzyme lowered kinase activity and significantly lowered the DNA content of endosperm nuclei[62].

The genetic basis of endosperm development has been illuminated by the isolation of *Arabidopsis* female gametophyte mutants assigned to the *fertilization-independent seed* (*fis*) class, which includes mutants designated as *medea* (*mea*), *fis2*, and *fertilization-independent endosperm* (*fie*). In these mutants, the endosperm develops autonomously in the absence of fertilization, indicating that endosperm development is suppressed by the wild-type function of these genes[63–65]. Most *FIS* genes encode proteins that are homologous to the polycomb group proteins. Moreover, the *FIS* genes are imprinted such that only the maternal copy of the gene is expressed in the endosperm[66–68]. This idea contravenes the expectation of equal participation of the genome inherited from both parents in development.

Perspectives

Although the above analysis of the changing research emphasis in the embryology of flowering plants during and after Maheshwari's time is not exhaustive, research on topics in plant embryology, especially embryogenesis and endosperm development, seems to have attained a degree of sophistication to take its place among the most exciting and active areas of study in plant development, well ahead of other areas of plant reproductive biology and on a level comparable to animal embryogenesis. The ease of genetic and molecular analysis in *Arabidopsis* now combined with the complete sequencing of its entire genome[69], ensures that this plant will remain an essential model organism for deciphering the genetic and molecular secrets of flowering plant reproduction. On the practical side, the impetus created by current research efforts has led to the creation of rice (*Oryza sativa*) genetically engineered to make β-caro-

tene in its endosperm cells. This rice known as 'golden rice' because of its pale yellow colour when polished as against the pearly white rice and its great humanitarian intent to improve the lives of millions who depend upon rice as a staple diet, has even caught the attention of the popular press[70]. Thus, Maheshwari's research on flowering plant embryology, part of which is embodied in his classical book *An Introduction to the Embryology of Angiosperms*[1], is a legacy that endures.

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