Functional Plant Science and Biotechnology ©2007 Global Science Books

In Vitro Fertilisation in Wheat (*Triticum aestivum* L.)

Zsolt Pónya[1*] • Beáta Barnabás[2] • Mauro Cresti[1]

[1] Università degli Studi di Siena Dipartimento di Scienze Ambientali "G. Sarfatti", Via P. A. Mattioli, 4, 53100 Siena, Italy
[2] Department of Cell Biology and Plant Physiology, Agricultural Research Institute of the Hungarian Academy of Sciences (ARI HAS), Brunszvik út 2., Martonvásár, 2462, Hungary

Corresponding author: * **ponyazs@yahoo.com**

ABSTRACT

Studying the early events of fertilisation and the first zygotic cleavage during zygotic embryogenesis in angiosperms requires meticulously designed, complex experimental approaches. In wheat, like in an overwhelming number of crop species, little is known about fertilisation and the early events that ensue concomitantly. Therefore, answering questions such as to what extent the wheat zygote is dependent on the endosperm for its normal development, what morhpogenetic stimuli come from the maternal tissues, what factors are necessary for the accomplishment of embryonic growth from the onset of zygote development, what intertwining pathways ensure egg activation triggered by fertilization is of paramount importance. Towards this goal a brief overview is provided as to the main achievements of *in vitro* fertilisation (IVF) in the best-described maize system. This paper summarizes experimental data obtained by the application of IVF and the microinjection method adopted in wheat to address some important issues such as the ultrastructural characterization of wheat egg cells isolated at different maturational stages; the competence of the wheat female gamete for transient expression of foreign genes; DNA dynamics during sperm-egg fusion and zygotic development; and cytoskeletal changes occurring during *in situ* egg cell development and in the course of fertilization. A summary is given about studies conducted to follow up structural changes in the endoplasmic reticulum identified to be the main calcium store in the female gamete in wheat. The present review concludes by depicting some future prospects pertaining to the potential offered by exploiting the sexual route for the improvement of wheat and delineates vistas opened up by the application of plant biotechnology tools in sexual plant reproduction research as well as in developmental biology of angiosperms.

Keywords: cell polarity, endoplasmic reticulum structure, F-actin dynamics, *in situ* egg cell development, microinjection, transient gene expression in egg cells

Abbreviations: CDKs, Cyclin-dependent kinases; **DAE**, days after emasculation; **DiI**, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate [$DiIC_{16}(3)$]; **ER**, endoplasmic reticulum; **HAP**, hours after pollination; **CLSM**, confocal laser scanning microscopy; **Rh-Ph**, rhodamine-phalloidin; **ZGA**, genomic gene activation

CONTENTS

Received: 23 August, 2007. Accepted: 4 November, 2007.

Invited Review

INTRODUCTION

The remarkable feature of the life cycle of angiosperms is the masterly treatment of the alternation of generations between a diploid sporophyte and a haploid gametophyte which is coupled with continuous somatic organogenesis. The switching from the haploid generation (the gametophyte phase) to the diploid sporophyte is achieved during the act of double fertilization, a phenomenon observable exclusively in angiosperms. This unique sexual reproduction strategy was discovered over one hundred years ago (Nawaschin 1898) and since then the perplexing complexity of the interactions occurring in the course of double fertilization between the male and the female gametophytes and the sporophyte has intrigued plant biologists. However, the underlying mechanisms of these intricate processes involving the delivery to the female gametophyte (the embryo sac) of two sperm cells (transmitted by the pollen tube) and their fusions with the egg cell and the central cell, possessing two nuclei, are relatively unexplored. This is mainly due to the inaccessibility of the female gametophyte deeply embedded in the nucellar tissue of the ovule which for a considerable period of time restricted the scrutiny of these events to microscopic observations performed on fixed and subsequently cut, dead tissues.

The isolation of viable gametes of opposite sexes, a prerequisite for *in vitro* fertilization of gamete pairs, has not been available until the early 1990s. Techniques allowing for the isolation of viable sperm cells (for review see Theunis *et al*. 1991) have preceded the elaboration of egg cell isolation methods and it was not until the commencement of the 1990s that isolation of viable egg cells was first achieved in maize (Kranz *et al*. 1991), followed by more examples to be included in the list of species in which viable egg cells could be obtained either employing a purely mechanical isolation procedure in barley, wheat and rape seed (Holm *et al*. 1994; Kovàcs *et al*. 1994; Katoh *et al*. 1997) for instance, or complementing microdissection with using mixtures of cell wall degrading enzyme solutions (Kranz *et al*. 1995).

Development of the zygote and that of the endosperm progenitor cell necessitate the successful completion of double fertilisation. How zygotic embryogenesis is accomplished giving rise to the embryo, and subsequently to the whole plant body, is largely unknown. The extensive developmental changes which unfold during zygotic embryogenesis and ultimately lead to the formation of a multicellular organism from the undifferentiated single-cell stage are commonly believed to be presaged by the cellular/molecular events that occur in the egg cell preceding and/or concomitantly upon fertilisation. Thus, questions of utmost importance in developmental biology as to the mechanisms of egg cell polarity, egg cell activation, gamete recognition, adhesion and fusion, block to polyspermy, zygote development, zygotic gene activation (ZGA), which governs the transition from the maternal to the embryonic control of development, asymmetric cell cleavage (occurring typically in the angiosperm zygote) and the pattern of *in vitro* zygotic embryogenesis warranted endeavours in establishing *in vitro* fertilization (IVF) as a technical tool to investigate these issues.

A closer look at the *in planta* situation is worthwhile in order to delineate the extent to which IVF may be deemed to be a suitable means in addressing these questions.

THE MECHANISMS OF DOUBLE FERTILISATION REVEALED BY *IN PLANTA* OBSERVATIONS IN DIFFERENT SYSTEMS

The cells participating in the process of double fertilisation in angiosperms possess unique features: the two male gametes are void of cell wall unlike the vegetative cell in which they are embedded. Their chromatin is highly condensed and few organelles (such as mitochondria, plastids, dictyosomes, endoplasmic reticulum) reside in a reduced cytoplasm (Raghavan 1997). The cytoskeleton of the pollen tube gains importance in delivering the male gametes as it seems that the sperm cells lack flagella or cilia hence implying that they are not motile cells unlike the male gametes of animals and that of other plants such as mosses and ferns (see review: Cai *et al*. 1997). The female gametophyte shows a wide range of variations in flowering plants the most common (and most probably the ancestral one) of which is the so-called *Polygonum*-type embryo sac (for review see: Huang and Russell 1992). It originates from only one of the four meiotic products of the megasporocyte and is therefore termed: 'monosporic'. The partner cells for the two sperm cells delivered into the embryo sac during fertilisation are the central cells, the egg cell and the synergids. These cells constitute a functional unit termed the Female Germ Unit (Dumas *et al*. 1984). The latter ones are located at the microplyar pole of the embryo sac where the pollen tube penetrates. The 'morhological polarity' of the egg cell, the nucleus of which is located either peripherically or centrally and it is partially surrounded by a cell wall, is believed to reflect the polarity axis of the embryos sac and is linked to the molecular machinery set in motion during the initiation of the asymmetric cell division of the zygote (West and Harada 1993). The central cell flanks with the egg cell and generally occupies most of the space in the embryo sac.

Transport of the male gametes into the embryo sac

Exploiting *Nicotiana tabacum* as a model system provided valuable information concerning how the male gametes may be transferred from the aperture of the pollen tube to the micropylar pole of the female gametophyton. Capitalising on the binding property of phallotoxins linked to fluorescent conjugates, several authors identified actin bands along the presumed path of the female gametes (Huang *et al*. 1993a; Russell 1993; Huang and Russell 1994). Further evidences supporting the presence of actin bands potentially interacting with myosin at the surface of the male gametes were supplied by Huang *et al*. (1993b) in *Plumbago zeylanica* and by Huang and Sheridan (1994) in *Zea mays* (for review see: Russell 1996). Although neither the involvement of the actin filaments in pulling the gametes nor the presence of myosin on the female gametes have been directly demonstrated hitherto, an extracellular acto-myosin based system is attractive. Pollen tubes have also been demonstrated to establish a gradient of intracellular cytosloic calcium towards the tip of the growing pollen tube hence suggesting a role for this versatile secondary messenger in guiding pollen tube growth and in sperm cell movement (Malhó 1998).

The conditions of the female gametophyte serving as a microenvironment for gamete adhesion and fusion

Upon release from the pollen tube the male gametes are purged into a very complex microenvironment made up of the degenerating synergid, into which the pollen tube is thought to discharge its content, pollen tube components, and most probably extracellular fluids. This presumably ever-changing microenvironment of the egg cell apparatus is of undefined pH and composition. The powerful method of X-ray microanalysis, however, revealed a high level of total calcium (Chaubal and Reger 1990, 1992a, 1992b, 1993) in the synergids, an observation congruous with the results obtained by using other methods to detect loosely sequestered calcium such as chlorotetracycline (CTC)-specific fluorescence imaging and the antimonate precipitate method (Huang and Russell 1997). From the data available to date, it seems that a high level of free or loosely bound calcium is required for the gamete fusion once the synergid

degenerates as it participates in the process of fusion between the two cells. Deciphering the features of the microenvironment of the fertilising sperm cell would enable us to make a great leap in better understanding gamete recognition and fusion. Furthermore, it would also contribute to the optimisation of the *in vitro* fusion systems elaborated thus far.

Adhesion and fusion of gametes

Questions such as: how do the gametes of opposite sexes adhere, what mechanisms control their fusion, whether are the fusions between the two male-female gamete pairs predetermined and what is the nature of the gamete-gamete recognition system are mainly unanswered. Only a limited body of information is available based mainly on transmission electron microscopy (TEM) observations made on *Plumbago zeylanica* (Russell 1983). Therefore, the process of the adhesion and fusion of the gamete pairs remains a conundrum leaving room for proposing several models for explaining the mechanisms of gamete recognition and adhesion. The most impressive hypotheses have been put forward by Russell (1996): according to this author's first model, it is in the potential of both male gametes to fuse with either one of the female cells (the egg cell and the central cell). This hypothesis assumes an inherent block to polyspermy, which would force the second sperm to fuse with the still unfertilised female gamete after the first fusion, has been completed. More sophistication is implied in the second model, which envisages a certain segregation of the two male gametes delivered by the pollen tube in such a manner that a specific order of release and a differing morphology would ensure a 'female gamete specific' transport towards either the egg cell or the central cell. This preferential fertilisation would not inevitably imply the establishment of a block to polyspermy, but probably it is acting in order that the secondly arriving sperm cell would be forced to fuse with the second, yet unfertilised, female gamete. In terms of preferential fusion the ultimate model surmises the existence of cell surface compounds, one set being characteristic of that sperm which is 'compatible' only with the egg cell, for instance, whereas the other endues the other sperm with complementarity to the central cell. This hypothesis, although allows for dimorphism of the two sperm cells, does not exclude similarity of the male gametes delivered by the same pollen tube. Should this scenario prove correct, a block to polyspermy would be optional.

In guessing which of the above-depicted models are to be favoured, one is left to rely on information obtained in *Plumbago zeylanica* in which the two sperms originating from the same male gametophyte are dimorphic. This distinction is expressed in the lack of plastids in that sperm which is adjacent to the vegetative nucleus, whereas in the cytoplasm of the other, plastids can be allocated that are smaller in size and more contrasted compared to the plastids of the egg cell (Russell 1985). One has to bear in mind, however, that there is no reason to suppose that this observation applies to other flowering species, in particular because *P. zeylanica* possesses an embryo sac that lacks synergids. Corroborating the preferential fertilisation scenario, it appears that in maize genotypes containing supernumerary chromosomes (termed: B-chromosomes) fusion is predetermined between the gamete pairs. It seems that B-chromosome containing sperm cells fuse significantly more frequently with egg cells than the ones lacking this 'extra' chromosome (Carlson 1986; Shi *et al*. 1996).

Hitherto there is no evidence to support the assumption of sperm dimorphism in wheat, however, a predetermined fusion scenario would have implications in the implementation of *in vitro* gamete fusion which could benefit from any morphological difference recurring consistently in isolated sperm cells so that mimicking closer the *in vivo* gamete fusion would become possible.

Integration of the male components into the female gametes

The mode of inheritance of plastids and mitochondria shows great variation among angiosperms. Nevertheless, in the majority of the species plastids are passed on either exclusively or prevailingly from the maternal partner cell. Uniparental inheritance is also predominant at least in those species investigated (for review see: Mogensen 1996). Further, quite many mechanisms ensure that during pollen formation, at the time of plasmogamy or following fusion, the male cytoplasm, male organelles and DNA content are severely reduced. For instance, enucleated bodies were found in the degenerating synergid receiving the pollen tube, adjacent to the egg and central cell in cotton and lily (Jensen and Fischer 1968; Janson and Willemse 1995) or next to the egg cell, in barley (Mogensen 1988). It was reasonable to suppose that these bodies were remnants of the male cytoplasm that had not integrated into the cytoplasm of the female gamete(s). It was suggested that still undefined mechanisms are responsible for either partly or entirely eliminating certain male organelles such as mitochondria and plastids.

Both the chronology and the mechanisms of the incorporation of the major male component, the nucleus, endowing the female gamete with the paternal genome, are appallingly unknown due mainly to the various times it takes the actual pollen tube to reach the micropilar pole of the embryo sac and to the fact that these events take place deep in inaccessible maternal tissues. Exploiting the gamete isolation and *in vitro* fusion system coupled with the microfluorimetric technique, Mogensen and Holm (1995) quantified DNA in the nuclei of mechanically isolated egg and zygote protoplasts in barley. Although the time course of karyogamy and cytological alterations upon sperm-egg cell fusion in the female gametoplast have been analysed in *in vitro* fused egg cell and sperm cell pairs (Faure *et al*. 1993, 2001), attempts to unravel double fertilisation and genetic imprinting at the organ level have failed hitherto (Nowack *et al*. 2007). Therefore, experimental systems closely mimicking *in vivo* double fertilisation hence allowing for studying thoroughly these intricate processes at the cellular and molecular level are still to be elaborated.

IN VITRO FERTILISATION AS A NOVEL MEANS IN STUDYING FERTILISATION

The first strategy in disentangling events triggered by fertilisation in the angiosperm egg employed microinjection of male gametes or nuclei into isolated embryo sacs (Keijzer *et al*. 1988; Keijzer 1992; Matthys-Rochon *et al*. 1994). Nevertheless, this approach did not yield regenerants; therefore it could not serve to fulfil the ultimate aim at dissecting the first events of zygotic embryogenesis leading to fully fertile, intact plants.

It was not until the commencement of the 1990s that methods allowing for fusing gametes of opposite sexes isolated from angiosperms became available. This technique ushered in a new era in addressing questions of utmost importance for deciphering the successive and well-concerted changes ensuing concomitantly upon fertilisation in the egg cell. In the wake of this method and by analogy with the thoroughly investigated animal systems including mammals (Lanzendorf *et al*. 1988; Palermo *et al*. 1995), it was tempting to attempt injecting sperm cells into isolated female gametes, but endeavours aimed at achieving this have thus far failed.

A more recent strategy focused on fusing gametes selected individually. Albeit the number of angiosperm species in which the isolation of both male and female gametes is solved is limited: *Hordeum Vulgare* (Cass 1973; Holm *et al*. 1994), *Lolium perenne* (van der Maas *et al*. 1993), *Zea mays* (Dupuis *et al*. 1987; Wagner *et al*. 1989), *Brassica napus* (Katoh *et al*. 1997), *Nicotiana tabacum* (Hu *et al*. 1985; Cao *et al*. 1996), *P. zeylanica* (Russell 1986; Cao and

Russell 1997), *Torenia fournieri* (Mól 1986; Keijzer *et al*. 1988) and *Alstroemeria* (Hoshino *et al*. 2006), fusion of gametic pairs resulted in invaluable data concerning the downstream processes induced by sperm-egg cell fusion.

As compared to egg cell isolation, sperm cell isolation is a relatively straightforward procedure: the generative cells are released from the pollen grains by an osmotic or pH shock (see review: Chaboud and Perez 1992) and even a large number of male gametes can be obtained by Percol or sucrose gradient centrifugations (for review see Russell 1991). However, selecting the individual sperm cells used later for the fusion and placing them in the vicinity of the isolated egg cells requires micromanipulation. Isolating the egg cells is by far more cumbersome and tedious and constitutes the limiting step in IVF. This is mainly due to the inaccessibility of the female gametophyte, the embryo sac, which is deeply embedded in the surrounding maternal tissues hence necessitating the manual microdissection of each ovules encasing the embryo sac, with or without prior enzymatic treatment of the tissues, depending on the species (Holm *et al*. 1994; see review: Huang and Russell 1992).

The first successful fusions of gametic pairs were carried out by Kranz *et al*. (1991) on maize. The system used was based on dielectrophoresis, which induces alignment of the two gametes and the membrane to membrane contact between the two cells to be fused preceding the trigger of one or multiple DC pulses resulting in cytoplasmic fusion. The fusion products of the gamete pairs gave rise to "artificial zygotes" produced by electrofusion which could be regenerated into intact and fertile maize plants (Kranz and Lörz 1993). Applying this system led to fusion products yielding multicellular structures also in wheat (Kovács *et al*. 1995).

Although this method proved relatively efficacious, it can merely be exploited for investigating gamete recognition as it is based on electrophysiological laws and the fusion of cells is brought about by "force". Thus, alternative ways suitable for unravelling gamete recognition were explored. Faure *et al*. (1994) and Kranz and Lörz (1994) employed 1 to 10 mM calcium chloride at low pH and 50 mM calcium chloride at high pH, respectively, to induce fusion of the sperm with the egg cell of maize. However, the regeneration of the fusion products has not been achieved as yet.

In vitro fertilisation with isolated angiosperm gametes

In vitro fertilisation of viable gametes isolated from higher plants was not possible until the early 1990s. The reason for this was that, whereas in animals and lower plants free-living gametes are naturally present, the gametes of land plants are confined to compact structures (the pollen grain and the embryo sac). Additionally, fertilisation takes place in the embryo sac, which is deeply encased in the ovular tissue, thus making the female gametes inaccessible. Consequently, during the procedure of egg cell isolation the nucellar tissue enclosing the embryo sac has to be removed with the other component cells of the female gametophyte (a central cell, some antipodals and generally two synergids). The newly emerging technique of *in vitro* fusion of isolated gametes, made it possible to regenerate fertile, intact plants from artificially produced zygotes through embryo-like structures. These techniques have repercussions not only on plant developmental biology, but also on cell cycle research and on exploring the sexual route as a means of delivering genes of foreign origin into the primary cell of the sporophytic generation, the zygote. The system elaborated by Kranz *et al*. (1991) allows for the examination of especially early developmental processes, which ensue concomitantly upon fertilisation, based on the isolation and fusion of gametes freed from their respective gametophytic tissue. Thus, events related to sexual reproduction like gamete recognition and interaction, egg cell capacitation triggered by the sperm cell and gametic hybridisation can be investigated. Further, single-cell molecular techniques (mRNA isolation from single cells, RT-PCR) offer the possibility of isolating gamete-specific and fertilisation-induced genes (Dresselhaus *et al*. 1994, 1996; Sauter *et al*. 1998). Clearly, since electrofusion brings about a "forced" incorporation of the two electrically polarisable cells aligned by alternating current field (AC-field), the electrophysical laws of which the membranes of the cells to be fused obey, the electric pulse can also be utilised for interspecific (generic) cell hybridisation to circumvent sporohytic incompatibility barriers; by the same token, any protoplast can be fused with gametes (gametosomatic hybridisation) (Desprez *et al*. 1995).

Since *in vivo* two fusions occur during double fertilisation, the *in vitro* situation (which involves a single fusion event between the egg cell and the sperm cell) differs from the *in planta* fertilisation. Electrofusion of defined pairs of gametoplasts under controlled conditions allows a relatively efficacious fusion yield hence obviating the need to rely on systems that require large quantities of cells for fusion (PEG-induced mass fusion, for example). Nevertheless, Faure *et al*. (1994) described a simple fusion medium containing 5 mM calcium in which isolated male-female gamete pairs of maize were reported to fuse at a very high frequency (80%) suggesting the involvement of calcium in inducing gametic fusion *in vivo*.

Although plant regeneration from single zygotes is laborious, sustained growth and differentiation of zygotes produced either *in vivo* or *in vitro* in cell culture offers the means to study the whole span of zygotic embryogenesis from the commencement of cell fusion. To achieve this goal, however, several criteria should be met: (1) the possibility of following zygote development under continuous microscopical visualisation, (2) working out single cell culture systems leading to plant regeneration under chemically defined conditions excluding nurse cells (based either on non-morphogenic suspension culture or highly embryogenic microspore culture), (3) obtaining zygotic embryos mimicking exactly the morhpogenetic programme of the fertilised egg cell. Thus far, however, only the first criteria could be fully met. Though the 3rd one is claimed to have been achieved (Kumlehn *et al*. 1997) by implanting *in vivo* zygotes into ovules, the experimental system is crippled by the lack of the possibility to monitor zygote development (which was found to mimic developmental pattern occurring during zygotic embryogenesis *in planta*) after implantation of the zygote.

The powerful techniques of CLSM (confocal laser scanning microscopy) and image analysis were employed to reveal the time course of karyogamy of the fusion products of maize gametes (Kranz and Lörz 1993; Tirlapur *et al*. 1995). In isolated barley zygotes DNA dynamics was traced down chronologically by applying microfluorometry (Mogensen and Holm 1995). In maize (Mól *et al*. 1994) in barley (Mogensen and Holm 1995) and in wheat (Pónya *et al*. 1999b) karyogamy was found to be premitotic. Cellular events related to fertilisation, for instance cell wall formation, which is considered to be involved in preventing polyspermy, were dissected in fusion products (Kranz and Lörz 1993; Tirlapur *et al*. 1995).

In vitro gamete fusion can also be used as a tool for obtaining gameto-somatic cell hybrids hence filling the hiatus between sexual crossing and somatic hybridisation. However, up to now information is lacking pertaining to interspecific, generic and intertribal hybrid and cybrid (formed by cytoplast-protoplast fusion) prototoplast fusions. Thus, the regeneration capacity is either absent or remains a very distant goal possibly due to the elimination of parts of, or entire chromosomes, polyploidisation or aneuploidy, or/and incongruity between the recipient nucleus and donor organelles of the remote species to be used. In addition, karyotypical instability was observed in hybrids after conventional crosses (Kalloo and Chowdhury 1992). Activation studies have revealed the instigation of early development in maize egg cells fused with female gametes of several cereal species (Kranz *et al*. 1995). Maize eggs, though, were found to be incompatible with *Brassica* sperms (Kranz and Dressel-

haus 1996) warning that post fertilisation barriers, as was found after *in vitro* pollination with ovules (Zenkteler 1995), are to be taken account of when gametic combinations involving distant species are produced.

Zygote culture and its potentials in developmental biology and transgene research

In order to study fertilisation and early zygotic embryogenesis, culturing isolated or *in vitro* produced zygotes permitting continuous observation of development is a prerequisite. However, hitherto only very few reports announced successful zygote culture (Kranz and Lörz 1993; Holm *et al*. 1994; Zhang *et al*. 1999). This is mainly due to the laborious isolation procedure of the zygote, which necessitates sophisticated micromanipulative tools as well as considerable experience.

The first plant species from which not only the isolation and culture of the *in vivo* fertilised zygotes could be achieved, but also their regeneration into mature plants, was barley (Holm *et al*. 1994). The zygotes extruded from the embryo sac developed into embryo-like structures and even gave rise to fully fertile, normal plants. Previously, the isolation of egg cells and sperm cells had been implemented in maize and using electric pulses their fusion yielded "artificial" zygotes, which subsequently could be regenerated into green plantlets (Kranz and Lörz 1993). Via enzyme digestion and microdissection, zygotes excised from the embryo sac of maize were also induced by Leduc and coworkers (Leduc *et al*. 1996) to form fertile plants. Interestingly enough, the same nurse culture of highly embryogenic microspores of barley was as effective in stimulating continued growth and divisions of the excised maize zygotes as in culturing barley zygotes. The "nurse effect" eliciting the division of the explanted zygotes may conceivably be mediated by the release of still unidentified nutrients or compounds needed for the cell cycle machinery to be set in motion. This zygote culture system can also be explored for utilising the sexual route for introducing alien genes into the ancestor cell of the next sporophytic generation. For example, Leduc *et al*. (1996) employing quantitative microphotometry showed DNA synthesis during the first cell cycle of maize zygotes microinjected with reporter genes (GUS gene and anthocyanin regulatory genes). In this study the GUS reporter gene put under the control of the maize histone H3C4 promoter and two anthocyanin regulatory genes driven by the 35S promoter were injected into isolated maize zygotes. However, the percentage of transgenic expression in the microinjected zygotes was rather low: on average, 3.5%. Thus, by combining transgenic technology with *in vitro* fertilisation and zygote culture offers elegant experimental designs allowing for marker gene-free delivery of foreign genes into crop plants. Some examples of recent developments in this field illustrating the emerging possibilities are given by Scholten and Kranz (2001). The studies summarized in this work focus on the transient expression of transgenes following microinjection of plasmids into egg cells or zygotes. The advantage of microinjection of transgenes into gametes/zygotes – provided that the problem of low transgene expression (surmised by Holm *et al*. 2000 to be attributable to the degradation of the foreign DNA) is circumvented – is that the need for the use of selectable marker genes could be superseded, as the regenerants could be screened directly for the presence of the transgene. This approach presupposes efficient and reproducible regeneration systems suitable for the *in vitro* culture of isolated zygotes, which were established for barley (Holm *et al*. 1994), wheat (Kumlehn *et al*. 1997, 1998; Bakos *et al*. 2003) and maize (Leduc *et al*. 1996). The technique of IVF coupled with efficient regeneration techniques of zygotes into mature and fertile plants opens up the possibility of manipulating gametes prior to unifying the maternal and paternal genome (Kranz and Lörz 1993).

Towards the direction set by attempting to gain an insight into the (nutritional) interaction between the zygote/the (pro)embryo and the endosperm, steps have been taken (Kranz *et al*. 1998) to produce "artificial" endosperm by fusing sperm cells with central cells of maize. The efficiency of these fusions was found to be very low, an observation that may be attributed to the low yield (and perhaps poor viability) of isolated central cells from the embryo sac of maize.

Although very demanding, microinjecting male gametes (Keijzer 1992) and sperm nuclei (Matthys-Rochon *et al*. 1994, 1998) into the cells of the female gametophyte also greatly contributed to the advance of our understanding of the cellular processes of double fertilisation.

THE PARADIGM OF EARLY EMBRYOGENESIS

The conundrum of early zygotic embryogenesis is the extent to which the gametes of opposite sexes may be deemed to be unique cells in the sense that the product of their fusion (the zygote) is programmed for a particular cell division pattern and for containing unique cytoplasmic determinants controlling embryo cell fate determination. Although angiosperm gametes possess characteristic features (specialized cell walls, ploidy level, etc.), which may account for "depositing" a stringent developmental cell fate in the zygote, the progenitor cell of embryogenesis, the fact that differentiated plant cells can acquire new cell fate, and more importantly, embryogenic competence when cultured *in vitro* off their adequate environment and deprived of their spatial context in the plant body, argues against any hypothesis supporting the view that either gamete carries unique templates required for the proper unfolding of the embryogenic program during (zygotic) embryogenesis. Thus, a critical analysis of the justifiability of IVF as a tool to dissect embryogenesis as such from early events onwards lies in pondering the extent to which zygotic embryo development can be paralleled with somatic embryogenesis. As somatic embryogenesis demonstrates, excised from their original ambience and supplied with new chemical information, somatic plant cells are capable of dedifferentiating and dividing subsequently giving rise to a polar embryo which eventually develops into an intact and fertile plant. Therefore, the question arises: are plant egg cells "specially" programmed so that the pattern of the first (typically asymmetrical) cell division of the zygote and the partitioning of pre-synthesised, developmental components act in unison to ensure the reinforcement or induction of developmental differences reflected in the differing cell fate of the daughter cells deriving from the first zygotic cleavage and the polarity of the zygotic embryo? The fact that somatic-and microspore-derived embryogenesis is possible under *in vitro* conditions does not support the tenet arguing for the "uniqueness" of the product of gamete fusion, i.e. the zygote. Nonetheless, it has been shown recently (Nowack *et al*. 2006) that a positive fertilisation signal deriving from the egg cell triggers endosperm proliferation hence suggesting that the embryo requires a specific microenvironment provided partly by the endosperm for normal embryogenesis to set in. Apart from its nutritive function, the endosperm is the tissue where checks for sexual reproduction are carried out in terms of both genomic imprinting and chromosome constitution (Grossniklaus *et al*. 2001). The finding that endosperm development is precocious as compared to the development of the embryo (Mól *et al*. 1994) corroborates the widely-held view that the presence of the endosperm cells may ensure adequate delivery of nutrients from the sporophytic tissues and signals the presence of the embryo. However, the jury is still out on the question as to what extent zygotic embryo development is dependent on extrazygotic influences. The observation that in several species mitotic activity commences earlier in the primary endosperm nucleus than in the zygote may hint at the early dependence of the embryo on endosperm development (Marinos 1970, Raghavan 1986). Nonetheless, by isolating T-DNA insertion mutants (cdc2a-1 and cdc2a-2) of *Arabidopsis thaliana* recently Nowack *et al*. (2006) reported unferti-

lised endosperm development demonstrating that the proliferation of the central cell was unleashed by a positive signal attributed to the fertilised egg cell.

Polarity during zygotic embryogenesis

The zygote, representing the unicellular stage of the multicellular organism of the next sporophytic generation, goes through well-concerted cell division, cell expansion, maturation and differentiation processes which are all involved in the progressive transformation of the fertilised egg cell into an embryo. The establishment of the apical-basal axis in the course of embryogenesis is a crucial event the signs of which are conspicuous from an early stage onwards and even before fertilisation. This evident polarity of the embryo of angiosperms defines an axis upon which the whole body plant is elaborated as revealed by morphological studies.

Little is known about how the embryonic polarity, seen as early as in the unfertilised egg cell (Schulz and Jensen 1968; Mansfield and Briarty 1991), unfolds. In this respect, it is to note that the egg cell, itself, is present in an asymmetrically organised female gametophyte with the egg cell apparatus occupying the micropylar pole of the embryo sac, while the antipodal cells reside at the opposite (chalazal) end (Reiser and Fischer 1993). Polarity in the angiosperm egg is manifested anatomically by the preferential location of a relatively large vacuole at its micropylar pole whereas its chalazal end is cytoplasm-dense. In several species, polarity in the egg cell (and subsequently in the zygote) is pronounced by the reorganisation of cytoplasmic components (Natesh and Rau 1984; Schulz and Jensen 1968). Consequently, since the asymmetry of the zygote seems to reflect the polar organisation of the egg, embryonic polarity may be established as early as during embryo sac development. However, the molecular machinery generating polarity in the embryo sac, egg cell and the zygote are obscure and the mechanisms maintaining this polarity are still to be deciphered since our current understanding of egg cell polarisation in angiosperms falls far behind that of the well-described animal systems such as *Drosophila* (Gonzales-Reyes *et al*. 1997). In this respect, the brown alga, *Fucus* possesses features which permit polarity issues to be addressed in an elegant system amenable to experimental manipulation. The fact that free-living egg cells and zygotes can be harvested, manipulated in large quantities and observed under the microscope has greatly facilitated gaining insight into the generation of polarity during early plant development (Hable and Kropf 2000). Even more importantly, the *Fucus* zygote can provide a model system for studying axis fixation and polarised growth during zygotic embryogenesis in angiosperms (Shaw and Quatrano 1996; Quatrano and Shaw 1997).

The typically asymmetric, first celldivision of the zygote yields two daughter cells with different cell fates. It is commonly believed that the polar axis of the developing embryo is determined by the defined pattern of this cell division. Therefore, domains that constitute the organisation of the plant body are proposed to be delineated during embryonic development, most probably stemming from the first unequal cell cleavage. Although no generalisations can be made pertaining to the ultrastructural "turmoil" that occurs in the egg following fertilisation (apart from the stunning "simplicity" of the zygote in terms of its structure, which almost betrays its function), a considerable body of information suggests that many properties of the egg cell change abruptly within the first hours (even minutes) concomitantly upon the accomplishment of fertilisation and karyogamy (Kranz *et al*. 1995). This does not preclude the assumption that changes taking place outside the cytoplasm of the fertilised egg also play a significant role in the evolution of the zygote. For instance, particular attention has been focused on the chalazal pole of the egg, which in many species lacks a cell wall in the unfertilised state. Most probably, at this stage the egg cell does not need to be isolated from the microenvironment of the embryo sac, whereas as fertilisation ensues, a proper insulation of the newly formed zygote from the potential influence of cells of a different genotype may be essential in the subsequent induction of the sporophytic cell divisions.

In several species quite a detailed "scenario" is available about the ultrastructural changes taking place in the egg following fertilisation. For example in *Gossypium hirsutum* cytoplasmic organelles such as plastids, ER (endoplasmic reticulum), mitochondria and ribosomes take up new positions at the chalasal end of the cell, around the nucleus. Nonetheless, this alteration in organelle position only accentuates the polarity conspicuous already in the unfertilised egg cell. Undoubtedly, the set of changes like the increase in the total amount of ER as well as in the tube-containing ER, the increase in size and number of starch grains in the plastids, the elaboration of additional cristae in the mitochondria and evolving new ribosomes, as they all seem to be a part of a series of cascade-like cellular events, usher in a new developmental vista for the fertilised egg cell (Jensen and Fisher 1968). Eggs of other plant species display much less discernible alterations. You and Jensen (1985) observed slight ultrastructural changes in the female gamete of *Triticum aestivum* after fertilisation. It may be envisaged that the cytological changes occurring during the formation of the zygote, although coordinated for the same purpose: rendering the fertilised egg cell a repository of information of developmental significance and consequence, bear little resemblance among species.

IN VITRO FERTILISATION IN WHEAT

Electrofusion

Electrofusion is a non-specific process that does not require membrane receptors. As a corollary, a wide variety of membranes can be fused by electric fields. However, there are numerous factors and variables that play major or minor roles in determining whether or not a given pair of membranes in contact will fuse.

Cell fusion mainly involves two major steps: membrane fusion and cytoplasmic fusion. To form a viable hybrid or syncytium, the fusing cells need to merge and reorganise their cytoplasms into one functional unit (cytoplasmic fusion). In AC fields, cells create local distortions in the electric field, causing the "ends" of cells to act as tiny electrodes. Consequently, when cells are put under dielectrophoretic force they become mutually attracted to each other. Several aspects of dielectrophoresis and cell alignment are particularly relevant to electrofusion: (1) dielectrophoresis and cell alignment are frequency dependent; (2) the rate of successful cell alignment depends on both the magnitude of the dielectrophoretic force and the radius of the cell (the smaller the cell, the larger the electric field that must be applied to achieve alignment-a criterion that has thorough implications for *in vitro* fertilisation since the size of the sperm cell to be fused with the egg cell is an order of magnitude smaller compared to the egg cell); (3) cell alignment is brought about most easily in media of low ionic strength (low conductivity). Low conductivity is also favourable for preventing turbulence due to convection induced by heating of the fusion medium. Although effective alignment occurs principally in two frequency ranges-one between 0.1 and 10 MHz and the other occurring at frequencies below 1 kHz-, at high AC frequencies, the permittivity difference between the cell and the medium is enhanced, and cells create greater field distortions when in media of low ionic strength, thereby enhancing mutual dielectrophoresis.

Once cell-cell contact has been established, fusion is triggered by application of one or more high-voltage DC pulse of microsecond duration. This brief pulse drives the membrane potential to such high values that the membrane undergoes physical breakdown, which subsequently leads to the formation of minute pores. Cell fusion ensues when membrane breakdown occurs in areas of cell-cell contact.

Membrane breakdown is reversible, and the cell retains its viability, as long as the DC pulse strength (voltage or pulse length) does not exceed a certain limit beyond which it can cause irreversible membrane damage and cell lysis.

When cells of significantly different sizes are to be electroporated (and fused), they are more likely to be irreversibly damaged by the electrical treatment since the larger cell would experience a large voltage drop across the membrane, whereas the smaller cell may not be electrically polarised as yet and have less chance to be porated. This fact has far-reaching consequences in those applications where a mixed population of cells (or cell pairs) of differing sizes are to be treated. Thus, conventional electroporation and electrofusion methods using typically a direct current (DC) electric field in the form of either an exponential decay pulse or a rectangular one, have severe shortcoming in those applications where a mixed populations of cells (or cell pairs) of differing sizes are to be treated. Consequently, the difficulty to optimise the conditions for electroporating non-homogenous cells (population) led Chang (1989) to develop a new method in which a pulsed electric field oscillating at radio frequency (RF) was employed for electroporation and electrofusion of cells. The main advantage of using such an oscillating electric field is to counterbalance the cell size-effect with an opposite effect of the so-called cell "relaxation" (for details see Chang 1989).

Physical factors influencing electrofusion

Numerous factors must be borne in mind when optimizing the conditions for electrofusion of gamete pairs. The effect of these factors on the fusion process is manifested in practical terms as fusion yield. The factors exerting impact on the success rate of fusion are themselves functions of other physicochemical variables such as: pulse parameters, membrane structure, ionic strength, changes induced by metabolism, presence of low concentrations of aqueous-soluble macromolecules, pH, heterogeneity of fusion substrate membranes, heating of the medium, electric turbulence among other (still unidentified) variables (Sowers 1989).

The most critical parameters that control electrofusion yield are the specification of the electric field pulse in terms of its strength (E: in the general range of 200-1000 V/cm) and duration. Adjustment of these factors can have at least a two-order-of magnitude effect on fusion yield. Data have been obtained to show that a reciprocity effect is present in electrofusion in terms of pulse duration and pulse strength (Zhelev *et al*. 1988; Sowers 1989), i.e. over some range, compensation for a reduction in pulse duration can be made by increasing pulse strength, and *vice versa*.

The fusion of wheat gamete pairs is performed in micropdroplets of fusion medium (with as low electric conductance as possible) to be optimised for iso-osmotic pressure and to be covered with mineral oil (to avoid evaporation) on a coverslip under microscopical observation (Koop and Schweiger 1985; Kranz *et al*. 1991; Kranz and Lörz 1994). The fusion is induced by AC-field (alternating current) alignment followed by DC-pulse(s) (direct current). Contact between the gametes to be fused is brought about efficiently by dielectrophoretic force. Alternative methods such as laser technique or chemically-induced (PEG: polyethylene-glycol) fusion can also be regarded as feasible approaches. However, a dubious drawback of applying chemicals may beset the procedure with further difficulties as regards a possible interaction between the membranes of the cells to be fused and the chemicals introduced into the fusion medium thus impairing cells' viability. Notwithstanding that the electrical conditions applied during electrofusion may appear rather artificial, high currents measured in plant cells (Bjorkman and Leopold, 1987) suggest that it may not be quite so. It may be conjectured that high voltage potentials may very well be present in the embryo sac serving as "inducers" for the fusion of gametic membranes.

Apoptosis in wheat egg cells: the age of the isolated gametes

The success rate of *in vitro* fertilisation and subsequently the survival of the artificially produced zygotes depend on the isolation (and fusion) of viable and competent female gametes. Isolating gametes at the right stage of their development seems to be essential for carrying out *in vitro* fertilization or various micromanipulations (including the delivery of DNA, RNA, proteins, etc. into isolated gametoplasts) in crops which may have implication in crop improvement (Leduc *et al*. 1996). Although methods for the isolation of viable egg cells of agronomically important monocots such as maize (Kranz *et al*. 1991), perennial ryegrass (van der Maas *et al*. 1993), barley (Holm *et al*. 1994) and wheat (Kovács *et al*. 1994) are already available, ultrastructural observations made to depict the ultrastructural characteristics of egg cell protoplasts (Faure *et al*. 1992; Cao and Russell 1997) are still very limited. Furthermore, these studies were mainly focused on female gametophytes and/or their constituents and involved gametes isolated at the time of anthesis. However, the life-span and receptivity of the female gametophytes may be very long and the strategy of avoiding self-fertilisation (protogyny) in many species including wheat poses the question as to the length of the time interval during which the egg cell is receptive.

Addressing the question of the time length during which the wheat egg protoplast is amenable to the IVF procedure and micromanipulation, Pónya *et al*. (1999a) isolated wheat egg cells at different developmental stages using the male gametophyte development as a reference for establishing the time span of *in situ* egg cell maturation. The morphological features and the ultrastructural characteristics of wheat egg cell protoplasts isolated from premature (3 days prior to anthesis) and overaged (12 days after anthesis) caryopses were compared. The adult egg revealed the typical features (membrane blebbing, autophagous vacuoles) of programmed cell death (PCD). From these data it was concluded that following a long life-span (approx. 18 days after anthesis) the cells of female gametophytes undergo apoptosis. The isolated egg protoplasts were found to be spherical in shape both in the young and old state. The pear shape characteristic of the *in situ* state of the wheat egg cell (You and Jensen 1985) seemed to have been lost upon isolation carried out using microdissection, presumably due to changes in the osmotic pressure and the loss of the compressing effect of neighbouring cells (synergids). Semi-thin (0.5-1 μm) sections prepared in the radial plane showed an increase in the volume of the egg cell which appeared to be accompanied with that of the nucleus and nucleolus together with peripheral vacuolisation more discernible in the case of young eggs (**Fig. 1A**). Images taken with a transmission electron microscope of egg cells isolated in the immature state 3 days prior to anthesis conformed with the structure observed in the *in planta* state (You and Jensen 1985): lipid bodies, mitochondria and amyloplasts containing starch were found among the peripheral vesicules in the cytoplasm. The overmature egg cell exhibited distinct features: in the nucleus chromatin degradation was found to occur; with the remaining chromatin adhering to the membrane and membrane blebbing could be observed (**Fig. 1B-D**). As compared to the immature egg, considerably larger quantities of lipid and reserve nutrients (starch granules, protein) were observed to accumulate in the cytoplasm. The mitochondria revealed normal structure, but lysis could be observed in the vesicules and autophagous vacuoles were seen (**Fig. 1D**) suggesting that similarly to animal cells (Cohen 1993) and certain plant suspensor cells (Jones and Dangl 1996), the characteristic symptoms of PCD can be observed in aging egg cells of wheat. These ultrastructural data corroborate the practical observation that the life-span of the female wheat gametophyte is rather long (approx. 2 weeks after anthesis) and hint that even the aging caryopsis can be activated by fertilisation. The *in vitro* fusibility of wheat egg

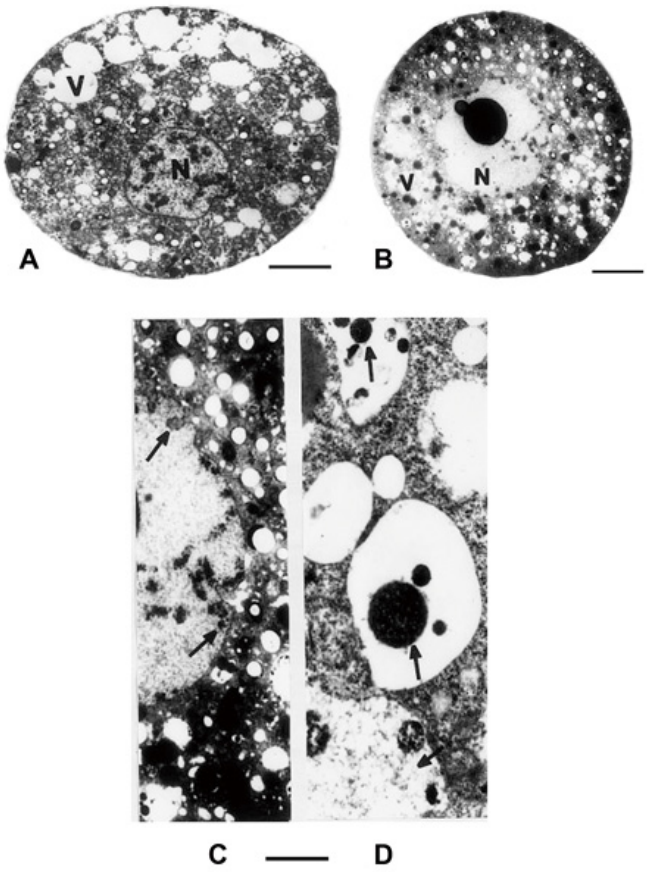

Fig. 1 Transmission electron microscopy micrographs of premature and overaged wheat egg cells. (**A**) Transmission electron microscopy (TEM) view of the young wheat egg cell. Note the central aggregation of organelles and the high peripheral vacuolization; (**B**) TEM view of an overaged female gametoplast. Note the deposition of the storage materials (lipid bodies, starch grains) and the degenerative nucleus. (N: nucleus, V: vacuole.) Bars: 12.8 μm; (**C-D**) Segments of the egg nucleus and cytoplasm with signs of programmed cell death, nuclear membrane blebbing and phagocytosis in the vacuoles (arrows). Bar: 5.3 μm. (Reprinted from: **Pónya Zs**, **Finy P, Fehér A, Dudits D, Barnabás B** (1999) *Sexual Plant Reproduction* **11**, 357-359, with kind permission of Springer Science and Business Media.)

cells isolated in various stages of development conforms to this view (Pónya unpublished results).

Microinjection of wheat egg cells

The impact of the maturational stage of isolated wheat egg cells on the transient expression rate of foreign DNA

Applying transgenic technology to isolated female gametes/zygotes of angiosperms exploiting the sexual route for gene integration presupposes the elaboration of techniques permitting the isolation of viable egg cells/zygotes and introducing foreign DNA into the egg prior to sperm-egg nuclear fusion (karyogamy) (Kranz *et al*. 2004). Microinjection has been suggested for long to be a potential means of transforming whole plants when coupled to IVF. In addition, the transgenic approach provides valuable experimental tools in elucidating various processes of double fertilization and early embryogenesis, for example by providing a way to dissect gene function by altering the expression level of a given gene (overexpression or expression of antisense RNA, e.g.) (Kranz *et al*. 2004). Numerous studies have demonstrated that these techniques are well suited to disentangle the role of key regulatory genes important for development, such as transcription factors (Ramachandran *et al*. 1994). Moreover, the green fluorescent protein (GFP) marker isolated from *Aequorea victoria*, opens up new possibilities in the field of investigations of this kind due to its non-toxic nature and the non-invasive visualisation by fluorescence microscopy hence allowing for spatial-temporal follow-up of dynamic changes in living cells. Additionally, GFP fusion protein technology can be exploited to study subcellular localisation, movements of proteins and organelles *in vivo* (Grebenok *et al*. 1997; Köhler 1998). Fusions of GFP with entire proteins of known or undefined function have been employed to reveal the localization and the translocation of proteins in question (Hanson and Köhler 2001). The GFP-based cameleon calcium indicator technique elaborated by Miyawaki *et al*. (1997) is an elegant system to characterize calcium dynamics during fertilisation and early embryonic development *in vivo*.

As evinced by the cytological characteristics of *in situ* egg cell development analysed at the ultrastructural level using TEM (**Figs. 1A, 1B**), changes clearly take place during egg cell maturation (Pónya *et al*. 1999a): in the cytoplasm of the premature egg cell a large number of mitochondria and numerous ribosomes and vacuoles can be seen (**Fig. 2A**), whereas in the mature (i.e. egg cell isolated 12 DAE) female gamete the storage materials (starch grains, lipid and protein bodies) are dominant and autophagous vacuoles appear indicating cell destruction (**Fig. 2B**). Therefore, it was conjectured that these developmental stages may influence the success rate of IVF and/or the "susceptibility" of the female gametoplasts to expressing foreign DNA. To test this hypothesis, Pónya *et al*. (1999b) developed an expeditious and efficacious technique of microinjection for introducing exogenous DNA into egg cells/zygotes of wheat isolated at precisely defined, consecutive intervals of three days after emasculation (DAE) performed on the florets when the spikes were just emerging and still sheathed by the flag leaf. This time 90% of the microspores were found to be at mid-uninucleate stage (Tímár *et al*. 1997). Capitalising on a novel method elaborated by Pónya *et al*. (1999b) which is based on exposing the female gametoplasts to be injected to high frequency AC-field for immobilisation (**Fig. 2C**), a significantly higher transient expression rate of the injected genes (46% and 52% on an average for egg cells and zygotes, respectively) could be achieved (**Tables 1, 2**) than reported previously for somatic plant protoplasts by Schnorf *et al*. (1991), thus, it was suggested by these authors that the impingement of high frequency alternating current on the cells (cell membranes) might account for such high expression rates; a finding which merits more thorough investigation. The transient expression frequencies of reporter genes (the GUS gene driven by the 35S promoter injected into *in vivo* produced zygotes and the GFP gene under the control of the ubiquitin promoter injected into egg cells (**Fig. 2D** and **2E**, respectively) seemed to vary according to the time of egg cell isolation (**Tables 1, 2**). Based on the ultrastructural observations on *in situ* developed and isolated egg protoplasts, it was suggested that this may reflect some stage-specific variation in competence of the egg cells to transcribe exogenous DNA. This finding appears to be corresponding with what was reported by Collas *et al*. (1989) in mouse, who demonstrated that the age of the oocytes is the most important factor involved in activation rates from close to zero using an electrical pulse at the time of ovulation to essentially 100% over a period of 12-14h. This change was not attributable to an abrupt elevation in cytosolic calcium level in response to the electrical field (an electromagnetic field is thought to cause activation of the oocyte by inducing an intracellular calcium rise; Whittingham 1980) the factor inhibiting activation in ovulated oocytes is thought instead to be a cytostatic factor, the protooncogene product c-mos (Sagata *et al*. 1989).

DNA dynamics in *in vivo* fertilised wheat egg cells

In order to establish an optimal time-window for the microinjection of DNA into the *in planta* fertilised wheat egg cell,

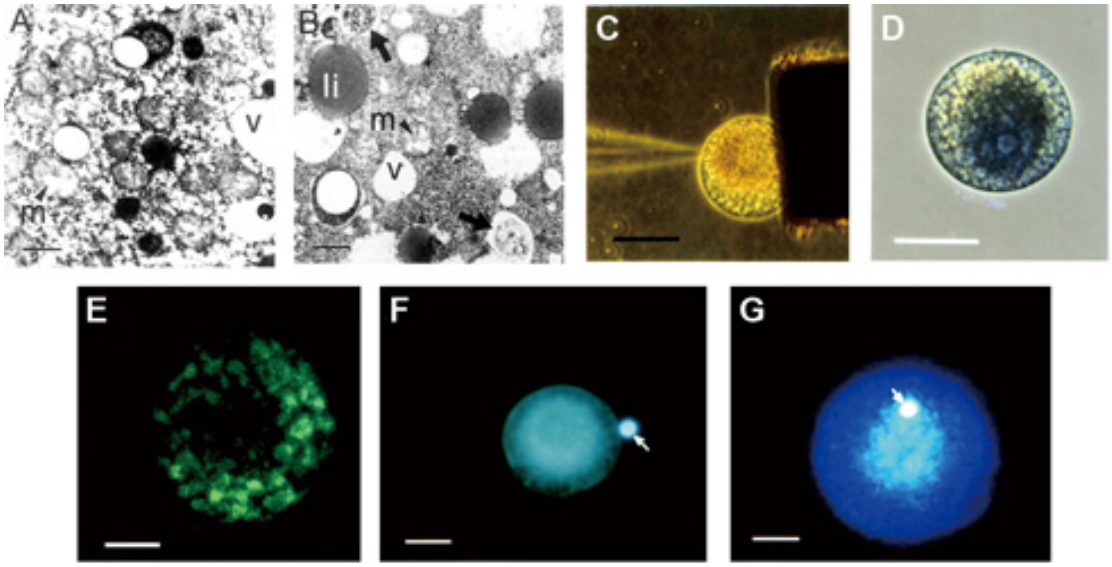

Fig. 2 Transient gene epression study in microinjected wheat egg cell/zygotes and gamete fusion. (**A**) Ultrathin section of a cytoplasm region of an egg cell of wheat isolated 6 days after emasculation (DAE), V: vacuole, m: mitochondrion. Bar: 1 μm; (**B**) Ultrathin section of a cytoplasm region of an egg cell of wheat isolated 12 DAE, V: vacuole, li: lipid body, m: mitochondrion, arrows: autophagous vacuoles. Bar: 1 μm; (**C**) Immobilisation of the wheat egg cell by high frequency AC-field. Bar: 30 μm; (**D**) GUS expression in a microinjected, fertilised egg cell after 48 h in culture. Bar: 30 μm; (**E**) GFP imaging in a microinjected, premature egg cell after 24 h in culture. Bar: 15 μm; (**F**) Sperm-egg cell contact (3 HAP), the sperm cell is indicated by arrow. Bar: 20 μm; (**G**) Karyogamy in a fertilised egg cell (6 HAP). The arrow indicates the sperm cell nucleus. Bar: 10 μm. (Reprinted from: **Pónya Zs, Finy P, Fehér A, Mitykó J, Dudits D, Barnabás B** (1999) *Protoplasma* **208**, 163-172, with kind permission of Springer Science and Business Media.)

Table 1 Transient expression of isolated egg cells of wheat after microinjection of GFP reporter gene.

Timing of microinjection*	№ of microinjected egg cells	№ of egg cells showing transient expression	Frequency of transient expression (%)
3	20	11	55.00
5	23	17	73.91
6	17	10	58.82
9	19	4	21.05
12	10	2	20.00
15	9	1	11.11

*Days after emasculation
(Reprinted from: **Pónya Zs, Finy P, Fehér A, Mitykó J, Dudits D, Barnabás B** (1999) *Protoplasma* **208**, 163-172, with kind permission of Springer Science and Business Media.)

Table 2 Transient expression of isolated zygotes of wheat after microinjection of GUS reporter gene.

No. of microinjected zygotes	No. of zygotes showing transient gene expression	Frequency of transient gene expression (%)
24	13	54.17
19	11	57.89
25	12	48.00
9	4	44.44

*Hours after pollination
(Reprinted from: **Pónya Zs, Finy P, Fehér A, Mitykó J, Dudits D, Barnabás B** (1999) *Protoplasma* **208**, 163-172, with kind permission of Springer Science and Business Media.)

the time course of DNA dynamics during zygotic development has been quantified via quantitative microspectro-fluorometry by Pónya *et al*. (1999b) (**Figs. 2F, 2G, 3A-I**).

DNA content in egg cells/zygotes isolated mechanically at defined times following hand pollination was estimated using microfluorometry. The data obtained clearly show that the nuclei of the egg cells prior to pollination or after 1 hr after pollination were at the 1C DNA level (**Fig. 3A**). Microfluorometry as a method to measure DNA quantities within the egg and zygote nuclei of higher plants had proven a reliable and reproducible means of obtaining consistent data pertaining to the DNA amounts in eggs and zygotes as had been demonstrated previously in barley by Mogensen and Holm (1995). In **Fig. 3** quantitative data showing the DNA quantity of egg cells isolated at various stages after pollination illustrate that karyogamy in wheat is in its incipient stage at 2-3 hours after pollination (HAP) and fusion of the male and female nuclei is completed by 180 min after pollination (**Fig. 3B**). This observation is consistent with the findings of Faure *et al*. (1993) who studied the time course of karyogamy in *in vitro cultured* zygotes produced by IVF. DNA levels began to rise (**Figs. 3C-E**) at 12 HAP and the 2C complement was doubled by 15 to 18 HAP (**Fig. 3F, 3G**). In some cases both karyo- and cytokinesis was found to be completed by 24 HAP (**Fig. 3I**) whereas in the bulk of the cells analysed the first zygotic cell cleavage occurred by 36 HAP. For the aim to effectuate a transformation system via the microinjection technique of foreign DNA into female gametes/zygotes of wheat, a particular focus of the quantitative analysis of the DNA level in egg cells excised from hand-pollinated wheat spikes was to establish the timing of the DNA synthesis (S) phase of the cell cycle. According to the data presented in (**Fig. 3F, 3G**) progression into the 'S' phase was found to occur at around 15 to 18 HAP (Pónya *et al*. 1999b). In wheat, the 4C DNA level was reached earlier than reported for either cultivar of barley observed by Mogensen and Holm (1995). In the course of the 15 to 18 HAP period there may have been some unfertilised egg cells (**Fig. 3G**) in the batch of cells analysed containing mainly zygotes still resting in G_1 or already completed 'S' phase.

The main advantage of the transformation method based on microinjection of zygotes isolated from crops is that the regenerants can be screened directly for the presence of the transgene hence superseding marker-gene based selection of the transfected population. This approach requires the elaboration of efficient regeneration systems for isolated zygotes of cultivated species which are the basis for this transformation method. These were developed for wheat (Kumlehn *et al*. 1997, 1998) and maize (Leduc *et al*. 1996). An efficacious regeneration system was also established for maize zygotes produced by IVF (Kranz and Lörz 1993). IVF makes it possible to inject DNA into egg gametoplasts prior to sperm-egg cell fusion performed *in vitro* which might provide a higher chance for the integration of foreign DNA into the genome of the target cell (Kranz *et al*. 2004).

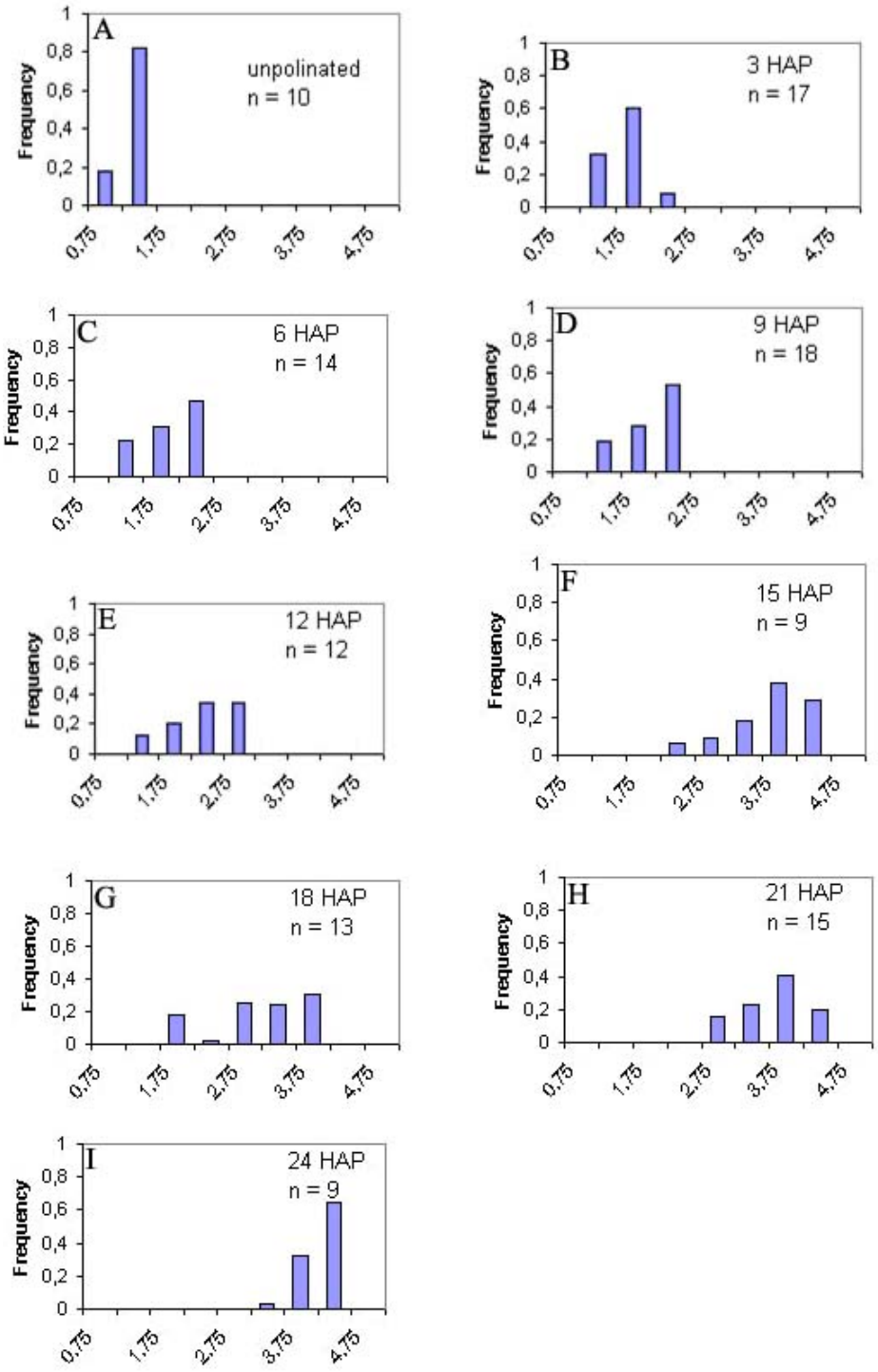

Fig. 3 Relative nuclear DNA quantities expressed as C values in wheat egg cells/ zygotes prior to and at different times after pollination. Frequency histograms show DNA levels of the nuclei analysed: (**A**) At time prior to pollination; (**B**) 3 hours after pollination (3 HAP); (**C**) 6 HAP; (**D**) 9 HAP; (**E**) 12 HAP; (**F**) 15 HAP; (**G**) 18 HAP; (**H**) 21 HAP; (**I**) 24 HAP. (Reprinted from: **Pónya Zs, Finy P, Fehér A, Mitykó J, Dudits D, Barnabás B** (1999) *Protoplasma* **208**, 163-172, with kind permission of Springer Science and Business Media.)

Microinjection as a means of studying *in vivo* F-actin dynamics in isolated egg cells of wheat

Rhodamine-phalloidin (Rh-Ph) microinjected into live plant cells and into cultured animal cells was reported to label the entire filamentous actin (F-actin) population present in the cell at the actual time of imaging (Schmit and Lambert 1990; Sanders and Wang 1991; Cleary 1995). To overcome the problem of the potential artefactual rearrangement of F-actin due to the extreme sensitivity of actin to permeabilization procedures and chemical fixation (Lloyd and Traas 1988), Pónya *et al*. (2001) utilised the *in vivo* microinjection technique for delivering minute amounts of the fluorescent Rh-Ph into live wheat egg cells. This method proved to be a useful tool for analysing F-actin dynamics of wheat egg cells and zygotes.

In order to minimise fading of the fluorescent signal and artefacts supposed to be caused by cytoskeletal alterations due to long exposure of the cells analysed to high energy UV-illumination (Wilson *et al*. 1987), an image acquisition system allowing for very fast integration was employed. This precaution has been deemed to be necessary as previously it had been shown by others (Collucio and Tilney 1984) that, if present in living cells for extended times, phalloidin can stabilise F-actin by acting against denaturing agents in a concentration-dependent manner, hence interfering with the F-actin/G-actin equilibrium. Imaging live cells injected with Rh-Ph has the inherent advantage over imaging actin arrays in fixed cells (Lloyd 1988; Baskin and Cande 1990) that it allows for actin dynamics to be studied and paralleled with specific cell cycle stages. For keeping the adverse effect of experimental manipulations (microinjection, imaging) on *in situ* cytoskeletal arrangement as low as possible, the lowest intracellular concentration (0.30 μM) of the fluorophore which still yielded good images has been established followed by visualisation of F-actin dynamics in excised egg cells and in those fertilised *in vitro*. In the course of the experiment no sequestration of the fluorescent

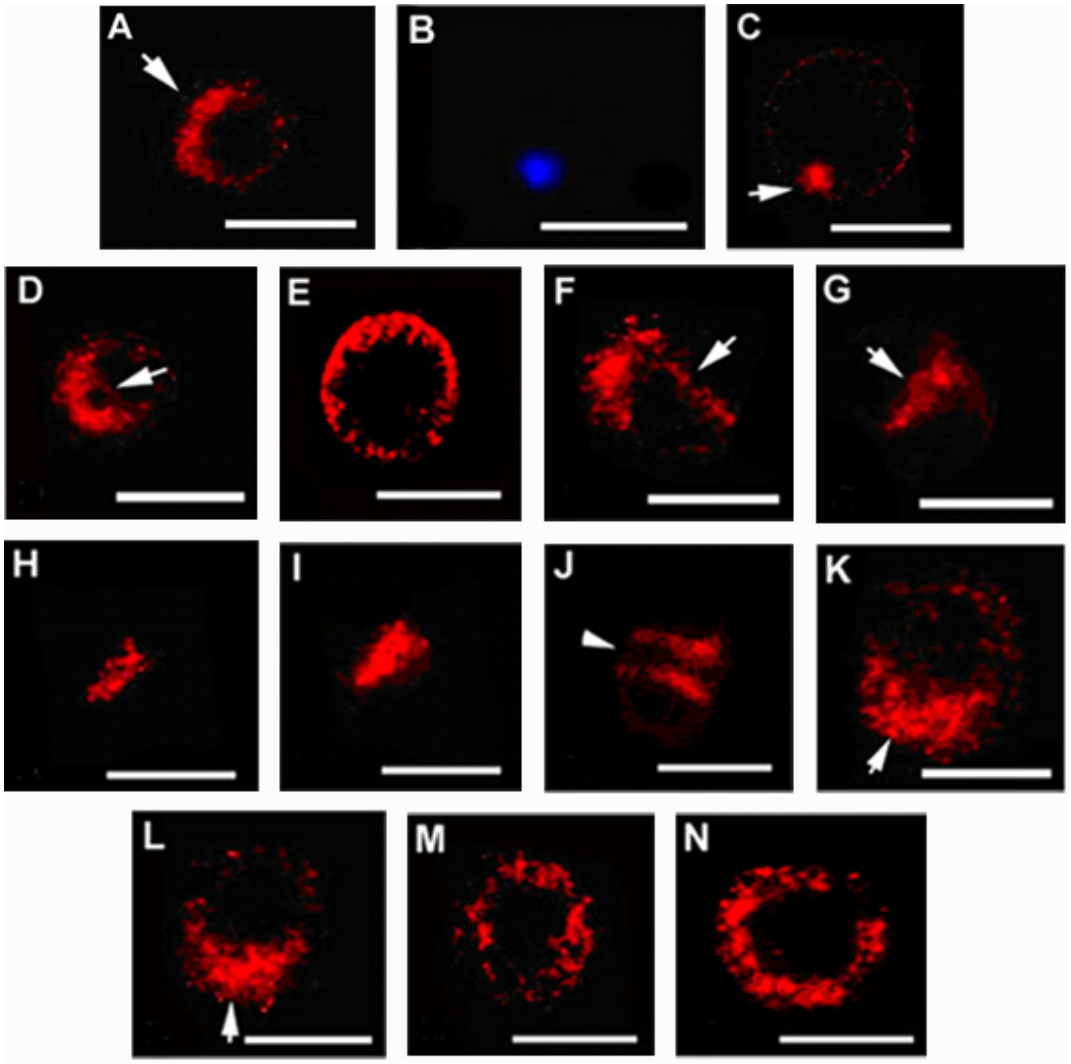

Fig. 4 Following *in vivo* F-actin dynamics in isolated wheat egg cells. (**A**) Mature (6 DAE) female gamete of wheat microinjected with Rh-Ph. Arrow shows the micropylar pole of the cell. Bar: 40 μm; (**B**) Male nucleus in the egg cytoplasm as revealed by DAPI-staining. Bar: 40 μm; (**C**) An actin patch in the same egg cell, labelled with Rh-Ph (arrow), colocalizes with the sperm nucleus. Bar: 40 μm; (**D**) *In vitro* fertilised egg cell of wheat 25 min following sperm-egg cell fusion. The arrow points to the sperm cell surrounded by an actin wreath. Bar: 40 μm; (**E**) Fusion product of sperm-egg cell fusion microinjected with Rh-Ph 14 hours after fertilisation (HAF). Bar: 40 μm; (**F**) *In vitro* fertilised wheat egg cell labelled with Rh-Ph 16 HAF, arrow points to the phragmoplast. Bar: 40 μm; (**G**) Egg cell of wheat fertilised *in vitro* and imaged 17 HAF following Rh-Ph injection. Arrow indicates the future divi-sion plane. Bar: 40 μm; (**H**) The same cell imaged 15 min later. Bar: 40 μm; (**I**) Zygote produced *in vitro* and imaged 18 HAF following Rh-Ph injection. Bar: 40 μm; (**J**) The two daughter cells of a zygote produced by electrofusion and labelled with Rh-Ph. The fluorescent signal indicates a distinct actin band on both sides of the common cell wall, arrow points to the common cell wall. Bar: 40 μm; (**K**) F-actin imaged in a wheat egg cell developed *in situ* and isolated at the receptive (7 DAE) stage. The arrow points to the micropylar pole of the cell. Bar: 40 μm; (**L**) *In vitro* cultured wheat egg cell made to adhere to a collagen-coated membrane surface. F-actin is revealed by Rh-Ph labelling. The attachment site is indicated by the arrow. Bar: 40 μm; (**M**) Immature (isolated 4 DAE) egg cell of wheat injected with Rh-Ph. Bar: 40 μm; (**N**) Rh-Ph labelling of F-actin in an overmature (12 DAE) egg cell of wheat. Bar: 40 μm. (Reprinted from: **Pónya Zs, Barnabás B** (2001) *Journal of Plant Physiology* **158**, 1527-1539, with kind permission of Elsevier GmbH.)

label, Rh-Ph, into the vacuoles could be observed.

In mature egg cells (i.e. those isolated 6 days after emasculation) F-actin localisation occurred to the micropylar pole of the cells (**Fig. 4A**). In wheat egg cells fertilised *in vitro* cortical F-actin was found to undergo rearrangement triggered by sperm delivery into the egg cytoplasm, leading to the cortical actin filaments' formation of a patch at the site of sperm cell entry within 10 min after sperm cell incorporation into the egg's cytoplasm (**Fig. 4B, 4C**). Fifteen minutes later reshuffled F-actin forms a crescent engulfing the integrating sperm cell from the pole of the sperm entry site (**Fig. 4D**). In wheat egg cells developed *in situ* and fertilised *in vitro,* fertilisation causes a reshuffling of the cortical actin wreath leading to a uniform distribution of filamentous actin at the cell periphery which emanate deep into the cytoplasm as mitosis proceeds 14 hours after IVF (**Fig. 4E**). The asymmetrical actin localisation observable in mature egg cells is also visible in fertilised egg cells and ensues from a uniformly distributed cortical actin ring and an actin furrow protruding from the cortical region and delineating the future division plane is observable 16 hours after *in vitro* fertilisation (**Fig. 4F**). In egg cells fertilised *in vitro* 17 hours after fertilisation (HAF) actin filaments were gradually accumulated in the phragmoplast in a continuously widening furrow, hinting at F-actin involvement in the regulation of cytokinesis during zygote development (**Fig. 4G-I**). After the completion of zygote cytokinesis a discernible actin band appeared along both sides of the common cell wall in the two-celled proembryo (**Fig. 4J**).

Spurred by the observation that *in planta* fertilised egg cells seemed to adhere strongly to the maternal tissue, hence suggesting that some directional cues deriving from the microenvironment of the embryo sac and communicated to the egg through the attachment site, isolated egg cells fertilised *in vivo* were cultured in Kao 90 medium (Holm *et al*. 1994) on collagen-coated membranes in order to investigate the effect of attaching the wheat zygote to a surface on actin distribution. Asymmetrical actin distribution, which is

widely held to be a sign of axis fixation (Baluska *et al*. 2000), could be observed in egg cells isolated at 7 DAE and immobilised egg cells cultured *in vitro,* at the attachment site (**Fig. 4K, 4L**). Intriguingly, unlike receptive egg cells, neither immature egg cells (isolated 4 DAE) nor overmatures ones (isolated 12 DAE) revealed a preferential actin localisation at a distinct site of the cell even if immobilised to a membrane (**Fig. 4M, 4N**). Asymmetrical F-actin distribution was also observed in egg cells isolated 7 DAE and monitored at various time intervals from *in vitro* culture, but if the cells were not immobilised by collagen, this fine meshwork of cortical actin was found to spread deep into the cytoplasm. However, in batches of egg cells which were cultured on collagen-coated membrane surfaces, the accumulation of F-actin at the attachment site was observed suggesting a putative role that actin localisation may play in axis selection/fixation of the developing (fertilised) egg cell inasmuch as adhering the cells to a surface may mimic plasmodesmata connections of the egg cell/zygote at the micropylar pole conjectured to represent one of the two poles (as contrast to the chalazal one) of the axis of the conspicuous polarity of the female gametophyte.

In egg cells fertilised *in vivo* a polar assembly of cortical F-actin was observed to colocalize with the micropylar region of the cell showing strong cell wall-specific fluorescent signal indicating the incipient build-up of the cell wall induced by fertilisation (**Fig. 5A, 5B**). Imaging of membrane-bound calcium-specific signals detected by using chlorotetracycline (CTC) (Kropf and Quatrano 1987) revealed co-localisation of calcium-accumulation at the site of F-actin-specific fluorescence, supporting the hypothesis that actin filaments may be involved in the calcium homeostasis of wheat egg cells (**Fig**. **6A, 6B**). These findings of Pónya *et al*. (2001) may hint at the possibility that the mechanisms acting in unfertilised wheat egg cells may be paralleled to those acting in zygotes of *Pelvetia compressa*, in which F-actin assembly at the presumptive rhizoid pole is conjectured to be involved in inducing cellular asymmetry (Kropf and Quatrano 1987).

Based on their observations, Pónya *et al*. (2001) suggested that a developmental axis may be selected in the receptive egg cell preceding fertilisation. However, whether this putative developmental axis is kept or changed after fertilisation according to sperm cell entry site (which may act as a marker for new axis selection) or the sperm incorporation site is chosen in conjunction with the already established polarity axis, remains to be determined. In any case, this apparently preset arrangement of F-actin becomes rearranged upon sperm entry during IVF (**Fig. 4B, 4C**), but if this phenomenon has significance in (re)defining the polarity axis during zygotic embryogenesis in wheat is not known. Pónya *et al*. (2001) argue that presumably this preselected accumulation site of F-actin may be involved in marking the micropylar pole of the cell, hence probably playing a significant role in specifying the orientation of the growth axis. The observation that the F-actin labelling was strongest in the examined egg cell apparatus at the micropylar region of the female gamete marked by synergid attachment lends credit to this assumption. Speculatively, if the alternative scenario is correct, i.e. the sperm entry site is to induce polarity, then, consequently, the fusion site must somehow be "imprinted" on the egg cytoplasm in such a way that the signal transduction cascade unleashed by sperm cell integration can lead to axis selection. Nevertheless, unravelling the complexity of the mechanisms inducing polarity in (fertilised) wheat egg cells is a deterring challenge on two accounts: upon isolation the egg cell is deprived of its microenvironment and subsequently of all the directional cues communicated to it by the surrounding cells and/or the maternal tissue; additionally, similarly to the well-described *Fucus* system polarity axis selection and fixation is a continuum of overlapping and intertwining events (Bouget *et al*. 1996) rendering their dissection difficult. Further, the external fertilisation in *Fucus* (egg cells are released into seawater) is significantly different from double fertilisation which poses the question as to whether the mechanisms conferring polarity on the Fucus zygote are similar to those events leading to polarised growth of the angiosperm zygotic embryo, in other words: are these events conserved and function generally in such phylogenetically distant species such as *Fucus* and angiosperms?

How the observed F-actin patch accumulates at a distinct region in the receptive wheat egg is obscure. Perhaps the mechanisms proposed by Jeffrey and Meier (1983) may be adopted in wheat: these authors hypothesise that the differential stabilisation of filaments are responsible for the presence of a special actin domain.

In the light of earlier reports (Kakimoto and Shibaoka 1987; McCurdy and Gunning 1990; Liu and Palevitz 1992; Cleary 1995) it is intriguing that F-actin preprophase band (PPB) was observed in wheat zygotes (Pónya *et al*. 2001) which may be recruited by actin-binding proteins or rhoGTPases to this site at a specific point in the cell cycle hinting at a stringent cytoskeletal control in assigning a distinct region of the wheat zygote as the presumably predetermined division plane (Fowler and Quatrano 1997). One may envisage a system of spatial and structural associations between F-actin domains acting in concert with non-filamentous actin and cortical membranes, supposedly through a preset cytoskeletal system that is organised to modulate the alteration of the egg cortex occurring concomitantly on fertilisation. According to this scenario, the cortical division site provides spatial guidance for the incipient phragmoplast presumably inserted at a predefined region of the cell wall and the cytoplasm which possess the necessary factors for the formation of the cell plate. A putative role played by transcellular ion currents in the selection/fixation of the polarity axis cannot be excluded and albeit electric currents of this sort were detected in fertilised *Pelvetia* eggs (Nucitelli 1978), they proved unstable and changed their position, thus Nucitelli was led to the conclusion that it appears improbable that the pattern of ionic fluxes alone mark the

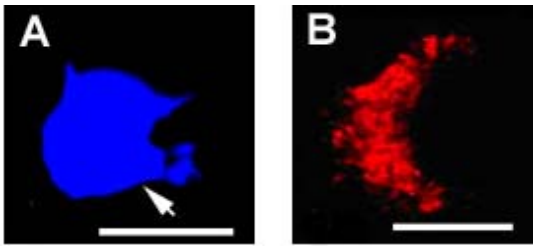

Fig. 5 Cortical F-actin accumulation colocalizes with the micropylar region of the *in vivo* fertilized wheat egg and coincides with the commencement of cell wall synthesis beginning at the micropylar pole. (**A**) Wheat egg cell isolated after *in vivo* fertilisation and stained with the cell wall-specific fluorescent dye, calcofluor white. Arow points to the micropylar pole. Bar = 40 μm; 19 b. (**B**) Image taken of the same cell labelled with Rh-Ph. Bar: 40 μm. (Reprinted from: **Pónya Zs, Barnabás B** (2001) *Journal of Plant Physiology* **158**, 1527-1539, with kind permission of Elsevier GmbH.)

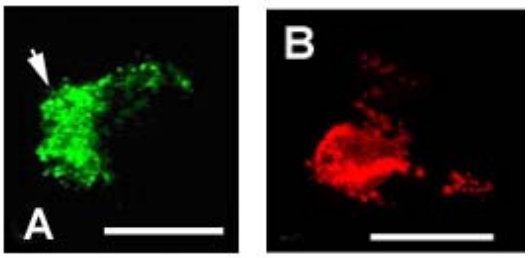

Fig. 6 Imaging of membrane-bound calcium using CTC as a fluorescent probe in mature egg cell microinjected with Rh-Ph. (**A**) Membrane-bound calcium accumulation is shown by the arrow. Bar: 40 μm; (**B**) The same cell injected with Rh-Ph. Bar: 40 μm. (Reprinted from: **Pònya Zs, Kristòf Z, Ciampolini F, Faleri C, Cresti M** (2004) *Sexual Plant Reproduction* **17**, 177-188, with kind permission of Springer Science and Business Media.)

developmental axis in the egg. Furthermore, it is worth mentioning that unlike the eggs of the brown algae which are fertilised off the mother plant (in the seawater), the angiosperm egg is encased deeply in the maternal tissues of the mother plant, so the mechanisms controlling axis selection and fixation may not be as flexible as those acting in the case of zygotes of the brown algae in which photopolarisation by unidirectional (blue) light is possible and axis selection can be artificially altered (Berger and Brownlee 1994).

The ultimate question is: how the visualisation of actin filaments achieved through Rh-Ph injection may shed light on the function of actin in (pre)determining the growth axis of the wheat zygote. It is surmised by Pónya *et al*. (2001) that these cortical microfilaments could act in unison with components of the cell wall patches via the formation of links with components of the cell wall patches and/or with the synergids. Ultrastructural data gained by You and Jensen (1985) seem to bolster this hypothesis by providing evidence for the existence of a common cell wall between the wheat egg cell and the synergids, which the actin cytoskeleton might be a part of. Such cross-bridges have already been depicted both in plant (Lancelle *et al*. 1987) and in animal cells (Horwitz *et al*. 1986). These connections have also been implicated in anchoring accessory proteins (e.g. actin-binding proteins) and integral membrane proteins (Horwitz *et al*. 1986).

The nature of mechanisms controlling the rapid spatial and temporal actin reorganisation concomitantly occurring upon wheat sperm incorporation is entirely unknown hitherto. Whether this spatial reorganisation of F-actin plays a role in axis selection/alteration (?) and/or functions later in the alignment of asymmetrical cell cleavage is ambiguous as yet. Pónya *et al*. (2001) surmise that a dynamic, actin-based cytoskeleton may regulate the viscoelastic properties of the egg cytoplasm, resulting in the alteration of the structure of the cortex of the activated egg triggered by fertilisation. This notion is in agreement with the supposition that a large pool of unpolymerized subunits may be available in the egg cytoplasm for new microfilament growth (Staiger *et al*. 1997). Alternatively, the rapid redistribution of actin filaments occurring subsequently on sperm-egg fusion may be due to the changed activity of the capping proteins known to block the rapidly-growing, barbed ends of microfilaments in many eurokaryotic cells. The change in the capping protein may be induced by cytoplasmic pH change or by intermediate elements of signal transduction pathways, such as Ca^{2+} and membrane polyphosphoinositides (Sun *et al*. 1995). However, the mechanical properties and composition of the actin-based cytoskeleton of the (fertilised) wheat egg cell calls for further investigation. In view of the observations made by Pónya *et al*. (2001) on the cortical F-actin rearrangements in wheat egg cells and zygotes, it is interesting to note that both the disruption of F-actin (Quatrano 1973) and enzymatic removal of the cell wall (Kropf *et al*. 1988) have been demonstrated to prevent axis fixation in *Fucus* zygotes.

Structural reorganisation of the endoplasmic reticulum during wheat egg cell development and fertilisation

One of the most fundamental issues in the developmental biology of angiosperms is how the egg cell prepares for fertilisation and what processes pave the way for egg activation unleashed by sperm cell binding and fusion of the two gametes. A "properly" activated angiosperm egg will subsequently have the faculty of maintaining/acquiring polarity which is thought to be crucial for the developmental pattern throughout zygotic embryogenesis and during early zygote development, in particular. For a considerable time, egg activation has been thoroughly investigated in lower plants such as *Fucus* (Roberts *et al*. 1994; Roberts and Brownlee 1995) and in many invertebrate and vertebrate species in the animal kingdom (Kubota *et al*. 1987; Miyazaki 1988; Miyazaki *et al*. 1993; Whitaker and Swann 1993; Ridgway *et al*. 1997). However, the mechanism by which the signal conveyed by the sperm cell is transduced in the egg cell of higher plants remains largely unknown.

Ca^{2+} implicated as a secondary messenger in the response of various plant cells to a wide range of stimuli has long been deemed a potential candidate for encoding the information brought by the incorporating sperm cell into the female gamete. Thus, Digonnet *et al*. (1997) addressed the issue whether calcium as a secondary messenger plays such a pivotal role in egg activation also in higher plants similarly as it does in the animal systems studied previously. In this study, a single $[Ca^{2+}]_{cyt}$ rise was observed in *in vitro* fertilised maize egg cells the fusing of which with male gametes was induced chemically by extracellular calcium in the fusion medium. This report prompted the development of the hypothesis that cytosolic calcium ($[Ca^{2+}]_{cyt}$) may act as a secondary messenger during egg activation in angiosperms, just as it marks activation in animal eggs. Theoretically, any $[Ca^{2+}]_{cyt}$ rise could originate from extracellular and/or intracellular calcium stores. Evolution "bestowed" on cells the ability to store Ca^{2+} in intracellular membranes and to alter the $[Ca^{2+}]_{cyt}$ level rapidly in a way that generates local $[Ca^{2+}]_{cyt}$ gradients within the cell which is considered to be a major achievement in cellular evolution.

In higher plant cells the dominant calcium store is the central vacuole; nevertheless, in the fully mature, receptive wheat egg cell few vacuoles can be observed (Pónya *et al*. 1999a), therefore it is unlikely that in the wheat egg the principal cell organelle performing a key role in the regulation of intracellular calcium is the vacuole. Spurred by this observation, Pónya *et al*. (2004) supposed another cell organelle, the endoplasmic reticulum (ER), to be the main calcium store in the wheat egg cell. To examine this hypothesis, they injected the ER-specific lipophilic, fluorescent dicerbocyanine dye, 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) [$DiIC_{16}(3)$], into wheat eggs isolated at different maturational stages. These authors demonstrated that in the course of *in situ* development of the wheat female gamete the distribution of the ER undergoes a series of structural reorganisation resulting in a polarised meshwork of the ER in the receptive egg cell. The accumulations also became labelled by DiI in fixed egg cells hence providing evidence that the membranes stained by the dye do belong to a continuous reticular network (and consequently the fluorescent signal is not due to membrane-trafficking). In 61 (89.70%) of 68 immature egg cells isolated 3 DAE and injected with DiI, the oil drop saturated with the dye remained brightly fluorescent for 60 min following injection (by which time it should have spread through the whole cell) indicating that at this developmental stage no continuous membrane network is present in the cell (**Fig. 7A**). At 4 DAE, throughout the perinuclear region of the female gamete a uniform distribution of membrane accumulation was observable throughout the perinuclear region of the cell and showed no sign of polarity (**Fig. 7B**), whereas in mature egg cells, isolated at the stage of anthesis, the ER membranes were markedly present at the cortical region at this stage and revealed remarkable polarity around the nucleus, as revealed by the dye (**Fig. 7C-E**). Intriguingly enough, the structural changes depicted by Pónya *et al*. (2004) in the ER during egg maturation of the wheat egg as had been previously observed in eggs of species such as sea urchin (Terasaki and Jaffe 1991; Terasaki and Sardet 1991), *Xenopus* oocytes (Gardiner and Grey 1983; Campanella *et al*. 1984), and mouse eggs (Mehlmann *et al*. 1995).

Based on their observations as to ER reorganization in the female gamete of wheat in the course of *in planta* development, these authors propose that the polar distribution of the ER membranes suggests a role in calcium regulation of the wheat egg. Pónya *et al*. (2004) speculated that the ER clusters in the cell cortex might suggest a presumptive sperm fusion site. The hypothesis is based on the reasoning that ER clustering in the mature angiosperm egg may have importance for Ca^{2+} release during fertilisation (Digonnet *et*

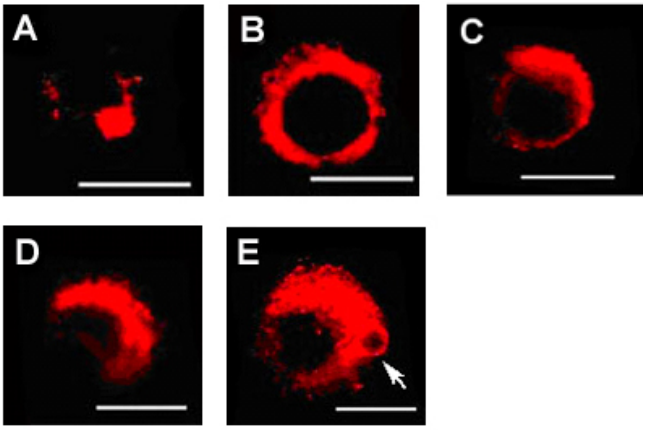

Fig. 7 Visualization of the endoplasmic reticulum (ER) in *in situ* developed and isolated wheat gametoplasts following labelling the ER by injection with 1,1'-dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). (**A**) Unfertilised wheat egg cell isolated 3 days after emasculation (DAE) and injected with DiI to visualise the ER. Bar: 20 µm; (**B**) Labelling of the ER network in a wheat egg cell isolated 4 DAE and injected with DiI. Note the perinuclear, uniformly distributed membrane accumulation of the ER around the nucleus. Bar: 10 µm; (**C**) Pronounced polarity of the ER as revealed by DiI in a mature egg cell of wheat. Bar: 30 µm; (**D**) The polarised network of the ER in a receptive egg cell isolated 6 DAE. Bar: 30 µm; (**E**) Thick, polarised membranes of the ER opposed to the vacuole-rich pole of the mature wheat egg cell. Arrowhead shows the delivery site of the oil droplet saturated with dye. Bar: 25 µm. (Reprinted from: **Pònya Zs, Kristòf Z, Ciampolini F, Faleri C, Cresti M** (2004) *Sexual Plant Reproduction* **17**, 177-188, with kind permission of Springer Science and Business Media.)

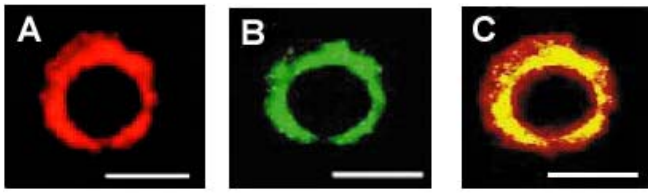

Fig. 8 Localisation of membrane-bound calcium in immature egg cells labeled for the ER. (**A**) DiI-specific fluorescence in an immature wheat egg cell isolated 4 DAE; (**B**) The same cell imaged after incubation in CTC using a filter combination suitable for detecting CTC fluorescence. (**C**) Merged image of (**A**) and (**B**). The yellow colour shows the co-location of red and green pixels. Bars: 15 µm. (Reprinted from: **Pònya Zs, Kristòf Z, Ciampolini F, Faleri C, Cresti M** (2004) *Sexual Plant Reproduction* **17**, 177-188, with kind permission of Springer Science and Business Media.)

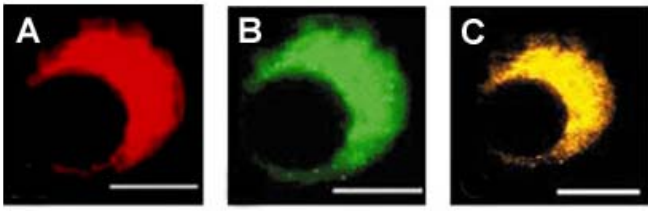

Fig. 9 Localisation of membrane-bound calcium in mature egg cells labeled for the ER. (**A**)Receptive wheat egg cell injected with DiI and examined 20 min later; (**B**) The same cell after incubation in CTC solution followed by incubation in the dark for 15 min; (**C**) Merged image of (**A**) and (**B**). Bars: 25 µm. (Reprinted from: **Pònya Zs, Kristòf Z, Ciampolini F, Faleri C, Cresti M** (2004) *Sexual Plant Reproduction* **17**, 177-188, with kind permission of Springer Science and Business Media.)

al. 1997) as higher $[Ca^{2+}]_{cyt}$ levels increase the sensitivity of $InsP_3$ (inositol-1,4,5-trisphosphate)-sensitive release channels to $InsP_3$ (Ferris and Synder 1992). Subsequently, at a predetermined (?) region of the egg cell in which the ER clusters are preponderant, more Ca^{2+} may be released from the ER membranes, resulting in higher local Ca^{2+} levels, which then leads to greater $InsP_3$ sensitivity. However, from the data obtained by these authors it cannot unequivocally be determined whether the sensitivity to $InsP_3$ or the capacity of Ca^{2+} stores, or both, increased. Furthermore, these authors hypothesize that phospolipase C that generates $InsP_3$ might be more concentrated in that domain of the plasma membrane where the ER accumulations are closer to the cortical region in the receptive wheat egg cell, so the polar localisation of the ER could also be a means for increasing $InsP_3$ sensitivity. Indeed, in the described animal systems the ability of the ER to store Ca^{2+} and release it at increasing levels as the oocyte matures is evinced by a number of experimental observations including the allocation of Ca^{2+} in the ER of live cells (Chiba *et al*. 1990) as well as by the direct measurement of cellular distributions using electron microprobe analysis (Jorgensen *et al*. 1988).

How the homogeneity of the meshwork of the ER during egg maturation is transformed to the pattern manifesting polarity characteristic of egg cells isolated at 6 DAE remains to be determined. Based on the results of Pónya and Barnabás (2001) who found that wheat egg cells microinjected with the fluorescent phallotoxin, rhodamine-phalloidin exhibited actin accumulations coinciding with sites revealing membrane-associated calcium stores, Pónya *et al*. (2004) put forward the idea of an actin-driven mechanism.

Membrane-bound calcium in wheat egg cells revealed by CTC

Pónya *et al*. (2004) used the chlorotetracycline (CTC)-staining method to investigate the membrane-associated calcium distribution during *in situ* development of wheat egg cells. The pattern revealed by the dye was identical to that seen in the same cell stained with DiI suggesting that the ER is the main Ca^{2+} store in the wheat egg, an observation which may have ramifications for explaining the structural changes of the ER during egg cell maturation and for the putative role of the ER in calcium signalling during egg cell activation. The membrane-bound calcium-specific fluorescent pattern exhibited by the egg cells at each developmental stage analysed was correspondent with that of the same cell injected with DiI so that when superimposed the two images of the same cell matched well. The level of this matching appeared to depend on the maturational stage analysed (compare **Figs. 8A-C, 9A-C**).

Transient fragmentation of the ER in in vitro fertilized wheat egg cells

When egg cells isolated at the receptive stage were fused to sperm cells, sequential changes could be observed in the organisation of the ER in the female gamete which led to a transient fragmentation of the polar accumulations of ER membranes (**Fig. 10A-G**). Whether this fragmentation has a role in the calcium release mechanism observed in the receptive egg after fertilisation (Antoine *et al*. 2000) is unknown, but these authors hypothesized that this transient fragmentation contributes to an increased calcium release capacity of the fertilised egg by increasing the curvature of the membranes from which calcium transport may be facilitated by calcium transport proteins. Alternatively or additionally, the biological significance of the transient loss of the ER connections observed to ensue after sperm-egg plasmogamy may facilitate the initial phases of the travel of the sperm cell in the egg's cytoplasm as its movement may be impeded by the meshwork of the ER present in the unfertilised egg cell, the breakdown of which would enable the sperm to move freely towards the egg nucleus. Moreover, the fragmentation of the ER in the angiosperm egg at fertilisation may lead to a transient decrease in cytoplasmic viscosity demonstrated to occur in eggs of several species, including frogs (Elinson 1983) and marine worms (Tyler 1932). As numerous proteins are synthesized following fer-

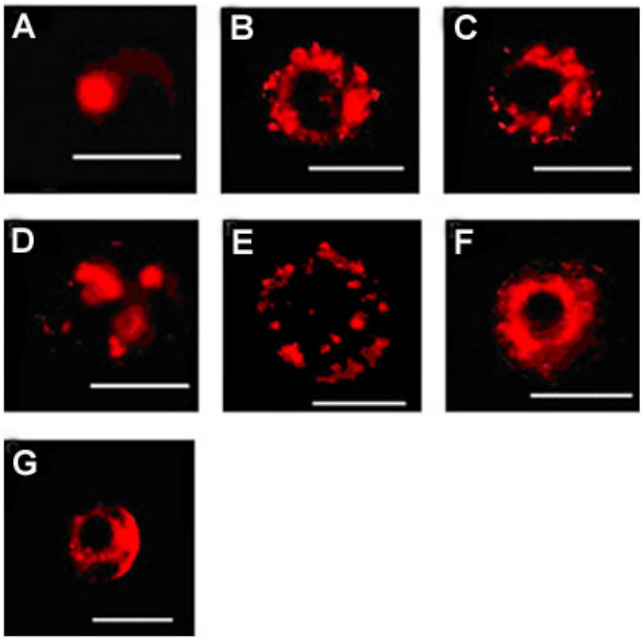

Fig. 10 Transient fragmentation of the ER during *in vitro* fertilisation. (**A**) Image of an *in vitro* fertilised mature wheat egg cell microinjected with DiI within 1 min of fusion of the sperm cell with the female gamete. Bar: 15 µm; (**B**) Receptive wheat egg cell injected with a DiI-saturated oil droplet 30 min prior to electrofusion and imaged 3 min postfertilisation. Bar: 50 µm. Successive images taken at 1 min intervals consecutively following fusion of the male gametes with the egg cell: (**C**) Egg cell injected with DiI, fertilised *in vitro* and imaged 4 min after fusion. Bar: 40 µm; (**D**) The same cell imaged 5 min postfertilisation. Bar: 40 µm; (**E**) The same cell imaged 1 min later. Bar: 45 µm; (**F**) DiI-distribution in an *in vitro* fertilised wheat egg cell imaged 10 min following gametic fusion. Bar: 35 µm; (**G**) The same cell imaged 2 min later Bar: 40 µm. (Reprinted from: **Pònya Zs, Kristòf Z, Ciampolini F, Faleri C, Cresti** M (2004) *Sexual Plant Reproduction* **17**, 177-188, with kind permission of Springer Science and Business Media.)

tilization, the fragmentation of the ER may somehow be involved in the intense protein synthesis process (Rees *et al*. 1995).

Can gamete manipulation and IVF prove to be useful tools in apomixis research?

Double fertilisation implies the fusion of two gametes of opposite sexes leading to the formation of the zygote as well as to the fertilisation of the central by the second sperm cell delivered into the embryo sac. This latter event gives rise to the endosperm, normally a triploid tissue possessing an unequal parental genomic contribution, which functions much rather like the placenta of mammals: it provides nutrients to the developing embryo. Thus, sexual fusion permits the exchange of the genetic material supplied by the parental genomes and through the production of a multitude of genotypes ensures the survival of the fittest ones favoured by natural selection. Albeit, according to this classical view, in evolutionary terms sexual reproduction has clear advantages for the survival of the actual species, several groups of plants have invented alternative propagation strategies based on asexual reproduction.

Under certain conditions a cell in the ovule may undergo fertilisation-independent embryogenesis the forms of which are almost as diverse as the species themselves known to follow mainly or totally asexual reproduction. Fertilisation-independent embryo development (parthenogenesis or "virgin birth") had been described in animals long before the hallmark components of apomixes (*apo* meaning "away from" and *mixis* "act of mixing"): egg cell formation produced without meiosis (apomeiosis), asexual embryogenesis and developmental alteration to ensure functional endosperm development, have been reported to occur in plants. Apomixes can be considered as integrated (sequential and simultaneous) processes resulting in the spatial and temporal deregulation of the sexual pathway. Stress is among the conditions under which reproduction is no longer associated with fertilisation and asexual reproduction becomes the more favourable survival strategy stress hinting that similar regulatory features may induce asexual embryogenesis in the angiosperm ovule *in vivo* (Rambaud *et al*. 1996) as those acting during somatic embryogenesis triggered by applying stress to somatic cell cultures (Mordhorst *et al*. 1997). Studies in *Arabidopsis* aimed at constructing models for the control of apomixis, and genetic and molecular analysis of *in vitro* somatic embryogenesis supplied clues pertaining to genes playing a role in rendering a plant cell competent to undergo embryogenesis. For instance, Hecht *et al*. (2001) found that overexpression of *Arabidopsis SERK* (*Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE*: *AtSERK*) enhances the embryogenic potential of cultured *Arabidopsis* cells. Intriguingly, *AtSERK* is expressed in regions of the developing ovule, including the egg cell, where embryogenesis may be unleashed in a selected cell upon activation by the appropriate signal (Hecht *et al*. 2001). Apparently, embryogenic competence conferred on either an unreduced egg cell or on any cell selected in the ovule may be under the control of several genes acting together in bringing about embryo initiation. Searching for clues as to what extent and in what ways zygotic and somatic embryogenesis follow identical or similar molecular pathways, it was observed that the overexpression of structurally unrelated transcription factors *BABY BOOM* (*BBM*), *LEAFY COTYLEDON 1* and *2* (*LEC1*, *LEC2*) or *WUSCHEL* (*WUS*) induces somatic embryogenesis in transformed plants (Stone *et al*. 2001; Boutilier *et al*. 2002; Zuo *et al*. 2002). However, whether any of these genes are expressed in the egg cell or zygote in the course of wild-type development of the female gametophyte is obscure. Mayer *et al*. (1998) hypothesize that their overexpression may cause some type of stress response that leads to the de-differentiation of specific cells, and embryogenesis subsequently ensues possibly due to embryonic identity acquired as the default state.

Thus, it seems that stress is among the factors required to enable a differentiated cell to acquire embryogenic capacity. Taking into consideration the morphological similarities observed during somatic versus zygotic embryogenesis, which suggest the presence of similar regulatory mechanisms during early stages of the two types of embryo development, it may be conjectured that the cellular responses induced upon stress applied in *in vitro* cultures of somatic cells trigger identical or similar cell-fate decision programmes in cells assigned to become embryogenic in the ovule during its apomictic development. Along the same line of reasoning, it may be also surmised that stress responses occurring when cells are treated *in vitro* with a temporarily high level of auxin (auxin "shock") (which leads to somatic embryogenesis in cell cultures) are similar to those allowing a stressed seedling to be rescued via somatic embryo formation in quite the same way as stress results in early flowering hence providing a "window of opportunity" for the individual to be rescued through precocious seed development.

In wheat, female gamete manipulation coupled with the so-called "Salmon wheat system" proved to be a powerful tool in delving into the nature of the mechanisms required to initiate embryogenesis of the wheat egg (Matzk 1996). A particular feature of the system is that while *in planta* usually an unreduced egg cell is the progenitor cell of parthenogenic development in the apomictis species, culture of isolated *reduced* egg cells from Salmon wheat plants leads to parthenogenesis (Matzk 1996) suggesting that the quiescent Salmon egg is already stockpiled with developmental information required to initiate embryogenesis. Although the sequential events inducing wheat egg activation and em-

bryo formation concomitantly occurring upon fertilisation or those leading to parthenogenesis from reduced Salmon egg cells remain unknown, ever more sophisticated *in vitro* culture systems allowing for single culture of isolated (and manipulated) egg cells could contribute to our understanding of the molecular mechanisms that trigger embryogenic development from the fertilised as well as from the unfertilised wheat female gamete.

The survival of both sexually or asexually produced embryos requires the development of the endosperm. Consequently, the formation of a viable embryo and successful seed germination are dependent on the second fertilisation product in the angiosperm seed (the endosperm) which supplies the embryo with nutrients. The endosperm is typically triploid and its proliferation is the result of releasing the central cell from a maternal repressive mechanism overcome by the second fertilisation event during which the homo-diploid central cell fuses with one of the two male gametes delivered into the embryo sac. However, using the *cdka;1* mutant of *Arabidopsis* as a pollen donor, Nowack *et al*. (2006) observed that endosperm development did take place even in the absence of the second fertilisation event hinting at the existence of a positive signal deriving from the fertilised egg cell. On the other hand, *Arabidopsis* mutants such as those belonging to the so-called *fertilisation-independent seed* class, *medea* (*mea*), *fis2* and *fertilisation-independent endosperm* (*fie*), revealed that endosperm proliferation is also possible in the absence of fertilisation (Ohad *et al*. 1996; Chaudhury *et al*. 1997; Grossniklaus *et al*. 1998; Grossniklaus and Vielle-Calzada 1998). Notwithstanding that *fis* class mutants produce seed-like structures, they eventually abort. However, fertilisation-independent fruit development occurs, suggesting that endosperm formation alone seems sufficient to induce fruit development and maturation. Fertilised seeds stemming from *fis* embryo sacs show defects in embryo and endosperm development and eventually abort suggesting that the *FIS* genes are essential in the control of cell proliferation (Grossniklaus *et al*. 2001). Therefore, fertilisation-independent endosperm proliferation is possible (as it was reported to occur in the *fis* mutants). Even though autonomous endosperm development is not an absolute hallmark of apomixis, it does occur in many apomictic species.

Whereas in the sexually produced triploid endosperm the ratio of paternal to maternal genomes (2m:1p) appears to be essential for normal seed development (as is evinced by the finding that many interploidy crosses result in seed abortion attributable to endosperm failure, for review see: Haig and Westoby 1991), apomicts are not sensitive to deviation from this paternal *versus* maternal genome ratio, so in their evolution they seem to have circumvented this m:p genome ratio impact on seed development mediated by mechanisms such as differential gene expression or differential allele-specific expression pattern dependent on parental origin (genomic imprinting) (Vinkenoog *et al*. 2001). Investigating these mechanisms is warranted by the intense commercial interest in apomixis technology. For instance, modifying sexual crop species into apomicts, assisted by enhancing our understanding of the formation of unbalanced endosperm in apomictic hybrids, the problem of high frequency seed abortion reported to occur in introgression hybrids might be overcome.

In the context of utilizing egg cell isolation and manipulation (microinjection and IVF) as tools for apomixis research in cereals and other crops, it is worth recalling that the formation of the female gametophyte and subsequent seed development ensuing upon fertilisation in sexual plants are dependent on the signals deriving from the surrounding sporophytic tissues (Gasser *et al*. 1998), therefore it seems reasonable to suppose that the activation of the quiescent egg cell triggered either by the fertilising sperm cell or via events resulting in parthenogenic development (Salmon wheat) are (at least partly) controlled also by maternal sporophytic and gametophytic tissues. Furthermore, the microenvironment of the embryo sac, which we know very little of, may ensure a characteristic hormonal, pH an ionic gradient, which may be established and maintained throughout gametogenesis, the subtle interplay of which may not be mimicked *in vitro*. However, the finding that reduced egg cells isolated from the Salmon wheat give rise to embryos via parthenogenesis under *in vitro* conditions suggests that the elements of the entire molecular machinery required for the initiation of embryogenesis are already present in the mature egg and the fusion with the male gamete may be necessary only for the bestowal of the male genetic material. Nevertheless, what events that occur during fertilisation-independent embryogenesis observed in the reduced Salmon egg obviate the need for the wheat male gamete to induce the cascade of the signalling mechanisms in the egg during sexual fusion remains unknown. Studies aimed at microinjecting isolated wheat egg cells (other than the Salmon type) with cloned genes known to have relevance in controlling entries at different stages of the cell cycle are likely to shed light on this issue.

In summary, female gamete isolation and IVF allow for continuous monitoring of egg cell development *in vitro* enabling one to unravel the mechanisms controlling egg activation and embryogenic potential provided that the signalling programme of parthenogenesis produces "end-products" whose depository becomes the egg cell and that the decoding of this developmental information is possible for the egg cultured off the maternal tissue. To dissect what triggering events lead to egg activation and to artificially induce them in unfertilised, reduced wheat egg cells will be the challenge of the years to come.

Molecular and genetic events during fertilisation and zygote development as revealed by IVF

From a developmental point of view the most important phase of ontogeny in angiosperms is concerned with progressive divisions of the zygote to form the embryo. This process has been thoroughly analysed almost exclusively by classical histological methods until the 1990s when insights into the associated cellular and molecular changes were provided by the electrofusion method developed by Kranz *et al*. (1991) and Kranz and Lörz (1993) for fusing single gamete pairs. In the early years of studying the development and physiology of zygotic embryos, considerable efforts have been made to focus on the morphological aspects of the first few rounds of zygotic division and on the subsequent morphogenesis of the generated cells which eventually give rise to the embryo. In this period, the ground has been set for the common tenet that each division plane of the fertilised egg and its descendants is actuated by taking the cue from a genetic blueprint characteristic of the given species. In the second period of the embryology of angiosperms, numerous studies were embarked upon to investigate the structural characteristics of embryos and their relationship to morphological development and function. One aspect in the forefront of these analyses was a concern centred on the ultrastructural changes that accompany the transformation of the zygote into a fully competent embryo. The third angle from which embryo development was studied attempted to shed light on a much debated and little understood area: the nutrition of the embryo. Experimental approaches were designed with the purpose to delve into the nutritional interactions during embryogenesis via perturbations of the growth of embryos that involved the isolation and culture of embryos at various stages of development.

Taken together, these analyses yielded only a limited understanding as pertaining to the underlying genetic and molecular mechanisms acting together to engender the manifestation of the programme of zygotic embryogenesis in angiosperms. However, in the wake of the 1990s novel and innovative techniques such as the isolation procedures of viable gametes in several species of higher plants (Theunis *et al*. 1991), micromanipulation of single cells, elaboration of the technique of fusing gametes of opposite sexes under controlled conditions (Kranz *et al*. 1991) and gene

expression studies permitting the identification of genes expressed at certain developmental stages on the single cell level (Sauter *et al*. 1998) ushered in a new era in the embryology of angiosperms. In the first half of the 1990s the main approach taken to address this issue has been to isolate and study mutants (Clark and Sheridan 1991; Jürgens 1995). An alternative way to study fertilisation-related and early embryogenesis-specific genes and their regulation was through exploiting the somatic embryogenesis system based on the observed similarities both at the morphological and genetic level of zygotic *versus* somatic embryogenesis induced by experimental manipulation (Dudits *et al*. 1991; Zimmerman 1993). These latter studies are, however, restricted to embryos at a multicellular stage leaving questions of paramount importance such as: how the first, typically asymmetrical cell cleavage of the zygote is triggered, what mechanisms ensure it and whether it can be reproduced *in vitro* off the maternal tissue, and what elements are incorporated into the intertwining cascade mechanisms of signal transduction of fertilisation, unanswered.

The technique of isolation of viable gametes from higher plants and *in vitro* fertilisation offer a new approach to address the question as to how zygotic embryogenesis unfolds at the cellular, biochemical and molecular level. The initial studies established the chronology of the cytological events of fertilisation and provided a time course for the formation of cell wall around the fertilsed egg cell and the division of *in vitro*-produced maize zygotes (Faure *et al*. 1993; Kranz *et al*. 1995). As a pioneering study to analyse gene expression in the course of fertilisation and early embryogenesis in higher land plants, representative cDNA libraries were generated from unfertilised egg cells of maize (Dresselhaus *et al*. 1994). These libraries were then analysed by differential screening methods and gene specific probes (Dresselhaus *et al*. 1996). In this manner, over 50 different up-or down-regulated transcripts have been identified some of which were found to be induced by fertilisation after several hours elapsed from gamete fusion (preceding the division of the zygote). Novel RT-PCR methods suitable for dissecting gene expression at the single cell level (Richert *et al*. 1996) revealed that the *cdc2* gene in maize is constitutively expressed prior to fertilisation and in the duration of zygotic development. Cyclins, however, exhibit a more varied pattern of gene expression: the mitotic B-type cyclin is newly expressed 24 h following fertilisation, whereas a second B-type cyclin was seen to oscillate (transcripts were detected between 12-14 h, 24-26 h and approximately 36 h after *in vitro* gamete fusion). The A-type cyclin (Zeama; CycA1;1) is first entirely down-regulated then abruptly induced 17 h following IVF. All identified maize A-and B-type cyclins (Renaudin *et al*. 1994, 1996) are presumably mitotic cyclins and expressed in G_2-phase of the cell cycle (Jacobs 1995). One, however, needs to be aware that all these molecular analyses have been impleented at the transcriptional level and consequently it needs to be taken into account that some transcripts might not be translated (or if so the proteins produced might not necessarily be active due to post-translational modification as is the case with the cell-cycle regulator cdc2 (Jacobs 1995). Among the isolated cDNAs generated from maize egg cells and zygotes two up-regulated clones were found to encode for proteins involved in RNA stability and processing. It seems that the union between the egg and the sperm cell is required for the expression of a gene encoding an endonuclease essential for DNA repair.

One of the cDNAs identified from the cDNA library is thought to have relevance in the calcium homeostasis of the fertilised egg cell as it was linked to encoding calreticulin. Intriguingly, its expression was demonstrated to be strongly correlated to cell cleavage (Dresselhaus 1996, 1999) and it is probably required as a chaperon to modify other proteins in the lumen of the endoplasmic reticulum (Dedhar 1994). In the light of this finding, the structural changes observed in the ER during the *in situ* development of the wheat egg cell may gain significance by assuming a key role for this cell organelle in the regulation of intracellular Ca^{2+} through the presence of Ca^{2+}-ATPases, Ca^{2+} storage proteins (calsequestrin, calreticulin) and Ca^{2+} release channels in the ER of the angiosperm egg. However, as compared to the wealth of the existing information shedding light on how the ER functions during the activation of the egg in animals studied thus far (Eisen and Reynolds 1985; Kline 1988; Han and Nuccitelli 1990; Kline and Kline 1992; Miyazaki *et al*. 1992; Terasaki *et al*. 1996), the plant kingdom remains unexplored. Numerous studies suggest that the ER is a highly dynamic structure interacting with a variety of cellular constituents, including cytoskeletal elements thus hinting that physiological changes through the modification of the ER play a crucial role in oocyte maturation in animals (Terasaki *et al*. 1986; Allen and Brown 1988; Kachar and Reese 1988; Terasaki and Jaffe 1991). Based on the observations of Pònya *et al*. (2004), the dynamic structure of the ER and the strong labelling of the ER membranes in wheat egg cells by the CTC dye suggest the presence of Ca^{2+} pumps tethered on the membrane meshwork of the endoplasmic reticulum similarly to Ca^{2+}-ATPase pumps in the sarcoplasmic reticulum in animal cells, which may be activated by sperm incorporation during fertilisation leading to the increase in $[Ca^{2+}]_{cyt}$ (cytosolic calcium). Whether the reorganization of the ER during egg cell maturation in wheat has importance for egg activation through calcium-releasing mechanisms is unknown. In animals, a rise in intracellular Ca^{2+} activity plays a substantial role in the activation of the oocyte, which seems to be a universal hallmark of activation of the animal egg. A hypothesis assuming the ER to be involved in calcium signalling in the angiosperm egg appears attractive as in most cells a transient increase in cytosolic Ca^{2+} is brought about by a calcium release from intracellular stores and influx across the plasma membrane. Substantial evidence has accumulated that implicates inositol 1,4,5-trisphosphate (IP_3) as a secondary messenger for stimulating the increase in cellular Ca^{2+} (Berridge 1993). The cellular source of IP_3 is hydrolysis of phosphatidylinositol 4,5-bisphosphate, and inositol lipid metabolism appears to be the pivotal component of numerous cellular responses (Ferris and Snyder 1992). In many of these cell systems the enzymatic pathway known to generate IP_3 appears to be linked to agonist action by GTP-binding proteins. Although the exact role of phosphorylation in plant morphogenesis has been intensely studied over the last couple of years, how G-protein coupled receptors, G-proteins, MAP kinase cascades and other signalling components interact during egg activation remains obscure (Tregear *et al*. 1996). Thus, further investigations should reveal the stimuli which trigger the activation mechanisms in the quiescent angiosperm egg cell upon fertilisation. In the light of the assumption that free calcium release originates from a hypothesized internal calcium store, which is surmised to be induced by IP_3 (Malhó 1998), the finding of Han *et al*. (2002) that microinjection of caged IP_3 triggers $[Ca^{2+}]_{cyt}$ elevation in the central cell of *Torenia fournieri*, is intriguing. Capitalising on the technique elaborated for the microinjection of isolated wheat egg cells, the issue of the signal transduction pathways unleashed by the sperm in the wheat egg during fertilisation could be dissected. Microinjection of caged IP_3 into isolated wheat egg cells and its subsequent photolysis coupled with measurement of $[Ca^{2+}]_{cyt}$ could shed light on the putative role of IP_3 and calcium in egg activation which may act in unison during signal transduction of fertilisation, the latter forming a 'fertilisation signal' (Musgrave *et al*. 1993; Digonnet *et al*. 1997). This approach may reveal if IP_3 plays a role in transducing the signal conveyed by the sperm cell to the intracellular calcium store of the wheat egg. A hypothesis assuming that Ca^{2+} release in the angiosperm female gamete is induced by a change in IP_3-receptors (such as an increase in the receptor number or a change in the phosphorylation state of the receptors) (Fujiwara *et al*. 1993) is supported by the observation that the amount of Ca^{2+} released upon IP_3 injection in the mouse egg can be elevated if IP_3-sensitive stores

are first sensitised by treatment with the sulfhydride reagent, thimerosal (Mehlmann and Kline, 1994), supposing that similar mechanisms are triggered in the angiosperm female gamete which act in the animal egg.

The finding that $[Ca^{2+}]_{cyt}$ rise was induced by sperm cell extract injected into *Torenia* central cells (Han *et al*. 2002) hints at the depletion of intracellular calcium store(s) causing cytoplasmic calcium elevation as opposed to hypothesizing the involvement of calcium influx in calcium rise. In addition, or alternatively, the ability of the angiosperm egg cell to develop a Ca^{2+} release mechanism during its maturation could be a consequence of the reorganisation of the ER. Therefore, as a result of the elevation of the IP_3 level, more Ca^{2+} may be released from the ER clusters, leading to locally higher Ca^{2+} levels, which would then entail higher IP_3 sensitivity. Thus, it may be speculated that the structural changes in the ER observed in the wheat egg during its *in situ* development may account for the pronounced ability of a mature egg to release Ca^{2+} in response to IP_3 or sperm integration. The application of the microinjection technique elaborated for isolated wheat egg cells could allow also for the introduction of sperm extract into isolated egg cells which subsequently would help disentangle whether the cytosolic calcium rise measured by Digonnet *et al*. (1997) in maize is triggered by gametic fusion or induced by an activation factor delivered by the sperm cell into the egg's cytoplasm. Attempts towards identification of the source of origin of the transient rise in $[Ca^{2+}]_{cyt}$ during fertilisation in egg cells of higher plants (Digonnet *et al*. 1997) could profit from experiments carried out in animals. For instance, Terasaki and Sardet (1991) demonstrated that the cortical ER network in the oocyte of sea urchin is a target of inositol 1,4,5 trisphosphate-mediated calcium release, and could also be implicated in the propagation of calcium waves. A particularly important aspect of ER organisation in the maturing wheat egg is its polar distribution (Pónya *et al*. 2004), which was first described in the *Xenopus* egg (Gardiner and Grey 1983), where differences in the density of ER-plasma membrane junctions were hypothesised to be associated with the preference for sperm entry in the animal hemisphere, and linked to the decreased velocity of the calcium wave at fertilisation in the vegetal hemisphere (Busa and Nuccitelli 1985; Kline and Nuccitelli 1985).

In searching for clues as to how the cell cycle machinery is activated in the angiosperm egg fertilised by the sperm, zygotic gene activation (ZGA) studies were launched in analogy to the animal systems in which ZGA has been demonstrated to be in command of expressing the abundant maternal transcripts stockpiled in the unfertilised egg cell. In mammals, the onset of ZGA has been shown to occur between the two-cell (mouse, hamster) to sixteen-cell stage (sheep, cow, for review see: Schultz 1993). Therefore, the embryo's own genome is inactive in the initial rounds of cell divisions. In other thoroughly examined animal species, like *Caenorhabditis elegans*, the primary embryonically transcribed RNAs were not detected until the three-four cell stage (Seydoux *et al*. 1996), or even later at the midblastula transition stage in *Xenopus* and zebra fish, by which developmental phase the embryo consists of thousands of cells (Newport and Kirschner 1982; Zamir *et al*. 1997). In *Drosophila* a wealth of information is available concerning ZGA: for example, the zygotic genome is not transcribed before the 11^{th} cell cycle round, when the embryo body is a syncytium comprising of a few thousand nuclei. Transcription is incipient only at cycle 14, at the beginning of the cellularisation stage (Edgar and Schubinger 1986; Orr-Weaver 1994).

The first division of the fertilised egg cell in higher plants as the initiating step of zygotic embryogenesis represents a typical example of asymmetric cell division the study of which is of utmost importance for the investigation of differentiation since it produces two daughter cells that are intrinsically different from the time they are produced hence providing a good system for cell lineage and differentiation studies. In gaining insight into the processes that regulate the zygotic cell division cycle in higher plants, IVF is a powerful method since, combined with molecular techniques allowing for gene expression studies at the single cell level, it can be exploited to define the time frame spanning from the time of gamete fusion to the proembryo stage in terms of cell cycle gene expression. Using gamete isolation and IVF, cyclin-dependent kinases (CDKs) as well as cyclins have been identified in several plant species (Renaudin *et al*. 1994; Jacobs 1995; Renaudin *et al*. 1996) and their function in plant development analysed (Hirt 1996; Shaul *et al*. 1996). These studies indicated that compared to the animal systems examined hitherto the switch from maternal to embryonic control occurs at a much earlier developmental stage in higher plants. In *Arabidopsis* used as a model system in studying zygotic gene activation and zygotic embryogenesis, the control of early stages of embryo and endosperm development is assumed to be mainly under maternal control (Vielle-Calzada *et al*. 2000). However, the typical sporophytic 3:1 segregation ratio observed in the phenotypes of numerous lethal *Arabidopsis* mutants embryos points to the possibility of no preponderant maternal effect in the course of early embryogenesis. Moreover, the expression of paternal alleles has been detected as early as 4 hours following *in vitro* fertilisation of maize egg cells, which, intriguingly, occurred at the time of chromatin decondensation preceding translational activity ensuing six hours after fertilisation (Weijers *et al*. 2001; Kohler *et al*. 2005). Analyses of *de novo* zygotic genome activity in maize revealed that the contribution to early embryogenesis and endosperm development of paternal *versus* maternal alleles varies significantly in terms of both the expression of genes and the timing of their expression (Sauter *et al*. 1998). Dissecting developmentally regulated mechanisms of cell differentiation from fertilisation onwards must be concerned also with epigenetic changes (modification of chromatin structure and/or DNA methylation and by modulating transcript stability or translation) (Steimer *et al*. 2004). The underlying genetic and molecular mechanisms of cell fate determination in angiosperms and decisions on cell specification during zygotic embryogenesis are largely unknown.

Different gene expression profiles of specific plant cells suggest that their specification can be traced back to mechanisms established during early embryonic development. For instance, the expression of ATML1 gene (*Arabidopsis thaliana* meristem L1 layer), which encodes a homeodomain protein, can be detected in the apical daughter cell deriving from zygotic division and in all proembryo cells until the eight-cell stage is reached, whereas at the 16-celled embryo stage, mRNA expression is confined to the protoderm. ATML1is thought to be part of the cell specification machinery controlling pattern formation based on cues delineating morphogenetic boundaries of positional information (Lu *et al*. 1996). Albeit a rigorous proof is still lacking as to coupling differences in sister cells with asymmetric distribution of molecules in the mother cell, a fundamental hypothesis of developmental biology is that specific molecules localised within regions of the cytoplasm of a mother cell can be distributed unequally to its own sister cells and can act as developmental determinants to cause these daughters to express distinct characteristics and have different fates as a result of asymmetric cell cleavage (Jürgens 2001). Cytoplasmic localisation appears to provide an appealing basis in calling for explanations for intrinsically determined asymmetric cell division, although other mechanisms may also be envisaged to play a role in producing daughter cells with different function. (For instance, the asymmetric placement of the mitotic spindle would entail daughter cells of different sizes, which would in turn account for differing function or fate.) Besides asymmetrically segregated cytoplasmic molecules, which could be considered to be developmental determinants and therefore useful in distinguishing sister cells, cell fate seems to depend also on positional signalling cues among which graded concentrations of auxin was shown to play an important role in the establishment of pattern formation of the young *Arabidopsis* embryo

(Jürgens 2001). An efflux-dependent auxin gradient was demonstrated to be involved in determining the apical-basal axis of *Arabidopsis* zygotic embryos (Friml *et al*. 2003) by ensuring a directional intracellular flow of auxin based on the polarised localisation of putative auxin transporters of the PINFORMED (PIN) family due to vesicle transport mediated by GNOM/EMB30 (GN) activity (Shevel *et al*. 1994; Busch *et al*. 1996; Steinmann *et al*. 1999; Geldner *et al*. 2001). Further support for the hypothesis arguing for an essential role of polar auxin transport in the establishnment of the developmental axis of the zygotic *Arabidopsis* embryo derived from the analysis of mutants which clearly showed that apical-basal pattern formation was altered by changes in PIN 1 location in mutants (Meyer *et al*. 1993). Analysis of the *Arabidopsis BODENLOS* (*BDL*) gene brought to light another particular auxin response during pattern formation: the auxin response protein encoded by the BDL gene inhibits MONOPTEROS (MP)-mediated embryo patterning (Hamann *et al*. 2002). The division plane, which appears to be under stringent cytoskeletal control in the wheat zygote (Pónya and Barnabás 2001), is altered in the apical daughter cell of the *Arabidopsis* zygote in *bdl* and *mp* mutants. Mutations in the MP and BDL genes also cause perturbation later on in the course of embryo development: the surmised hypophysis fails to cleave asymmetrically and subsequently to produce the precursors of the quiescent center and the lower-tier stem cells of the root meristem (Berleth and Jürgens 1993; Hamann *et al*. 1999). *Arabidopsis* with its complete genome sequenced not only offers a unique possibility for genetic studies, but its fixed pattern of embryo formation allows for tracing the origin of seedling structures back to the region in the early embryo (Mansfield *et al*. 1991; Laux *et al*. 2004) through mutant analysis.

However, a detailed description of early embryogenesis in *Arabidopsi*s has to rely on indirect (mutant) analysis since the female gametophyte of higher plants is not easily accessible due to the "dual nature" of the tissues and cells surrounding the developing zygote/proembryo: the sap of the embryo sac is rather liquid-like meanwhile the sporophytic ovular tissues and the ovary wall are solid and full of cuticular tissues. However, based on the newly developed gamete isolation techniques in the early 1990s (Kranz *et al*. 1991) it became possible to isolate viable gametes of opposite sexes in maize and via their elecrofusion the production of *in vitro* zygotes became possible. The "artifical" zygotes produced in this manner give rise to proembryos the unequal division of which mimics the asymmetrical cell cleavage of the two-celled embryo *in planta* (Kranz *et al*. 1991; Kranz and Lörz 1993; Kranz *et al*. 1995) enabling one to observe directly this cell division typically occurring in the zygote of higher plants (Scholten *et al*. 2002; Hoshino *et al*. 2004; Okamoto *et al*. 2004). In wheat, however, this cleavage of the fusion product is a symmetrical division (Okamoto and Kranz 2005), which calls for explanation. In addition, recently a procedure was elaborated for the isolation of the apical and basal cells from maize proembryos creating the basis for the analysis of up- or down-regulated genes in the apical *versus* the basal cell (Okamoto *et al*. 2005).These micromanipulation techniques performed at the single cell level, when combined with the most advanced technologies in proteomics, proved to be a powerful approach in molecular analyses and in protein identification in the apical and basal cell of the two-celled maize embryo (Okamoto *et al*. 2004). cDNAs synthesized from egg cells, apical cells, basal cells, two-celled embryos and multicellular embryos were used as templates for performing randomly amplified polymorphic DNA (RAPD) PCR (Okamoto and Kranz 2005). Based on the patterns of the DNA-bands detected in the gels, these authors categorised the expression patterns into six groups. According to this classification *Group 1* contained a DNA-band, which was detected in the apical cell, proembryo and multicellular embryo of maize, whereas it was not detectable in the egg cell or basal cell, therefore it was assumed to be derived from a gene transcript that was up-regulated exclusively in the apical cell. *Group 2* represented a DNA-band detectable in the basal cell, two-celled embryo and multicellular embryo, but not in the egg cell or apical cell, thus it was supposed to stem from a gene transcript that was up-regulated only in the basal cell. In *Group 3* a DNA-band was observed in the apical cell, basal cell, two-celled embryo and multicellular embryo, but not in the egg cell hence suggesting a gene transcript synthesised *de novo* in both the apical and basal cells following fertilisation.

In *Group 4*: A DNA-band observed in the egg cell-, basal cell-, the proembryo and the multicellular embryo-derived samples, but not in the apical cell, was surmised to originate from a gene transcript that was down-regulated only in the apical cell after sperm-egg fusion. *Group 5* revealed a DNA-band detected in the egg cell, apical cell, two-celled embryo and multicellular embryo, but not in the basal cell indicating a gene transcript that was assumed to be down-regulated only in the basal cell. In *Group 6* a DNA-band appeared in all cell/embryo types, consequently it was judged to be amplified from a gene transcript that was constitutively expressed in all cell/embryo types. Following sequencing of the DNA-bands detected and expression pattern verification, BLAST analysis revealed cDNA clones (amplified DNA-bands) one of which (Clone 72) was found to bear close resemblance to the farnesyltransferase (FTase) β-subunit, which is involved in the prenylation of proteins. Interestingly, the *Arabidopsis* FTase β-subunit, encoded by the *ERA1–WIGGUM* gene, is expressed in the embryo proper throughout embryogenesis and had been found to be essential for meristem formation (Yalovsky *et al*. 2000; Ziegelhoffer *et al*. 2000). Clone one showed similarity to an *Arabidopsis* ubiquitin-specific protease demonstrated to be involved in early embryo development (Doelling *et al*. 2001). Clone 97 showed (low) homology to the B subunit of the CCAAT-binding factor previously found to be a crucial embryogenesis regulator in *Arabidopsis* (Lotan *et al*. 1998; Kwong *et al*. 2003; Lee *et al*. 2003). In the light of the key role that auxin plays in cell polarity and cell fate decision-related mechanisms, the finding that Clone 29 showed similarity to the *Arabidopsis* ubiquitin-related protein termed TIR1 protein (which functions in the auxin response pathway via the COP9 signalosome) is particularly intriguing (Ruegger *et al*. 1998; Schwechheimer *et al*. 2001, 2002.). Its expression was suppressed only in the apical cell after fertilisation suggesting that the auxin-responsive capability of the apical *versus* basal cell differs.

Further analysis of these up- or down-regulated genes putatively involved in controlling early embryogenesis with special regard to the monitoring of the temporal expression of key regulatory genes which is possible by employing IVF coupled with the technique permitting the isolation of the apical and basal cell of the 2-celled embryo paves the way for identifying embryogenesis-related genes that have not yet been characterized as yet. Perhaps the greatest advantage that the system elaborated by Okamoto and Kranz (2005) is that it makes it possible to study the underlying mechanisms of cell polarity induction and unequal division during zygote/proembryo development in higher plants. Coupled with what we already know about auxin response regulating proteins, IVF and single cell monitoring of egg cells, zygotes and proembryonic cell pairs may become a powerful system allowing for the subcellular localisation of molecules putatively involved in polarity induction through either immunocytological methods conducted on the single egg cell and zygotes level (Hoshino *et al*. 2004) or by a transgenic approach using green fluorescent proteins (Scholten and Kranz 2001). Another approach capitalising on this experimental system would be the subcellular localization of mRNA molecules in analogy to organisms such as *Fucus* and developing animal embryos in which localisation of transcripts proved to be a powerful tool in unraveling mechanism of cell polarity through targeting gene products to a specific region of a cell or embryo (Choi *et al*. 2000; Kloc *et al*. 2002; Hamada *et al*. 2003).

Analysis of this kind conducted in the angiosperm zy-

gote enable one to investigate the mechanisms responsible for cytoplasmic localisation of molecules involved in regulating development similarly to studies aimed at dissecting the importance of localised RNA and protein products within the egg of *Drosophila* (Driever and Nüsslein-Volhard 1988). For example, genes found to be up-regulated in the apical or basal cell of the maize proembryo and expressed in the early zygote suggest that their transcripts are localised in the putative apical and basal region of the zygote, although a fast degradation of the transcripts immediately after zygotic cleavage cannot be unequivocally excluded (Haecker *et al*. 2004; Okamoto *et al*. 2005). As regards down-regulated genes in the apical or basal cell, they hint at the possibility that the transcripts from these genes may exhibit a uniform distribution in the maize female gamete and become distributed to the apical and basal regions of the zygote upon fertilisation, or alternatively these transcripts may reveal subcellular localisation already in the egg cell. Localisation of mRNA molecules in isolated egg cells/zygotes is supposed to be an essential part of the events leading to the selection/fixation of polarity axis and may be crucially important in preparing for asymmetrical division of the zygote, thus microinjection of RNAs conjugated to a fluorescent label and their subsequent visualisation may provide clues in elucidating the mechanisms of cell polarity and asymmetrical cell division. A transgenic approach making use of GFP-based gene technology assisted by the availability of egg cell/zygote specific promoters coupled with IVF and zygote isolation procedures should reveal whether to what extent and in what ways these mechanisms are under the control of the maternal tissue.

IVF as a tool to identify proteins in the angiosperm egg

Since the set of major proteins present in differentiated cells reflects cell identity and function, identification of the major protein components in different cell types facilitates shedding light on cellular processes and helps decipher the interactions of various proteins in a plant cell. Therefore, investigating the protein profile of the angiosperm egg provides clues as to female gametogenesis, fertilisation and proembryo formation in higher plants. Based on this approach, Okamoto *et al*. (2004) identified the major protein components expressed in isolated maize egg cells with the aim of likening the major proteins identified in the animal *versus* the plant female gamete and of setting the ground for the localisation of the putatively polar distribution of maternally expressed egg proteins which was envisioned to be a vital part of the mechanisms leading to the asymmetrical division of the zygote in higher plants. Their work supplied valuable information on the protein profile of the maize female gamete. Protein analyses of major proteins in maize egg cells have revealed that, unlike mammal oocytes, the angiosperm egg does not contain chaperons as major protein components (Okamoto *et al*. 2004) hence showing a considerable difference between the mammal and the angiosperm egg. Nonetheless, a major similarity between the eggs of the two kingdoms is that they both can be categorised as so-called "catering-type" eggs as they do not contain yolk proteins as storage proteins (Okamoto *et al*. 2004), a finding which is in line with the tenet that the nutrients throughout embryo development and seed formation are continuously supplied by the mother plant. By using scaled-down one-and two-dimensional polyacrylamide gel electrophoresis, highly sensitive liquid chromatography and tandem mass spectrometry (LC-MS/MS) technology, Okamoto *et al*. (2004) detected traceable amounts of proteins derived from a few egg cells isolated from maize. According to the experimental data, the maize egg cell abounds in cytosolic glycolytic enzymes, mitochondrial ATP synthase β-subunit, adenine nucleotide translocator (Winning *et al*. 1992) and annexin p35. The comparison of the expression profiles of annexin p35 in the zygote, 2-celled embryo, central cells and cultured cells revealed strong expression only for egg cells (Okamoto *et al*. 2004), an interesting result in the light of the many versatile functions the members of this group had been demonstrated to have: they are mainly Ca^{2+}-and phospholipid-binding proteins, but are also implicated in membrane trafficking, ion transport, mitotic signaling, cytoskeleton rearrangement and DNA replication in animal cells (for review see: Gerke and Moss 2002). Importantly, annexins found in plants bear structural similarity to their animal counterparts reflected also by sharing the basic properties as Ca^{2+}-dependent membrane-binding molecules (Pirck *et al*. 1994; Clark and Roux 1995; Battey *et al*. 1996). Further, extensive studies have also assigned a role for annexin in exocytosis and Golgi-mediated secretion of newly synthesized plasma membranes and cell wall materials in plant cells (Carroll *et al*. 1998; Battey *et al*. 1999; Clark *et al*. 2001). Annexin p35 identified in the unfertilised maize egg hints at a potential involvement of the protein in the rapid formation of the cell wall around the zygote (cell wall formation around the zygote starts 30 seconds! after *in vitro* fusion of the gametes of opposite sexes) (Kranz *et al*. 1995) suggesting that cell wall materials are stockpiled in the unfertilised egg cell prior to fertilisation the secretion of which may be facilitated possibly by exocytosis which may be mediated by annexin p35. Based on the Ca^{2+}-binding properties of annexins, this supposition seems likely since it is widely known that Ca^{2+} has a regulatory impact on exocytosis both in plants and in animals (Bush 1995; Battey *et al*. 1999). In support of this hypothesis stands the calcium rise measured in the cytoplasm of isolated maize eggs during their fusion with the sperm induced by extracellular calcium putatively through extracellular calcium influx (Digonnet *et al*. 1997) (which may have its source in the degenerated synergid *in planta*). Alternatively, or additionally, a hypothesis envisaging an intracellular calcium rise having its origin from an intracellular calcium store is equally attractive. Based on the results of Pónya *et al*. (2004) that the endoplasmic reticulum appears to be the main calcium store accumulating considerable amount of calium in the ripe wheat egg lends credit to this assumption.

The cytosolic glycolytic enzymes identified from the bands (cytosolic glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and triosephosphate isomerase) known to be involved in glycolysis (Plaxton 1996) were recently shown to be associated with the outer membranes of mitochondria, hinting that this spatial compartmentation of glycolysis permits pyruvate supply directly into the mitochondrion (Giege *et al*. 2003). The presence of mitochondrial proteins identified as major proteins in maize eggs (Okamoto *et al*. 2004) may be accounted for by the energy-demanding series of early cytological zygotic events such as: migration of cytoplasmic organelles towards the nucleus, cell wall formation and nuclear division ensuing following IVF (Kranz *et al*. 1995). The giant mitochondria observed in the maize egg (Faure *et al*. 1992) (and in geranium, Kuroiwa and Kuroiwa 1992) give further support to this hypothesis. In addition, unlike animal mitochondria, which respire fatty acids and glycolytically derived pyruvate, plant mitochondria rarely rely on fatty acids as energy source (reviewed in Plaxton 1996) suggesting that glycolysis is the preponderant 'fuel'-supplying pathway in the plant cell. Intriguingly, glycolysis has been shown to be activated by sperm incorporation into the mouse oocyte (Urner and Sakkas 1999).

Experimental approaches to study double fertilisation in model species

A detailed description of egg cell maturation *in situ* before and after anthesis is hindered by the female gametophyte being deeply encased by the sporophytic maternal tissues. However, based on the newly developed isolation techniques in the early 1990s it became possible to observe egg cells, synergids and central cells microscopically under *in vitro* conditions.

The elaboration of the embedding technique of female

gametes in ultra low gelling temperature agarose made it possible to study the ultrastucture of isolated egg cells and zygotes and the dynamics of nuclear quantities of the fertilised egg cell of barley has been established using microfluorometry (Mogensen and Holm 1995). The quantisation of DNA content during sperm-egg fusion and karyogamy has been performed also in wheat by Pónya *et al*. (1999) using the same technique suitable for DNA content analysis at the individual cell level. In order to clarify the ultrastructure of rice zygotic embryogenesis *in vivo* and compare the disclosed phenomena with the ultrastructural changes during somatic embryogenesis *in vitro* Maeda E and Maeda K (1990) studied the egg cell apparatus of rice after anthesis developing an ultra-thin sectioning procedure and could obtain results concerning the development of zygotic embryos of *Orzya sativa*. By capitalising on a clearing method, the process of double fertilisation in *Zea* mays and the concomitant events were analysed by Mól *et al*. (1994). The investigation was aimed at giving a cytological basis for *in vitro* experimental systems via establishing uniform and controlled conditions (*in vitro* pollination, fusion of isolated gametes and molecular studies upon fertilisation). Although earlier studies described the entry of pollen tubes into maize embryo sac and the fusion of male and female nuclei in the egg cell and the central cell at the light microscopy level, the ultractructure during, and not only before and after, fertilisation has been studied more recently (Mól *et al*. 1994). The development of techniques applicable to isolation of living embryo sac from ovules of several species including *Heliantus anuus* L. (Zhou 1987) opened up new vistas in analysing live female gametophyton which led to new approaches in disentangling the process of double fertilisation, the studying of which until the first half of the 1980s was based on fixed material (Zhou and Yang 1982). These studies focused on the complicated morphological changes taking place during zygotic embryogenesis and on the early events following *in vivo* double fertilisation in higher plants. You and Jensen (1985), who, assuming the possibility of other patterns of the nuclear fusion present in the duration of double fertilisation, described fusion of the sperm nucleus with that of the egg in wheat. However, it was not until the middle of the 1990s (Kranz *et al*. 1995) that the question of the time scale of nuclear fusion in *in vitro* fused gametes of maize was addressed after elaborating the technique of *in vitro* fusing isolated male gametes with female gametoplasts of maize (Kranz *et al*. 1993). These authors demonstrated karyogamy during *in vitro* fertilisation of maize gametes of opposite sexes and by conducting the fixation of the fusion products at precise time intervals after fusion induced by electric pulses they could follow the fate of the male nucleus after cytoplasmic fusion. Early events (such as cell wall formation, first nuclear division and the first division of the zygote) taking place after the commencement of zygote development *in vitro* were also examined (Kranz *et al*. 1995).

The main reason why so few reports are presented in the relevant literature on zygote /proemryo culture is that they must be isolated from the inner sanctum of the ovule in order to be cultured and this is a tedious procedure indeed. Adding up to this difficulty is the very low yield of cells to be analysed during *in vitro* culture thus thwarting attempts to optimise the culture conditions (as no monitoring can be carried out on a mass of cells). From this perspective, the culture of ovaries, ovules and even embryo sacs can be regarded as particularly suitable for fulfilling the purpose of analysing the growth requirements of proembryos and zygotes. Obviously, the exacting task to optimise ovule/embryo culture can be paralleled with the efforts made on devising an appropriate set of culture conditions for the culture of proembryos. In this domain, a pioneering study was done by Maheshwari (1958) who isolated ovules of *Papaver somniferum* containing either zygotes or two-celled proembryos. Rapid growth of the nascent sporophyte was obtained in a medium supplemented with caseine hydrolyzate, yeast extract or kinetin. Other examples such as *Zephyranthes* sp. (Kapoor 1959), *Capsella bursa-pastoris* (Lagriffol and Monnier 1985), *Hordeum vulgare* (Töpfer and Steinbiss 1985; Holm *et al*. 1994), and *Triticum aestivum* (Zenkteler and Nitzsche 1985; Comeau *et al*. 1992) can be cited as successful applications of ovary/ovule culture for inducing the development of enclosed zygotes or proembryos. A perspicuous sign of the success of ovule culture in relation to the development of the growth of the confined embryo is the accelerated growth of the ovular tissues. Stewart and Hsu (1977) found that post fertilisation ovules of cotton can be cultured with a reasonable degree of efficiency if grown on a medium containing low concentrations of IAA, kinetin and GA together with 15 mM NH_4^+. The key to the success of the combined use of hormones applied at a low level and ammonium ions was the discovery that the addition of hormones alone enables the ovules to swell, whereas NH_4^+ was found to promote growth and division of the zygote enclosed in the ovule. More recently certain intermediate systems have been elaborated with the aim of obtaining *in vitro* growth of zygotes and proembryos. One approach involves the culture of zygote-containing embryo sacs of *Z. mays* surrounded by the nucellus with or without the endosperm (van Lammeren 1988; Campenot *et al*. 1992; Mól *et al*. 1993, 1995) and the culture of embryo sacs of *Cucumis* sp. with proembryos and free nuclear endosperm (Custers and Bergervoet 1990). The subsequent transfer of embryos to a medium of a different composition preceded by an initial culture of embryo sacs of *Z. mays* enclosing zygotes or two-celled proembryos is a relatively straightforward procedure for regenerating plants *in vitro* from zygotes (Mól *et al*. 1993, 1995).

The likely extracellular calcium store for the wheat egg is the degenerating synergid (which receives the penetrating pollen tube) as is suggested by Chaubal and Reger (1992b), who, by using freeze-substitution fixation, scanning electron microscopy, and energy-dispersive X-ray microanalysis examined post-pollination calcium distribution in the sister synergids of wheat. This study revealed that both synergids contained relatively high concentrations of calcium. Following pollen-tube discharge, typically one of the sister synergids was found to be devoid of high concentration of calcium, whereas the other synergid retained high concentrations of the element indicating that calcium is rapidly lost from one of the sister synergids after pollen-tube discharge. Based on these findings, it might be speculated that *in planta* the calcium (stored at high concentration prior to fertilisation in the synergids) depletion occurring dramatically in wheat synergids following sperm-egg fusion, as was demonstrated by Chaubal and Reger (1992b), serves the purpose of supplying extracellular calcium needed for the cytoplasmic calcium rise observed by Digonnet *et al*. (1997). In this IVF system, extracellular calcium (5 mM $CaCl_2$), contained in the fusion medium and demonstrated to induce the fusion of the unfertilised maize egg cell with the sperm, is assumed to mimic the *in planta* situation (relatively high extracellular calcium concentration). Therefore, it may be hypothesised that if calcium is present in the synergid that receives the pollen tube, just prior to fertilisation, it may be required to serve as the calcium source for egg activation and alternatively or additionally it may be needed to prepare the sperm cell for fusion with the female gamete as it is suggested by Chaubal and Reger (1992b). The work presented by Antoine *et al*. (2000) argues for the former scenario. Exploiting the Ca^{2+}-selective vibrating probe to measure calcium fluxes during the IVF process in maize, these authors demonstrated a calcium influx in maize eggs induced by the fertilising sperm. The calcium influx, in the form of a wavefront, appeared to propagate from the sperm entry site. Furthermore, artificial induction of calcium influxes unleashed a number of post-fusion events such as cell wall formation lending credit to this assumption. The demonstration of a long-lasting Ca^{2+} influx (Antoine *et al*. 2000) may explain the cytoplasmic rise observed independently by Digonnet *et al*. (1997) in *in vitro* fertilised maize female gametes. However, direct correlation between these two

events and the chronology of these calcium changes in the cytoplasm of the maize egg have yet to be established.

Information on cytoskeletal rearrangements in higher plant zygotes is scarce (Pónya *et al*. 2001), by using single cell manipulation techniques, Hoshino *et al*. (2004) addressed the issue of putative fertilisation-induced changes in microtubular organisation in the fertilised maize egg cell by employing an immunocytochemical approach. In this study, isolated and cultured maize egg cells and developing zygotes of maize were incubated with anti-α-tubulin antibodies in order to examine the subcellular localisation of microtubules. In isolated single egg cells a few cortical microtubules were observed but well organised microtubules were rarely detected whereas distinct cortical microtubules and strands of cytoplasmic microtubules radiating from the nucleus to the cell periphery were observed in developing zygotes. Solely cortical microtubules were observed in zygotes up to 7 h after *in vitro* fertilisation. After this time, radiating microtubules additionally appeared and persisted during zygote development. These results indicate early and pronounced fertilisation-induced changes in microtubular organization in the fertilised egg cell of maize.

CONCLUSIONS AND FUTURE PROSPECTS

In vitro fertilization is a powerful technique in addressing questions of overriding importance in developmental biology of angiosperms. Coupled to *in vitro* culture systems IVF allows for monitoring egg cells/zygotes/artificially produced fusion products deriving from gamete pairs fused *in vitro* hence making the direct observation and continuous monitoring of the developmental pattern of individual cells possible.

However, a purely single-cell culture system superseding the use of nurse cells would be highly desirable in order to follow up the *in vitro* development of the wheat zygote ideally mimicking the *in planta* events leading to morphologically normal and functionally competent zygotic embryos. Once achieved, this *in vitro* cell-culture system could facilitate a great improvement in our understanding as to the extensive changes taking place during *in vivo* zygotic embryogenesis which, by spanning the undifferentiated stage to a multicellular organism, ultimately lead to the formation of a new adult plant. Combining this system with the use of GFP-based marker genes designed for studying subcellular structures permits to analyze cytological characteristics during fertilization and in the course of early zygotic development.

Adapting to wheat the method elaborated by Kranz *et al*. (1998) for producing *in vitro* maize endosperm tissue via using the electrofusion technique would represent an elegant approach for dissecting the coordinated events of double fertilisation in wheat with special regard to establishing what particular characteristics the nutritive tissue may have for ensuring normal zygotic development to occur.

In wheat zygotes the first cleavage is an invariably asymmetrical division, the molecular and cellular mechanisms of which are thought to trigger normal embryonic development unfolding as a well-concerted programme of cellular differentiation. Exploiting IVF coupled with microinjection (which allows for exogenous DNA delivery timed in synchrony of cell cycle stages of the fertilised egg cells or before IVF is carried out) of isolated wheat egg cells, the cascade mechanisms of fertilisation-induced signal transduction may be dissected with particular regard to cellular events such as cytoskeletal changes, membrane trafficking and nuclear movement. Pónya *et al*. (2001) suggest that actin may be involved in polarising the unfertilised female wheat gamete, although it is still to be elucidated whether this polarity is "localised polarity" at the presumable micropylar pole of the cell or one of the signs of "overall polarity". The next challenge will be to monitor spatial and temporal relationships between ion channels, membrane proteins, cytoskeletal anchoring proteins and the cytosolic gradients of ions such as H^+ and Ca^{2+}.

In transgenic research, wheat sperm cells have immense potential as natural agents for DNA delivery to the egg cell. Besides the advantage that the female gametes may be attractive target cells for genetic transformation (through microinjection) upon their isolation as they represent the basis for marker-free selection systems, the attractiveness of the male gametophyte is enhanced by the possibility of superseding tissue culture manipulations altogether (direct DNA uptake into pollen grains or sperm cells prior to pollination). In this respect, wheat evokes much interest. Importantly, studies directed at analyzing modes of nuclear fusion (Tian *et al*. 2005) would facilitate the optimisation of IVF as well as nuclear resynchronization for cloning studies in wheat. Additional studies are required to decipher the relationship between cell cycle stage, DNA levels and karyogamy in wheat for conducting investigations of this kind.

It would be intriguing to combine the calcium-induced fusion system worked out by Faure *et al*. (1994) and Kranz and Lörz (1994) for studying gamete recognition with the experimental approach of Pónya *et al*. (2004) suitable for live imaging of ER to determine whether the sperm-egg fusion mediated by extracellular calcium in the fusion medium occurs at the presumptive "preferential" site. Although the pattern of changes depicted by Pónya *et al*. (2004) in the structure of the ER during the *in situ* development of wheat egg cells may be different in other angiosperm species, the transition by which a young egg is endowed with a highly organised and polarised array of cortical ER clusters might be a common feature of egg cell development. This would point to universal calcium signalling during the fertilisation of the angiosperm egg and would have ramifications in the identifications of intracellular calcium stores in the female gamete of higher plants. The next step taken in the direction of investigations aimed at dissecting the controlling mechanisms of polar axis fixation and cell fate determination would be to identify other molecules with signalling capacities and depict the elements of the signalling cascade of fertilisation. Subsequently, data generated in these studies would help clarify the intricate issue of egg activation which would then assist parthenogenesis research having utmost significance in molecular breeding.

Electrofusion also offers the possibility of fusing female wheat gametes with sperm cells of incompatible species hence circumventing sporophytic incompatibility barriers. Should post-zygotic incompatibility not occur in these artificially produced (inter-generic/inter-specific) hybrid cells, agronomic traits (e.g. cold hardiness) of wheat could be improved.

Although the way by which the angiosperm zygote "spells out" the syllabary of its developmental programme remains a conundrum, combined with what we already know about double fertilisation, experimental results obtained in wheat may help fathoming into the abyss of egg activation as well as may give an impetus to future endeavours aimed at shedding light on sperm-egg interaction in higher land plants.

ACKNOWLEDGEMENTS

Part of the work summarized here was financially supported by the European Commission Research Directorates General under the arrangements of the Marie Curie Individual Fellowship Programme (contract number: QLK5-CT-2002-51591). The assistance of Mrs. Brigitta Varga in the preparation of the manuscript is hereby acknowledged.

REFERENCES

Allen NS, Brown DT (1988) Dynamics of the endoplasmic reticulum in living onion epidermal cells in relation to microtubules, microfilaments, and intracellular particle movement. *Cell Motility and the Cytoskeleton* **10**, 153-163

Antoine AF, Faure JE, Cordeiro S, Dumas C, Rougier M, Feijó JA (2000) A calcium influx is triggered and propagates in the zygote as a wavefront during *in vitro fertilization* of flowering plants. *Proceedings of the National Academy of Sciences USA* **97**, 10643-10648

Bakos F, Darkó É, Pónya ZS, Barnabás B (2003) Regeneration of fertile wheat (*Triticum aestivum* L.) plants from isolated zygotes using wheat microspore culture as nurse cells. *Plant Cell, Tissue and Organ Culture* **74**, 243-247

Baluska F, Volkmann D, Barlow PW (2000) Actin-based domains of the "cell periphery complex" and their associations with polarized "cell bodies" in higher plants. *Plant Biology* **2**, 253-267

Baskin TI, Cande WZ (1990) The structure and function of the mitotic spindle in flowering plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 277-315

Battey NH, James NC, Greenland AJ (1996) cDNA isolation and gene expression of the maize annexins p33 and p35. *Plant Physiology* **112**, 1391-1396

Battey NH, James NC, Greenland AJ, Brownlee C (1999) Exocytosis and endocytosis. *Plant Cell* **11**, 643-660

Berger F, Brownlee C (1994) Photopolarysation of the *Fucus* sp. zygote by blue light involves a plasma membrane redox chain. *Plant Physiology* **105**, 519-527

Berleth T, Jürgens G (1993) The role of the MONOPTEROS gene in organizing the basal body region of the *Arabidopsis* embryo. *Development* **118**, 575-587

Berridge MJ (1993) Inositol triphosphate and calcium signaling. *Nature* **361**, 315-325

Bjorkman T, Leopold C (1987) An electric current associated with gravity sensing in maize roots. *Plant Physiology* **84**, 841-846

Bouget FY, Gerttula S, Shwa L, Quatrano RS (1996) Localization of actin mRNA during the establishment of cell polarity and early cell divisions in *Fucus* embryos. *The Plant Cell* **8**, 189-201

Boutilier K, Offringa R, Sharma VK, Kieft H, Ouellet T, Zhang L, Hattori J, Chun-Ming Liu CH, van Lammeren AAM, Brian LAM, Custers JBM, van Lookeren Campagne MM (2002) Ectopic expression of *BABY BOOM* triggers a conversion from vegetative to embryonic growth. *Plant Cell* **14**, 1737-1749

Busa, WB, Nuccitelli R (1985) An elevated free cytosolic Ca^{2+} wave follows fertilization in eggs of frog, *Xenopus laevis*. *Journal of Cell Biology* **100**, 1325-1329

Bush DS (1995) Calcium regulation in plant cells and its role in signaling. *Annual Review of Plant Physiology and Plant Molecular Biology* **46**, 95-122

Busch M, Meyer U, Jürgens G (1996) Molecular analysis of the *Arabidopsis* pattern formation gene GNOM: Gene structure and intragenic complementation. *Molecular and General Genetics* **250**, 681-691

Campenot MK, Zhang G, Cutler AJ, Cass DD (1992) *Zea mays* embryo sacs in culture. I. Plant regeneration from 1 day after pollination embryos. *American Journal of Botany* **79**, 1368-1373

Campanella C, Andreucetti P, Taddei C, Talevi R (1984) The modification of cortical endoplasmic reticulum during *in vitro* maturation of *Xenopus laevis* oocytes and its involvement in cortical granule exocytosis. *Journal of Experimental Zoology* **229**, 283-293

Cai G, Moscatelli A, Cresti M (1997) Cytoskeletal organization and pollen tube growth. *Trends in Plant Science* **2**, 86-91

CaoY, Reece A, Russell SD (1996) Isolation of viable sperm cells from tobacco (*Nicotiana tabacum*). *Zygote* **4**, 81-84

Cao Y, Russell SD (1997) Mechanical isolation and ultrastructural characterization of viable egg cells in *Plumbago zeylanica*. *Sexual Plant Reproduction* **10**, 368-373

Carlson WR (1986) The B chromosome of maize. *Critical Review in Plant Science* **3**, 201-226

Caroll AD, Moyen C, van Kesteren P, Tooke F, Battey NH, Brownlee C (1998) Ca^{2+}, annexins, and GTP modulate exocytosis from maize root cap protoplasts. *Plant Cell* **10**, 1267-1276

Cass DD (1973) An ultrastructural and Nomarski-interference study of the sperms of barley. *Canadian Journal of Botany* **51**, 601-605

Chaboud A, Perez R (1992) Generative cells and male gametes: Isolation, Physiology and biochemistry. *International Review in Cytology* **140**, 205-232

Chang DC (1989) Cell poration and cell fusion using an oscillating electric fields. *Journal of Biophysics* **56**, 641-652

Chaubal R, Reger BJ (1990) Relatively high calcium is localized in synergid cells of wheat ovaries. *Sexual Plant Reproduction* **3**, 98-102

Chaubal R, Reger BJ (1992a) Calcium in the synergid cells and other regions of pearl millet ovaries. *Sexual Plant Reproduction* **5**, 34-46

Chaubal R, Reger BJ (1992b) The dynamics of calcium distribution in the synergid cells of wheat after pollination. *Sexual Plant Reproduction* **5**, 206-213

Chaubal R, Reger BJ (1993) Prepollination degeneration in mature synergids of pearl millet: An examination using antimonite fixation to localize calcium. *Sexual Plant Reproduction* **6**, 225-238

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES, Peacock WJ (1997) Fertilization-independent seed development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences USA* **94**, 4223-4228

Chiba K, Kado RT, Jaffe LA (1990) Development of calcium release mechanisms during starfish oocyte maturation. *Developmental Biology* **140**, 300-306

Choi SB, Wang C, Muench DG, Ozawa K, Franceschi VR, Wu Y, Okita TW (2000) Messenger RNA targeting of rice seed storage proteins to specific ER subdomains. *Nature* **407**, 765-767

Clark GB, Roux SJ (1995) Annexins of plant cells. *Plant Physiology* **109**, 1133-1139

Clark GB, Sessions A, Eastburn DJ, Roux SJ (2001) Differential expression of members of the annexin multigene family in *Arabidopsis*. *Plant Physiology* **126**, 1072-1084

Clark JK, Sheridan WF (1991) Isolation and characterization of 51 embryo-specific mutations of maize. *Plant Cell* **3**, 935-951

Cleary AL (1995) F-actin redistributions at the division site in living *Tradescantia* stomatal complexes as revealed by microinjection of rhodamine-phalloidin. *Protoplasma* **185**, 152-165

Cohen JJ (1993) Apoptosis. *Immunology Today* **14 (3)**, 126-130

Collas P, Balise JJ, Hofman GA, Robl JM (1989) Electrical activation of mouse oocytes. *Theriogenology* **32**, 835-844

Collucio LM, Tilney LG (1984) Phalloidin enhances actin assembly by preventing monomer dissociation. *Journal of Cell Biology* **99**, 529-535

Comeau A, Nadeau P, Plourde A, Simard R, Maës O, Kelly S, Harper L, Lettre J, Landry B, St. Pierre CA (1992) Media for the *in voulo* culture of proembryos of wheat and wheat-derived interspecific hybrids or haploids. *Plant Science* **81**,117-125

Custers JBM, Bergervoet JHW (1990) *In vitro* culture of embryos of *Cucumis* spp.: heart-stage embryos have a higher ability of direct plant formation than advanced-stage embryous. *Sexual Plant Reproduction* **3**, 152-159

Dedhar S (1994) Novel functions of calreticulin: interaction with integrins and modulation of gene expression. *Trends in Biochemical Sciences* **19**, 269-271

Desprez B, Chupeau MC, Vermeulen A, Delbreil B, Chupeau Y, Bourgin JP (1995) Regeneration and characterization of plants produced from mature tobacco pollen protoplasts via gametosomatic hybridization. *Plant Cell Reports* **14**, 204-209

Digonnet C, Aldon D, Leduc N, Dumas C, Rougier M (1997) First evidence of a calcium transient in flowering plants at fertilization. *Development* **124**, 2867-2874

Doelling JH, Yan N, Kurepa J, Walker J, Vierstra RD (2001) The ubiquitin-specific protease UBP14 is essential for early embryo development in *Arabidopsis thaliana*. *The Plant Journal* **27**, 393-405

Dresselhaus T, Lörz H, Kranz E (1994) Representative cDNA libraries from few plant cells. *The Plant Journal* **5**, 605-610

Dresselhaus T, Hagel C, Lörz H, Kranz E (1996) Isolation of a full-size cDNA encoding calreticulin from a PCR-library of *in vitro* zygotes of maize. *Plant Molecular Biology* **31**, 23-34

Dresselhaus T, Cordts S, Heuer S, Sauter M, Lörz H, Kranz E (1999) Novel ribosomal genes from maize are differentially expressed in the zygotic and somatic cell cycles. *Molecular and General Genetics* **261**, 416-427

Driever W, Nüsslein-Volhard C (1988) The *bicoid* protein determines position in the *Drosophila* embryo in a concentration-dependent manner. *Cell* **54**, 95-104

Dudits D, Bögre L, Györgyey J (1991) Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *Journal of Cell Science* **99**, 475-484

Dumas C, Knox RB, McConchie CA, Russell SD (1984) Emerging physiological concepts in fertilization. *What's New in Plant Physiology* **15**, 17-20

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

Edgar BA, Schubiger G (1986) Parameters controlling transcriptional activetion during early *Drosophila* development. *Cell* **44**, 871-877

Eisen A, Reynolds GT (1985) Source and sinks for the calcium released during fertilization of single sea urchin eggs. *Journal of Cell Biology* **127**, 641-652

Elinson RP (1983) Cytoplasmic phases in the first cell cycle of the activated frog egg. *Developmental Biology* **100**, 440-451

Faure JE, Mogensen HL, Kranz E, Doggonet C, Dumas C (1992) Ultrastructural characterization and three-dimensional reconstruction of isolated maize (*Zea mays* L.) egg cell protoplasts. *Protoplasma* **171**, 97-103

Faure JE, Digonnet C, Dumas C (1994) An *in vitro* system for adhesion and fusion of maize gametes. *Science* **263**, 1598-1600

Faure JE, Dumas C (2001) Fertilization flowering plants. New approaches for an old story. *Plant Physiology* **125**, 102-104

Faure JE, Mogensen HL, Dumas C, Lörz H, Kranz E (1993) Karyogamy after electrofusion of single egg and sperm cell protoplasts from maize: cytological evidence and time course. *The Plant Cell* **5**, 747-755

Ferris CD, Snyder SH (1992) Inositol 1,4,5-trisphosphate-activated calcium channels. *Annual Review of Physiology* **54**, 469-488

Fowler JE, Quatrano RS (1997) Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. *Annual Review of Cell Developmental Biology* **13**, 697-743

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G (2003) Efflux-dependent auxin gradients establish the apicalbasal axis of *Arabidopsis*. *Nature* **426**, 147-153

Fujiwara T, Nakada K, Shirakawa H, Miyazaki S (1993) Development of inositol trisphosphate-induced calcium release mechanism during maturation of hamster oocytes. *Developmental Biology* **156**, 69-79

Gasser CS, Broadhvest J, Hauser BA (1998) Genetic analysis of ovule deve-

lopment. *Annual Review of Plant Physiology* and *Plant Molecular Biology* **49**, 1-24

Gardiner DM, Grey RD (1983) Membrane junctions in *Xenopus* eggs: their distribution suggests a role in calcium regulation. *Journal of Cell Biology* **96**, 1247-1255

Geldner N, Friml J, Stierhof YD, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425-428

Gerke V, Moss SE (2002) Annexins: from structure to function. *Physiological Reviews* **82**, 331-371

Giege P, Heazlewood JL, Roessner-Tunali U, Millar AH, Fernie AR, Leaver CJ, Sweetlove LJ (2003) Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. *Plant Cell* **15**, 2140-2151

Gonzales-Reyes A, Elliott H, St. Johnston D (1997) Oocyte determination and the origin of polarity in *Drosophila*: the role of the spindle genes. *Development* **124**, 4927-4934

Grebenok RJ, Pierson E, Lambert GM, Gong FC, Afonso CL, Haldermann-Cahill R, Carrington JC, Galbraith DW (1997) Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *The Plant Journal* **11**, 573-586

Grossniklaus U, Spillane C, Page DR, Kohler C (2001) Genomic imprinting and seed development: endosperm formation with or without sex. *Current Opinion in Plant Biology* **4**, 21-27

Grossniklaus U, Vielle-Calzada JP (1998) Parental conflict and infanticide during embryogenesis. *Trends in Plant Science* **3**, 328

Grossniklaus U, Vielle-Calzada JP, Hoeppner MA, Gagliano WB (1998) Maternal control of embryogenesis by *MEDEA*, a *Polycomb*-group gene in *Arabidopsis*. *Science* **280**, 446-450

Hable WE, Kropf DL (2000) Sperm entry induces polarity in fucoid zygotes. *Development* **127**, 493-501

Haecker A, Groß-Hardt R, Geiges B, Sarkar A, Breuninger H, Herrmann M, Laux T (2004) Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development* **131**, 657-668

Haig D, Westoby M (1991) Genomic imprinting in endosperm: its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. *Philosophical Transactions of the Royal Society of London Series B* **333**, 1-13

Hamada S, Ishiyama K, Sakulsingharoj C, Choi SB, Wu Y, Wang C, Singh S, Kawai N, Messing J, Okita TW (2003) Dual regulated RNA transport pathways to the cortical region in developing rice endosperm. *Plant Cell* **15**, 2265-2272

Hamann T, Benkova E, Bäurle I, Kientz M, Jürgens G (2002) The *Arabidopsis* BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes and Development* **16**, 1610-1615

Hamann T, Mayer U, Jürgens G (1999) The auxin–insensitive bodenlos mutation affects primary root formation and apical-basal patterning in the *Arabidopsis* embryo. *Development* **126**, 1387-1395

Han JK, Nuccitteli R (1990) Inositol 1,4,5-trisphosphate-induced calcium release in the organelle layers of the stratified, intact egg of *Xenopus laevis*. *Journal of Cell Biology* **110**, 1103-1110

Han YZ, Huang BQ, Guo FL, Zee SY, Gu HK (2002) Sperm extract and inositol 1,4,5-triphosphate induce cytosolic calcium rise in the central cell of *Torenia fournieri*. *Sexual Plant Reproduction* **15**, 187-193

Hanson MR, Köhler RH (2001) GFP imaging: methodology and application to investigate cellular compartmentation in plants. *Journal of Experimental Botany* **356**, 529-539

Hecht V, Vielle-Calzada JP, Hartog MV, Schmidt ED, Boutilier K, Grossniklaus U, de Vries SC (2001) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology* **127**, 803-816

Hirt H (1996) In and out of the plant cell cycle. *Plant Molecular Biology* **31**, 459-464

Holm PB, Olsen O, Schnorf M, Brinch-Pedersen H, Knudsen S (2000) Transformation of barley by microinjection into isolated zygote protoplasts. *Transgenic Research* **9**, 21-32

Holm PB, Knudsen S, Mouritzen P, Negri D, Olsen FL, Roué C (1994) Regeneration of fertile barley plants from mechanically isolated protoplasts of the fertilized egg cell. *Plant Cell* **6**, 531-543

Horwitz A, Duggan K, Buck C, Beckerle MC, Burridge K (1986) Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature* **320**, 531-533

Hoshino Y, Murata N, Shinoda K (2006) Isolation of individual egg cells and zygotes in *Alstroemeria* followed by manual selection with a microcapillary-connected micropump. *Annals of Botany* **97**, 1139-1144

Hoshino Y, Scholten, S, von Wiegen P, Lörz H, Kranz E (2004) Fertilization-induced changes in the microtubular architecture in the maize egg cell and zygote – an immunocytochemical approach adapted to single cells. *Sexual Plant Reproduction* **17**, 89-95

Hu SY, Li LG, Zhu C (1985) Isolation of viable embryo sacs and their protoplasts of *Nicotiana tabacum*. *Acta Botanica Sinica* **27**, 343-347

Huang BQ, Russell SD (1992) Female germ unit: Organization, isolation, and function. *Inernational Review in Cytology* **140**, 233-293

Huang BQ, Pierson ES, Russell SD, Tiezzi A, Cresti M (1993a) Cytoskeletal organisation and modification during pollen tube arrival, gamete delivery and fertilisation in *Plumbago zeylanica*. *Zygote* **1**, 143-154

Huang BQ, Strout GW, Russell SD (1993b) Fertilization in *Nicotiana tabacum* – Ultrastructural organization of propane-jet-frozen embryo sacs *in vivo*. *Planta* **191**, 256-264

Huang BQ, Russell SD (1994) Fertilization in *Nicotiana tabacum*: cytoskeletal modifications in the embryo sac during synergid degeneration. *Planta* **194**, 200-214

Huang BQ, Sheridan WF (1994) Female gametophyte development in maize: microtubular organization and embryo sac polarity. *Plant Cell* **6**, 845-861

Huang BQ, Russell SD (1997) Calcium distribution in fertilized and unfertilized ovules and embryo sacs of *Nicotiana tabacum* L. *Planta* **1**, 93-105

Jacobs TW (1995) Cell cycle control. *Annual Review of Plant Physiology* **46**, 317-339

Janson J, Willemse MTM (1995) Pollen tube penetration and fertilization in *Lilium longiflorum* (Liliaceae). *American Journal of Botany* **82**, 186-196

Jeffrey WR, Meier S (1983) A yellow crescent cytoskeletal domain in *ascidian* eggs and its role in early development. *Developmental Biology* **96**, 125-143

Jensen WA, Fisher DB (1968) Cotton embryogenesis: the entrance and discharge of the pollen tube in the embryo sac. *Planta* **78**, 158-183

Jones AM, Dangl JL (1996) Logjam at the Styx: programmed cell death in plants. *Trends in Plant Science* **4**, 114-119

Jorgensen AO, Broderick R, Somlyo AP, Somlyo AV (1988) Two structurally distinct calcium storage sites in rat cardiac sarcoplasmic reticulum: an electron microprobe analysis study. *Circulation Research* **63**, 1060-1069

Jürgens G (1995) Axis formation in plant embryogenesis: cues and clues. *Cell* **81**, 467-470

Jürgens G (2001) Apical-basal pattern formation in *Arabidopsis* embryogenesis. *The EMBO Journal* **20**, 3609-3616

Kachar B, Reese T (1988) The mechanism of cytoplasmic streaming in characean algal cells: sliding of endoplasmic reticulum along actin filaments. *Journal of Cell Biology* **106**, 1545-1552

Kakimoto T, Shibaoka H (1987) Actin filaments and microtubules in the preprophase band and phragmoplast of tobacco cells. *Protoplasm*a **140**, 151-156

Kalloo G, Chowdhury JB (Eds) (1992) *Distant Hybridization of Crop Plants. (Monographs on Theoretical and Applied Genetics)* Vol 16, Springer-Verlag, Berlin, 271 pp

Kapoor M (1959) Influence of growth substances on the ovules of *Zephyranthes*. *Phytomorphology* **9**, 313-315

Katoh N, Lörz H, Kranz E (1997) Isolation of viable egg cells of rape (*Brassica napus* L.). *Zygote* **5**, 31-33

Keijzer CJ (1992) The isolation of sperm cells, their microinjection into the egg apparatus and methods for structural analysis of the injected cells. In: Cresti M, Tiezzi A (Eds) *Sexual Plant Reproduction*, Springer-Verlag, Berlin, pp 161-172

Keijzer CJ, Reinders MC, Leferinkten Klooster HB (1988) A micromanipulation method for artificial fertilization in Torenia. In: Cresti M, Gori P, Pacini E (Eds) *Sexual Reproduction in Higher Plants*, Springer-Verlag, Berlin, pp 119-124

Kline D (1988) Calcium-dependent events at fertilization of the frog egg: Injection of a calcium buffer blocks ion channel opening, exocytosis, and formation of pronuclei. *Developmental Biology* **126**, 346-361

Kline D, Kline JT (1992) Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Developmental Biology* **149**, 80-89

Kline D, Nuccitelli R (1985) The wave of activation current in the *Xenopus* egg. *Developmental Biology* **111**, 471-487

Kloc M, Zearfoss NR, Etkin LD (2002) Mechanisms of subcellular mRNA localization. *Cell* **108**, 533-544

Kohler C, Page DR, Gagliardini V, Grossniklaus U (2005) The *Arabidopsis thaliana* MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting. *Nature Genetics* **37**, 28-30

Köhler RH (1998) GFP for *in vivo* imaging of subcellular structures in plant cells. *Trends in Plant Science* **3**, 317-320

Koop HU, Schweiger HG (1985) Regeneration of plants after electrofusion of selected pairs of protoplasts. *The European Journal of Cell Biology* **39**, 46-49

Kovács M, Barnabás B, Kranz E (1994) The isolation of viable egg cells of wheat (*Triticum aestivum* L.). *Sexual Plant Reproduction* **7**, 311-312

Kovács M, Barnabás B, Kranz E (1995) Electro-fused isolated wheat (*Triticum aestivum* L.) gametes develop into multicellular structures. *Plant Cell Reports* **15**, 178-180

Köhler RH (1998) GFP for *in vivo* imaging of subcellular structures in plant cells. *Trends in Plant Science* **3**, 317-320

Kranz E, Bauter J, Lörz H (1991) *In vitro* fertilization of single, isolated gametes of maize mediated by electrofusion. *Sexual Plant Reproduction* **4**, 12-16

Kranz E, Dresselhaus T (1996) *In vitro* fertilization with isolated higher plant gametes. *Trends in Plant Science* **1**, 82-88

Kranz E, Lörz H (1993) *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *Plant Cell* **5**, 739-746

Kranz E, Lörz H (1994) *In vitro* fertilization of maize by single egg and sperm

cell protoplast fusion mediated by high calcium and high pH. *Zygote* **2**, 125-128

Kranz E, von Wiegen P, Lörz H (1995) Early cytological events after induction of cell division in egg cells and zygote development following *in vitro* fertilization with angiosperm gametes. *The Plant Journal* **8**, 9-23

Kranz E, von Wiegen P, Quader H, Lörz H (1998) Endosperm development after fusion of isolated, single maize sperm and central cells *in vitro*. *The Plant Cell* **10**, 511-524

Kranz E, Hoshino Y, Okamoto T, Scholten S (2004) Double fertilisation *in vitro* and transgene technology. In: Srivastava PS, Narula A, Srivastava S (Eds) *Plant Biotechnology and Molecular Markers*, Anamaya Publishers, New Delhi, pp 31-42

Kropf DL, Kloareg B, Quatrano RS (1988) Cell wall is required for fixation of the embryonic axis in *Fucus* zygotes. *Science* **239**, 187-190

Kropf DL, Quatrano RS (1987) Localization of membrane-associated calcium during development of fucoid algae using chlorotetracycline. *Planta* **171**, 158-170

Kubota HY, Yoshimoto Y, Yoneda M, Hiramoto Y (1987) Free calcium wave upon activation in *Xenopus* eggs. *Developmental Biology* **119**, 129-136

Kumlehn J, Brettschneider R, Lörz H, Kranz E (1997) Zygote implantation to cultured ovules leads to direct embryogenesis and plant regeneration of wheat. *The Plant Journal* **12**, 1473-1479

Kumlehn J, Lörz H, Kranz E (1998) Differentiation of isolated wheat zygotes into embryos and normal plants. *Planta* **205**, 327-333

Kuroiwa H, Kuroiwa T (1992) Giant mitochondria in the mature egg cell of *Pelargonium zonale*. *Protoplasma* **168**, 184-188

Kwong RW, Bui AQ, Lee H, Kwong LW, Fischer RL, Goldberg RB, Harada JJ (2003) LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *The Plant Cell* **15**, 5-18

Lagriffol J, Monnier M (1985) Effects of endosperm and placenta on development of *Capsella* embryos in ovules cultivated *in vitro*. *Journal of Plant Physiology* **118**, 127-137

Lancelle SA, Cresti M, Hepler PK (1987) Ultrastructure of the cytoskeleton in freeze-substituted pollen tubes of *Nicotiana alata*. *Protoplasma* **140**, 141-150

Lanzendorf SE, Malony MK, Veeck LL, Slusser J, Hodgen GD, Rosenwaks Z (1988) A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertility and Sterility* **49**, 835-842

Laux T, Wurschum T, Breuninger H (2004) Genetic regulation of embryonic pattern formation. *Plant Cell* **16**, S190-202

Leduc N, Matthys-Rochon E, Rougier M, Mogensen L, Holm P, Magnard JL, Dumas C (1996) Isolated maize zygotes mimic *in vivo* zygotic embryonic development and express microinjected genes when cultured *in vitro*. *Developmental Biology* **177**, 190-203

Lee H, Fischer RL, Goldberg RB, Harada JJ (2003) *Arabidopsis* LEAFY COTYLEDON1 represents a functionally specialized subunit of the CCAAT binding transcription factor. *Proceedings of the National Academy of Sciences USA* **100**, 2152-2156

Liu B, Palevitz BA (1992) Organisation of cortical microfilaments in dividing root cells. *Cell Motility Cytoskeleton* **23**, 252-264

Lloyd CW, Traas JA (1988) The role of F-actin in determining the division plane of carrot suspension cells. Drug studies. *Development* **102**, 211-222

Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell* **93**, 1195-1205

Lu P, Porat R, Nadeau JA, O'Neill SD, (1996) Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell* **8**, 2155-2168

Maeda E, Maeda K (1990) Ultrastructure of egg apparatus of rice (*Oryza sativa*) after anthesis. *Japanese Journal of Crop Science* **59**, 179-197

Maheshwari N (1958). *In vitro* culture of excised ovules of *Papaver somniferum*. *Science* **127**, 342

Malhó R (1998) Role of 1,4,5-inositol triphosphate-induced Ca^{2+} release in pollen tube orientation. *Sexual Plant Reproduction* **11**, 231-235

Mansfield SG, Briarty LG (1991) Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. *Canadian Journal of Botany* **69**, 461-467

Marinos NG (1970) Embryogenesis of the pea (*Pisum sativum*). The cytological environment of the developing embryo. *Protoplasma* **70**, 261-279

Matthys-Rochon E, Mól R, Haizmann P, Dumas C (1994) Isolation and microinjection of active sperm nuclei into egg cells and central cells of isolated maize embryo sac. *Zygote* **2**, 29-35

Matthys-Rochon E, Piola F, LeDeunff E, Mol R, Dumas C (1998) *In vitro* development of maize immature embryos: A tool for embryogenesis analysis. *Journal of Experimental Botany* **49**, 839-845

Matzk F (1996) The 'Salmon' system of wheat- a suitable model for apomixes research. *Hereditas* **125**, 299-301

Mayer KF, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T (1998) Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95**, 805-815

McCurdy DW, Gunning BES (1990) Reorganisation of cortical actin microfilaments and microtubules at preprophase and mitosis in wheat root-tip cells: a double label immunofluorescence study. *Cell Motility Cytoskeleton* **15**, 76-87

Mehlmann LM, Kline D (1994) Regulation of intracellular calcium in the mouse egg: Calcium release in response to sperm or inositol trisphospahte is enhanced after meiotic maturation. *Biology of Reproduction* **51**, 1088-1098

Mehlmann LM, Terasaki M, Jaffe LA, Kline D (1995) Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte. *Developmental Biology* **170**, 607-615

Meyer U, Büttner G, Jürgens G (1993) Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. *Development* **117**, 149-162

Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, Tsien RY (1997) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. *Nature* **388**, 882-887

Miyazaki S (1988) Inositol 1,4,5-triphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *Journal of Cell Biology* **106**, 345-353

Miyazaki S, Shirakawa H, Nakada K, Honda Y (1993) Essential role of the inositol 1,4,5-trisphosphate receptor/ Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. *Developmental Biology* **158**, 62-78

Miyazaki Si, Yuzaki M, Nakada K, Shirakawa H, Nakanishi S, Nakade S, Mikoshiba K (1992) Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol-1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science* **257**, 251-255

Mogensen HL (1988) Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. *Proceedings of the National Academy of Sciences USA* **85**, 2594-2597

Mogensen HL (1996) The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany* **83**, 383-404

Mogensen HL, Holm PB (1995) Dynamics of nuclear DNA quantities during zygote development in barley. *Plant Cell* **7**, 487-494

Mordhorst AP, Toonen MAJ, de Vries SC (1997) Plant embryogenesis. *Critical Reviews in Plant Sciences* **16**, 535-576

Mól R (1986) Isolation of protoplasts from female gametophytes of *Torenia fournieri*. *Plant Cell Reports* **3**, 202-206

Mòl R, Matthys-Rochon E, Dumas C (1993) *In vitro* culture of fertilized embryo sacs of maize: zygotes and two-celled proembryos can develop into plants. *Planta* **189**, 213-217

Mól R, Matthys-Rochon E, Dumas C (1994) The kinetics of cytological events during double fertilization in *Zea mays* L. *Plant Journal* **5**, 197-206

Mòl R, Matthys-Rochon E, Dumas C (1995) Embryogenesis and plant regeneration from maize zygotes by *in vitro* culture of fertilized embryo sacs. *Plant Cell Reports* **14**, 743-747

Musgrave A, Schuring F, Munnik T, Visser K (1993) Inositol 1,4,5-trisphosphate as fertilization signal in plants: testcase *Chlamydomonas eugametos*. *Planta* **191**, 280-284

Natesh S, Rau MA (1984) The embryo. In: Johri BM (Ed) *Embryology of Angiosperms*, Springer, Berlin, pp 377-443

Nawaschin SG (1898) Resultate einer Revision der Befruchtungsvorgange bei *Lilium martagon* und *Fritillaria tenella*. *Bulletin de l'Académie Impériale des Sciences de St. Pétersbourg Series* **33**, 39-47

Newport J, Kirschner M (1982) A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell* **30**, 687-696

Nowack MK, Grini PE, Jakoby MJ, Lafos M, Koncz Cs, Schnittger A (2006) A positive signal from the fertilization of the egg sets off endosperm proliferation in angiosperm embryogenesis. *Nature Genetics* **38**, 63-67

Nowack MK, Shirzadi R, Dissmeyer N, Dolf A, Endl E, Grini PE, Schnittger A (2007) Bypassing genomic imprinting allows seed development. *Nature* **44**, 312-315

Nucitelli R (1978) Ooplasmic segregation and secretion in the *Pelvetia* egg is accompanied by a membrane-generated electrical current. *Developmental Biology* **62**, 13-33

Ohad N, Margossian L, Hsyu YC, Williams C, Repetti P, Fischer RL (1996) A mutation that allows endosperm development without fertilization. *Proceedings of the National Academy of Sciences USA* **93**, 5319-5324

Okamoto T, Higuchi K, Shinkawa T, Isobe T, Lörz H, Koshiba T, Kranz E (2004) Identification of major proteins in maize egg cells. *Plant Cell Physiology* **45**, 1406-1412

Okamoto T, Kranz E (2005) *In vitro* fertilization – a tool to dissect cell specification from a higher plant zygote. *Current Science* **89**, 1861-1869

Okamoto T, Scholten S, Lörz H, Kranz E (2005) Identification of genes that are up- or down-regulated in the apical or basal cell of maize two-celled embryos and monitoring their expression during zygote development by a cell manipulation and PCR-based approach. *Plant Cell Physiology* **46**, 332-338

Orr-Weaver TL (1994) Developmental modification of the *Drosophila* cell cycle. *Trends in Genetics* **10**, 321-327

Palermo GD, Cohen JJ, Alikani M, Adler A, Rosenwaks Z (1995) Intracytoplasmic sperm injection: A novel treatment for all forms of male factor infertility. *Fertility and Sterility* **63**, 1231-1240

Pirck M, Hirt H, Heberle-Bors E (1994) The cDNA sequence encoding an annexin from *Medicago sativa*. *Plant Physiology* **104**, 1463-1464

Plaxton W (1996) The organization and regulation of plant glycolysis. *Annual*

Review of Plant Physiology and Plant Molecular Biology **47**, 185-214

Pónya Zs, **Finy P, Fehér A, Dudits D, Barnabás B** (1999a) Morphological characterisation of wheat (*T. aestivum*) egg cell protoplasts isolated from immature and overaged caryopses. *Sexual Plant Reproduction* **11**, 357-359

Pónya Zs, Finy P, Fehér A, Mitykó J, Dudits D, Barnabás B (1999b) Optimisation of introducing foreign genes into egg cells and zygotes of wheat (*Triticum aestivum* L.) via microinjection. *Protoplasma* **208**, 163-172

Pónya Zs, Barnabás B (2001) Microinjected fluorescent phalloidin *in vivo* reveals F-actin dynamics in isolated egg cells of wheat (*Triticum aestivum*, L.) developed *in situ* and fertilised *in vitro*. *Journal of Plant Physiology* **158**, 1527-1539

Pònya Zs, **Kristòf Z, Ciampolini F, Faleri C, Cresti M** (2004) Structural change in the endoplasmic reticulum during the *in situ* development and *in vitro* fertilisation of wheat egg cells. *Sexual Plant Reproduction* **17**, 177-188

Quatrano RS (1973) Separation of processes associated with differentiation of two-celled *Fucus* embryos. *Developmental Biology* **30**, 209-213

Quatrano RS, Shaw SL (1997) Role of the cell wall in the determination of cell polarity and the plane of cell division in *Fucus* embryos. *Trends in Plant Science* **2**, 15-21

Raghavan V (1986) *Embryogenesis in Angiosperms*, Cambridge University Press, Cambridge, 303 pp

Raghavan V (1997) *Molecular Embryology of Flowering Plants*, Cambridge University Press, NY, 712 pp

Ramachandran S, Hiratsuka K, Chua NH (1994) Transcription factors in plant growth and development. *Current Opinion in Genetics and Development* **4**, 642-646

Rambaud C, Blervacq AS, Devaux P, Dubois T, Dubois J, Lammin F, Vasseur J (1996) There is no somatic meiosis in embryogenic leaves of *Cichorium*. *Annals of Botany* **78,** 223-232

Rees BB, Patton C, Grainger JL, Epel D (1995) Protein synthesis increases after fertilization of sea urchin eggs in the absence of an intracellular pH. *Developmental Biology* **169**, 683-698

Reiser L, Fischer RL (1993) The ovule and embryo sac. *The Plant Cell* **5**, 1291-1301

Renaudin JP, Colasanti J, Rime H, Yuan Z, Sundaresan V (1994) Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins. *Proceedings of the National Academy of Sciences USA* **91**, 7375-7379

Renaudin JP, Doonan JH, Freeman D, Hashimoto J, Hirt H, Inzé D, Jacobs T, Kouchi H, Rouzé P, Sauter M, Savouré A, Sorrell DA, Sundaresan V, Murray JAH (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organisation. *Plant Molecular Biology* **32**, 1003-1018

Richert J, Kranz E, Lörz H, Dresselhaus T (1996) A reverse transcriptase-polymerase chain reaction assay for gene expression studies at the single cell level. *Plant Science* **114**, 93-99

Ridgway EB, Gilkey JC, Jaffe LF (1997) Free calcium increases explosively in activating medaka eggs. *Proceedings of the National Academy of Sciences USA* **74**, 623-627

Roberts SK, Brownlee C (1995) Calcium influx, fertilisation potential and egg activation in *Fucus serratus*. *Zygote* **3**, 191-197

Roberts SK, Gillot I, Brownlee C (1994) Cytoplasmic calcium and *Fucus* egg activation. *Development* **120**, 155-163

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes and Development* **12**, 198-207

Russell SD (1983) Fertilization in *Plumbago zeylanica*: Gametic fusion and fate of the male cytoplasm. *American Journal of Botany* **70**, 416-434

Russell SD (1985) Preferential fertilization in Plumbago: Ultrastructural evidence for gamete-level recognition in an angiosperm. *Proceedings of the National Academy of Sciences USA* **82**, 6129-6132

Russell SD (1986) Isolation of sperm cells from the pollen of *Plumbago zeylanica*. *Plant Physiology* **81**, 317-319

Russell SD (1991) Isolation and characterization of sperm cells in flowering plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 189-204

Russell SD (1993) The egg cell: development and role in fertilization and early embryogenesis. *Plant Cell* **5**, 1349-1359

Russell SD (1996) Attraction and transport of male gametes for fertilization. *Sexual Plant Reproduction* **9**, 337-342

Sagata N, Watanable N, Vande Woude GF, Ikawa Y (1989) The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature* **342**, 512-518

Sanders MC, Wang YL (1991) Assembly of actin-containing cortex occurs at distal regions of growing neurites in PC12 cells. *Journal of Cell Science* **100**, 771-780

Sauter M, von Wiegen P, Lörz H, Kranz E (1998) Cell cycle regulatory genes from maize are differentially controlled during fertilization and first embryonic cell division. *Sexual Plant Reproduction* **11**, 41-48

Schmit AC, Lambert AM (1990) Microinjected fluorescent phalloidin *in vivo* reveals the F-actin dynamics and assemlby in higher plant mitotic cells. *The Plant Cell* **2**, 129-138

Schnorf M, Neuhaus-Url, Galli A, Iida S, Potrykus I, Neuhaus G (1991) An improved approach for transformation of plant cells by microinjection: Molecular and genetic analysis. *Transgenic Research* **1**, 23-30

Scholten S, Kranz E (2001) *In vitro* fertilization and expression of transgenes in gametes and zygotes. *Sexual Plant Reproduction* **14**, 35-40

Scholten S, Lörz H, Kranz E (2002) Paternal mRNA and protein synthesis coincides with male chromatin decondensation in maize zygotes. *The Plant Journal* **32**, 221-231

Schultz RM (1993) Regulation of zygotic gene activation in the mouse. *Bio Essays* **15**, 531-538

Schulz R, Jensen WA (1968) *Capsella* embryogenesis: the egg, zygote and the youn embryo. *American Journal of Botany* **55**, 807-819

Schwechheimer C, Serino G, Callis J, Crosby WL, Lyapina S, Deshaies RJ, Gray WM, Estelle M, Deng XW (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science* **292**, 1379-1382

Schwechheimer C, Serino G, Deng XW (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell* **14**, 2553-2263

Seydoux G, Mello CC, Pettitt J, Wood WB, Priess JR, Fire A (1996) Repression of gene expression in the embryonic germ lineage of *C. elegans*. *Nature* **382**, 713-716

Shaul O, Van Montagu M, Inzé D (1996) Regulation of cell division in *Arabidopsis*. *Critical Reviews in Plant Sciences* **15**, 97-112

Shaw SL, Quatrano RS (1996) The role of targeted-secretion in the establishment of cell polarity and the orientation of the division plane in *Fucus* zygotes. *Development* **122**, 2623-2630

Shevel DE, Leu WM, Gilimor CS, Xia G, Feldmann KA, Chua NH (1994) EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec 7. *Cell* **77**, 1051-1062

Shi L, Zhu T, Mogensen HL, Keim P (1996) Sperm identification in maize by fluorescence *in situ* hybridization. *Plant Cell* **8**, 815-821

Sowers AE (1989) The study of membrane fusion and electroporation mechanisms. In: Allen MJ, Cleary SF, Hawkridge FM (Eds) *Charge and Field Effects in Biosystems 2*, Plenum Press, New York, pp 315-337

Staiger CJ, Biggon BC, Kovar DR, Zonia LE (1997) Profilin and actin-depolymerizing factor: modulators of actin organisation in plants. *Trends in Plant Science* **2**, 275-280

Steimer A, Schob H, Grossniklaus U (2004) Epigenetic control of plant development: new layers of complexity. *Current Opinion in Plant Biology* **7**, 11-19

Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Gälweiler L, Palme K, Jürgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316-318

Stewart JM, Hsu CL (1977) *In-ovulo* embryo culture and seedling development of cotton (*Gossypium hirsutum* L.). *Planta* **137**, 113-117

Stone SL, **Kwong LW, Kelly MY, Pelletier J, Lepiniec L, Fischer RL, Goldberg RB, Harada J** (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that induces embryo development. *Proceedings of the National Academy of Sciences USA* **98**, 11806-11811

Sun HQ, Kwiatkowska K, Yin HL (1995) Actin monomer binding proteins. *Current Opinion in Cell Biology* **7**, 102-110

Terasaki M, Chen LB, Fujiwara K (1986) Microtubules are highly interdependent structures. *Journal of Cell Biology* **103**, 1557-1568

Terasaki M, Jaffe LA (1991) Organization of the sea urchin egg endoplasmic reticulum and its reorganization at fertilization. *Journal of Cell Biology* **114**, 1069-1078

Terasaki M, Jaffe LA, Hunnicutt GR, Hammer JA (1996) Structural change of the endoplasmic reticulum during fertilization: Evidence for loss of membrane continuity using the green fluorescent protein. *Developmental Biology* **179**, 320-328

Terasaki M, Sardet C (1991) Demonstration of calcium uptake and release by sea urchin egg cortical endoplasmic reticulum. *Journal of Cell Biology* **115**, 929-940

Theunis CH, Pierson ES, Cresti M (1991) Isolation of male and female gametes in higher plants. *Sexual Plant Reproduction* **4**, 145-154

Tian HQ, Yuan T, Russell SD (2005) Relationship between double fertilization and the cell cycle in male and female gametes of tobacco. *Sexual Plant Reproduction* **17**, 243-252

Tirlapur U, Kranz E, Cresti M (1995) Characterisation of isolated egg cells, *in vitro* fusion products and zygotes of *Zea mays* L. using the technique of image analysis and confocal laser scanning microscopy. *Zygote* **3**, 57-64

Tímár I, Kristóf Z, Barnabás B (1997) Comparative studies on the male and female gametophyte development in three different *Triticum* species. *Plant Science* **126**, 97-104

Töpfer R, Steinbiss HH (1985) Plant regeneration from cultured fertilized barley ovules. *Plant Science* **41**, 49-54

Tregear J, Jouannlc S, Schwebel-Dugue N, Kreis M (1996) An unusual protein kinase displaying characteristics of both the serine/threonine and tyrosine families is encoded by the *Arabidopsis thaliana* gene *ATN1*. *Plant Science* **117**, 107-119

Tyler A (1932) Changes in volume and surface of *Urechis* eggs upon fertilization. *Journal of Experimental Zoology* **63**, 155-173

Urner F, Sakkas D (1999) Characterization of glycolysis and pentose phosphate pathway activity during sperm entry into the mouse oocyte. *Biology of Reproduction* **60**, 973-978

van der Maas HM, Zaal MACM, de Jong ER, Krens FA, van Went JL (1993) Isolation of viable egg cells of perennial ryegrass (*Lolium perenne* L.). *Protoplasma* **173**, 86-89

van Lammeren, AM (1988) Observations on the structural development of immature maize embryous (*Zea mays* L.) during *in vitro* culture in the presence or absence of 2,4-D. *Acta Botanica Neerlandica* **37**, 49-61

Vielle-Calzada JP, Baskar R, Grossniklaus U (2000) Delayed activation of the paternal genome during seed development. *Nature* **350**, 241-243

Vinkenoog R, Spielmann M, Scott RJ (2001) Autonomous endosperm development in flowering plants: how to overcome the imprinting problem? *Sexual Plant Reproduction* **14**, 189-94

Wagner VT, Song YC, Matthys-Rochon E, Dumas C (1989) Observation on the isolated embryo sac of *Zea mays* L. *Plant Science* **59**, 127-132

Weijers D, Geldner N, Offringa R, Jürgens G (2001) Early paternal gene activity in *Arabidopsis*. *Nature* **414**, 709-710

West MAL, Harada JJ (1993) Embryogenesis in higher plants: An overview. *Plant Cell* **5**, 1361-1369

Whitaker M, Swann K (1993) Lighting the fuse at fertilization. *Development* **117**, 1-12

Whittingham DG (1980) Parthenogenesis in mammals. *Oxford Review of Reproduction Biology* **2**, 205-223

Wilson P, Fuller E, Forer A (1987) Irradiations of rabbit myofibrils with an ultraviolet microbeam. II. Phalloidin protects actin in solution but not in myofibrils from depolymerization by ultraviolet light. *Biochemistry and Cell Biology* **65**, 376-385

Winning BM, Sarah CJ, Purdue PE, Day CD, Leaver CJ (1992) The adenine nucleotide translocator of higher plants is synthesized as a large precursor that is processed upon import into mitochondria. *The Plant Journal* **2**, 763-773

Yalovsky S, Kulukian A, Rodriguez-Concepcion M, Young CA, Gruissem W (2000) Functional requirement of plant farnesyltransferase during development in *Arabidopsis*. *The Plant Cell* **12**, 1267-1278

You R, Jensen WA (1985) Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (*Triticum aestivum*). *Canadian Journal of Botany* **68**, 44-48

Zamir E, Kam Z, Yarden A (1997) Transcription-dependent induction of G_1 phase during the zebra fish midblastula transition. *Molecular Cell Biology* **17**, 529-536

Zenkteler M (1995) Self and cross pollination of ovules in test tubes. In: Terzi M, Cella R, Falavigna A (Eds) *Current Issues in Plant Molecular and Cellular Biology*, Kluwer Academic Press, Dordrecht, pp 191-199

Zenkteler M, Nitzsche W (1985) *In vitro* culture of ovules of *Triticum aestivum* at early stages of embryogenesis. *Plant Cell Reports* **4**, 168-171

Zhang J, **Dong WH, Galli A, Potrykus I** (1999) Regeneration of fertile plants from isolated zygotes of rice (*Oryza sativa*). *Plant Cell Reports* **19**, 128-132

Zhelev DV, Dimitrov DS, Doinov P (1988) Correlation between physical parameters in electrofusion and electroporation of protoplasts. *Bioelectrochemistry and Bioenergy* **20**, 155-167

Zhou C (1987) A study of fertilization events in living embryo sacs isolated from sunflower ovules. *Plant Science* **52**, 147-151

Zhou C, Yang HY (1982) Enzymatic isolation of embryo sacs in angiosperms: isolation and microscopical observation on fixed materials. *Acta Botanica Sinica* **24**, 403-407

Ziegelhoffer EC, Medrano LJ, Meyerowitz EM (2000) Cloning of the *Arabidopsis* WIGGUM gene identifies a role for farnesylation in meristem development. *Proceedings of the National Academy of Sciences USA* **97**, 7633-7638

Zimmerman JL (1993) Somatic embryogenesis: A model for early development in higher plants. *The Plant Cell* **5**, 1411-1423

Zuo J, Niu QW, Frugis G, Chua NH (2002) The *WUSCHEL* gene promotes vegetative-to-embryonic transition in *Arabidopsis. The Plant Journal* **30**, 349-59