Val Raghavan • **Double Fertilization**

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Double Fertilization

Embryo and Endosperm Development in Flowering Plants

With 75 Illustrations, Including 16 Color Plates

Springer

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For
Lakshmi Raghavan and Anita Raghavan

About the Author

Since 1978, Val Raghavan has been a Professor at The Ohio State University, Columbus, Ohio (USA), where he is currently affiliated with the Department of Plant Cellular and Molecular Biology. After obtaining a Ph.D. degree from Princeton University, Dr. Raghavan held post-doctoral positions at Harvard University, Rockefeller University, and Dartmouth College, and faculty appointments at the University of Malaya, Kuala Lumpur and the National University of Singapore. He has published extensively on various aspects of the development of vascular plants, especially on zygotic and asexual embryogenesis in flowering plants.

Preface

Double fertilization is hailed as a unique event in the life cycle of flowering plants. Defined as the union of one sperm with the egg on the one hand, and of a second sperm with the diploid fusion nucleus on the other, double fertilization sets in motion the chain of events that result in the formation of the embryo and endosperm. Whereas recognition of the importance of these two fusion episodes in seed formation in flowering plants is as old as the discovery of double fertilization itself, their central role in the development of the embryo and endosperm in seeds and grains of crop plants used widely in human and animal nutrition came to be recognized only in later years.

The study of the development of the embryo and endosperm from their single-celled beginnings under the rubric of embryology has occupied an important position in the multifaceted investigations on the reproductive biology of flowering plants undertaken during most of the past century. In recent years, descriptive studies of embryo and endosperm development have been overshadowed by the increasing use of genetic and molecular approaches to study flowering plant embryology, led by the work in the model plant *Arabidopsis thaliana*. Although some of these studies have been reviewed periodically in multiauthored volumes, my objective in writing this book is to provide an overview of past accomplishments in the field, and a sense of the outstanding future problems as they relate to the products of double fertilization. Admittedly, molecular and genetic studies in conjunction with screening of mutants, isolation of genes, and identification of their protein products, are emphasized to some extent at the expense of structural and developmental studies. The main reason for this is that I have tried to write a book on investigations that reflect a rethinking of the way that we have viewed embryogenesis and endosperm development as the end product of a series of stereotypical divisions. In my opinion, these recent studies with molecular overtones have brought us close to an understanding of the critical details that control the transformation of these cellular domains of the ovule into mature tissues of the seed or grain.

The book begins with an account of the history of the discovery of double fertilization, which must surely find a place in a volume dealing with that topic. The details of how body plans of eudicot and monocot embryos are established occupy the next chapter, which sets the stage, in the following three chapters, for a discussion of notable advances made in the identification of the genetic and molecular factors that control the development of the embryo (Chaps. 3, 5) and suspensor (Chap. 4) during progressive embryogenesis. The last chapter to deal wholly with embryos (Chap. 6) describes their general strategies during quiescence or dormancy. The main body of the book concludes with accounts of the developmental, genetic, and molecular studies on the endosperm covered in Chaps. 7 and 8, and, in the final chapter, descriptions of apomixis, somatic embryogenesis, and pollen embryogenesis illustrating embryogenesis and partial endosperm development in the absence of double fertilization. The level of exposition of the topics in the different chapters is considered suitable for graduate students who want get a coherent view of the current perspectives on embryogenesis and endosperm development in flowering plants, and for researchers in the field who plan fresh attacks on unsolved problems on the topics covered.

In conclusion, I would like to thank the many publishers/authors who gave me permission to use illustrations from published articles in my book. Besides myself, no one contributed more to the preparation of the final manuscript than Mr. Eduardo Acosta, Webmaster of my Department. He transformed my rough pencil sketches into professional black and white drawings or into images in gorgeous colors, and was also responsible for transferring all of the illustrations into their electronic versions suitable for printing. It is my pleasure to acknowledge my indebtedness to Eduardo for this help. On the producing side at Springer, Heidelberg, I appreciate the editorial advice and suggestions given from time to time by Dr. Jutta Lindenborn, desk editor, and the professional expertise, critical judgments, and interest in the

subject matter of the book that Dr. Helen Rothnie, copy editor, brought to the job. Last, but not least, I thank my wife Lakshmi for her appreciation of my interests, which allowed me to spend long hours in my office and laboratory where I felt comfortable to pursue scholarly activities. My daughter, Anita, was generous with her sense of good humor, often transmitted by remote control from London, England, during the preparation of this book.

Columbus, Ohio V. Raghavan
August 2005

Contents

Abbreviations

GENERAL ABBREVIATIONS

ABA	abscisic acid
APC	anaphase-promoting-complex
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
2,4-D	2,4-dichlorophenoxyacetic acid
EMS	ethylmethane sulfonate
ER	endoplasmic reticulum
GA	gibberellic acid
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GlyRS	glycyl tRNA synthetase
GUS	β-glucuronidase
IAA	indoleacetic acid
ICL	isocitrate lyase
JIM8	a monoclonal antibody
MS	malate synthase
MYB	recognition site in the genome identified with myeloblastosis-associated viruses
NAA	naphthaleneacetic acid
NPA	naphthylphthalamic acid
pcd	programmed cell death
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
T-DNA	transferred DNA
TIBA	triiodobenzoic acid
TUNEL	terminal deoxyribonucleotidyl transferase-mediated dUTP-fluorescein nick end labeling

LIST OF cDNA CLONES, GENES, MUTANTS, AND PROTEIN PRODUCTS

Listed below are the cDNA clones, genes, mutants, and protein products and their abbreviations in the form in which they are first mentioned in the text. With a few exceptions, abbreviations and names of wild-type genes are given here and in the text in italicized capital letters; mutants are indicated in italicized lowercase letters. Abbreviations of protein products are given in capital letters.

AAP	*AMINO ACID PERMEASE*
aba	*ABA-deficient*
ABC	ATP-binding cassette
ABI	*ABA-INSENSITIVE*
abp	*auxin-binding protein*
Ac	*Activator*
adl	*Arabidopsis dynamin-like proteins*
AGL	*AGAMOUS-Like*
AGO	*ARGONAUTE*
AHAP3	*Arabidopsis* HAP3
ALDP	adrenoleukodystrophy protein
ALE	*ABNORMAL LEAF SHAPE*
aml	*Arabidopsis Minute-like*
ANT	*AINTEGUMENTA*
AP2	*APETALA2*
ARF	ADP-ribosylation factor; auxin response factor
ARL2	a relative of the ARF-family of proteins
AS	*ASYMMETRIC LEAVES*
ask	*Arabidopsis thaliana Skip-like1*
ASKη	*Arabidopsis shaggy*-related protein kinase *etha*
ASKζ	*Arabidopsis shaggy*-related protein kinase *dzeta*
Atcul	*Arabidopsis thaliana cullin*
AtEm	*Arabidopsis thaliana Em*
Athb	*Arabidopsis thaliana HOMEOBOX*
AtLTP	*Arabidopsis thaliana LIPID TRANSFER PROTEIN*
ATML	*Arabidopsis thaliana MERISTEM L1 LAYER*

AtpA	*atp1*, *ATPase1*; a mitochondrial gene
AtPIN	*Arabidopsis thaliana PIN-FORMED*
AtRPS5	mutated gene of *aml1*
ATS	*ARABIDOPSIS THALIANA SEED*
AtSERK	*Arabidopsis thaliana SERK*
At2S3	*Arabidopsis thaliana 2S ALBUMIN*
AX92	a gene of *Brassica napus* embryos and seedlings
axr	*auxin-resistant*
B22E	a barley endosperm gene
BAP	*BASAL LAYER ANTIFUNGAL PROTEINS*
BBM	*BABY BOOM*
bdl	*bodenlos*
BETL	*BASAL ENDOSPERM TRANSFER LAYER*
bga	*borgia*
bio	biotin mutant
BIO2	biotin synthase gene
BOP	*BLADE-ON-PETIOLE*
BP	*BREVIPEDICELLUS*
bt	*brittle*
bZIP	basic leucine zipper class of transcriptional regulators
C1	a gene in the anthocyanin pathway of maize
cab	gene encoding chlorophyll *a*/*b* binding protein
cap	*capulet*
CBF	CCAAT-box-binding transcription factor
CDC	*CELL DIVISION CYCLIN*
CDK	cyclin-dependent kinase
C-ESE	*CARROT EARLY SOMATIC EMBRYOGENESIS*
CHAPERONIN-60α	an *Arabidopsis* gene
CHI	*CHITINASE*
CHO	*CHAMPIGNON*
CLE	*CLAVATA-Like*
clv	*clavata*
CNA	*CORONA*
cox	gene of cytochrome-*c* subunit
CPC	*CAPRICE*
cph	*cephalopod*
cr	*crinkly*
CRC	*CRUCIFERIN*
cts	*comatose*
CUC	*CUP-SHAPED COTYLEDON*
CUL	CULLIN
CYCD	*CYCLIN D*
CycZme1	*Zea mays* mitotic cyclin belonging to the subgroup *Zeama;CycB1*
cyd	*cytokinesis-defective* mutant of pea
cyt	*cytokinesis-defective* mutant of *Arabidopsis*
DcSERK	*Daucus carota SERK*
DDM	*DECREASE IN DNA METHYLATION*
dek	*defective kernel*
DEM	*DEFECTIVE EMBRYO AND MERISTEMS*
des	*defective seedling*
dex	*defective endosperm expressing xenia*
dgr	*distorted growth*
dme	*demeter*
DOM	*DOMINO*
dsc	*discolored*
dzr	a post-transcriptional regulator of zein
E1, E2	embryonic proteins
edd	*embryo-defective development*
EED	*EMBRYONIC ECTODERM DEVELOPMENT*
EEL	*ENHANCED Em LEVEL*
Em	*EARLY METHIONINE-LABELED*
EMB	*EMBRYO-DEFECTIVE*
emb	*embryo-specific*
eml	*embryoless*
emp	*empty pericarp*
END	*ENDOSPERM*
EP	*EXTRACELLLULAR PROTEIN*
ERG	*ERA-RELATED GTPases*
ESC	*EXTRA SEX COMBS*
Esr	*EMBRYO SURROUNDING REGION*
F644	an *Arabidopsis* gene
FBP	*FLORAL BINDING PROTEIN*
fer	*feronia*
FIE	*FERTILIZATION-INDEPENDENT ENDOSPERM*
FIL	*FILAMENTOUS FLOWER*
FIS	*FERTILIZATION-INDEPENDENT SEEDS*
fist	an *Arabidopsis* embryo mutant

FK	*FACKEL*
fs	*fass*
FUS	*FUSCA*
FWA	a late-flowering *Arabidopsis* gene
GAI	*GIBBERELLIN-INSENSITIVE*
gcs	*glucosidase*
gk	*gurke*
GL	*GLABRA*
GLA	*GLOBULAR ARREST*
GLM	*GOLLUM*
glo	*globby*
GlyRS	glycyl-tRNA synthetase
gn	*gnom*
GRAS	transcription factors encoded by *SHR*, *SCR* , *GAI* and *RGA* genes
GRP94	a chaperone protein
HAL	*HALLIMASCH*
HAP3	heme-activated protein 3
hbt	*hobbit*
hik	*hinkel*
HMG	high mobility group protein
HOS	*HOMEOBOX GENE OF ORYZA SATIVA*
HSP	heat shock protein
HYD	*HYDRA*
ig	*indeterminate gametophyte*
iku	*haiku*
JAG	*JAGGED*
KAN	*KANADI*
KAPP	kinase associated protein phosphatase
keu	*keu*
KIS	*KIESEL*
kn	*knolle*
KN	*KNOTTED*
knf	*knopf*
KTi	Kunitz trypsin inhibitor
lachrima	a maize gene
LEA	LATE EMBRYOGENESIS ABUNDANT
LEC	*LEAFY COTYLEDON*
L1L	*LEC1-LIKE*
LLP	ligand-like protein
LTP	*LIPID TRANSFER PROTEIN*
MADS-box	floral organ identity genes
MAT	*MATURATION*
MEA	*MEDEA*
MEG	*MATERNALLY EXPRESSED GENE*
MET	*METHYL TRANSFERASE*
MET1 a/s	*METHYL TRANSFERASE* antisense
mic	*mickey*
mgo	*mgoun*
mp	*monopteros*
msi	*multicopy suppressor of IRA* (inhibitory regulator of Harvey sarcoma virus oncogene RAS-cAMP pathway)
MtSERK	*Medicago truncatula SERK*
Mu	*Mutator*
nam	*no apical meristem*
NRP	*NO APICAL MERISTEM (NAM)-RELATED PROTEIN*
OLEO	*OLEOSIN*
ORG	*ORIGIN RECOGNITION COMPLEX*
OSH	*ORYZA SATIVA HOMEOBOX*
OsKn1	*Oryza sativa KNOTTED1-like*
PAP85	an *Arabidopsis* gene encoding a vicilin-like protein
PAS	*PASTICCINO*
PEI	an *Arabidopsis* gene
PER	*PEROXIREDOXIN*
PFI	*PFIFFERLING*
PGA	*PLANT GROWTH ACTIVATOR*
PHB	*PHABULOSA*
PHE	*PHERES*
PIC	*PINOCCHIO*
PID	*PINOID*
PILZ	a group of *Arabidopsis* genes
PIN	*PIN-FORMED*
pkl	*pickle*
PLS	*POLARIS*
PLT	*PLETHORA*
PNH	*PINHEAD*
pol	*poltergeist*
POR	*PORCINO*
PP2C	PROTEIN PHOSPHATASE 2C
PRL	*PROLIFERA*
pt	*primordial timing*
pZE40	a barley endosperm gene
R	*RED* (a gene controlling pigmentation of maize aleurone cells)
RAB	*RESPONSIVE TO ABA*
rbcL	gene of the large subunit of Rubisco
REV	*REVOLUTA*
RGA	*REPRESSOR OF GA*
rgf	*reduced grain filling*

RINO	*myo*-inositol-1-phosphate synthase gene
Roc	*rice outermost cell-specific*
Rop	Rho-like GTPase
RPS16	ribosomal protein S16
RSH	*ROOT-SHOOT-HYPOCOTYL-DEFECTIVE*
rsw	*radially swollen*
rsy	*raspberry*
sal	*supernumerary aleurone*
SCF	SKP1 [SUPPRESSOR OF KINETOCHORE PROTEINS1]/CDC53 [or CULLIN], *F*-box protein
SCR	*SCARECROW*
SCZ	*SCHIZORIZA*
SCE7	a member of the ARF nucleotide exchange factors
seg	*shrunken endosperm caused by the maternal genotype*
SERK	*SOMATIC EMBRYOGENESIS RECEPTOR KINASE*
SET domain	proteins encoded by *SUPPRESSION OF VARIEGATION*, *ENHANCER OF ZEST*, and *TRITHORAX* genes
sex	*shrunken endosperm expressing xenia*
sh	*shrunken*
SHAGGY	a gene that encodes a protein kinase in *Drosophila*
SHD	*SHEPHERD*
shl	*shootless*
SHR	*SHORT ROOT*
sin	*short integument*
slp	*schlepperless*
sml	*shootmeristemless*
smt	*sterol methyl transferase*
SNAP	a vesicle trafficking gene
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SOL	*SUPPRESSOR OF LLP*
SPÄTZLE	a maize gene involved in endosperm cellularization
SPL	*SPOROCYTELESS*
srn	*siréne*
SSR16	*SMALL SUBUNIT RIBOSOMAL PROTEIN S16*
stm	*shoot meristemless*
su	*sugary*
sus	*suspensor*
TCP	Teosinte branched1, Cycloidea, and PCF1 genes which encode transcription factors
TFC	tubulin folding cofactor
ton	*tonneau*
TOR	*TARGET OF RAPAMYCIN*
tpl	*topless*
tps	*trehalose phosphate synthase*
TTG	*TRANSPARENT TESTA GLABRA*
TTN	*TITAN*
twn	*twin*
vcl	*vacuoleless*
VP1	*VIVIPAROUS1*
Vp1-R	wild type viviparous gene
vp1-R	mutant allele of *Vp1-R*
Vpp	a gene that encodes a type of vacuolar H^+-translocating inorganic pyrophosphatase
Wee1	a protein kinase
WER	*WEREWOLF*
WOL	*WOODEN LEG*
WOX	*WUSCHEL*-related homeobox
wus	*wuschel*
wx	*waxy*
XTC	*EXTRA COTYLEDON*
YAB	*YABBY*
YEC2	yeast protein of unknown function
Zeama; *CycA1, B1, B2*	groups of the *Zea mays mitotic cyclin* gene
ZLL	*ZWILL*
ZmAE	*Zea mays ANDROGENIC EMBRYOS*
ZmEBE	*Zea mays embryo sac/basal endosperm transfer layer/embryo surrounding region*
ZmHox	*Zea mays homeobox*
ZmMRP	*Zea mays MYB-RELATED PROTEIN*
ZmOCL	*Zea mays OUTER CELL LAYER*
ZmPRPL 35	*Zea mays PLASTID RIBOSOMAL PROTEIN L35*
ZmSERK	*Zea mays SERK*
*Zm*Wee1	a maize homolog of Wee1

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FIGURES

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Fig. 7.3a,b Kowles RV, Phillips RL (1988) Endosperm development in maize. International Review of Cytology 112:97–136 © 1988, reprinted with permission from Elsevier

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PLATES

Plate 1, Fig. a–d Williams JH, Friedman WE (2002) Identification of diploid endosperm in an early angiosperm lineage. Nature 415:522–526 © 2002, reprinted by permission, Macmillan Publishers Ltd

Plate 1, Fig. e–i Fu Y, Yuan M, Huang B-Q, Yang, H-Y, Zee S-Y, O'Brien TP (2000) Changes in actin organization in the living egg apparatus of *Torenia fournieri* during fertilization. Sexual Plant Reproduction 12:315–322 © Springer, Berlin Heidelberg New York

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Plate 16, Fig. h–k Touraev A, Ilham A, Vicente O, Heberle-Bors E (1990) Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Reports 15:561–565 © Springer, Berlin Heidelberg New York

1 Double Fertilization – A Defining Feature of Flowering Plants

The expression fertilization may be used in an abstract or a concrete sense. In the abstract it denotes the process by which characters from two individuals are transmitted to a single organism in the succeeding generation. This phenomenon is almost universal throughout the animal and vegetable kingdoms, and its effects have been observed by many successive generations of breeders both of animals and of plants. In this way a considerable body of evidence has accumulated, and it has been found that certain laws are universally true of organisms which thus spring from a double stock. Such an organism passes through its complete life history, which may include more than one cycle of development. It exhibits a combination of characters drawn from both parents. The offspring of the same pair differ from each other: some resemble one parent, some the other, and those of mixed appearance may lean to either side. But a balance is maintained in each generation between the two stocks, so that neither parent has on the whole greater weight than the other.

E. Sargant 1900

This book is about post-fertilization reproductive development in the most evolutionarily successful and wonderfully diverse group of plants on the face of the earth: angiosperms or flowering plants. Angiosperms, along with four different groups of living representatives of gymnosperms, namely, cycads, Ginkgoales (which includes the monotypic *Ginkgo biloba*), conifers, and Gnetales, are also known as seed plants. Seeds of angiosperms are enclosed within a fruit instead of being produced as exposed units on the surface of sporophylls or similar structures as they are in gymnosperms. Although study of the reproductive biology of angiosperms has a long history, sustained cellular and molecular investigations of this topic constitute a modern development.

Fertilization, besides its obvious role in genetic recombination, essentially denotes the fusion of the egg and sperm to form a zygote and, as will soon become clear, the word does not capture the full scope of events that occur in flowering plants. The traditional setting for fertilization in flowering plants is the sanctum sanctorum of the female gametophyte – more popularly known as the embryo sac – which itself is wrapped in several layers of cells of the nucellus and integuments constituting the ovule. A typical embryo sac initially has two groups of four haploid nuclei embedded within it, one at the micropylar end and the other at the opposite, chalazal end. The demarcation of groups of

three nuclei at each end, each nucleus surrounded by its own cytoplasmic domain as a distinct, compartmentalized, membrane-bound cell, is the primary determinant of form of the mature embryo sac. The three cells at the micropylar pole are organized as the egg apparatus, consisting of a large egg cell flanked on either side by a cellular synergid. The three cells at the opposite pole become the antipodals. The main body of the embryo sac remaining after the egg apparatus and antipodals are cut off is the central cell consisting of the two orphaned nuclei from either pole, which may remain separate, side-by-side, as unfused haploid nuclei, or fuse to form a diploid polar fusion nucleus. The mature embryo sac is thus a seven-celled, eight-nucleate supercell in which fertilization occurs (Fig. 1.1). This type of embryo sac development, which is prevalent in about 70% of angiosperms, is known as the 'normal' type, and, because it was first described in *Polygonum divaricatum* (Polygonaceae), it is conventionally designated as the 'Polygonum' type (Maheshwari 1950). In the context of fertilization, the term female germ unit has been proposed for the egg apparatus and the central cell (Dumas et al. 1984), but it is not widely used.

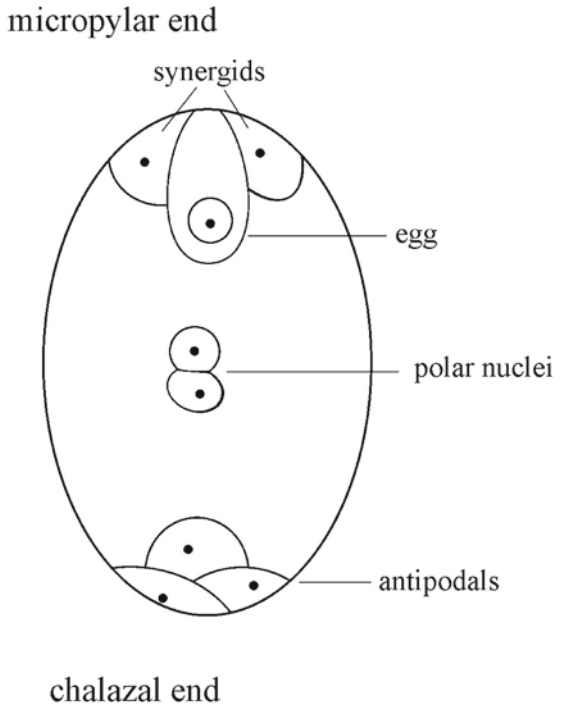
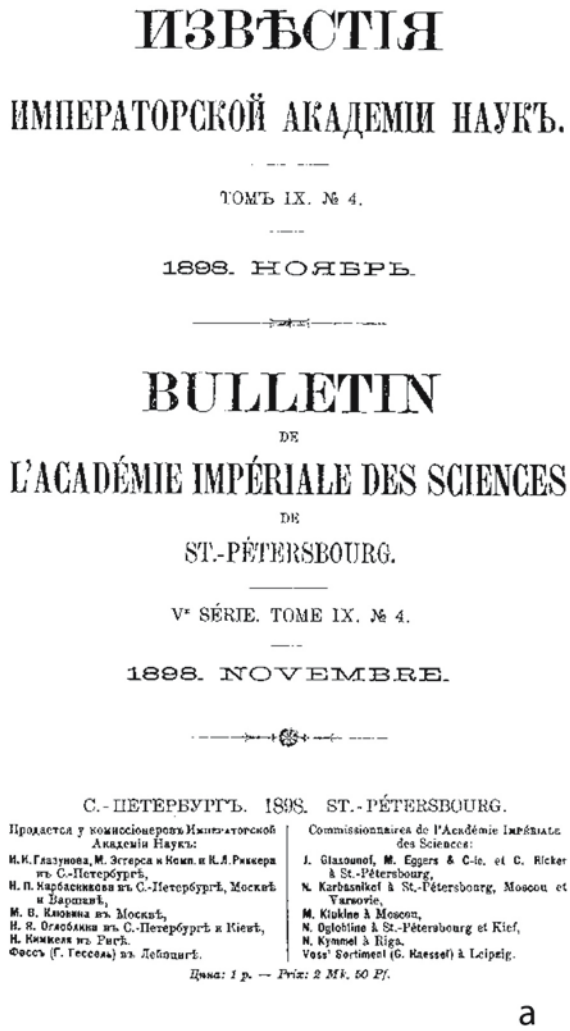


Fig. 1.1 Diagram of a 'Polygonum' type of embryo sac showing the disposition of cells

The process of fertilization in flowering plants, including the encounter of the male and female gametes and the actual fusion of gametic nuclei, presents a degree of complexity not found in other groups of plants. Pollination, resulting in the transfer of pollen grains from the anther to the stigmatic surface of the appropriate flower type, is the beginning of a cascade of events that delivers the male gamete to the vicinity of the egg. Following germination of pollen grains on the stigma, the resulting pollen tubes carrying the two male gametes (produced by a mitotic division of the generative cell of the pollen grain) navigate through the carbohydrate-rich matrix of the stigma, style, and the ovular tissues, and reach the vicinity of the embryo sac. Fusion of the male and female gametes takes place when the pollen tube enters the embryo sac and releases the sperm. Hitherto partially or totally uncharacterized extracellular matrix components of the stigma and style spring into action to sustain pollen tube growth, and the ever-present signaling molecules generated by the diploid cells of the ovule or the haploid cells of the embryo sac for pollen tube attraction contribute to successful fertilization (Johnson and Preuss 2002). Following fertilization, the ovule develops into the seed enclosed in the ovary, which becomes the fruit. Although these facts – the bare bones of the reproductive biology of flowering plants – have long been known, perspectives on the molecular genetics of the individual phases involved have come from recent cell biological studies and analyses of female gametophytic mutants of *Arabidopsis thaliana* (Brassicaceae; hereafter referred to by genus name only). The purpose of this chapter is to present an overview of the peripheral and central events of fertilization in flowering plants with a focus on both old and new literature.

1.1 Discovery of Double Fertilization

Unambiguous proof of the actual fusion of the male and female gametes embodied in fertilization in flowering plants can be traced to a monographic publication of Strasburger (1884). This work was devoted mostly to the nuclear cytology of pollen grains and pollen tubes of plants belonging to a wide range of families, and to the fate of male gametes delivered by pollen tubes in the embryo sacs



ИЗВѢСТІЯ
ИМПЕРАТОРСКОЙ АКАДЕМІИ НАУКЪ.

ТОМЪ IX. № 4.

1898. НОЯБРЬ.

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Resultate einer Revision der Befruchtungsvorgänge bei Lilium Martagon und Fritillaria tenella.

Von **Sergius Nawaschin.**

(Vorgelegt der Akademie am 30. September 1898.)

In der Versammlung der russischen Naturforscher und Aerzte, die Ende August dieses Jahres in Kiew tagte, habe ich meine Beobachtungen über die Befruchtung bei *Lilium Martagon* und *Fritillaria tenella* unter Demonstration von zahlreichen Zeichnungen und Präparaten vorgetragen. Da ich jetzt für eine lange Frist nach Buitenzorg abreise und deswegen die erwähnte Arbeit nicht ausführlich behandeln kann, so will ich in der vorliegenden kurzen Publikation die Hauptresultate meiner Untersuchung weiteren Kreisen mittheilen.

Ich habe das Studium der Befruchtung bei den genannten Pflanzen, denen bekanntlich innerhalb der letzten acht Jahre wohl mehr als irgend welcher anderen Pflanze von vielen Seiten Aufmerksamkeit geschenkt worden, in der Absicht vorgenommen, mich auf Grund meiner eigenen Erfahrung an diesen vielfach untersuchten Objecten in den Studien der Befruchtung bei den «Apetalen» richtig orientiren zu können. Ich habe meine Untersuchung des fraglichen Vorgangs bei der Wallnuss wegen ausserordentlicher Schwierigkeit des Objects (die männlichen Sexualkerne sind hier sehr winzig, und die Samenanlagen lassen sich mit keinem von den üblichen Mitteln genügend fixiren) einstweilen aufgegeben in der Hoffnung, auf dieselbe mit besserem Erfolge erst später zurückzukommen.

Es wurden kleine Stückchen der Fruchtknoten von *Fritillaria tenella* aus dem hiesigen botanischen Garten und von *Lilium Martagon*, das in der Umgebungen von Kiew wild wächst, hauptsächlich in die Flemming'sche Lösung eingelegt. Nach dem bekannten Flemming'schen Dreifärbungsverfahren wurden zahlreiche Schnittserienpräparate angefertigt. Die beiden Pflanzen wurden auch in vorgerückterer Jahreszeit mehrmals geprüft. Diese Prüfung zeigte, dass die Samen von *Fritillaria* sich eine Zeitlang ganz normal, d. h. unter Bildung eines normalen Embryo und

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b

Fig. 1.2a,b Discovery of double fertilization. **a** Cover page of the journal in which Nawaschin's discovery of double fertilization was first published. **b** First page of the article describing double fertilization

of *Gloxinia hybrida* (Gesneriaceae), *Himantoglossum hircinum*, *Orchis latifolia* (Orchidaceae), and *Monotropa hypopitys* (Pyrolaceae). The most complete, illustrated details were provided on *M. hypopitys*, in which it was shown that one of the two male gametes conveyed by the pollen tube fused with the nucleus of the egg. At that time the male gametes were known as generative nuclei and it was uncertain whether these gametes were true cells or naked nuclei. However, the observation that a male gamete fused with the egg in the act of fertilization was contrary to a previous puzzling finding that this event was orchestrated by the diffusion of the cytoplasmic contents of the pollen tube (see Maheshwari 1950). Although Strasburger's 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 source of origin of the endosperm (albumen), remained major hurdles in gaining a complete insight into the dynamics of fertilization in angiosperms.

1.1.1 Who Discovered Double Fertilization?

The breakthrough in the discovery of double fertilization occurred when S. Nawaschin in Russia showed that, in ovules of *Lilium martagon* and *Fritillaria tenella* (Liliaceae), both male gametes from the pollen tube penetrated the embryo sac; whereas

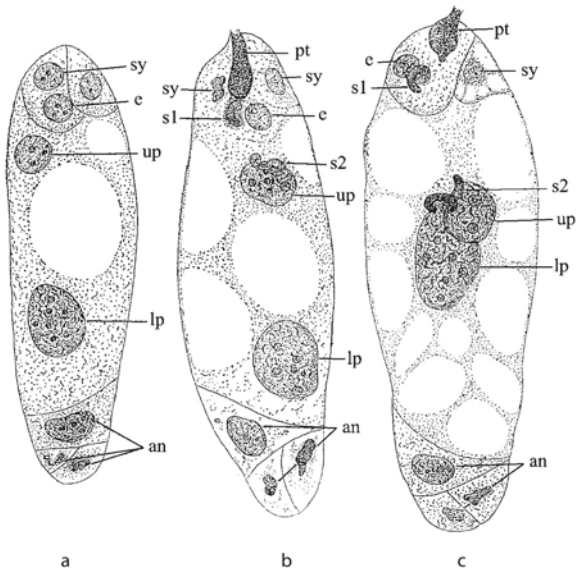

Fig. 1.3a–c Double fertilization in *Lilium martagon*. **a** Mature embryo sac showing the egg apparatus, consisting of the egg and synergids, antipodals, upper polar nucleus, and lower polar nucleus. **b** Mature embryo sac after discharge of male gametes from the pollen tube. The nucleus of one sperm has entered the egg and that of the second sperm is in contact with the upper polar nucleus. The nucleus of one of the synergids is disintegrating. **c** Union of one sperm with the egg nucleus and of the second sperm with the two polar nuclei. *an* Antipodals, *e* egg cell, *lp* lower polar nucleus, *pt* pollen tube, *s1* sperm that fuses with the egg, *s2* sperm that fuses with the polar nucleus, *sy* synergid, *up* upper polar nucleus. (Reprinted from Guignard 1899a)

one of them fused with the nucleus of the egg cell, the other fused with the polar fusion nucleus (at that time known as the definitive nucleus) floating in the central cell, initiating a second fertilization event (Nawaschin 1898, 1899). The results of this work were presented orally on 24 August 1898 to the botanical section of the "Naturforscherversammlung" held in Kiew, Russia (20–30 August 1898) and published as an abstract in the following year (Nawaschin 1899); the full paper appeared a few months after the meeting (Nawaschin 1898). Thus, reverent credit is due to Nawaschin for this legendary discovery of the two fusion events during fertilization in flowering plants (Fig. 1.2a,b). The phenomenon observed by Nawaschin was also independently confirmed in *L. martagon* and *Lilium pyrenaicum* by L. Guignard (1899a, 1899b) in France. The account of this investigation was communicated to the Academy of Sciences in Paris on 4 April 1899 and was published soon afterwards in its Report ("Comptes Rendus") (Guignard 1899a). Exactly the same paper, with a footnoted reference to the earlier paper with volume number and a middle page number, was also published in another journal in the same year (Guignard 1899b). The work described in these two papers, which included a reference to Nawaschin's 1899 abstract, was accompanied by a series of illustrations in the form of line drawings showing the two fusion events (Fig. 1.3a–c). Guignard's description and figures portrayed a precise two-step sequence of events involving the fusion of the second sperm with the upper polar nucleus, followed by integration of this fusion product into the lower polar nucleus. Within a few months of the publication of Guignard's papers, full confirmation of the startling discovery of fusion of the second sperm with the polar fusion nucleus came from a reexamination of previously prepared slides of fertilized ovules of *L. martagon* by E. Sargant in England (Sargant 1899). The coincident choice of ovules of species of *Lilium* and *Fritillaria* by investigators working in three European countries as the classic experimental system in these pioneering studies is not surprising because of the relatively large size of the embryo sac and its equally conspicuous nuclei as seen in microscopic preparations of ovules of these two genera. Indeed, because of this and other advantages, slides demonstrating embryo sac development in various species of *Lilium* and *Fritillaria* have been popular in the teaching of general plant biology; species of these genera have also been favored systems of subsequent investigators because embryo sac development in them appeared to be a simplified version of a complex series of nuclear fusions and divisions that did not have parallels in other plants studied (Maheshwari 1950). To designate the two fertilization events that occur at the inception of the sporophytic phase in flowering plants, Guignard (1899a, 1899b), in a seemingly visionary act, used the term 'double copulation' in the title of the first two papers and 'double fécondation' in later publications. Strasburger (1900) referred to the two fertilization events as 'doppelten Befruchtung' in the title of a paper, and nearly the same term ['die doppelte Befruchtung' and 'двойное оплодотвореніе' (in Russian)] appeared in the text of two papers by Nawaschin (1900a, 1900b). The term 'double fertilization' now in universal use was first employed in the title of a paper by Thomas (1900) and in the

text of a paper by Sargant (1900). Putting to rest the prevalent assumption that the endosperm was generated by fusion of the two polar nuclei, the abovementioned investigators also concluded correctly that the product of fusion of the second sperm with the polar fusion nucleus gives rise to the endosperm, typically constituted of cells with chromosomes of biparental origin from the coalescence of three nuclei. The discovery of double fertilization in the liliaceous species, and the confirmation of its occurrence in many other angiosperms, including both monocotyledons (monocots) and dicotyledons (eudicots), within a period of just over a year – for example, additional species within the Liliaceae such as *Fritillaria meleagris*, *Scilla bifolia*, *Lilium candidum*, *Tulipa celsiana*, *Tulipa gesneriana*, and *Tulipa sylvestris* (Guignard 1899c, 1900a, 1900b), *Narcissus poeticus* of the Amaryllidaceae (Guignard 1900a), and *Himantoglossum hircinum*, *Orchis latifolia*, *Orchis maculata*, and *Orchis mascula* of the Orchidaceae (Strasburger 1900) (all monocots), *Erigeron philadelphicus*, *Erigeron strigosa*, *Guizotia oleiflora*, *Helianthus annuus* (sunflower), *Heliopsis patula*, *Rudbeckia grandiflora*, *Rudbeckia laciniata*, *Rudbeckia speciosa*, *Silphium integrifolium*, *Silphium laciniatum*, *Silphium terebinthinaceum*, and *Spilanthes oleracea* of the Asteraceae (Guignard 1900a; Land 1900; Nawaschin 1900a, 1900b), *Hibiscus trionum* of the Malvaceae (Guignard 1900a), *Anemone nemorosa*, *Caltha palustris*, *Clematis viticella*, *Delphinium elatum*, *Helleborus foetidus*, *Nigella sativa*, and *Ranunculus flammula* of the Ranunculaceae (Guignard 1900a; Nawaschin 1900a, 1900b; Thomas 1900), *Reseda lutea* of the Resedaceae (Guignard 1900a), *Juglans* sp. of the Juglandaceae (Nawaschin 1900a, 1900b), and *Monotropa hypopitys* of the Pyrolaceae (Strasburger 1900) (all eudicots) – may be said to have ushered in twentieth century plant embryology, paving the way for what will surely go down as the golden age in the study of reproductive biology of flowering plants. Appropriately, the centennial of this discovery has been marked by the publication of several reviews on this topic (Jensen 1998; Erdelská and Dubová 2000; Faure 2001; Koul 2001; Friedman 2001b; Raghavan 2003b). Besides paying tribute to Nawaschin and Guignard, these articles show how their discovery has driven the field of plant embryology for more than a century, including most current research in this field.

1.1.2 Universality of Double Fertilization in Flowering Plants

The momentum created in the waning years of the nineteenth century to establish double fertilization as a ubiquitous feature in the reproductive biology of flowering plants was followed by a sustained effort in the first 2 years of the twentieth century leading to the discovery of this phenomenon in additional members of the Ranunculaceae (Guignard 1901c), Liliaceae (Ikeda 1902), Juglandaceae (Karsten 1902), and Pyrolaceae (Shibata 1902), as well as in plants belonging to Poaceae (Guignard 1901a), Najadaceae (Guignard 1901b), Solanaceae, Gentianaceae (Guignard 1901d), Asclepiadaceae (Frye 1902), Brassicaceae (Guignard 1902), and Ceratophyllaceae (Strasburger 1902). Guérin (1904), in a monograph devoted entirely to the topic of fertilization in seed-bearing plants, and Coulter and Chamberlain (1912) in their classic book on the *Morphology of Angiosperms*, refer to 16 families of angiosperms, encompassing about 40 genera and over 60 species definitely known to have a second fertilization event; these two publications surveyed the literature up to the end of 1902. From that time onwards, along with the presence of a reduced female gametophyte and embryo-nourishing endosperm, the occurrence of double fertilization was accepted as a general feature of the reproductive biology of angiosperms. Indeed, under this assumption, there were only occasional references to double fertilization in the numerous publications dating from the early 1900s to the present dealing with the variability and diversity of reproductive processes in flowering plants with special reference to their embryogenesis and endosperm development (Johansen 1950; Maheshwari 1950; Davis 1966; Johri et al. 1992). However, this period was notable for providing the first glimpses of electron microscopic details of double fertilization in several plants, including cotton (*Gossypium hirsutum*; Malvaceae; Jensen and Fisher 1967), maize (*Zea mays*; Poaceae; Diboll 1968; van Lammeren 1986), barley (*Hordeum vulgare*; Poaceae; Cass and Jensen 1970; Mogensen 1982, 1988), *Linum catharticum* (Linaceae; d'Alascio Deschamps 1974), spinach (*Spinacia oleracea*; Chenopodiaceae; Wilms 1981), *Plumbago zeylanica* (Plumbaginaceae; Russell 1982, 1983),

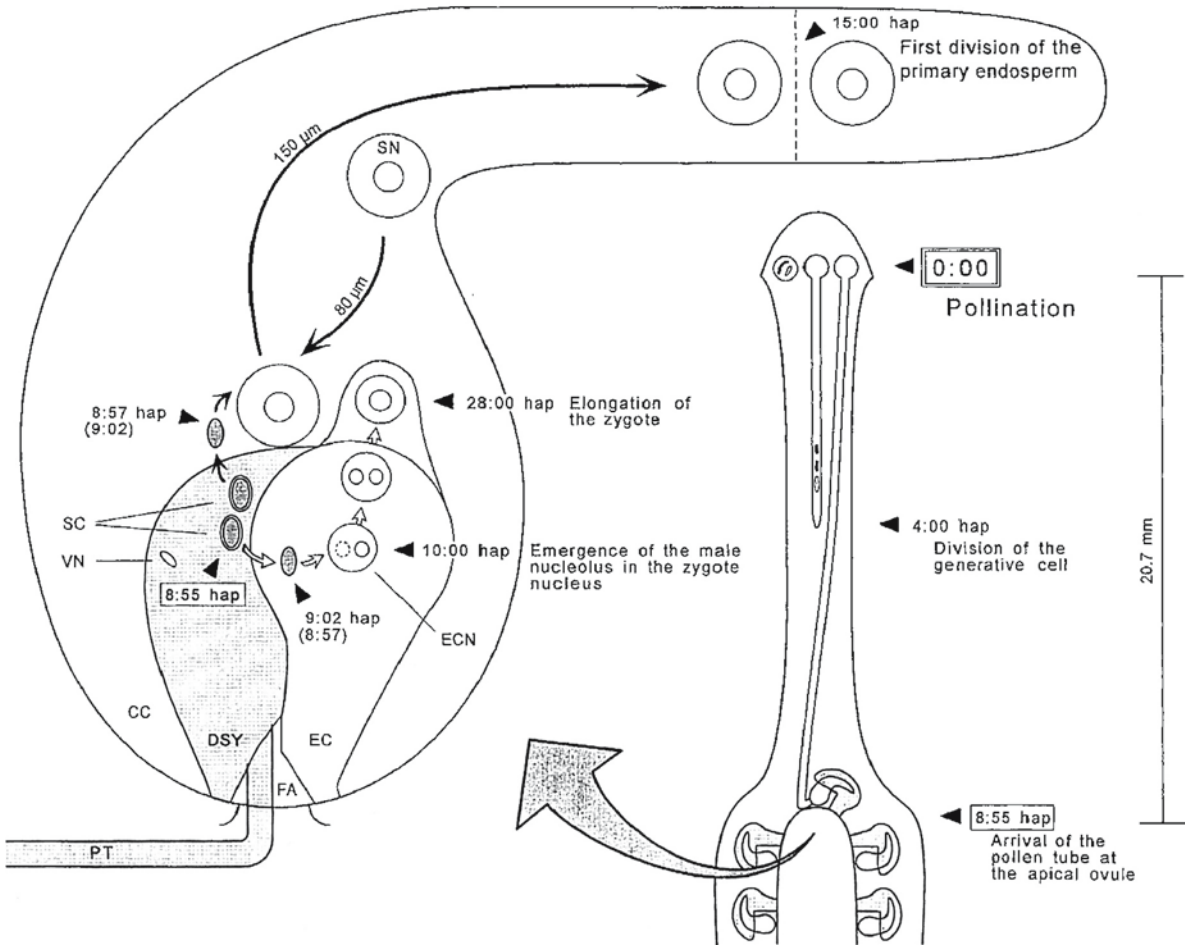

Fig. 1.4 A diagrammatic representation of the time course of double fertilization in *Torenia fournieri*. The time is indicated in hours after pollination (*hap*). Part of the carpel is shown on the right and the embryo sac of the apical ovule is on the left. *CC* Central cell, *DSY* degenerating synergid, *EC* egg cell, *ECN* egg cell nucleus, *FA* filiform apparatus, *PT* pollen tube, *SC* sperm cells, *SN* second polar nucleus, *VN* vegetative cell nucleus. (Reprinted from Higashiyama et al. 1997)

wheat (*Triticum aestivum*; Poaceae; You and Jensen 1985; Gao et al. 1992), *Triticale* (Poaceae; Hause and Schröder 1987), *Populus deltoides* (Salicaceae; Russell et al. 1990), and tobacco (*Nicotiana tabacum*; Solanaceae; Yu et al. 1994).

Almost all observations on double fertilization were made using fixed and/or fixed and sectioned materials. Over the years, complementary powerful insights into isolated aspects of double fertilization were provided by observations of living material of *Monotropa hypopitys* (Strasburger 1900), *Monotropa uniflora* (Shibata 1902), *Calanthe veitchii*, *Cypripedium insigne*, *Dendrobium nobile* (Orchidaceae; Poddubnaya-Arnoldi 1960), *Jasione montana* (Campanulaceae), *Galanthus nivalis* (Amaryllidaceae; Erdelská 1974, 1983), *Torenia fournieri* (Scrophulariaceae; Higashiyama et al. 1997), and *Arabidopsis* (Faure et al. 2002). It is believed that in *M. hypopitys* the male gametes find their way to the egg and the polar fusion nucleus by passively navigating between the cytoplasmic strands that criss-cross the embryo sac (Strasburger 1900). Cinematographic observations of ovules of *J. montana* and *G. nivalis* poised for double fertilization have provided data on the timing of movements of the two sperm in the central cell and on some hitherto unrecorded changes in size and shape of the embryo sac elements (Erdelská 1983). Because the naked embryo sac protrudes from the micropyle of the ovule, *T. fournieri* has proved an especially useful system for live monitoring of the fusion events of fertilization unhindered by the presence of ovu-

lar tissues (Fig. 1.4). Here the polar fusion nucleus engages in two targeted movements in the embryo sac. First is its slow migration from a region of the embryo sac to one side of the egg apparatus to await the arrival of the pollen tube with its cargo of male gametes. Second, after fertilization this nucleus is propelled from the vicinity of the egg apparatus to another specific site in the embryo sac (Higashiyama et al. 1997). These nuclear movements have raised wider questions about the involvement of specific signaling molecules during double fertilization, but their identity remains obscure. Using pollen grains from a transgenic line of *Arabidopsis* expressing the green fluorescent protein (GFP) fused with a pollen-specific promoter in the vegetative cell, Faure et al. (2002) have determined the precise time-course of the fertilization processes. Most importantly, this work has opened up the potential use of GFP, tagged to as yet unidentified sperm-cell- and embryo-sac-specific promoters, to follow labeled gametes during double fertilization in vivo without invasive manipulations.

1.2 Seed Development without Double Fertilization

One family of flowering plants whose members do not indulge in double fertilization is the Podostemaceae. Kapil (1970), beginning with relatively early studies, reviewed some of the problems in the embryology of members of the Podostemaceae, including the contradictory reports on the occurrence of double fertilization in members of this family. Compared with most other flowering plants, members of this family have a thalloid plant body that resembles an alga, lichen, or a liverwort. This, along with several other features in their vegetative and reproductive life, makes the Podostemaceae an extraordinary family of flowering plants (Mohan Ram and Sehgal 2001). The final configuration of the mature embryo sac in Podostemaceae studied from time to time initially influenced the reasons for attributing the absence of double fertilization to this family. Typically, the organized embryo sac is four-celled, consisting of a large egg cell and one or two small synergids constituting the egg apparatus, and a central cell harboring a polar nucleus or one or two antipodals. In some species with two synergids in the egg apparatus, the nucleus of the central cell has been shown to degenerate either before the pollen tube enters the embryo sac or before fertilization, or to survive as an antipodal (Battaglia 1971; Nagendran et al. 1976, 1980); in others in which the egg is flanked by only one synergid, the remaining two nuclei are designated as antipodals (Mukkada 1963, 1964; Arekal and Nagendran 1975). The implication is that the absence of a true polar nucleus in the embryo sac precludes fusion of the second male gamete initiating another fertilization event and formation of the endosperm. Understanding the reasons for the absence of double fertilization in this family is a real challenge because mechanical factors such as failure of the pollen tube to discharge the second sperm are probably also involved (Chopra and Mukkada 1966; Mukkada 1969). As double fertilization is a complex process requiring coordinated action of the component cells of the female gametophyte in concert with the male gametes, it is difficult to reconcile some of these observations with what may be actually happening, and hence more studies are required to understand the basis for the absence of double fertilization in the Podostemaceae; a great deal will be revealed by studying the widest possible selection of species.

Conclusive evidence of double fertilization is also lacking in most of the primitive angiosperms so far investigated. In spite of much research, views on the origin and early evolution of angiosperms have remained controversial, and it has not been possible to identify the earliest angiosperms from classifications based on morphological and physiological criteria and limited molecular systematic studies. Over a period of time, these studies designated groups such as Magnoliales, Ceratophyllaceae, and Chloranthaceae as candidates for the earliest angiosperms. However, a series of recent and concurrent investigations on angiosperm relationships inferred from phylogenetic analyses of DNA sequences that combined mitochondrial, chloroplast, ribosomal, and phytochrome genes have shown persuasively that the monotypic genus *Amborella trichopoda* (Amborellaceae), Nymphaeales (Nymphaeaceae and Cabombaceae), and the Illiciales-Trimeniaceae-Austrobaileyaceae complex (together known as the "ANITA" grade) are basal to the common ancestor of monocots and eudicots (Mathews and Donoghue 1999; Soltis et al. 1999; Qiu et al. 1999; Parkinson et al. 1999; The Angiosperm Phylogeny Group 2003). This conclusion was soon reinforced by molecular

comparisons of additional chloroplast genes (Graham and Olmstead 2000). The current contenders for the earliest angiosperm lineages are Nymphaeales and Austrobaileyales (Illiciaceae, Schisandraceae, Trimeniaceae, and Austrobaileyaceae; Friedman et al. 2003). However, our knowledge of fertilization processes has not kept pace with the recognition of these new branches of angiosperm evolution, and it has not been definitely established that a representative selection of the earliest lineages of flowering plants identified by molecular phylogenetic analyses displays double fertilization. The limited contributions to the reproductive biology of basal angiosperms currently available pertain mostly to descriptive accounts of their floral morphology and comparative embryology (Friedman 2001a; Friedman and Floyd 2001). The closest that published studies in the comparative embryology of some basal angiosperm lineages such as *Illicium anisatum* (Illiciaceae; Hayashi 1963), *Brasenia schreberei* (Cabombaceae; Khanna 1965), and *Euryale ferox* and *Nymphaea stellata* (Nymphaeaceae; Khanna 1964, 1967) have come is to assume the existence of double fertilization and the formation of an endosperm, but without photographic or other convincing documentation. An exception is provided by studies showing that the embryo sac of *Nuphar polysepalum* (Nymphaeaceae) is typically four-celled, made up of an egg cell flanked by two synergids and a uninucleate central cell (Williams and Friedman 2002; Friedman and Williams 2003). Besides providing striking fluorescent micrographs of the fusion of the sperm nucleus with the haploid central cell nucleus, the authors of these reports have shown by DNA quantitation that the biparental endosperm generated by the second fusion event is diploid (see Plate 1, Fig. a–d). Two additional studies have followed the development of the endosperm from its single-celled origin in *A. trichopoda* and *Illicium floridanum*, but the ploidy level of the tissue has not been determined (Floyd and Friedman 2000, 2001). An investigation of female gametogenesis in *Kadsura japonica* (Schisandraceae) has revealed the development of a four-celled embryo sac, with a haploid central cell nucleus, with the clear implication of the origin of a diploid primary endosperm nucleus following double fertilization (Friedman et al. 2003). It will obviously be of great interest to establish unambiguously by refined microscopic methods the existence of double fertilization in other basal angiosperms, and to ascertain the ploidy level of the resulting endosperm to evaluate the evolutionary significance of this process and the origin of the embryo-nourishing tissue in flowering plants.

Despite the well-known advantages of sexual recombination in the transmission of hereditary characters, plants have also evolved various mechanisms for propagation of the progeny while remaining innocent of sex. In the context of double fertilization, the phenomenon known as apomixis leads to the formation of seeds enclosing a fertilization-independent embryo and, in some cases, an autonomously developing endosperm. Apomictic plants display prefertilization deviations from the normal sexual developmental program by aberrations in female meiosis to produce an unreduced diploid embryo sac enclosing an egg and polar fusion nucleus already endowed with a full complement of both male and female genomes (Ramachandran and Raghavan 1992; Koltunow et al. 2002). Whereas attempts to unravel the genetic control of apomixis in natural apomicts have not led to the isolation of genes involved in the process, mutational studies in the sexually reproducing *Arabidopsis* have provided new insights into the role of genes controlling certain steps in the cascade leading to an apomictic-type seed phenotype. Loss-of-function mutations in a cluster of genes now known as *FERTILIZATION-INDEPENDENT SEEDS2* (*FIS2*) (Chaudhury et al. 1997), *FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*, allelic to *FIS3*) (Ohad et al. 1996, 1999; Luo et al. 1999), and *MEDEA* (*MEA*, allelic to *FIS1*, *F644*) (Ohad et al. 1996, 1999; Grossniklaus et al. 1998; Kiyosue et al. 1999; Luo et al. 1999) have been shown to initiate a substantial program of seed development resulting in the generation of a free-nuclear or a cellular endosperm, seed coat formation, and even partial embryogenesis in the absence of fertilization as in the case of some apomicts. Because embryo and endosperm development in the wild-type plants typically follows double fertilization, these genes have been justifiably assigned a role as suppressors of autonomous divisions in the prefertilization egg nucleus and polar fusion nucleus. As described in Chaps. 5 and 8, in addition to their ability to initiate partial embryo and endosperm developmental programs in the absence of fertilization, *fis2*, *fie*, and *mea* mutants (referred to as *fis* class mutants; Gross-