MUTANT ANALYSIS OF ENDOSPERM DEVELOPMENT IN *ARABIDOPSIS*

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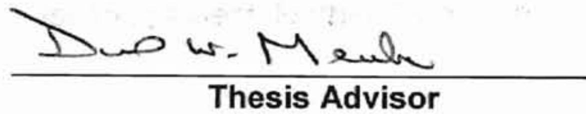

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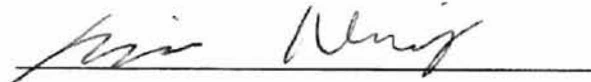

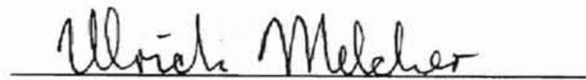

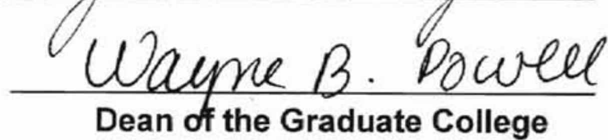

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CHAPTER 1

RESEARCH ON *ARABIDOPSIS* DEVELOPMENTAL GENETICS

1. INTRODUCTION

Model systems are important in investigating fundamental biological principles conserved among organisms. In the plant kingdom, traditional model systems include such agricultural species as maize, tomato, pea, rice, barley, petunia and snapdragon (Meinke, 1993). However, each of the above-mentioned species has limitations, such as long life cycle, large growing space requirements, or large and complex genomes. For many years, there was not one single species generally recognized by plant biologists as the best plant model for a wide range of studies (Meinke et al.,1998).

Arabidopsis thaliana was applied to plant research initially by Laibach in Europe forty years ago, and was studied more carefully by Rédei in the U.S. thirty years ago. It has gradually become a suitable model system for plant physiology, biochemistry, molecular biology, development and genetic research. The first detailed classical genetic map of *Arabidopsis* was published in the early 1980s. Beginning in 1990, the multinational *Arabidopsis* community began to include many laboratories in the U.S., Europe, Australia and Japan. The Multinational Coordinated *Arabidopsis thaliana* Genome Research Project was

initiated to sequence the entire genome by the end of 2000 and subsequently analyze every gene essential for plant growth and development. Since then, standardized BAC and YAC libraries and physical maps have been constructed, and more than 50 Mb of genomic DNA has been sequenced. Genetic stock centers, databases, newsgroups, internet resources and advanced technologies have also been established (Meinke et al., 1997; Meinke et al., 1998). The advances made in *Arabidopsis* research may be applied to other agriculturally-important plant species, other eukaryotes and even human beings. The successful sequencing of *Arabidopsis* genome will also facilitate the human genome sequencing project that is scheduled to be accomplished by 2003.

2. *ARABIDOPSIS* IS A GOOD MODEL SYSTEM FOR GENETIC RESEARCH

2.1 General features of *Arabidopsis*

Arabidopsis thaliana is a small weed in the mustard family (Brassicaceae) that grows in sandy soils throughout Europe, Asia and North America. This plant has many favorable features convenient for genetic analysis. *Arabidopsis* is easy to grow and maintain in laboratory. It starts to produce flowers two or three weeks after planting. The plant reaches maturity in several weeks later. The 2mm long flowers are composed of four sepals, four white petals, six stamens and a central gynoecium (Fig1). Flowers are normally self-pollinating, which greatly facilitates isolating recessive mutants. Cross-pollination can be done in the lab by removing the immature stamens and pollinating manually under a dissecting microscope. Each successfully pollinated flower can produce 20 to 60 seeds, with length about 0.5mm, and located in 1cm long fruits called siliques. One plant can produce several hundred siliques resulting in a total of 5000 seeds.

2.2 Advantages in genetic research

Arabidopsis is a particularly suitable organism for studying the molecular basis of plant developmental genetics via identification and analysis of mutants (Meyerowitz 1987, 1989). *Arabidopsis* is a diploid species, so mutations in many genes are likely to cause recognizable phenotypes. Several thousand mutations

which disrupt many developmental and physiological processes have been identified (Meinke et al., 1998; Meyerowitz and Somerville, 1994). *Arabidopsis* has five pairs of chromosomes and a 120Mb genome, containing approximately 20,000 genes. There is less repetitive DNA in *Arabidopsis* compared to other species, which makes the cloning of genes by chromosome walking feasible. The three interrelated maps of each chromosome (classical genetic, recombinant inbred and physical maps) have been established and published (Meinke et al., 1998). The segregation pattern of F2 plants after self-fertilization of the F1 generation reveals the recombination frequency between genes of interest. The Meinke lab is responsible for gene nomenclature and updating the classical genetic map through the Web, and has cataloged the location of more than 460 mutant genes on this map. The markers including restriction fragment length polymorphism (RFLPs), simple sequence length polymorphisms (SSLPs), cleaved ampli*fie*d polymorphic sequences (CAPs), and cloned genes, expressed sequence tags (ESTs), and the ends of bacterial (BAC) and yeast (YAC) artificial chromosomes (Meinke 1998) have been located on the RI map. Up-to-date information on physical and RI maps is also available through the AtDB website based at Stanford University (http://genome-www.stanford.edu/Arabidopsis). The establishment of a variety of computer resources reflecting the most recent data allows more efficient information exchange and use of resources among researchers all over the world.

3. USING MUTANT ANALYSIS TO STUDY SEED DEVELOPMENT in *ARABIDOPSIS*

3.1 Mutagenesis and mutant analysis of *Arabidopsis*

Mutagenesis and mutant analysis are powerful tools in genetic research. Through studying the mutant phenotype, we can deduce the specific function of a given gene and the way organisms respond to the removal of certain components. There are several kinds of effective tools for mutagenesis in *Arabidopsis*. X-ray was the first method used to cause DNA deletions (Müller, 1963). Ethylmethanesulphonate (EMS) chemical mutagenesis has the advantage of causing a high frequency of point mutations, so that allelic mutations ranging from partial to total loss of function can be produced (Meinke, 1985). However, in order to clone genes disrupted by the above-mentioned two methods, a large amount of work for chromosome walking is required.

The availability of insertional mutagenesis with T-DNA from *Agrobacterium tumefaciens* has greatly facilitated the cloning process. The result of this transformation is that the T-DNA is randomly introduced into wild-type genes and disrupts their normal function (Feldmann and Marks, 1987; Feldmann, 1991; Feldmann and Meinke, 1991; Castle et al., 1993). Moreover, with the introduction of markers conferring antibiotic resistance, the successfully transformed plant can be selected by survival on antibiotic medium. The plant gene sequences flanking the insert can then be recovered by plasmid rescue

(Yanofsky et al., 1990), inverse PCR (Deng et al., 1992), and library construction (Herman and Marks, 1989).

Over 5000 transgenic families produced by T-DNA insertional mutagenesis have been screened by the Meinke lab (Castle et al., 1993). That does not include a more extensive screen recently done in collaboration with Novartis Crop Protection in North Carolina. 36% of the 115 mutants examined in detail by Castle et al. (1993) was tagged. Six classes of mutant embryo phenotypes were defined in preliminary studies: preglobular, globular, transition, cotyledon, fusca and others. The insertional pattern could be diverse and complicated. Approximately 50% of the mutants contain a single site of T-DNA insertion; the other half contained two or more T-DNA inserts. Some T-DNA inserts contain inverted or tandem repeats, or exhibited other complex patterns. Some mutants from this large screening were placed on the classical genetic map and some were cloned by plasmid rescue. However, results did show that some chromosome aberrations were produced by this method. The frequency of untagged mutants was also higher than previously expected (Castle et al., 1993).

3.2 Stages of embryogenesis

Embryogenesis differs greatly between animals and plants. Animals establish their body organization essentially by the end of embryogenesis, while plant embryogenesis only constructs a primary body organization. New structures then keep developing from shoot and root meristems. Detailed studies of plant embryogenesis have been reviewed in many articles (Goldberg et al., 1994; Meinke 1991a, b).

The earliest study on *Arabidopsis* embryogenesis was published by Misra (1962). Embryo differentiation in *Arabidopsis thaliana* follows the classical *Capsella* model of dicot embryo development (Mansfield and Briarty, 1991; Castle and Meinke, 1993; Meinke, 1994); the whole process takes 8-12 days. Embryonic stages (globular, heart, torpedo, and cotyledon stages) are defined by the shape of the embryo (Fig2), which results from position-dependent mitosis, orientation of cell-division planes, and directional cell expansion. After fertilization, cytoplasmic polarization is maintained in zygote. The zygote divides asymmetrically to give rise to a larger basal cell and a smaller apical cell. The former will carry on repeated transverse divisions to form the six- to eight- cell suspensor and hypophysis, while the latter will form the embryo proper. During the globular stage, the hypophysis differentiates into the root meristem and root cap. Cell division in the suspensor finishes at the early globular stage. The suspensor serves as the mechanical support of embryo proper and passes necessary nutrients, metabolites, and growth substances from maternal tissue to embryo proper through plasmodesmata. The suspensor degenerates by the time the seed matures (Castle and Meinke, 1993; Lyndon, 1990).

The apical cell divides 3 times to produce the octant-stage embryo. The first two divisions are longitudinal and the third is transverse. By this stage, the total cell volume is increased very little (Mansfield and Briarty, 1991). Then the first tissue differentiation occurs to form the protoderm which gives rise to epidermis in the future. The continuous cell divisions form the globular stage embryo. The transition from globular to heart stage is called triangular stage,

when the embryo's symmetry switches from radial to bilateral and the cotyledon primordia appear. Also, ground tissue and vascular tissues are being formed by triangular stage. The shoot meristem, which will produce leaves after seed germination, gradually forms between the two cotyledons in the heart stage. Chloroplasts mature at the torpedo stage when two cotyledons elongate and become green. The elongated cotyledons fold down parallel to the hypocotyl at the mature stage--cotyledon stage (Fig 2). Desiccation and dormancy happen before seeds meet appropriate germination conditions. After germination, the root meristem produces the main root. The internal layer of pericycle cells gives rise to the lateral primordia. The shoot meristem forms shoot, rosette leaves and flower primordia after germination.

The *Arabidopsis* inflorescence meristem produces a series of flowers along the growing main stem, so that a succession of siliques with seeds in different embryonic stages are produced, from the older ones to the younger ones, which enables researchers to investigate embryogenesis in different stages within a single plant. Our laboratory has analyzed genes crucial for *Arabidopsis* embryogenesis through mutant isolation and characterization (Meinke 1991a). There are three types of *Arabidopsis* mutants categorized by the Meinke lab: embryonic lethal, embryo defective, and pigment mutant (Meinke 1991b). In the following section, I will review some recent work on embryonic mutations.

4. EMBRYONIC MUTATIONS OF *ARABIDOPSIS*

4.1 Embryonic mutations

In order to identify every gene with an essential function during embryogenesis and understand the complex physiological and biochemical pathways and gene interactions involved, it is important to establish extensive resources of mutants defective in many different genes. A large number of embryonic mutants have been created and studied in detail (Müller, 1963; Meinke and Sussex 1979; Meinke, 1985; Meinke 1986; Errampalli et al., 1991; Mayer et al., 1991; Meinke 1991b). There have been more than one thousand embryo-defective mutants isolated in labs all over the world. Over 250 embryo-defective mutants collected in the Meinke lab show diverse abnormalities in basic cellular metabolism, body pattern and organization, signal transduction pathways, replication, transcription and translation, and regulation of development (Meinke, 1996). The effort to locate the corresponding genes on the classical genetic maps adds more classical markers which facilitates map-based cloning strategies. Different classes of embryo-defective mutants of *Arabidopsis* are reviewed in the next section.

4.2 Review of research on embryo-defective mutants

Deficiencies in basic cellular functions during embryogenesis result in a variety of embryo-defective mutants with distinguishable morphological or physiological abnormalities. Embryo-defective mutants with metabolic defects would die if the deficiency were not compensated, such as the auxotrophic

mutant embryos that survive only on enriched media. Two mutants, *bio1* and *bio2*, disrupted in the biotin synthesis pathway have been studied in Meinke lab (Baus et al., 1986; Schneider et al., 1989; Patton et al., 1998). These mutant embryos range from globular to cotyledon stages and develop into normal plants only when supplied with biotin in culture. The *bio1* mutant is defective in the 7,8-disminopelargonic acid step of biotin synthesis pathway (Shellhammer, 1991; Shellhammer and Meinke, 1990). Since a wild-type copy of the BIOA gene from E. coli could greatly reduce the biotin defect symptoms in *Arabidopsis,* it was apparent that the biotin synthetic pathway was conserved between plants and bacteria (David Patton and Eric Ward, Personal communication; Meinke, 1996). Usually, *bio2* embryos are smaller and arrested earlier (mostly before torpedo stage) than *bio1* mutants which can reach cotyledon stage. The final step of biotin synthesis, from desthiobiotin to biotin, is disrupted in this case (Patton et al., 1998). In Addition, *Arabidopsis* autotropic mutants for thiamine and tryptophan have been isolated as seedling lethals (Li and Rédei, 1969; Last et al., 1991). Diffusion of nutrients from surrounding maternal tissue or the function of redundant genes might be the survival reason for the viability of heterozygous seeds.

By the heart stage, the basic body plan of *Arabidopsis* has been set up. Mutations in the regulatory genes for body pattern development can cause disruption of apical-basal patterns (shoot meristem-cotyledon-hypocotyl-root meristem), radial patterns (protoderm-ground tissue-vascular system), or seedling shapes (Mayer et al., 1991; Castle et al., 1993). Mutants with distorted

cotyledon, hypocotyl, or suspensor have been found in the Meinke lab (Meinke 1985; Baus et al., 1986; Patton and Meinke 1990). Cell division, division plane orientation and expansion all greatly affect body pattern. Mutants altered in genes involved in these processes have been isolated. For example: *titan1*, a mutant that repeats cycling between S phase, interphase and prophase during embryo cell division (Liu and Meinke, 1998); and a *cyt* mutant defective in embryo cytokinesis and cell-wall architecture (Nickle and Meinke, 1998). Mutants defective in early cell division such as *emb30*/gnom, which was thought to have an essential function in directing the initial asymmetrical division of the zygote during early embryogenesis (Jürgens 1994; Jürgens et al., 1994), was shown to facilitate transporting materials to the cell surface (Shevell et al., 1994). Castle and Meinke (1993) proposed two possible roles for body organization genes, which are directly regulating morphogenesis or indirectly affecting morphogenesis through regulation of cellular process (Mayer et al. 1991).

Analysis of embryo-defective mutants can also lead to better understanding of signal transduction pathways. *Fusca* is a group of mutants with inappropriate anthocyanin accumulation in immature cotyledons, which is normally a light regulated process. Mutants of this type were first found by Müller (1963). Twelve *fusca* loci have been identified so far (Castle and Meinke, 1994; Baumlein et al., 1994). Castle found that *FUS6* is closely related to a human sequence, which can rescue a yeast G-protein-mediated signal transduction pathway mutant. *FUS6* plays a role as the repressor of morphogenesis in darkness.

Normally cell divisions in the suspensor stop when it reaches six to eight cells. The suspensor degenerates by the time the seed matures (Lyndon, 1990; Castle and Meinke, 1993; Yeung and Meinke, 1993; Schwartz at al., 1994, 1997). But *sus* mutants have much larger suspensor than normal. Cloning the tagged gene responsible for the *sus2* mutation revealed the gene product to be an important spliceosome assembly factor in RNA processing. There are also other mutants playing roles in DNA replication, transcription and translation processes, such as the *prolifera* mutant involved in DNA replication and another EMB gene encoding a ribosomal protein (Meinke, 1996).

Loss of regulatory functions of genes results in striking phenotypes of many mutants. The *leafy cotyledon* mutant and *twin* embryo mutants are two good examples. Homeotic mutant *leafy cotyledon* has vegetative leaf-like features in place of embryonic cotyledons, which is the consequence of the failure to activate a series of embryo-specific pathways. Mutations in at least three different genes are known to produce this phenotype (Meinke, 1992; Meinke et al. 1994; West et al. 1994; Baumlein et al. 1994; Keith et al. 1994). Those genes positively regulate cell differentiation during embryogenesis and negatively regulate the embryonic cotyledons' potential of turning to vegetative leaves. LEC1 gene was recently isolated. It showed homology to a transcriptional factor, the CCAAT box-binding factor HAP3 subunit (Lotan et al., 1998). Twin embryo and seedlings are generated from the pleiotropic *twin* mutant with incomplete penetrance (Vernon and Meinke 1994, 1995). In this mutant, the extra embryo develops from suspensor cells indicating the

suspensor's further potential besides simply supporting development of the embryo proper. The wild-type gene may function to inhibit the suspensor's potential of become an embryo and therefore regulating morphogenesis of the embryo proper (Vernon and Meinke 1994).

5. BACKGROUND OF RESEARCH IN ENDOSPERM DEVELOPMENT

5.1 General properties of endosperm

Much work has been done to investigate embryogenesis, but researchers have not forgotten other major components of the seed. Endosperm, the product of the second fertilization event and a distinctive feature for flowering plants, has drawn scientists' interests in its evolutionary origin, its developmental processes, its differentiation from the embryo, and its economic value.

Double fertilization, an evolutionary hallmark of angiosperms, was first traced by Nawaschin (1898) one hundred years ago. During the process of fertilization, the pollen tube grows through the micropyle of the embryo sac into one of the two synergids and releases two sperm cells. One sperm fuses with the egg to form the zygotic embryo and the second sperm fertilizes two polar nuclei to give rise to the triploid endosperm. In the case of *Arabidopsis*, seven cells form the female gametophyte: one egg cell and two synergids at the micropylar end, the central cell with two nuclei at the middle of the embryo sac, and three antipodal cells at the other end (Mansfield et al. 1991).

The endosperm is a nutrient storage tissue essential for embryo growth and germination. But the nourishing role is not the only role endosperm plays. It is also involved in hormonal regulation of embryo growth, maintenance of a high osmotic potential around the embryo, mechanical support during early embryo growth, and storage of reserves. Recent studies showed that in very early stages

of embryogenesis, when the endosperm itself is actively dividing and without much storage nutrient accumulation, the proembryo gets most nutrients from tissue other than the endosperm (Vijayaraghavan and Prabhakar, 1984). Furthermore, not all angiosperms have endosperm; *Orchidaceae*, *Podostemaceae*, and *Trapaceae* do not have endosperm. In some plant species such as *Arabidopsis*, the endosperm is utilized by the developing embryo and is degraded by the time the seed matures. These species are called exalbuminous. In these seeds, reserves can be stored in some parts of the embryo like cotyledons. In peas, the endosperm is absorbed in the free nuclear state before cell walls are formed. In cereal plants and maize, the endosperm is a major component of the mature seed and makes up most of the mature kernel weight (Schweizer et al., 1995). Endosperm accumulates starch, protein, oil, carotenoid, polysaccharides and macronutrients for embryo and early seedling development (Lopes and Larkins, 1993).

According to the cellularization pattern, endosperm development is classified to three types: nuclear, cellular, and helobial. The most common type is the nuclear pattern occurring in maize, barley and rice. Free nuclear division without cytokinesis takes place initially. Then cellularization starts at a certain stage and progresses centripetally toward the central vacuole until the whole endosperm is cellularized (Vijayaraghavan and Prabhakar, 1984). Mitosis and cytokinesis follow the initial endosperm nuclear division in the cellular pattern of endosperm development and persists throughout the whole process. In the intermediate helobial type development, two unequal chambers form after the

first nuclear division. The larger micropylar chamber has the free-nuclear type division, while the smaller chalazal chamber either remains undivided or only divides a limited number of times.

Endosperm cells are compactly arranged without intercellular spaces. They consist of only one or two cell types: living reserve cells and/or aleurone cells. The reserve cells are surrounded by up to four layers of aleurone cells. The reserve cells perform the main storage function, while the aleurone cells are thick-walled, living, nucleated cells. Abundant protein bodies surrounded by lipid bodies appears in aleurone cells (Demason, 1997). The aleurone complex cells play a major role in later stages of grain filling.

5.2 Evolutionary origin of endosperm

At the beginning of this century, there were two major hypotheses about the evolutionary origin of endosperm. Strasburger (1900) thought that the second fertilization was only an extension of vegetative female gametophyte development (Friedman, 1994). However, Sargant (1900) proposed that double fertilization in the ancestors of angiosperms generated a second embryo, which evolved into a body with storage function (the endosperm). This transformation might be due to a second female nucleus joining the second fertilization event (Lopes and Larkins, 1993). Sargant's theory is now more favored based on recent research findings. After determining that helobial- type endosperm development is derived from the other two types, researchers were trying to answer the question: which type of endosperm is the earliest form, free nuclear or cellular? There is no fossil record available to explain this event. Based on

the belief that double fertilization first appears in the common ancestor of the angiosperms and its closest living relatives, the *Gnetales (Ephedra, Gnetum and Welwitschia*), Friedman (1994) carried out comparative developmental analysis of reproduction trying to decide which development pattern was used by the progenitor of endosperm, the supernumerary embryo. *Ephedra* also possesses double fertilization feature. The difference is that the products of both fertilization events are embryos (Friedman, 1992). So the second product is called a supernumerary zygote nucleus. In this case, the developing embryo obtains nutrients from the large multicellular female gametophyte, supporting the hypothesis of Sargant. The second fertilization in *Ephedra* fertilizes the ventral canal nucleus instead of the chalazal polar nucleus as in angiosperms, the common ancestor of flowering plants and *Gnetales* may maintain the ventral canal nucleus during fertilization. It was proposed that the ancestors of angiosperms have female gametophytes as their nourishing resource. After double-fertilization and the supernumerary embryo appeared, the modification on supernumerary embryo make it to nourish the normal embryo. There might be an intermediate condition in which both female gametophyte and the second fertilization product function as nourishing source. Finally, with the continuously increasing importance of the endosperm, the female gametophytes lost the function of nourishment and were reduced to the embryo sac. So, Friedman stressed the point of Sargant (1900) that the endosperm should no longer be seen as a tissue; instead, it should be recognized as " a highly modified evolutionary homologue of an embryo: or in other words, as an organism."

(Friedman, 1994). The primitive extant seed plants have the free-nuclear pattern of embryogeny. Both fertilization products of *Ephedra* show a cellular pattern of embryogeny which suggested the developmental loss of free nuclear embryogeny may happen before the appearing of double fertilization and the supernumerary embryo (Friedman, 1994). Therefore, the progenitor of endosperm possessed cellular pattern of development, so the later evolved endosperm is also characterized by cellular pattern.

5.3 Questions in endosperm research and future prospects

There are still many enigmas in endosperm research drawing scientists' attention for both basic research and practical purposes. For instance: What are the different aspects of endosperm function and endosperm / embryo interaction (DeMason, 1997)? How are the events in the transition from a coenocytic to a cellularized endosperm regulated (Olsen, 1998)? How is the process of programmed cell death regulated during maturing of starch endosperm cells of cereals? What is the pattern of storage material accumulation? What is the mechanism of desiccation tolerance / intolerance? What are biological capabilities of endosperm?

Endopolyploidization or endoreduplication is a phenomenon present in many species, which means DNA replicates without mitosis. In maize, it is an important contributor to grain yield. Endoreduplication happens mainly in the central cells of the endosperm. It is usually associated with cell expansion or the initiation of stored reserve accumulation (DeMason, 1997). The function of endoreduplication is still debatable. Many suggestions have been summarized

by DeMason (1997). It is interesting if we try to correlate endoreduplication with the evolutionary advantage of endosperm. We might find some consistency: Brink and Cooper (1947a, b) speculated that the endosperm has gained the advantages of heterosis through double fertilization, and endosperm's triploid nature may also bring genetic advantage in an environment of diploid cells (Lopes and Larkins, 1993). Endoreduplication can amplify both advantages. Due to the great economic value of endoreduplicationin maize, Schweizer et al. (1995) developed two mathematical models describing the kinetics of endosperm growth, which provided a way to quantify the complex pattern of endosperm development.

The utilization of new techniques can improve our understanding of endosperm development. For example: Kranz et al. (1998) have developed the technique of *in vitro* fusion of maize central cells with sperm cells, which overcame the obstacle that the central cell is deeply embedded in embryo sac and not accessible for experimentation. The early events of double fertilization and endosperm development can thus be better analyzed. Further investigations using with this technique will hopefully help to identify the specific cytoskeletal components involved in central cell polarization, nuclear migration, and signal transduction, or downstream events of endosperm development at the molecular level (Olsen, 1998).

In terms of practical efforts made to improve seed quality and use, scientists try to gain a better understanding regarding endosperm cellular organization and functional roles (Lopes and Larkins, 1993). The bioengineering

of endosperm-specific expressed genes will hopefully help to express high levels of nutritionally desirable proteins encoded by introduced genes, which will cause a nutritional revolution in food industry. Scientists are also trying to manipulate the rate-limiting step of starch biosynthesis in order to improve yield.

5.4 Normal *Arabidopsis* endosperm development

Arabidopsis endosperm development is similar to *Capsella* which has been studied in detail by Schulz and Jensen (1974). Marsden and Meinke (1985) published some light microscopy study details of *Arabidopsis* endosperm formation. The process is thoroughly described by Mansfield and Briarty (1990 a; 1990 b). Since the micropylar and chalazal chambers of endosperm are very differently developed and structured, Mansfield (1994) defined *Arabidopsis* endosperm as the helobial pattern.

Two to four hours after fertilization (HAF), the primary endosperm nucleus divides for the first time to two daughter nuclei. The two nuclei migrate to opposite poles of the embryo sac. Free-nuclear divisions follow and form a mass of nuclei and cytoplasm without cell walls. In late-globular stage, the free-nuclear endosperm reaches the maximum of development. Endosperm nuclei accumulate around the periphery of the embryo sac, and a large central vacuole exists in the center of embryo sac. Cellularization starts around 60HAF, is mainly complete by 84HAF, and may continue up to 120HAF. At the late globular to early heart stage of embryo development, wall formation starts at the micropylar edge, and expands centripetally towards the embryo along the periphery of the embryo sac. Events proceed differently at chalazal chamber. Of the 4 nuclei

formed after the second division of primitive nucleus, one migrates to the chalazal pole of the embryo sac. Its cytoplasm keeps proliferating without nuclear divisions until more than 24 HAF the chalazal nucleus starts to divide. Nuclear diameters in the chalazal end are usually larger than at the micropylar end (Schulz and Jensen, 1971). Chalazal endosperm has not yet cellularized at 96HAF. A limited number of divisions then follow, and it becomes totally cellularized by 120HAF. The chalazal endosperm shows no orderly cell pattern. Schulz and Jensen (1974) proposed that the nearby embryo proper and suspensor affect the developmental pattern of the micropylar endosperm, while the chalazal proliferating tissue, degenerated antipodals and nucellus affect the chalazal endosperm. In *Arabidopsis*, endosperm is broken down and absorbed by the growing embryo. A single layer of endosperm cells containing storage reserves persists during seed desiccation (Fig. 3).

5.5 Purpose and value of this dissertation

Mutant identification, isolation and characterization play the same important role in studying endosperm development as in embryo research. Neuffer and Sheridan (1980) defined a large number of EMS-induced defective kernel (dek) mutations in maize. Bosnes et al. (1987) have identified various induced *shrunken* endosperm mutations in barley. But only a limited number of *Arabidopsis* endosperm mutants have been studied in detail. In order to gain a better understanding of genetic control of endosperm development, we need to set up a reasonable collection of endosperm mutants defective in various aspects.

In this dissertation, I try to answer some fundamental questions in endosperm development. Four aspects are discussed in the following chapters:

1. The nature of embryo-endosperm interaction: Does early endosperm development need signaling or growth substance from the embryo? To what extent does the endosperm rely on the enlarging embryo? Is cotyledon initiation of embryo development correlated with the transition from free-nuclear endosperm to cellularized endosperm? Is endosperm cellularization regulated by certain hormones or other signal from the late-globular to early-heart stage embryo?

2. The cell cycle control in endosperm: Cell cycle control is an exciting topic in biology. In animal and human systems, it has been carefully examined and much progress has been made in the past ten years. Revealing the secrets in cell cycle control may help us to conquer cancer. Plant scientists have now also started to explore this theme. The striking family of mutants, *titans*, we observed in our lab provides a good opportunity for investigating cell cycle control in plants. In this part of my dissertation, I describe a new titan mutant isolated from a population of T-DNA insertion lines.

3. Endosperm cellularization: What are the triggering factors for the start of cellularization? How is the process regulated? What decides the specific cellularization pattern? How is cellularization different for micropylar endosperm and chalazal endosperm? How are phragmoplasts formed and microtubles arranged?

4. The female gametophytic expression: Mutations affecting the female gametophyte functions can produce abnormalities during embryo and endosperm development. The comprehensive analysis of a group of such mutants in our lab combined with the findings from other labs may shed light on an important part of angiosperm sexual reproduction and provide a better understanding of gene imprinting.

CHAPTER 2

EMBRYO-ENDOSPERM INTERACTION

1. INTRODUCTION

A genetic balance between embryo, endosperm and maternal tissue is crucial for normal seed development (Lopes and Larkins, 1993). It is generally agreed that the endosperm plays a nourishing role in seed development. In addition to this well-known role, it also provides the necessary physical, chemical and hormonal environment for embryo differentiation (DeMason, 1997). But it is not at all stages of embryo development that endosperm acts as the nutrition source. At very early stages of embryogenesis, before cellularization occurs in the endosperm, conditions exist contradicting this supposition of a nourishing role. Instead, synergids, the suspensor, and integuments could be structures from which the embryo gains nutrition.

Since there is no symplastic continuity between the embryo sac and maternal tissue or between embryo and endosperm, there must be transfer cells or transfer-cell type wall ingrowths to facilitate the uptake activity of the embryo sac and embryo. At the micropylar end of the synergids, there are transfer-cell type walls. One synergid that does not degrade immediately upon double fertilization may transfer metabolites from the embryo sac to the very early

embryo (Newcomb, 1973). Wall ingrowths adjacent to the integuments and endosperm are also found in the suspensor, which may transfer nutrients from the surrounding integuments and endosperm to the embryo. The nutrient flow at early stages of embryogenesis could be from the parent plant to the seed coat to the suspensor then to the embryo proper. Or, it is also possible that nutrients can move from the testa through the nucellus, endosperm, and into the embryo (DeMason, 1997).

In later stages of embryogenesis, the endosperm accumulates reserves for the developmental need of the embryo. Extensive tissue culture studies have shown that mature embryos are autotrophic and only need simple media with mineral salts, a carbohydrate source and vitamins (Raghavan and Srivastava, 1982). Younger embryos need greater sucrose concentrations and higher osmolarity in the medium, which might make the nutrient flow into the embryo more effective. Endosperm also provides necessary hormones for the growing embryo (DeMason, 1997).

In the process of studying *defective kernel (dek)* mutants of maize, important interactions between embryo and endosperm are proved. Chang and Neuffer (1994) proposed four kinds of interactions between the embryo and endosperm: unidirectional interaction from normal endosperm to mutant embryo; unidirectional inhibition from mutant endosperm to normal embryo; unidirectional interaction from normal embryo to mutant endosperm (in maize, it seems that the endosperm development is affected by embryo regulation.); and bi-directional

interactions. It was also shown that auxin from endosperm is important in the regulation of embryo development.

More details were studied in free-nuclear type endosperm development. Fewer characteristics are known about the cellular and helobial types of endosperm development. A majority of the endosperm is maintained at seed maturity in maize. But in *Arabidopsis* the endosperm is collapsed and degraded during embryo growth. Which factor controls the timing of endosperm degradation? What regulates the totally different fates of embryo and endosperm after double fertilization? What kind of biochemical exchanges exists between embryo and endosperm? Is there a joint effort from both embryo and endosperm for transporting nutrients? What is the genetic mechanism integrating the structural and physiological adaptations balancing embryo and endosperm development? There are still so many questions remaining unsolved.

A novel mutant was isolated from large-scale screening at Novartis, which was named *emb1002*. Mutant seeds of *emb1002* at maturity have well-developed cellular endosperm and an extremely early arrested embryo possessing only 1 or 2 cells. The extreme phenotype of this mutation provided a good opportunity for studying the nature of embryo-endosperm interactions, and help to answer question such as: Does early endosperm development need signaling or growth substances from the embryo? In this chapter, detailed mutant characterization and classical genetic mapping procedures are described. Light, dissecting, and Nomarski microscopy was used to carry out phenotypic and histological analyses.

2. METHODS AND MATERIALS

2.1 Growth of plant materials

Plants were grown under 40W fluorescent lights in a room around 23°C. The lights are on a 16 hour light and 8 hour dark cycle. We plant 9 plants per 3-inch pot with soil containing 12 parts coarse vermiculite (Terra Lite, W. R. Grace Co., Cambridge, MA), 3 parts potting soil (Redi-Earth Peat-Lite, W. R. Grace Co., Cambridge, MA), and 1 part sterile sand. The fertilizer solution we use for daily watering consists of 1.6g/l of 7-16-19 All Purpose Hyponex (Hyponex Co., Fort Wayne, IN) and 0.1g/l 15-16-17 Peat Lite Special (Peters Co., Allentown, PA) in Reverse Osmosis water.

2.2 Mutant phenotypic analysis

emb1002 was generated in the Columbia ecotype seed by T-DNA insertional mutagenesis via *Agrobacterium* transformation at Novartis in North Carolina. Hygromycin resistance was used in this case to detect the presence of functional T-DNA insert.

The phenotype of mutant seeds in siliques was observed under a Wild dissecting microscope with the light cast onto the dissecting stage by desktop spot illuminators. Two pairs of fine tip forceps are used to open siliques, isolate mutant seeds and dissect seeds. Mutant seed sizes were measured with a micrometer under dissecting microscope and compared to wild-type seed sizes.

In order to study the internal morphology of mutant seeds, we used 1 part water: 2 parts Hoyer's solution (containing 7.5g gum arabic, 100g chloral

hydrate, 5ml glycerine per 30ml water) to clear both wild-type and mutant seeds overnight on a glass slide with a Fisherbrand 1 ½cm coverslip. The next morning, when the seed structures had become transparent, seeds were observed with an Olympus BH-2 compound microscope equipped with Nomarski optics for embryo and endosperm morphology. The *emb1002* endosperm nucleolar numbers per seed at globular, heart, torpedo, cotyledon stages were counted with a 450X450μm grid (under magnification 20X) to compare with normal endosperm nucleolar numbers per seed of wild-type. The grid is composed of 100 4.5 x 4.5 um squares. The microscope could be adjusted to focus on different layers of the endosperm and the numbers of endosperm nucleoli in each small square were counted first. Finally, the total numbers out of 100 small squares were added up, which was the total endosperm nucleolus number of the entire seed. Endosperm nucleolar diameters in cleared *emb1002* seeds at globular, heart, torpedo stages were measured (under magnification of 40X) with an ocular micrometer and then converted to actual length to compare with normal endosperm nucleolar diameters of wild-type seeds. Under magnification of 40x, the smallest division of the ocular micrometer stands for 1.2μm, the major division stands for 12μm, and the total division stands for 225μm. The endosperm nucleolar number of wild-type seed at globular, heart, torpedo stages was counted and wild-type endosperm nucleolar diameters at globular, heart, torpedo stages were measured with the same method. The endosperm nucleolar number and diameter of wild-type seed at cotyledon stage was not examined, because *Arabidopsis* endosperm is degraded during cotyledon stage. Mutant

embryo proper widths at the globular, heart, torpedo, and cotyledon stages were measured (under magnification of 40X) with an ocular micrometer. Pictures of mutant seeds under Nomarski microscope (magnification of 20X) were taken with Kodak Tmax100 or Technical Pan 100 black-and-White film by an Olympus C-35AD-2 camera installed on the microscope.

Histological analysis including seed fixation, dehydration, embedding, sectioning and staining were performed according to the protocol of Liu (1998). Mutant seeds in siliques of different stages were picked up with a pair of fine forceps. Seeds were transferred directly to fixation solution containing 4% formaldehyde (EMS, Electron Microscopy Sciences, Fort Washington, PA, USA) and 2.5% glutaraldehyde (EMS) in 50mM potassium phosphate buffer. Seeds were fixed overnight at room temperature. The next morning, seeds were washed three times with 0.05M potassium phosphate buffer, 15 minutes each, followed by washing with double distilled water 15 minutes per time for three times. Dehydration was by 30%, 50%, 70%, 95%, 100%, and100% ethanol for one hour at each step. Then the ethanol was replaced by 3:1, 1:1, and 1:3 ethanol: L.R. White Resin (EMS) each for one hour. Finally three one-hour washes with pure L.R. White were performed. The seeds were transferred to capsules filled with L.R. White and a printed label. Polymerization took 16 hours at 60° before the plastic blocks were formed. The blocks were sectioned to 1.5μm thick slices with a Leica 2045 Microtome (Leica, Bellevue, WA). The freshly made glass knifes were prepared with a LKB 7801B knifemaker (LKB, Sweden). A boat for water filling was glued to the glass knives with dental wax.

Thin plastic sections were transferred with a fine-wire ring to water drops on a 10-ring immunostaining slide, 4 to 6 sections per ring. After the slides were dried on a hot plate, they were put in a staining rack (TissueTek) and immersed in 200ml 1% periodic acid for 30 minutes at room temperature. A wash with running water was applied to the slides for 5 minutes, then double distilled water was used to wash the slides twice. Schiff's reagent treatment at room temperature was 30 minutes prior to a 10-minute wash with running water. The slides were rinsed with double distilled water twice and dried on a hot plate. Counterstaining was performed on a hot plate with 1% amido black 10B. The slides were mounted with 3 drops of ACCU-MOUNT 60 mounting medium (Stephens Scientific, Division of Cornwell Co., Riverdale, NJ) after rinsing and drying on a hot plate. The slides were observed with an Olympus BH-2 microscope using Kohler illumination. Pictures of sections of mutant seeds were taken with Kodak Tmax100 or Technical Pan 100 black-and-White film by an Olympus C-35AD-2 camera installed on the microscope.

2.3 Classical genetic mapping

Classical genetic mapping procedures are described as follows. Flowers of heterozygous mutant line *emb1002* were emasculated before self-pollination. Pollen from visible-marker lines Dp23, Dp24, Dp28/hy2, and W_6*sti* were transferred to heterozygous female stigma (Patton et al., 1991). Marker line DP23 contains markers *ch1, er, gl1, cer2,* and *tt3*, located on chromosomes 1 through 5 respectively. Other lines have markers in regions not covered by DP23. Dp24 contains markers *er, bp, yi* and *ttg*. DP28 was first constructed by

Patton containing *dis1, clv2, er,* and *tt5,* Later hy2 was crossed with DP28 and DP28/hy2 was formed. W_6*sti* was composed of *sti, cp2, er, as,* and *cer8*. Only *sti* and *cer8* were examined in my experiment. F_1 seeds were dry-harvested from the cross-produced siliques and planted. Siliques of F_1 plants were screened for heterozygotes. The ones containing 25% mutant seeds are desired (VIS emb/ vis EMB). F_2 seeds arising from self-fertilization of F1 heterozygous plants were dry-harvested. Depending on the rate of germination, at least three tubs of plants (27 pots with nine plants per pots) were planted for each cross in order to make an F_2 population of about 200. F_2 plants were screened for both visible markers and embryo phenotypes. Details of mapping procedures were described by Franzmann et al. (1995).

The computer program GeneMapping written by Wang Li. (Computer Science Department, Oklahoma State University) was used to analyze screening data. The older computer functions BUILD, SUMMARY, CHI, RECF2 were used occasionally to confirm the accuracy of the GeneMapping program. The gene mapping program sums the total number of plants in each of the phenotypic classes, calculates the Chi-square to determine whether the segregation ratios were significantly different from that expected for unlinked genes. Subsequently, an estimate of the percentage recombination between *emb* and certain visible markers was obtained. The internal Chi-square printed out shows the consistency of mapping data. The theory of the program was explained in detail by Patton (1991). Recombination rate can be converted into map distances in centimorgans using the Kosambi mapping function.

3. RESULTS

emb1002 is a T-DNA insertion-tagged mutant based on genetic analysis of other members in the Mienke lab. Mature *emb1002* mutant seeds in siliques are white in color, half-translucent and somewhat watery. Table 3 illustrated that *emb1002* seed size and shape are uniform and close to those of wild-type seeds. The embryo cannot be seen by dissection under dissecting microscope.

Endosperm nuclear number, size, distribution, morphology are good standards for studying *Arabidopsis* endosperm development. Because nucleoli are more prominent than nuclei under Nomarski microscope (Liu and Meinke, 1998), I have examined nucleoli in most detail. There is usually one nucleolus per nucleus in *Arabidopsis* seed endosperm. During the progress of my research, I have collected data on nucleolar number and diameter for normal *Arabidopsis* endosperm development, which is very useful for comparative mutant analysis and characterization (Table 1, 2). Under Nomarski compound microscope, the cleared *emb1002* mature seeds are seen to have well-developed endosperm with normal cellularization. Endosperm nucleoli are evenly dispersed throughout the seed. Endosperm nucleolus number of *emb1002* seeds at globular, heart, and torpedo stages were counted (the stages were defined by wild-type seed embryonic stages in the same siliques) to compare with normal endosperm development in wild-type seeds. At globular and heart stages, *emb1002* endosperm nucleolar numbers are close to those of wild-type seeds. It seemed that there could some degradation of nucleoli happening after

torpedo stage because there were some smaller endosperm nucleoli appearing and number of normal size endosperm nucleoli reduced after torpedo stage. By cotyledon stage, the normal-size endosperm nucleoli are much fewer (Table 4). The purpose of diameter measurement here is to test how normal the emb1002 endosperm nucleolus sizes are compared to those of wild-type (Table 5). They are shown to be close to those of wild-type. On the other hand, *emb1002* have extremely early arrested embryos. The embryos are very small and distorted (Table 6). It is difficult to count the cell number in embryo proper and suspensor. But there are no more than 4 cells in embryo proper and usually one to two cells. The mutant phenotype is shown in Fig. 4.

Histological study showed normal endosperm morphology and normal cellularization in *emb1002* endosperm when wild-type seeds were at cotyledon stage (Fig. 5). The embryo proper and suspensor are composed of few cells, which is consistent with the Nomarski microscope observations.

Linkage analysis is summarized in Table 7. Marker name, their positions on chromosome, the number of plants screened and recombination percentage between visible markers and *emb1002* generated by the GeneMapping program are listed. The *emb1002* gene showed linkage with visible markers *ttg, tt3* and *yi.* It seems that gene *EMB1002* is localized on lower half of chromosome 5 about 6 centimorgans below *tt3* and 25 centimorgans above *yi.*

4. DISCUSSION

emb1002 is a T-DNA tagged mutant with very early arrested embryo and advanced endosperm. It provides us a good opportunity to study the nature of embryo-endosperm interaction, especially the impact of the embryo on endosperm development in early embryogenesis. The above- mentioned results of Nomarski microscope and histological study show the endosperm morphology to be mainly normal in terms of nucleolar number, size and endosperm cellularization. So, *emb1002* seems to encode an embryo-specific gene required for early embryo development, whose defect leads to the early death of embryo, without seriously affecting the endosperm. From detailed mutant analysis and characterization, many interesting questions were answered, such as: Is the late-globular to early-heart stage embryo directly responsible for the initiation of endosperm wall formation? Dose cotyledon initiation of embryo development causes the transition from free-nuclear endosperm to cellularized endosperm? Is endosperm cellularization regulated by certain hormones or other signals from the late-globular to early-heart stage embryo (Mansfield and Briarty, 1990b)? Since the endosperm develops to a quite advanced stage and carries on normal cellularization and the embryo dies at zygotic stage, we could suggest that endosperm cellularization does not greatly rely on signals from the embryo. However, we cannot exclude the possibility that the developing embryo does have some impact on endosperm development.

Based on morphology, It seems that the normal size endosperm nucleoli of *emb1002* begin to degenerate to smaller ones at the torpedo stage. At this stage, the number is still basically consistent with wild-type except that smaller endosperm nucleoli begin to appear. Comparing the number of normal-size *emb1002* endosperm nucleoli at cotyledon stage with that of *emb1002* seed at torpedo stage, we can see the former is much fewer than the latter. It might be due to the endosperm degradation. It is interesting to consider what factor regulates endosperm degradation. Wild-type *Arabidopsis* endosperm is degraded during cotyledon stage. In the case of *emb1002*, since the embryo is dead at a very early stage, it may not be the embryo giving the signal to initiate endosperm degradation at the equivalent to torpedo stage. We do notice that even if the endosperm nucleoli seemed to be breaking down to smaller ones, the cellularization structure remained normal in sections (WT=cotyledon stage). Therefore, is it possible that the endosperm has a self-regulated timing mechanism which produces substances assisting endosperm lysis around torpedo stage? And that a growing embryo might provide external mechanical pressure to facilitate this crushing process? The growing embryo continuously withdrawing nutrients from endosperm could also promote the endosperm degradation. Further biochemical and molecular experimentation needs to be done in order to prove these hypotheses raised simply from microscope observations.

The results of mutant analysis will also help to explain the determining factor of seed size. The seed sizes of *emb1002* are basically similar to wild-

type's. Since this mutant has extremely early arrested embryo and advanced developed endosperm and normal seed size, it appears that seed size of Arabidopsis is determined more by the extent of endosperm development situation than by embryo development.

CHAPTER 3

CELL CYCLE CONTROL IN ENDOSPERM

1. INTRODUCTION

Cell cycle control, an area of great research interests, has been studied in detail in yeast, animal and human systems. The progress of these efforts helps us not only to develop new biomedical strategies such as cancer therapy, but also to understand principles of growth and development better (Liu and Meinke, 1998).

The mitotic cell cycle is composed of a DNA synthesis phase S, a mitotic cell division phase M and the phases between them, G_1 and G_2. Mitosis can be divided into the prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. Two daughter cells are formed after the cycle. On one hand, many fundamental mechanisms of cell cycle control are conserved among eukaryotes. On the other hand, plants do have many unique features related to cell cycle different from other eukaryotes, such as the potency of non-dividing cells to re-enter the cell cycle (Shaul et al., 1996).

Cyclin-dependent kinases (CDKs) are important regulators driving cell cycle. CDKs need to combine with their regulatory proteins, cyclins, to function.

Cyclins' levels fluctuate during the cell cycle, and the accumulation of mitotic cyclins activated the mitosis. The activity of the CDK-cyclin complexes is controlled by phosphorylation and dephosphorylation events. The activation of the CDK-cyclin complex leads to the activation of a transcription factor, which subsequently turns on the transcription of certain genes required for the next cell cycle step (Hartwell and Kastan, 1994). Plant CDK and cyclin homologous have been found in several species including *Arabidopsis* (Ferreira et al., 1991; Hemerly et al., 1992; Doerner et al., 1996; Hirayama and Shinozaki, 1996). *Arabidopsis* CDK gene *Cdc2aAt* and cyclin genes *cyc1At* and δ-type cyclins are the most studied to date. It seems that the activation of *cdc2aAt* expression might correlate with the competence to divide rather than actual cell division, while *cyc1At* expression is restricted to dividing cells. The δ-type cyclin expression is not restricted to dividing cells and seems induced by external and internal stimuli to plant cells at early stages (Shaul et al., 1996).

CDK and cyclins are not the only factors regulating plant cell cycle. Mitosis and cytokinesis include events such as chromosome condensation, sister chromatid cohesion, chromosome separation, spindle pole duplication, separation and migration, spindle formation and elongation, nuclear envelope breakdown and reformation (Liu and Meinke, 1998). How are these complicated events regulated harmoniously to make sure the cell cycle is carrying on in order? Mutant analysis plays a valuable role in understanding this complex network. There have been many examples of the central role of mutant characterizations in yeast and *Drosophila* cell cycle research (Stern and Nurse,

1996; Duronio et al., 1998). Recently, the isolation of a class of *Arabidopsis* mutants, *titan*, defective in mitosis during seed development will hopefully shed light on plant cell cycle control (Liu and Meinke, 1998).

Three non-allelic *titan* mutants have been studied carefully by Liu and Meinke (1998). The name "*titan*" came from the striking phenotype common to the three recessive mutants: giant endosperm nuclei resulted from continuous DNA synthesis without nuclear division. The individual mutants do have their own unique phenotypes. The *titan1* mutant seeds have distorted embryos with a few greatly enlarged cells and huge nuclei. The enlarged endosperm nuclei are also defective at migration to the chalazal end of the embryo sac early in development. It seems that nuclei are blocked at prophase. The repeated cycles among DNA synthesis and prophase lead to the above-mentioned phenotypes. The *ttn2* is an embryo lethal mutant with embryo composed of several small cells. The endosperm of *ttn3* has the common *titan* feature at globular and heart stages: large mitotic figures with multiple chromosomes were seen. However, endosperm cellularization occurs at later stages. In particular *ttn3* has a normal embryo and develops into viable plants which produce 100% defective seeds after self-pollination. Study of *titian3* will possibly help us to answer the evolutionary question of endosperm identity: how do endosperm and embryo establish distinct patterns of mitosis and cytokinesis after the point of double fertilization. It seems that the failure of maintenance of normal ploidy levels in the developing endosperm does not have a crucial impact on pattern formation in the early embryo (Liu and Meinke, 1998).

Liu and Meinke (1998) recently cloned the *TTN3* gene using the TAIL-PCR method in Meinke laboratory. It encodes a member of the SMC2 family of condensin proteins required for chromosome condensation. Mitotic condensation is responsible for chromosome folding. Factors such as *cis* sites, histones, topoisomerase II and SMC proteins play important roles in chromosome condensation. SMC proteins have been found in bacteria, fungi, invertebrate and vertebrate animals. They are very possibly one of the crucial components in establishing the chromosome structures (Strunnikov et al., 1995). Defects in the SMC family of genes cause failure to segregate chromosomes in mitosis. The SMC2 subgroup includes the ScII, XCAPE, and cut-14 proteins (Strunnikov et al., 1995; Koshland and Strunnikov, 1996). This discovery of the plant homologue of SMC2 revealed again that SMC proteins are evolutionary conserved.

Another member of the *titan* class of mutants, *titan4*, was isolated following *titan*1,2 and 3. *titan4* is tagged with T-DNA insert. Careful mutant characterization of *TTN4* in this dissertation could hopefully enrich our knowledge upon *titan* mutants and plant cell cycle control.

2. METHODS AND MATERIALS

2.1 Growth of plant materials

Same as Chapter 2.

2.2 Mutant phenotypic analysis

The mutant *titan4* was created in the Columbia ecotype by Agrobacterium-mediated seed transformation at Novartis in North Carolina. Hygromycin resistance was used in this case to detect the presence of functional T-DNA insert.

The methods are mainly the same as in Chapter 2. Mutant analyses under dissecting and Nomarski microscopes were performed. Endosperm nucleolar numbers at globular and cotyledon stages were counted. Endosperm nucleolar sizes were measured. Because of the irregular shapes of *titan4* endosperm nucleoli, endosperm nucleolar length and width were measured instead of diameter. Endosperm nucleoli at cotyledon stage with length above 10μm and with length below 10μm were measured separately. Cell numbers of the *titan4* embryo-proper and suspensor were counted.

In addition, complementation crosses were performed to determine the allelic relationships between *titan 4* and *titian 2*, *titan 4* and *titan1*, *titan4* and embryo-defective mutant 12.25 (from Lukowitz and Somerville at the Carnegie Institute, Stanford, CA, USA), *titan4* and 19.22 (also from Lukowitz and Somerville). Due to the apparently different phenotypes of *titan4* and *titan3*, they are least possible to be allelic. Complementation tests between *titan4* and *titan3*

were not performed. The crossing procedure is the same as mentioned in Chapter 2. *titan4* was usually used as the female parent but was used as the male parent in cross with the *ttn2* as female. The resulting siliques were examined 10 days after crossing. If the seeds in silique all appear wild-type, the two mutants crossed are not allelic. If there are 25% mutant seeds in the silique, the crossed mutants are allelic and defective in the same gene.

2.3 Classical genetic mapping

Techniques used were mainly the same as described in Chapter 2, except that multiple marker lines DP24, DP28/hy2, W_6*sti* and single marker *clv1* were used for mapping. Since linkage has been found before using DP23, DP23 was not further applied in the mapping process in this case.

3. RESULTS

emb1002 is a T-DNA insertion-tagged mutant based on genetic analysis of other members in the Mienke lab. Mutant seeds in siliques are watery and glassy. Seed sizes usually are slightly smaller than wild-type seeds. Embryos can't be seen by dissection under dissecting microscope.

Under the Nomarski compound microscope, greatly enlarged endosperm nucleoli are shown with irregular shapes and unusual morphologies. Nuclear migration is present and normal. There are endosperm nucleoli observed at the chalazal end of the embryo sac. Endosperm nucleolar numbers of 28 seeds at cotyledon stages were counted to compare with normal endosperm of wild-type. The giant nucleolus number usually ranged from 1 to 20. The average was about 10. This number was much less than the endosperm nucleoli number of wild-type seeds (see Table1 for comparison). The deficiency of nucleolar division was one of the evidence that this mutant is defective in cell cycle control. There were also some relatively-small-size endosperm nucleoli (endosperm nucleolar diameters are under 10μm). The ratio of giant to smaller-size nucleoli varied from seed to seed. Some *titan*4 seeds were examined at the globular embryo stage equivalent, and only giant endosperm nucleoli were seen. Mainly, the giant nucleolus numbers are less than 20. So, it seems that the giant endosperm nucleoli observed in later stages do not arise from the fusion of many small endosperm nucleoli at a young stage. In the extreme case there were approximately 40 giant endosperm nucleoli inside a seed. Endosperm nucleolus

sizes at the cotyledon stage were measured (Table 2; Table 8). Twenty giant endosperm nucleolus sizes (length x width) and 20 small endosperm nucleolus sizes at cotyledon stage were measured to compare with the normal endosperm nucleolar sizes of wild-type seeds. These data are shown in Table 8. The results indicate the dramatic increase in size and reduction in number of *titan4* endosperm nucleoli, which support the model that *titan4* is cell cycle control mutant defective in mitosis.

The *ttn*4 embryo is arrested very early. The typical *ttn4* embryo proper usually contains no more than 4 cells, but on average, only 2. In some cases, there is no embryo seen but giant endosperm nucleoli are seen. This could be because either incomplete clearing with Hoyer's or the embryo was arrested at a very early stage. Cell sizes are usually small. In a few cases, large bubble-shaped cells occur in the embryo. But they are not as large as those of *titan*1. Cell number in the suspensor ranges from 2 to 8; the average is 4. Sizes and shapes of the suspensor cells are variable, including long narrow cells, bubble-shaped large cells, short and narrow ones. Some suspensor cells have 2-3 nucleoli. Sometimes, there appears to be a division between the nucleoli. Some nucleoli in the suspensor are larger than normal, but not as large as those of *titan* 1. See Figure 6 for mutant phenotypes.

Histological studies showed there is no cellularization of the endosperm at cotyledon stage (Fig. 7). The embryo proper and suspensor are only composed of a few cells, which is consistent with Nomarski microscope observations.

Complementation test results showed that *titan 4* is not allelic to *titian 2*, *titan1*, 12.25, and 19.22, because they can complement each other in all cases and produce 100% phenotypically normal seeds in siliques.

Linkage analysis is summarized in Table 9. Marker name, their positions on chromosome, the number of plants screened and recombination percentage between visible markers and *titan4* generated by the GeneMapping program are listed. It seems that the gene *TITAN4* is localized at bottom of chromosome1 about 12 centimorgans below *clv2* and 15 centimorgans above *clv1*.

4. DISCUSSION

The *titan* family of mutants is disrupted in the mitotic cell cycle with striking defects. Ten to fifteen *TTN* genes were estimated to exist in the *Arabidopsis* genome based on the mutant frequency observed so far (Liu and Meinke, 1998). Only one allele for each of the five *titan* mutants was obtained in Meinke lab collection. Recently, 23 *titan*-like mutants representing 4 different genes named as *CHAMPIGNON, HALLMASCH, PFIFFERLING*, and *PORCINO* were identified (Mayer et al., 1999). Based on the very similar phenotypic description and close mapping positions, the gene *CHAMPIGNON* is possibly allelic to *TITAN1*, and the gene *PFIFFERLING* is possibly allelic to *TITAN4*. A new mutant, *titan5*, was isolated and proved to be tagged at Novartis recently. *titan5* is also allelic to the *titian* type mutants 12.25 and 19.22 isolated by Lukowitz and Somerville of the Carnegie Institute, Stanford.

The embryonic lethal mutant *titan4* has features common to the phenotype of other *titans,* specifically the giant endosperm nucleoli from DNA duplication without division. Like *ttn1* and *ttn2,* the *ttn4* gene seems important for both embryo and endosperm development, while *ttn3* seems to be endosperm-specific. *titan4* resembles *titan2* most closely, although the complementation tests proved it is not allelic to *titan2*. *titan4* also has some distinct features. The comparisons of *titan* 1, 2, 3, and 4 are listed in Table 10. *titan 1, 2, 3,* and *4* were mapped to different locations on classical genetic map. *titan1* was located on the bottom of Chromosome 3. *titan2* is at the top of Chromosome 1. *titan3* is at the

bottom of chromosome 5. And *titan4* is at the bottom of Chromosome 1. So, it seems that members in *titan* family form a heterogeneous group of genes playing important roles at different stages of the mitotic cell cycle in plants (Liu and Meinke, 1998).

TTN gene could play essential roles in cell cycle control or in the structure and mechanics of mitosis (Liu and Meinke, 1998). The *TTN3* gene appears to encode a member of the SMC2 family of condensins essential for chromosome condensation, which fits the structural mechanics of mitosis model. *TTN1, TTN4* and *TTN5* genes are still in the process of molecular cloning (*TTN2* is not tagged). Their exact functions are not known yet. First, they possibly play roles as cell cycle checkpoint regulators. As mentioned in the introduction, there have been some *Arabidopsis* cell cycle genes isolated (Doerner et al., 1996; Hirayama and Shinozaki, 1996; Shaul et al., 1996). Also, as *TTN3*, there may also be other *TTN* genes required during the processes of chromosome condensation, sister chromatid cohesion, chromosome separation, spindle pole duplication, separation and migration, spindle formation and elongation (Strunnikov et al., 1995; Koshland and Strunnikov, 1996). In particular, *ttn*1's defect in nuclear migration provided further support for this model. Hopefully the investigation of *titans* will also help us dissect the genetic control of cytoskeletal organization in plants. One promising approach to support this model would be using chemical inhibitors of cytoskeletal organization to phenocopy *titan* defects (Liu and Meinke, 1998).

Many questions remain to be answered. The nucleolus is the most distinguished feature in most eukaryotic nuclei, and the question of nucleolar organization is intriguing and only partially solved. Shaw at al. (1996) studied carefully the organization of nucleolar activity in plants using electron, confocal microscopy and *in situ* hybridization. There are two nucleolar organizer regions (NORs) occurring on Chromosome2 and 4, which are composed of clustered randomly repeated blocks of ribosomal RNA (Haberer et al., 1996). What do the ring shapes and the irregular structures of the giant endosperm nucleoli mean? There is little known about the genetic regulation of embryo cell size. *ttn* mutants provide us a good opportunity to explore in this subject. *ttn1* has greatly enlarged cells in embryo–proper and suspensor, while *ttn2* has small cells in the early aborted embryo. *ttn3* has normal cells in the normally-developed embryo. *ttn4* in most cases has small cells like those of *ttn*2. But large bubble like cells occur in *ttn4* embryos and suspensors occasionally. Liu and Meinke (1998) posed the question of the regulation of cell size in relation to nuclear volume and basic metabolism during plant growth and development. All *ttns* have similar endosperm defects. Why were very different cell sizes observed in their embryo? The underlying molecular basis of the control of cell size during seed development requires further investigation. Furthermore, evolutionary questions including endosperm identity, and differences between endosperm and embryo developmental regulation will be hopefully addressed through careful mutant analysis of *titan*s. Molecular characterization, such as gene expression pattern

gene products with other gene products in the series of cell cycle events will shed light on plant cell cycle control.

CHAPTER 4

CELLULARIZATION IN ENDOSPERM

1. INTRODUCTION

Cytokinesis is the last step of mitosis, when two identical daughter cells arise from one mother cell, a process common to organisms from yeast to animal and plants. Much research has been carried out in yeast and *Drosophila* to understand the details of this process (Miller and Kiehart, 1995; Chang and Nurse, 1996).

Plant cell division is different in many respects from animal cell division. Plasma membrane constriction causes cell division in animals, whereas in plants two daughter cells are separated by a cell wall. Animal cell migration regulates morphogenesis, while morphogenesis in plants is regulated by cell division and elongation (Varner and Lin, 1989). In plants, the phragmoplast, a complex structure including microtubules (MTs), microfilaments (MFs), Golgi derived vesicles, and endoplasmic reticulum, gives rise to the cell plate. The cell plate then extends to form the cell wall (Staehelin and Hepler, 1996).

Biochemical, molecular genetic, and microscope observations have been done to analyze plant cytokinesis. Again, identification of cytokinesis mutants became a great tool for dissection of this complicated process. Examples include

the *cyd* mutant of pea, and the *keule*, *knolle* and *cyt* mutants of *Arabidopsis,* which act during embryogenesis and at later vegetative developmental stages (Lauber et al., 1997; Nickle and Meinke, 1998). The *KNOLLE* gene was found encoding a membrane-associated protein related to vesicle-docking syntaxins (Lauber et al.,1997). Two allelic mutants *cyt-1* and *cyt-2* have been analyzed in detail by Nickle and Meinke (1998). There have been other allelic *cyt* mutants isolated by Lukowitz and Somerville at the Carnegie Institute, Stanford, CA. *Cyt* is an embryo lethal mutant with cell-wall outgrowths containing excessive periodic acid-Schiff reagent (PAS)-positive polysaccharides. Unesterified pectins diffuse throughout the cell wall. Callose formation in both intact and incomplete cell walls was observed (Nickle and Meinke, 1998). Also the *cyt1* embryo cannot be rescued in culture. The herbicide dichlobenil, which disrupts cellulose biosynthesis, can phenocopy some of cyt1 defects. These facts suggest that *CYT1* is important in the formation of plant cell-wall architecture. There were different models proposed for *CYT1* function. *CYT1* was thought to encode another catalytic subunit of cellulose synthase or a sucrose synthase subunit associated with the cellulose synthase complex. *CYT1* is possibly functioning during the callose-rich stage of cell-plate formation, and *CYT1* also could function in a biochemical pathway or signal transduction network which influences some other essential functions (Nickle and Meinke, 1998).

Besides the above-mentioned study in embryo cellularization, endosperm cellularization has also drawn scientists' attention. Endosperm cellularization is closely related with its development. Recent combination of immunofluorescence

methods and confocal laser scanning microscopy facilitated the exploration of endosperm cellularization. It seems that this process is highly conserved among barley, wheat, rice and maize, which includes the process of nuclear migration, polarization, first anticlinal wall formation, alveolation, periclinal cell division, and the subsequent differentiation of peripheral aleurone initials (which will produce alpha-amylase at germination) and inner starchy endosperm initials (Olsen et al., 1992; Brown et al. 1994; Olsen et al. 1995; Olsen, 1998). *Arabidopsis* endosperm cellularization has many similarities with other angiosperms.

In the mutant collection of the Meinke lab, *cyt* represents one class of mutant defective in embryo cellularization, while another type of mutants defective at cellularization in developing endosperm has now been isolated. The mutant characterization of *emb99* and *emb1309* will be discussed in the following section.

2. MATERIALS AND METHODS

2.1 Growth of plant materials

Same as Chapter 2.

2.2 Mutant phenotypic analysis

The mutant *emb99* was generated in the Wassilewskija ecotype by Agrobacterium-mediated seed transformation (Feldmann and Marks, 1987). Kanamicin resistance was used as the selectable marker in this case. The mutant *emb1309* was created in the Columbia ecotype by same method at Novartis in North Carolina. Basta resistance was used in this case.

The methods are mainly the same as in Chapter 2. Mutant analyses under dissecting microscope and Nomarski microscope were performed. Endosperm nucleolus numbers of *emb99* at globular, torpedo and cotyledon stages were counted. Endosperm nucleolus diameters at globular, heart, torpedo and cotyledon stages were measured. Embryo and endosperm nuclear and nucleolar diameter in sections of seeds at late torpedo stages were measured. For *emb1309*, only descriptive work was done.

In addition, complementation crosses were performed to determine the allelic relationships between *emb99* and *emb1309*. The crossing procedure is the same as mentioned in Chapter 3. *emb1309* was used as female parent in 4 crosses , and *emb99* was used as female parent in 9 crosses.

2.3 Classical genetic mapping

Method is mainly same as described in Chapter 2. Multiple marker lines DP23, DP24, DP28/hy2, W_6*sti* were used for mapping *emb99*. DP23, DP24, DP28/hy2, W_6*sti* were used for mapping *emb1309*.

3. RESULTS

Tagging status of *emb99* is unsolved because of multiple insertions. Tagging status of *emb1309* is not examined yet. In both two mutants, seeds in siliques are usually watery and glassy. Seed sizes are slightly reduced. In *emb1309* some seed sizes are only 1/3-1/2 of the wild-type's size. There are occasionally a few spontaneously aborted seeds in siliques. In typical mutant seeds a green torpedo stage embryo can be seen after dissection under the microscope.

The *emb99* endosperm nucleoli are consistently round and evenly dispersed throughout the seed. Nuclear migrations are present and normal. Endosperm nucleolus number of *emb99* seeds at globular, torpedo and cotyledon stages were counted (Table 11). Parts of the data shown are from the work of Amy Davis in the Meinke laboratory. The average endosperm nucleolus number of *emb99* at torpedo stage was more than twice of that of wild-type at globular stage, was about one tenth higher than that of wild-type at heart stage and is one- third lower than that of wild-type at torpedo stage. Endosperm nucleolar diameters of *emb99* at globular, heart, torpedo and cotyledon stages were measured. The endosperm nucleolus diameter was about twice that of wild-type (Table 2; Table 12). The nucleolar diameters of wild-type become progressively smaller at later stages, while the nucleolar diameters of *emb99* become larger at older stages. The *emb99* embryos are arrested at torpedo

stage with mainly normal morphology and cellularization in the embryo proper and suspensor (Fig 8a).

emb1309 is similar to *emb99*, but it also has some unique aspects. *emb1309* endosperm nucleolar sizes are more variable than *emb99*'s, ranging from 2.4μm to 21.6μm. The average is 10.2μm (average of 30 nucleoli counted at cotyledon stages). Sometimes irregular-structured endosperm nucleoli were seen. There are about 50-100 nucleoli distributed evenly in each seed. Small seeds which sometimes appear in siliques have enlarged endosperm nucleoli or normal endosperm nucleoli; sometimes embryos were arrested at globular stages; sometimes embryos were arrested at heart to torpedo stages. So, these small seeds can be mutant seeds, development-delayed wild-type seeds or spontaneously aborted seeds. The ratio of small seeds to normal size mutant seeds to total seeds is 52/69/653 in twenty siliques. *emb1309* embryos are arrested at heart to torpedo stage, and their cotyledons are not as long as those of *emb99*. Usually the two cotyledons are widely open. Suspensors are mostly normal (Fig 8b).

The results of histological study in both cases are consistent with the Nomarski microscope observations. No endosperm cell wall formation is seen in sections (Fig 9). Nuclear and nucleolar diameter of *emb99* embryo and endosperm in sections of seeds at late torpedo stages were measured (Table 13). The comparison of embryo nuclear and nucleolar size between *emb99* and wild-type seed shows no significant differences. So the embryo morphology and cellularization are normal. Measurements of *emb99* and wild-type endosperm

nuclear diameters were taken on sections, which cannot be done in cleared seeds because nuclei are not prominent under Nomarski microscope. The size of endosperm nucleoli of *emb99* in sections is basically consistent with the size seen in cleared seeds, which is two to three times bigger than that of wild-type seed endosperm.

Linkage analysis is summarized in Table 14 and Table 15. Marker name, their positions on chromosome, the number of plants screened and recombination percentage between visible markers and *emb99* or *emb1309* generated by the GeneMapping program are listed. Mapping with DP23, DP24 and DP28/hy2 were done by Amy Davis. This part of data is not shown in Table 14. *EMB99* gene only showed certain kind of linkage with clv2 and gl2, but not others. The Chi-square is always high (data not shown) and recombination data are not perfectly consistent, so the precise mapping position of *EMB99* is still questionable. Based on the mapping data we obtained to date, *EMB99* gene is most likely localized on the lower half of chromosome 1. It is a little puzzling that *EMB1309* is also mapped to the lower half of chromosome 1 below clv2. But complementation test between *emb99* and *emb1309* showed they are not allelic. There were 178 wild-type seeds among 192 total seeds obtained through the complementation crosses. The rest are small spontaneously aborted seed. *EMB1309* showed some linkage with ch1 and clv2. Since there were many plants with ch1 phenotype (pale green plants) died before screening, the linkage data with ch1 might not be very accurate.

4. DISCUSSION

emb99 and *emb1309* represent a class of endosperm cellularization defective mutants with a normal pattern of inheritance (about 25% defective seeds). These mutants are in contrast to *cyt*, which represents an embryo cellularization defective class of *Arabidopsis* mutants. There is no endosperm cellularization of *emb99* observed after heart stage. It appears that *emb99* and *emb1309* are not mutants whose endosperm development simply stops before cellularization at late globular to early heart stage. Instead, they appear to have more specific defects in endosperm cellularization. The reason is that the average endosperm nucleolus number of *emb99* at torpedo stage is higher than wild-type at globular and heart stages. In some extreme cases, *emb99* endosperm nucleolus number is higher than wild-type's at torpedo stage. So, if the endosperm development simply stopped, there should not be so many syncytial nucleoli divided. Moreover, since *emb99* endosperm nucleoli number at torpedo stage is lower than those of wild-type, the nuclear division of *emb99* syncytium-endosperm is less frequent besides the cellularization deficiency at later stages comparing to wild-type.

emb99 displays slightly enlarged endosperm nucleoli from the globular stage onward. Since *emb99* endosperm nucleolar number remains similar from torpedo to cotyledon stage, It seems there are neither further divisions nor degeneration of *emb99* endosperm nucleoli after torpedo stage. And noticeably, the nucleolar diameter of wild-type becomes smaller at older stages, while the

nucleolar diameter of *emb99* becomes greater at older stages. So, DNA synthesis without division probably still continues after the torpedo stage.

Despite the total lack of cell wall formation in endosperm, the *emb99* embryo carries on normal development until it is arrested at torpedo stage. The *emb1309* embryo shows certain kind of distortion when it is arrested at torpedo stage. *emb99* embryo/endosperm nuclear/nucleolar diameter in sections of seeds at late torpedo stages were measured. Because the space in the embryo cell is small and it is difficult to tell difference between nucleus and cytoplasm, the embryo nucleus size shown might include partial cytoplasm. The comparison of embryo nuclear and nucleolar size between *emb99* and wild-type seed shows no significant differences. So the embryo morphology and cellularization are normal until torpedo stage, when the embryo is arrested. Thus, there could also be some defective factors in the embryo which are important for the embryo to continue maturing. Alternatively the lack of endosperm cellularization may hinder further development of embryo.

The above-mentioned mutant analyses were limited to the morphological level. Further biochemical and molecular investigations need to be performed to define the stage at which endosperm cellularization could go wrong in these mutants. Immunohistochemistry methods and confocal laser scanning microscopy could be used to observe whether there is any nucleocytoplasmic domain (NCD) formation or polarization. After defining the exact step where the endosperm cellularization is stopped, we could put our emphasis on those specific factors involved in these steps, which might help us to answer questions

like: what is the triggering factor for endosperm cellularization? The molecular cloning of the mutated gene is the best way to reveal the gene function. The T-DNA tagging status of these two mutants need to be resolved.

Complementation tests demonstrated that *emb99* and *emb1309* are not allelic. So it seems that there are a number of genes in the *Arabidopsis* genome causing similar defects in endosperm cellularization. *emb1309* shows titan-like endosperm nucleoli structures (more irregular and larger endosperm nucleoli) which were not usually found in *emb99*. We can also propose that *emb1309* represents an intermediate type of mutant between *emb99* and titans. Isolation of further mutants in this class will hopefully give more insight into the topic of endosperm cellularization and cell cycle regulation.

CHAPTER 5

FEMALE GAMETOPHYTE EXPRESSION

1. INTRODUCTION

The plant life cycle is composed of a haploid gametophyte phase and a diploid sporophytic phase. Angiosperms have two kinds of reduced gametophytes compared to lower plants: the male gametophyte within the anther and female gametophyte (or embryo sac) in the ovule. The male gametophyte consists of two sperm cells encased within one vegetative cell. The most common type of female gametophyte is the *Polygonum* type, which is comprised of seven cells: two synergid cells, one egg cell, three antipodal cells and one central cell with two polar nuclei inside (Drews et al. 1998; Christensen et al. 1998). Female gametophyte development includes two phases: megasporogenesis and megagametogenesis. During megasporogenesis, four haploid megaspores arise from a diploid megaspore mother cell; three of them die. In *Arabidopsis*, during the process of megagametogenesis, the only surviving megaspore undergoes three rounds of mitosis and gives rise to an eight-nucleate cell. Then, nuclear migration, nuclear fusion and cellularization

produce the seven-celled *Polygonum* type female gametophyte. The antipodal cells degenerate before fertilization.

The female gametophyte plays several important roles in plant sexual reproduction, including those of directing the pollen tube to the ovule, directing double fertilization, inducing seed development and affecting embryo and endosperm development (Drews et al 1998). Molecules produced by genes expressed within the female gametophyte or in the surrounding sporophytic tissues of the ovule could both affect female gametophyte development. Large-scale screening for female gametophyte mutants is a good way to identify genes with specific functions in female gametophyte development and function. There are three kinds of gametophytic mutations: female gametophyte specific mutations which are not transmitted through the egg, male gametophyte specific mutations which are not transmitted through the sperm, and general gametophytic mutations which affects both the female gametophyte and the male gametophyte and cannot be transmitted to subsequent generation (if they are fully penetrant). Female gametophyte specific mutations show non-Mendelian 3:1 segregation patterns and produce 50% mutant seeds regardless of the pollen genotype. There are two criteria combined together to identify female gametophyte mutants: reduced seed set (50% desiccated or defective seed), and segregation distortion for the ratio of Kanamycin-resistant to kanamycin-sensitive plants among T-DNA insertional mutagenized lines (Drews et al. 1998; Christensen et al. 1998).

The recent use of the confocal laser scanning microscope facilitates the study of female gametophyte development and function. Mutations at different stages have been isolated. Megagametogenesis mutants affecting specific steps were analyzed by Christensen et al. (1998). Non-allelic mutants *fem1 and fem2* are female gametophyte specific mutants, while fem3 and fem4 are general gametophytic mutants. Some other mutants *gfa2, gfa3, gfa4, gfa5, and gfa7* were also studied, which are all general gametophytic mutants. The *fem2, fem3, gfa4, gfa5* mutants are arrested very early at the one-nucleate stage, which indicated that "these haploid-expressed genes are required very early in megagametogenesis" (Drews et al. 1998; Christensen et al. 1998). The *fem1, fem4, gfa2, gfa3, and gfa7* mutants are defective in later events such as polar nuclei fusion and cellularization

Normally, seed development starts after fertilization in angiosperms. In order to explore the specific factors inducing seed development, mutant screens for fertilization-independent seed development were carried out and female gametophytic mutants, *fie, fis1, fis2, fis3* and *medea* were found correspondingly by Ohad et al (1996) and Chaudhury et al (1997) and Grossniklaus et al. (1998). *fis* mutants have long siliques and developing seeds in the absence of fertilization. The *fis* seed coat development is close to that of wild-type. *fis*3 totally lack egg transmissibility and embryo, while *fis*1 and *fis*2 only have low-level egg transmissibility and a low frequency embryo-like structure at the globular stage. *fis*3 endosperm development stops before cellularization, while *fis*1 and *fis*2 endosperm have partial cellularization. *fis*1 has a similar phenotype

to *medea*, and both were mapped to the top of chromosome 1. *fis*2 was mapped to the middle of chromosome 2. *fis*3 and *fie* have similar phenotypes and were located to the upper half of chromosome 3. After pollination by pollen from either *FIE/FIE*, *FIS/FIS* or *MEDEA/MEDEA* plants individually, 50% mutant seeds with embryo arrested at the torpedo stage and aberrant endosperm are formed in *fie, fis, medea* sliques correspondingly (*FIE/fie*, *FIS/fis* or *MEDEA/medea*) (Ohad et al.1996; Chaudhury et al.1997; Grossniklaus et al. 1998). The gametophytic nature of these mutants eliminated the possibility of complementation tests. But recent molecular cloning revealed that the protein encoded by *FIS*1 encodes is homologous to the *Drosophila Polycomb* group gene Enhancer-*of-zeste* and is allelic to *MEDEA* gene (Grossniklaus et al. 1998; luo et al. 1999). In animals, *Polycomb* group proteins are recognized as repressors of homeotic genes that ensure the stable expression patterns through cell division and regulate cell proliferation (Grossniklaus et al. 1998). The difference between *fis*1 and *medea* is that *fis*1 is a null allele lacking all the protein domains, while the *medea* protein only misses the SET domain (Luo et al. 1999). The name "SET" came from the three founding members of the family in *Drosophila-Suppressor of variegation 3-9 [Su(var)3-9], E(z), and trithorax (trx)* (Grossniklaus et al. 1998). *FIS*2 gene was also cloned by the same group of researchers. It encodes a C_2H_2 zinc-finger transcriptional factor (Luo et al. 1999). The *FIE* protein is also a homolog of the *Polycomb* proteins from *Drosophila* and mammals (Ohad et al. 1999). There have been nine alleles of recessive *fie* mutants isolated so far. *FIE* is a single-copy gene in the *Arabidopsis* genome and is expressed both in reproductive and

vegetative tissues. It is likely to be an allele of *fis*3; molecular cloning of *fis*3 is now needed.

In summary, the study of gametophytic mutations will give insights to many crucial events of the angiosperm reproduction, such as: "the establishment of female gametophyte polarity, specification and differentiation of the female gametophyte cells, polar nuclei migration and fusion, antipodal cell and synergid cell death, pollen tube guidance, fertilization and induction of seed development, and gametophytic maternal control of seed development (Drews et al. 1998)". We have established a collection of female gametophyte mutants in the Meinke lab. *emb173, emb299, emb1188, emb1173, emb1005* discussed in the following sections have been studied at the genetic level.

2. MATERIALS AND METHODS

2.1 Growth of plant materials

Same as Chapter 2.

2.2 Mutant phenotypic analysis

The mutant *emb173* was produced in the Wassilewskija ecotype by Agrobacterium-mediated seed transformation (Feldmann and Marks, 1987). Kanamicin resistance was used as the selectable marker in this case. The mutant *ebm299, emb1188, emb1173, emb1005* were created in the Columbia ecotype by same method at Novartis in North Carolina. Hygromicin resistance was used for *emb299* and *emb1005*. Basta resistance was used for all the other mutants.

The methods are mainly the same as in Chapter 2. Mutant analyses under dissecting microscope and Nomarski microscope were performed. Endosperm nucleolus numbers were estimated. Endosperm nucleolus diameters at cotyledon stages were measured.

In addition, complementation crosses between these mutants and wild-type were performed to determine the female gametophyte mutant nature. The crossing procedure is the same as mentioned in Chapter 3. *emb173, ebm299, emb1188, emb1173, emb1005* were used as female parents.

2.3 Classical genetic mapping

Method is mainly the same as described in Chapter 2. Multiple marker lines DP23, DP28/hy2 were used for mapping *emb1188*. Since genetic linkage has shown before screening DP24 crossed plants, the screening of DP24 crossed plants was not performed.

3. RESULTS

emb173 is untagged according to genetic analysis of other members in the Meinke lab. The tagging status of *emb299*, *emb1188* and *emb1005* is still unresolved due to multiple T-DNA insertions. The tagging status of emb1173 is not examined. In all cases, mutant seeds in siliques are usually half-translucent with sticky seed coat. Inside the seed, the liquid is thick and sometimes contains white particles. Seed sizes are usually enlarged or similar to those of wild-type. A green torpedo stage embryo can be seen by dissection under the dissecting microscope. The ratios of defective seeds to wild-type seeds in siliques are close to 1:1 in all cases.

These mutants have many similarities in phenotype including: enlarged endosperm nucleoli, long, irregular stripes present in endosperm with larger and more condensed endosperm nucleoli at the chalazal end. Endosperm nucleolar numbers of these mutants are approximately 200-300. It seems that when the mutant seeds mature, the endosperm nucleoli are more enlarged. When wild-type seeds are at heart stages, the mutant endosperm nucleoli are smaller than those of later stages. Sometimes several large cytoplasmic masses with condensed nucleoli inside were seen in the endosperm. Sometimes the distribution is more even. Subtle differences were also observed among these mutants. *emb299* endosperm nucleoli are only slightly enlarged from 4.8 μm to 12 μm and usually with round shapes. *emb173* has been described by Castle et

al. (1993). *emb173*'s endosperm nucleoli are enlarged more. Sizes vary from 6μm to 22.8μm. A few could be quite large and *titan*-like. Endosperm nucleolar sizes of *emb1188* are relatively consistent and are intermediate between those of *emb299* and *emb173*. Size ranges from 6.0μm to14.4μm. Most nucleoli are round shaped; ring, and irregular structures are seen sometimes. *emb1173* endosperm nucleoli are slightly enlarged and similar to those of *emb299*. Sizes vary from 1.2 μm to 13.2 μm. Sometimes many ring- and irregular structured nucleoli were seen, which are relatively larger. *emb1005* endosperm nucleoli are also slightly enlarged; Sizes vary from 3.6 μm to 13.2 μm. Ring-structure nucleoli were seen in this mutant. Average of endosperm nucleolar diameter was summerized in Table 16. In this case endosperm nucleolar diameters were rounded up to the nearest whole number.

Embryos were arrested at heart to early torpedo stages. Embryo cellular organizations are mainly normal except for some increased cell proliferation and irregular cell divisions. Vacuolated cells were seen often. Suspensors are mainly normal (Fig10).

Histological study showed no cellularization in sections when wild-type was at torpedo to early cotyledon stages. The desiccating seeds were not examined. The phenotypes of embryo proper and suspensor are consistent with the Nomarski microscope observations (Fig 11).

50% mutant seeds resulted from complementation test with wild-type pollen, which proved that all of these mutants are female gametophyte mutants. *Emb173* was mapped by Castle et al. (1993) to the top of chromosome 1.

emb299 was mapped by Amy Davis to the top1/3 of chromosome 5. *emb1188* was mapped to Chromosome 3 below hy2 and above gl1 recently (Table 17).

4. DISCUSSION

Maternal control of seed development in angiosperms includes gametophytic maternal control and sporophytic maternal control. Female gametophytic mutations affecting gametophytic maternal control of embryo or endosperm development produce 50% defective seed and 50% wild-type seeds in siliques (Drews et al., 1998). All such mutations, including the Meinke lab collection, display maternal effect embryo lethality regardless the pollen genotype (Ohad et al.1996; Chaudhury et al.1997; Grossniklaus et al. 1998). It has been proved through molecular cloning that *fis*1 is allelic to *medea*. We also suspect they are allelic to *emb173* from the very similar phenotypes and close mapping positions. For the same reason, the cloned *FIE* gene is possibly allelic to *FIS*3. But the final conclusions remain until further cloning results are shown. *FIS*1, *MEDEA*, and *FIE* gene products are all members of *Polycomb* protein group. Cloned *FIS*2 gene product was shown to be a zinc-finger transcriptional regulator (Grossniklaus et al. 1998; Luo et al. 1999; Ohad et al. 1999). One of the model among groups of researchers for the actions of these genes is that these gene products form multiprotein complexes with other unknown gene products to repress transcription of genes required for seed development. Mutation in any of these genes could lead to derepression of the seed development genes (SDG). In male gametes, however, one or more gametophytic maternal control related genes could be silenced epigenetically due to genomic imprinting. During fertilization, the derepressed SDGs are

introduced from pollen and lead to initiation of seed development (Luo et al. 1999). When both the male and female allele are present but function unequally in the embryo, the parental-dependent traits are produced, which is called genomic imprinting (Barlow, 1995). Genomic imprinting is very important in mammal embryonic development and has also been shown to exist in endosperm of angiosperms (Haig and Westoby, 1991; Scott et al. 1998). DNA methylation is the most likely mechanism so far to explain how genes are imprinted. Overall, these gametophytic maternal control related genes function as a part of the signal transduction pathway of the induction for seed development.

Identification of these mutants also gives insights into the topics of embryo and endosperm development. Since embryo development is generally arrested and endosperm development is aberrant in these mutants, the *FIE*, *FIS* and *MEDEA* genes seem to be required for normal embryo and endosperm development (Ohad et al. 1999). It is also possible that the abortion of the embryo is due to the abnormal diploid endosperm development. Since *fie* and *fis*3 allow the initiation of endosperm development without fertilization, but do not allow the embryo development at all, the mechanisms regulating embryo and endosperm development may be different (Ohad et al. 1996). Ohad et al. (1999) suggested *FIE Polycomb* protein functions to suppress endosperm development without fertilization. Since seed coat and siliques of mutant plants are similar to those of wild-type, it seems that the maternal seed coat and silique formation is the consequence of stimulation by endosperm development.

These mutant phenotypes are not transmitted through the female gametophyte. The reason for this could be that the egg and central cell of the mutant plant do not function or interact properly after fertilization, or due to gene dosage or imprinting which means a single wild-type paternal *FIE* gene, or a silenced wild-type paternal *FIE* gene may not be able to rescue two mutant *fie* alleles from the maternal parent during double fertilization (Ohad et al. 1996). But the gene dosage experiment done by Grossniklaus et al. (1998) eliminated the possibility of the gene dosage hypothesis. The imprinting hypothesis is still possible.

The *medea* phenotype is consistent with the parental conflict theory for the evolution of parent-of-origin-specific effects or gene imprinting, which means paternal excess of paternally expressed imprinted loci is usually associated with enhanced growth of the endosperm and embryo, while the maternal excess of maternally-expressed imprited loci inhibits endosperm and embryo development (Haig and Westoby, 1991; Scott et al. 1998). As the consequence of the lost function of the *MEDEA* gene, which is under maternal control, an embryo with increased cell proliferation results (Grossniklaus et al, 1998).

Finally, the identification of *FIE*, *FIS* and *MEDEA* could shed light on autonomous apomixis naturally occurring in some plant species. The fertilization-independent phenotype of these mutants implied that the altered activities or regulation of these genes could be related to the fertilization-independent reproductive development in nature.

The female gametophytic mutants in the Meinke lab collection possess related but slightly different phenotypes resembling those of *FIE*, *FIS* and *MEDEA*. Further molecular cloning needs to be performed following tagging status analysis in order to identify the molecules affecting female gametophyte function and to decide the allelic relationships between these mutants and with the mutants studied in other labs. Eventually this research can lead us to a better understanding of angiosperm sexual reproduction.

Figure 1. Drawing of *Arabidopsis thaliana* from Ross-Craig(1948)
A. Mature plant showing rosette leaves, main stem and lateral branches with terminal infloresence; B. Trichomes on the surface of rosette leaves; C. Flower at the time of pollination; D. Floral petal; E, F. stamens; G. compound pistil; H. Mature silique with valves split in the middle to show two rows of seeds; I. Mature seed.

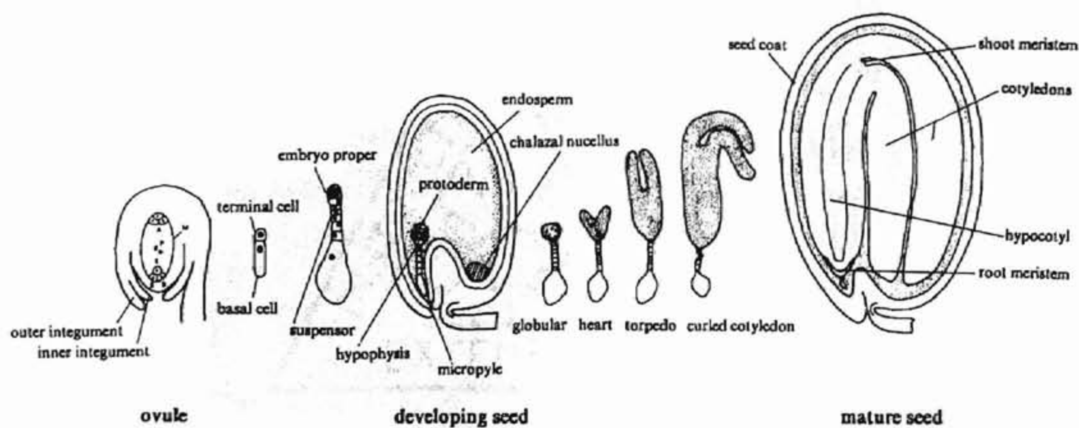

Fig2. Reproduced from Castle and Meinke (1993). Embryo and seed development in *Arabidopsis*. The megagametophyte (M) within the ovule contains three antipodals (A), two polar nuclei (P) in the central cell, two synergids (S) and the egg (E). Double fertilization of the egg and polar nuclei leads to a diploid zygote and triploid endosperm. The zygote divides asymmetrically to produce the terminal cell, which becomes the embryo proper, and the basal cell, which gives rise to the hypophysis and suspensor. The embryo goes through globular, heart, torpedo and cotyledon stages before seed maturity. The mature embryo contains a shoot meristem between the cotyledons and a root meristem below the hypocotyl.

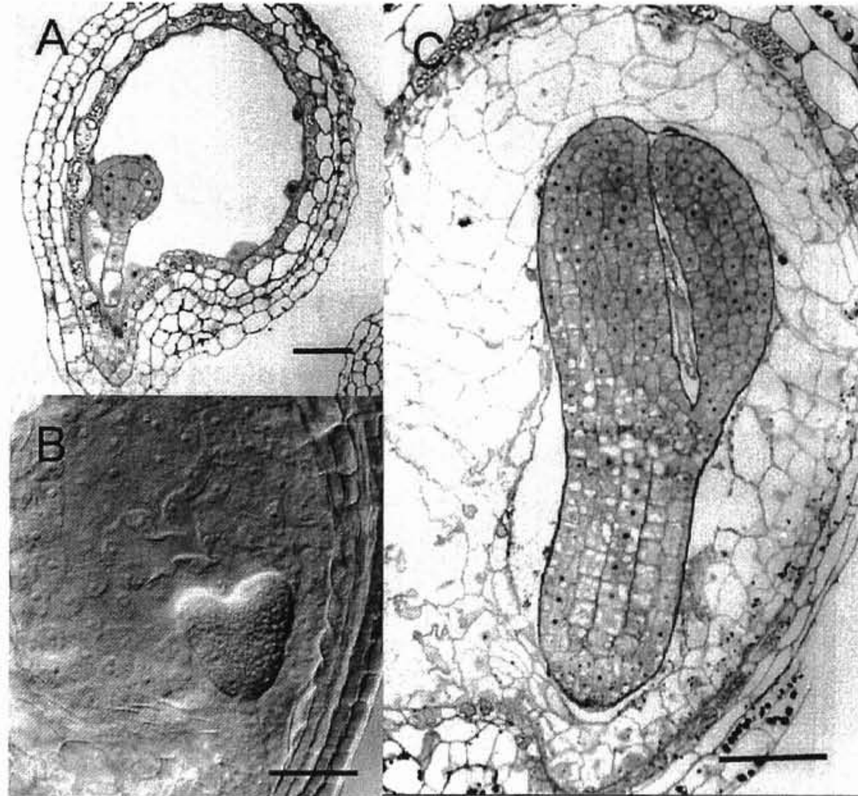

Fig 3. Normal endosperm development of wild-type Arabidopsis seed at: A. globular stage (image under light microscope of plastic section); B. heart stage(image under Nomarski scope of cleared seed); C. cotyledon stage (image under light microscope of plastic section). Scale bar=48um

Normal *Arabidopsis* Endosperm Development

Table 1. Endosperm Nucleolar Number of Wild-type *Arabidopsis* Seeds

Stage	Endosperm Nucleolus Number	
	Average	Range
Globular	98 ± 31	49 - 140
Heart	215 ± 56	141 - 313
Torpedo	320 ± 95	195 - 495

The endosperm nucleolar numbers of 10 seeds were counted at each stage

Table 2. Endosperm Nucleolar Diameter of Wild-type *Arabidopsis* Seeds

Stage	Diameter of Endosperm Nucleoli (μm)	
	Average	Range
2 cell EP[a]	5.3 ± 0.6	4.2 - 6.0
4-8 cell EP	5.1 ± 0.7	3.6 - 6.0
Early Glob[b]	4.8 ± 0.4	4.2 - 4.8
Late Glob	5.0 ± 0.7	3.6 - 6.0
Heart	4.2 ± 0.5	3.6 - 4.8
Torpedo	3.0 ± 0.6	2.4 - 3.6

[a] EP: embryo proper
[b] Glob: Globular stage

Table 3. Comparison of Seed Size of *emb1002* and Wild Type seeds

Genotype	Stage	Length (μm)		Width (μm)	
		Average	Range	Average	Range
Wild-type	Cotyledon	593 ± 34	529 - 656	415 ± 38	345 - 483
Emb1002	Cotyledon	557 ± 39	483 - 644	431 ± 40	357 - 529

Sizes of 20 seeds for each of *emb1002* and wild-type were measured with a micrometer under dissecting scope.

Table 4. Comparison of Endosperm Nucleolar Number of *emb1002* and wild-type seeds

Genotype	Stage	Endosperm Nucleolus Number	
		Average	Range
emb1002	Globular	87 ± 32	60 - 135
	Heart	202 ± 123	141 - 344
	Torpedo	327 ± 91	181 - 415
	Cotyledon	181 ± 56	135 - 255
Wild-type	Globular	98 ± 31	49 - 140
	Heart	215 ± 56	141 - 313
	Torpedo	320 ± 95	195 - 495

The endosperm nucleolar numbers of 5 emb1002 and 10 wild-type seeds at each stage were counted

Table 5. Comparison of Endosperm Nucleolar Diameter of *emb1002* and wild-type seeds

Genotype	Stage	Diameter of Endosperm Nucleoli (μm)	
		Average	Range
emb1002	Globular	4.7 ± 0.8	3.6 - 6.0
	Heart	5.7 ± 0.9	3.6 - 7.2
	Torpedo	3.8 ± 1.0	2.4 - 6.0
wild-type	Late Glob	5.0 ± 0.7	3.6 - 6.0
	Heart	4.2 ± 0.5	3.6 - 4.8
	Torpedo	3.0 ± 0.6	2.4 - 3.6

The diameter of 20 endosperm nucleoli were measured at each stage

Table 6. *emb1002* Embryo Proper Width

Stage	Width of Embryo Proper (μm)	
	Average	Range
Globular	14.2 ± 5.0	6.0 - 20.4
Heart	19.0 ± 3.4	14.4 - 25.2
Torpedo	19.7 ± 2.0	16.8 - 24.0
Cotyledon	17.3 ± 2.9	13.2 - 21.6

The embryo proper width of 10 *emb1002* seeds were Measured.

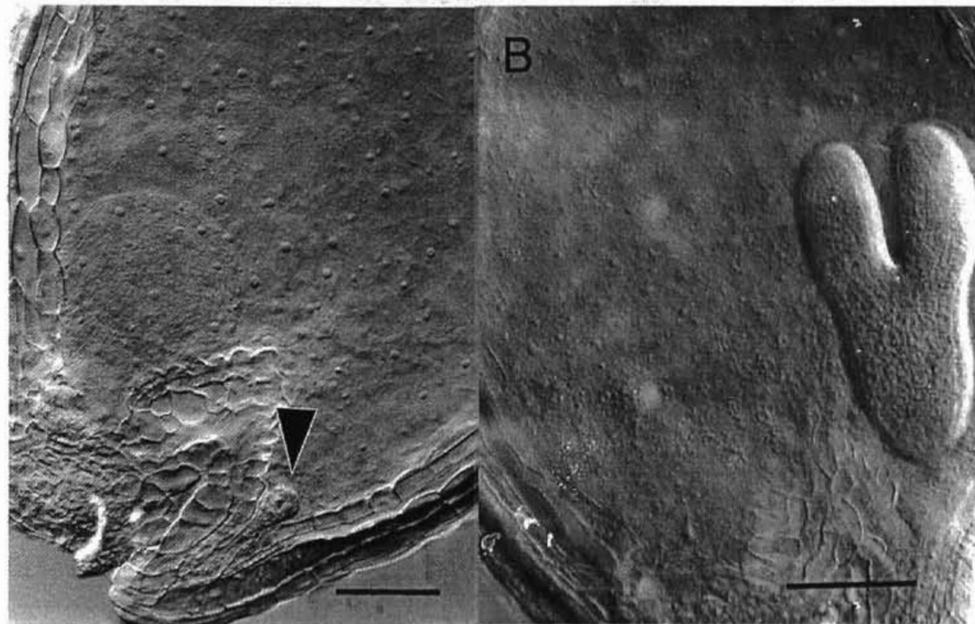

Fig 4. emb1002 mutant phenotype under Nomarski compound scope compared with wild-type. A.emb1002 at torpedo to cotyledon stage (which is defined by the stage of wild-type seeds in the same silique of the mutant seed); B. wild-type seed at late torpedo to early cotyledon stage. Scale bar=48um

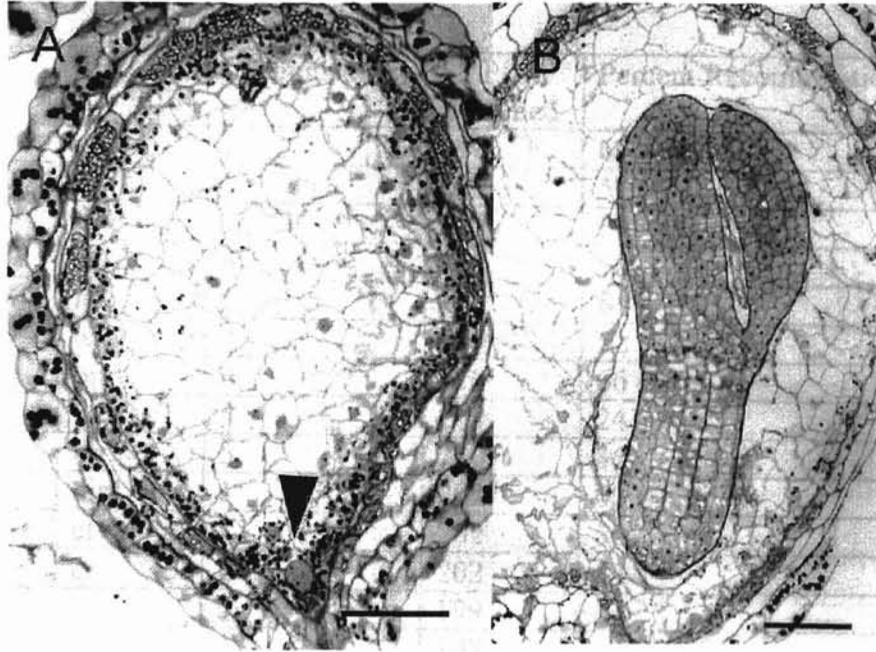

Fig 5. *emb1002* mutant phenotype in plastic sections compared to wild-type. A. *emb1002* at mature stage with extremely early arrested embryo (which is pointed by arrow-head); B. wild-type seed at late torpedo to early cotyledon stage. Scale bar=48um

Table 7. Recombination Estimates Between *emb1002* and Visible Markers

Marker		Chromosome	F2 Plant Screened	Percent Recombination[a]
DP23:	ch1	1	198	69
	er	2	199	57
	gl1	3	199	64
	cer2	4	197	58
	tt3	5	191	6
DP24:	er	2	209	52
	bp	4	210	66
	yi	5	209	24
	ttg	5	210	36
DP28/hy2	dis1	1	202	60
	clv2	1	202	56
	er	2	202	53
	tt5	3	199	49
	hy2	3	198	66
W_6sti	sti	2	218	41
	cer8	2	211	56

[a] From the analysis results of the GeneMapping program.
In all cases, the AD Method of estimation of recombination percent in the GeneMapping program was used.

Table 8. *titan4* Endosperm Nucleolar Size

Stage	Length (μm)		Width (μm)	
	Average	Range	Average	Range
CotL[a]	22.3 ± 8.1	12.0 – 42.0	15.4 ± 5.5	6.0 - 30.0
CotS[b]	4.0 ± 1.6	1.2 - 7.2	3.5 ± 1.6	1.2 - 6.0

[a] The endosperm nucleoli at cotyledon stage with length above 10μm.
[b] The endosperm nucleoli at cotyledon stage with length below 10μm.
The diameter of 20 endosperm nucleoli were measured in each case.

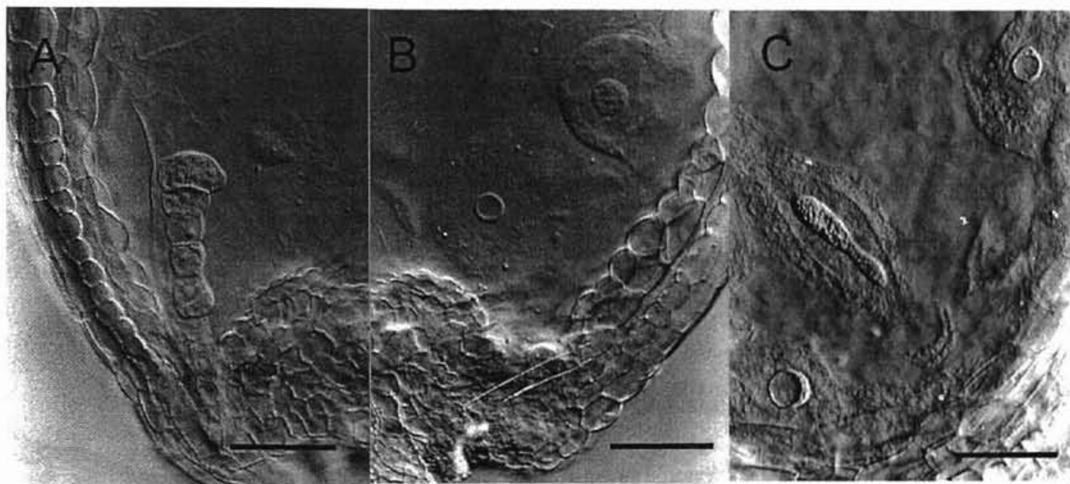

FIg 6. *titan4* mutant phenotype under Nomarski compound scope. A. *titan4* early-arrested embryo in mature seed; B. *titan4* giant endosperm nucleoli with ring- and irregular shapes; C. *titan4* giant endosperm nucleoli with ring- and irregular-shapes. Scale bar=48um.

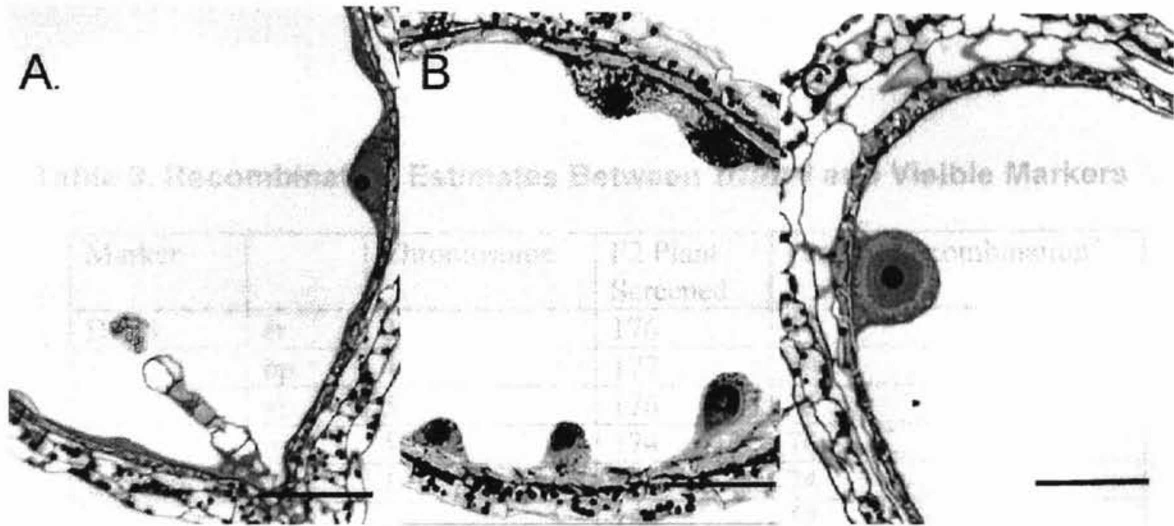

Fig 7. *titan4* mutant phenotype in plastic sections. A. *titan4* early-aborted embryo in mature seeds; B. C. *titan4* giant endosperm nucleoli in sections. Scale bar=48um

Table 9. Recombination Estimates Between *titan4* and Visible Markers

Marker		Chromosome	F2 Plant Screened	Percent Recombination[a]
DP24:	er	2	176	54
	bp	4	177	49
	yi	5	176	35
	ttg	5	174	74
DP28/hy2	dis1	1	186	74
	clv2	1	184	12
	er	2	186	64
	tt5	3	181	49
	hy2	3	185	60
W_6sti	sti	2	204	51
	cer8	2	192	61
	clv1	1	191	15

[a] From the analysis result of the GeneMapping program.
In all cases, AD Method of estimation of recombination percent in the GeneMapping program was used.

Table 10. Comparison of *titans*

Mutant Phenotypes	*titan1*	*titan2*	*titan3*	*titan4*	*Wild-type*
Endosperm:					
Nuclei Size	Giant	Giant	Giant	Giant	Normal
Mitotic Figures	Absent	Absent	Giant	Absent	Present
Nuclear Migration	Absent	Present	Present	Present	Present
Cell Wall Formation	Absent	Absent	Present	Absent	Present
Embryo:					
Nuclei	Giant	Normal	Normal	Normal	Normal
Cells	Giant	Aborted	Normal	Aborted	Normal
Viability	Aborted	Aborted	Viable	Aborted	Viable
Mapping Position	Bottom of Chrom. 3	Top of Chrom. 1	Bottom of Chrom. 5	Bottom of Chrom. 1	-----

Table 11. *emb99* and Wild-type Endosperm Nucleolar Number Comparison

Genotype	Stage	# seed Measured	Endosperm Nucleolus Number	
			Average	Range
emb99	Globular	5	103 ± 28	76 - 144
	Torpedo	5	230 ± 71	135 - 334
	Cotyledon	10	233 ± 67	120 - 346
Wild-type	Globular	10	98 ± 31	49 - 140
	Heart	10	215 ± 56	141 - 313
	Torpedo	10	320 ± 95	195 - 495

Table 12. *emb99* Endosperm Nucleolar Diameter

Stage	Diameter of Endosperm Nucleoli(μm)	
	Average	Range
Globular	8.3 ± 1.3	6.0 - 12.0
Heart	8.5 ± 1.3	7.2 - 12.0
Torpedo	9.2 ± 1.2	7.2 - 12.0
Cotyledon	10.0 ± 1.3	7.2 - 12.0

The diameter of 20 endosperm nucleoli were measured at each stage

Fig 8. *emb99* and *emb1309* mutant phenotype under Nomarski compound scope compared to wild-type. A.. *emb99* embryo is mainly normal at heart stage, but the endosperm nucleoli are enlarged; B. The cotyledons of *emb1309* are wider open than usual. The endosperm nucleoli are enlarged; C. wild-type at heart stage. Scale bar=48um

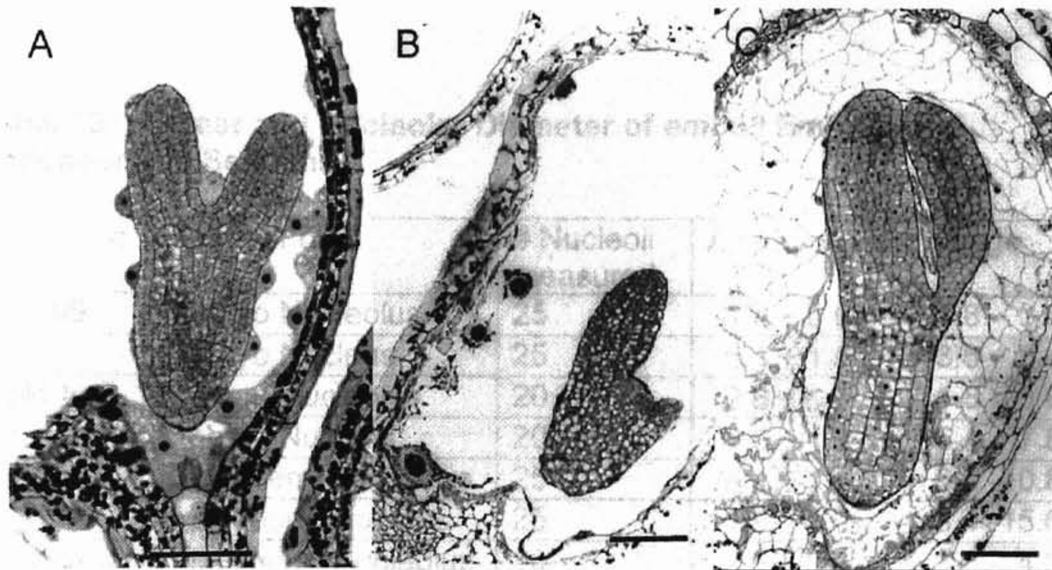

Fig 9. *emb99* and *emb1309* mutant phenotype in plastic sections compared to wild-type. A. *emb99* embryo is mainly normal when it is arrested at torpedo stage, while endosperm nucleoli are enlarged and no cellularization is observed in endosperm; B. The cotyledons of *emb1309* are wider open than usual. The endosperm nucleoli are enlarged and no cellularization is observed in endosperm; C. wild-type seed at torpedo stage. Scale bar=48um

Table 13. Nuclear and Nucleolar Diameter of *emb99* Embryo and Endosperm in Sections

Genotype	Diameter of	# Nucleoli measured	Average(μm)	Range
emb99	Embryo Nucleolus	25	2.4 ± 0.6	1.8 - 3.6
	Embryo Nucleus	25	6.1 ± 1.5	3.6 - 8.4
Wild-type	Embryo Nucleolus	20	2.6 ± 0.5	1.8 - 3.8
	Embryo Nucleus	20	6.6 ± 1.0	5.4 - 7.8
emb99	Endosperm Nucleolus	25	8.4 ± 1.4	6.0 -10.8
	Endosperm Nucleus	25	11.8 ± 1.9	8.4 -15.6
Wild-type	Endosperm Nucleolus	10	2.5 ± 0.8	1.8 - 4.2
	Endosperm Nucleus	10	8.2 ± 2.6	4.2 - 13.8

Seed stage: late torpedo – cotyledon

Table 14. Recombination Estimates Between emb99 and Visible Markers

Marker	Chromosome	F2 Plant Screened	Percent Recombination[a]
gl2	er	207	46
	gl2	209	21
Clv2	clv2	140	23
W_6sti	sti	218	41
	cer8	211	56

[a] From the analysis result of the GeneMapping program.
In all cases, AD Method was used.

Table 15. Recombination Estimates Between *emb1309* and Visible Markers

Marker		Chromosome	F2 Plant Screened	Percent Recombination[a]
DP23:	ch1	1	183	39
	er	2	177	48
	gl1	3	183	44
	cer2	4	181	53
	tt3	5	163	67
DP24:	er	2	258	50
	bp	4	258	56
	hy2	5	252	75
	ttg	5	256	52
DP28/hy2	dis1	1	210	51
	clv2	1	209	26
	er	2	210	56
	tt5	3	187	67
	hy2	3	208	49

[a] From the analysis results of the GeneMapping program.
In all cases, the AD Method of estimation of recombination percent in the GeneMapping program was used.

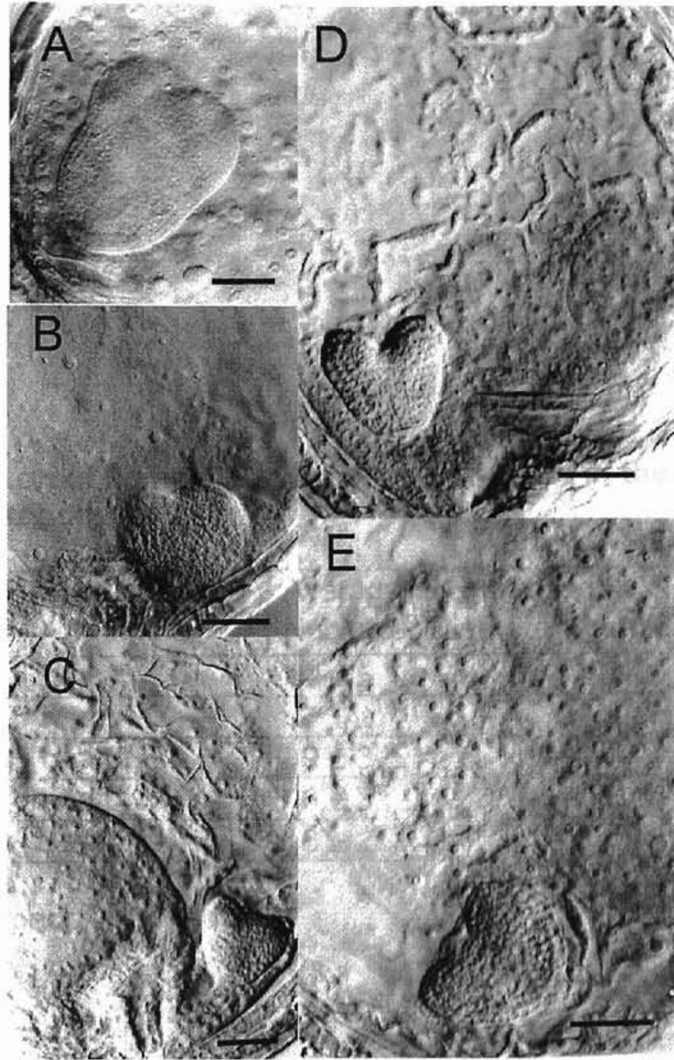

Fig 10. Mutant Phenotypes of *emb173, emb299, emb1188, emb1173*, and *emb1005* under Nomarski compound scope. A. *emb173;* B. *emb299*; C. *emb1188*; D. *emb1173*; E. *emb1005*. Scale bar=48um

Table 16. Comparison of Female Gametophytic Mutants in the Meinke Lab Collection

Genotype	Stage Embryo Arrested	Average of Endosperm Nucleolar Diameter (μm)
emb173	heart	11 ± 5
emb299	heart	7 ± 2
emb1188	heart	9 ± 3
emb1173	heart	6 ± 3
emb1005	heart	6 ± 2

Seed stage: late torpedo – cotyledon

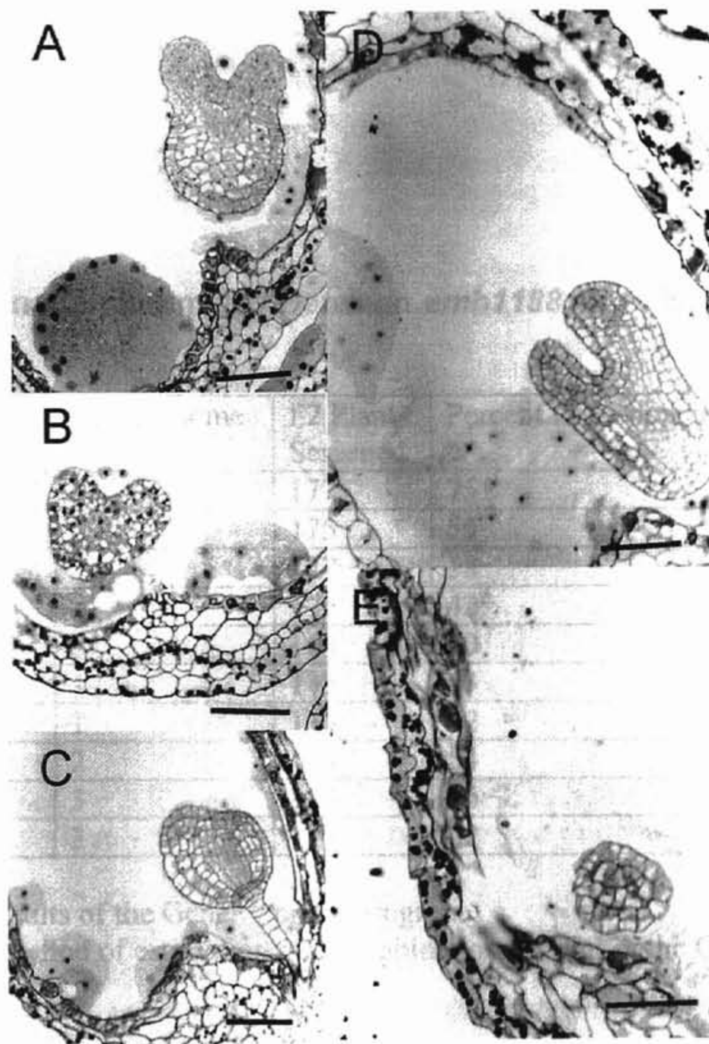

Fig 11. Mutant phenotypes of *emb173, emb299, emb1188, emb1173*, and *emb1005* in plastic sections. A.. *emb173*; B. *emb299*; C. *emb1005*; D. *emb1188*; E. emb1173. Scale bar=48um.

Table 17. Recombination Estimates Between *emb1188* and Visible Markers

Marker		Chromosome	F2 Plant Screened	Percent Recombination[a]
DP23:	ch1	1	177	75
	er	2	175	55
	gl1	3	177	18
	cer2	4	176	42
	tt3	5	164	50
DP28/hy2	dis1	1	135	53
	clv2	1	135	72
	er	2	135	54
	tt5	3	128	56
	hy2	3	134	22

[a] From the analysis results of the GeneMapping program.
In all cases, the AD Method of estimation of recombination percent in the GeneMapping program was used.

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