Analysis of communication pathways during seed development in *Arabidopsis thaliana*

Inaugural-Dissertation

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I. CONTENTS

II. ZUSAMMENFASSUNG

Während der Samenentwicklung in Blütenpflanzen müssen die einzelnen Entwicklungsprozesse des Embryos, des Endosperms und der umgebenen maternal, sporophytischen Integumenten, die von genetisch unterschiedlichem Ursprung sind, untereinander abgestimmt werden. Bisher ist nur wenig über die Koordination zwischen endospermalen und embryonalen Wachstum bekannt. In *Arabidopsis* entsteht auf Grund von Mutationen im Zellzyklusregulatorgens *"CYCLIN DEPENDENT KINASE A;1"* (*CKDA;1*)' Pollen, der nur die Eizelle befruchtet. Die Samen, die aus der Kreuzung mit *cdka;1* entstehen, beinhalten rein maternales Endosperm. In dieser Arbeit wurde *cdka;1* mutanter Pollen zur Entschlüsselung der einzelnen Prozesse während der frühen Samenentwicklung verwendet.

Kreuzungen von 14 *Arabidopsis* Ökotypen mit *cdka;1* mutanten Pollen wiesen eine breite genetische Varianz in Hinblick auf Endospermentwicklung ohne paternalen Genombeitrag auf. In den hierdurch entstandenen Samen lief die embryonale Entwicklung zunächst autonom ab, wurde jedoch später durch die Menge an endospermalen Nuklei beschränkt. Sechs Komponenten, die die Entwicklung von unbefruchtetem Endosperm beeinflussen, wurden in einer QTL Analyse unter Verwendung einer rekombinanten Inzuchtpopulation zwischen den beiden *Arabidopsis* Ökotypen Bayreuth-0 und Shadara entdeckt. Insgesamt wurden vier QTLs detektiert, die von zwei haupt und vier komplexen Loci gebildet wurden. Die Gene zweier DNA N-Glykosylasen ROS1 und DME, die die Demethylierung von symmetrischen Cytosinmethylierungen katalysieren, lagen in den Intervallen von einem der zwei komplexen QTLs. Durch eine phänotypische Analyse konnte ein neuer funktionaler Aspekt von *ROS1* und *DME* in der Beschränkung der Proliferation von unbefruchtetem Endosperm gezeigt werden. *ros1-dme* Doppelmutanten retteten den durch *cdka;1* Befruchtung hervorgerufenen Samenabort in Abhängigkeit von der Aktivität der DNA Methyltransferase MET1 während der sporophytischen Entwicklungsphase. Die fehlende Co-Transmission des mutanten *dme* Allels in den geretteten Samen wies auf einen ‚Trans-Effekt' durch *ros1* und *dme* hin. Eine nähere Untersuchung ergab, dass der mutanten Phänotyp in einer segregierenden Population paramutationsähnlich vererbt wurde. Auf Grund der Messung von fast 100% relativem Methylierungsgehalt auf Sequenzabschnitten von *PHE1* und *AGL36* in *ros1-dme* Doppel- im Vergleich zu Einzelmutanten und Col-0 Wildtyppflanzen wurde Hypermethylierung als Ursache für den mutanten Phänotyp angenommen. Diese Hypermethylierung wurde wahrscheinlich durch Trans-Interaktionen zwischen homologen DNA-Sequenzen auf unterschiedlichen Chromosomen in paternal

dominanter Art und Weise hervorgerufen. Die in dieser Arbeit dargestellten Ergebnisse bestätigen vormalige Ergebnisse, dass Endospermentwicklung unter anderen durch die Veränderung des Methylierungslevels vor der Befruchtung beeinflusst werden kann. Darüber hinaus deuten die gewonnen Erkenntnisse darauf hin, dass paternale DNA-Methylierungsmuster übertragen werden können. Die vorliegenden Daten zeigen, dass vermutlich nicht nur der epigenetische Status des Sporophyten, sondern auch der des hierauf folgenden Gametophyten und die Samenentwicklung nach erfolgter Befruchtung von paternaler Seite beeinflussbar sind.

III. ABSTRACT

Seed development in flowering plants requires coordination between the two genetically different fertilization products, the embryo and the endosperm and the surrounding maternal tissues, the integuments. However, little is known about the coordination of endosperm and embryo growth. In *Arabidopsis*, mutations in the cell cycle regulator *CYCLIN DEPENDENT KINASE A;1* (*CKDA;1*) result in pollen that only successfully fertilizes the egg cell and seeds generated from crosses with *cdka;1* pollen develop endosperm with solely maternal contribution. Here, fertilization by the cdka;1 mutant pollen was used to dissect early seed development. Crosses of 14 *Arabidopsis* accessions pollinated with *cdka;1* mutant pollen revealed a large natural genetic variation with regard to the development of endosperm without paternal contribution. This work revealed a surprisingly large degree of autonomy in embryo growth, but also showed the embryo's growth restrictions with regard to endosperm size. By using a recombinant inbred line population between the two *Arabidopsis* accessions Bayreuth-0 and Shahdara four QTLs were discovered, two main and four complex loci that influence the development of unfertilized endosperm. The genes of two DNA N-glycosylases ROS1 and DME, which catalyze the demethylation of symmetrical cytosine methylation, lay inside the two intervals of one of the two complex QTLs. A functional analysis revealed a new aspect of *ROS1* and *DME* in restricting the proliferation of unfertilized endosperm. Moreover, *ros1-dme* double mutants could rescue the observed seed abortion upon *cdka;1* pollination dependent on the activity of the methyltransferase MET1 during the sporophytic phase. Surprisingly, the rescue was independent of *dme* co-transmission, indicating an effect of *ros1* together with *dme* in trans. The inheritance pattern of the mutant phenotypes revealed a paramutation-like phenomenon and the detection of almost 100% relative methylation levels on *PHE1* and *AGL36* sequence loci in *ros1-dme* double compared to the single mutants and Col-0 wild-type plants suggested that hypermethylation caused the mutant phenotype. The observed hypermethylation is likely to be established by in trans interactions between homologous DNA sequences on different chromosomes in a dominant paternal manner. These findings confirm previous results that endosperm formation is, beside other factors, triggered by the alteration of methylation levels prior to fertilization. Furthermore, DNA methylation patterns can probably be transferred via the paternal gametes, influencing not only the epigenetic status of the sporophyte, but also of the following gametophyte and affecting seed development after subsequent fertilization.

IV. PUBLICATIONS

Natural variation in the degree of autonomous endosperm formation reveals independence and constraints of embryo growth during seed development in *Arabidopsis thaliana*

Ungru, A., M. K. Nowack, M. Reymond, R. Shirzadi, M. Kumar, S. Biewers, P. E. Grini and A. Schnittger (2008). Genetics 179(2): 829-841.

- Apart of the KS22:GFP pictures taken by M.K.N, the vanillin staining done by R.S & P.E.G and the seed weight and size measurements by M.K. & S.B., I did all the work for this paper.

Reproductive cross-talk: seed development in flowering plants

Nowack, M. K., A. Ungru, K. N. Bjerkan, P. E. Grini and A. Schnittger (2010). Biochem Soc Trans 38(2): 604-612.

- Apart of the pictures designed by K.N.B, the review was written in teamwork by all listed authors

Genome-wide Transcript Profiling of Endosperm without Paternal Contribution Identifies Parent-of-origin Dependent Regulation of *AGAMOUS-LIKE36*

Shirzadi, R., E. D. Andersen, K. N. Bjerkan, B. M. Gloeckle, A. Ungru, C. Koncz, R. B. Aalen, A. Schnittger and P. E. Grini (submitted).

- For this paper, I measured the expression of *AGL36* by qRT-PCR in *dme* and *mea* mutant and Col-0 wild-type seeds.

V. ABBREVIATIONS AND GENE NAMES

%	percent
°C	degree Celsius
3'	three prime end of a DNA fragment
35S	35S promotor from the Cauliflower Mosaic virus
5'	five prime end of a DNA fragment
AGL	*AGAMOUS-LIKE*
AGO4	*ARGONAUTE4*
An-1	*Arabidopsis thaliana* Antwerp-1 accession
ANOVA	Analysis of variance, statistical method
ARF2/MNT	*AUXIN RESPONSE FACTOR 2/MEGAINTEGUMENTA*
Atg	*Arabidopsis thaliana* genome
Bay-0	*Arabidopsis thaliana* Bayreuth-0 accession
BGA	*BORGIA*
bp	base pair
Bur-0	*Arabidopsis thaliana* Burren-0 accession
C	DNA content of a haploid genome
CAP2	*CAPULET*
CC	central cell
CDKA;1	*CYCLIN-DEPENDENT KINASE A1*
cDNA	complementary DNA
CMT3	CHROMOMETHYLASE 3
Col-0	*Arabidopsis thaliana* Columbia-0 accession
Cvi-0	*Arabidopsis thaliana* Cape Verde Islands-0 accession
DAE	days after emasculation
DAP.	days after pollination
DCL3	*DICER-LIKE 3*
DDM1	*DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1*
DME	*DEMETER*
DML2	*DEMETER LIKE 2*
DML3	*DEMETER LIKE 3*
DNA	desoxyribonucleic acid
DRM1	*DOMAINS REARRANGED METHYLTRANSFERASE 1*
DRM2	*DOMAINS REARRANGED METHYLTRANSFERASE 2*
E(z)	*Enhancer of zeste (Drosophila melanogaster)*
EC	egg cell

EDTA	ethylenediaminetetraacetic acid
EDV	endosperm division value
Esc	*Extra sex combs (Drosophila melanogaster)*
ESR	embryo-surrounding region
Est-1	*Arabidopsis thaliana* Estland-1 accession
et al.	*et alii* / *et aliae* [Lat.] and others
ETCs	endosperm transfer cells
EZA1	*ENHANCER OF ZESTE-LIKE 1*
F1, F2, F3	first, second, third... filial generation after a cross
FIE	*FERTILIZATION-INDEPENDENT ENDOSPERM*
Fig.	Figure
FIS2	*FERTILIZATION-INDEPENDENT SEED 2*
FIS-class	proteins forming the core of the FIS-PRC2
FIS-PRC2	Arabidopsis PRC2 containing MEA, FIS2, FIE, and MSI1
FWA	*FLOWERING WAGENINGEN*
gene-/-	homozygous mutant of a gene
gene+/-	heterozygous mutant of a gene
GFP	green fluorescent protein
GLC	*GLAUCE*
GUS	BETA-GLUCURONIDASE
HADC	histone deacetyltransferase
HATs	histone acetyltransferases
H3K27	Lysine residue 27 of the histone H3
i.e.	Id est (Lat.) that is
IKU2	*HAIKU 2*
ISR	intergenic region
KAL	*KALYPSO*
Kas-1	*Arabidopsis thaliana* Kashmir-1 accession
kb	1000 base pairs
KIR	*KIRKE*
KYP	*KRYPTONITE*
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession
MEA	*MEDEA*
MET1	*METHYLTRANSFERASE 1*
MN1	*MINIATURE*
MPC	*MATERNALLY EXPRESSED PAB C-TERMINAL*
MSI1	*MULTICOPY SUPPRESSOR OF IRA 1*

Mt-0	*Arabidopsis thaliana* Martuba-0 accession
n	Number
Nd-1	*Arabidopsis thaliana* Niederzenz-1 accession
No-0	*Arabidopsis thaliana* Nossen-0 accession
ORF	OPEN READING FRAME
PcG	Polycomb-group
PCR	polymerase chain reaction
PHE1	*PHERES1*
PRC2	Polycomb Repressive Complex 2
qRT-PCR	quantitative Real-time PCR
QTL	quantitative trait loci
RBR1	*RETINOBLASTOMA RELATED 1*
RdDM	RNAi directed DNA methylation
RNA	ribonucleic acid
RDR2	*NA-DEPENDENT RNA POLYMERASE2*
ROS1	*REPRESSOR OF SILENCING1*
RT PCR	reverse transcription followed by a polymerase chain reaction
S phase	synthetic phase of the cell cycle
SC	Sperm cell
Sha	*Arabidopsis thaliana* Shahdara accession
siRNAs	small interfering RNA
SWN	*SWINGER*
t-DNA	transferred DNA
Tris/HCl	buffer containing 2-amino-e-hydroxymethyl-1,3-propanediol and HCl
TTG2	*TRANSPARENT TESTA GLABRA 2*
UTR	untranslated region
WS-0	*Arabidopsis thaliana* Wassilewskija-0 accession
wt	wild-type
x	crossed to (crosses are always indicated in the order: female x male)

The nomenclature for plant genes follows the *Arabidopsis* standard: *GENES* are written in upper case italics, while mutant *genes* are indicated in lower case italics. PROTEINS appear in upper case regular letters, mutant proteins in lower case regular letters.

VI. FIGURE AND TABLE INDEX

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1 INTRODUCTION

1.1 The Seed

In flowering plants, seeds form a highly intricate unit comprised of three parts: integuments, endosperm and embryo. The latter represents the next generation and its growth is supported by the surrounding endosperm. The construction of the seed serves three purposes. First of all, seeds are dispersal units in space and time. A hard shell and an extremely decreased metabolism allow them to survive under unfavorable conditions for lengthy periods of time, until acceptable conditions allow for germination. The distribution of the progeny depends on the shape of the surface, which can enable them to fly or swim. Finally, seeds are filled with nutrients providing the developing embryo and often also the germinating seedling with energy reserves. Within a plant, seeds are one of the most complex structures, representing tissues of three different genetic origins. In diploid plants two of them are fertilization products, which are the triploid endosperm coming from the fertilization of the homodiploid central cell, and the diploid embryo resulting from the fertilization of the haploid egg cell. This event is called double fertilization and is unique to flowering (Sitte et al. 2002). The third genetically different seed structure is the seed coat comprised of several layers of sporophytic origin contributed by the mother plant. Thus, a temporal and spatial coordination of growth between these different tissues is critical for reproductive success.

1.2 Endosperm development

Endosperm has specialized functions to support proper seed development: it supplies the embryo with nutrients, it initiates integument growth and differentiation, and plays a major role in further developmental processes such as dormancy and germination (Holdsworth et al. 2008; Nowack et al. 2010). Furthermore, endosperm size might function as a reproductive border to avoid inbreeding and interspecies hybridization (Bushell et al. 2003; Kinoshita 2007; Walia et al. 2009). One probable basis for this reproduction barrier is the responsiveness towards gene dosage effects. The genes controlling this process are regulated especially by epigenetic

mechanisms such as genomic imprinting. Cytosine methylation of the nuclear DNA and lysine methylation on histone H3 tails assure correct gene expression levels (Chan et al. 2005; Guitton and Berger 2005; Baroux et al. 2007; Huh et al. 2007; Ikeda and Kinoshita 2009).

Immediately after fertilization the endosperm undergoes a phase of free nuclear divisions followed by differentiation into distinct compartments with specific functions. These different functional units can best be seen in cereals. Here, endosperm transfer cells (ETCs) are located next to the maternal tissue and do uptake nutrients from the mother plant (Becraft 2001; Gutierrez-Marcos et al. 2004; Olsen 2004). The nutrient transfer from the ETCs to the embryo is mediated by the embryo-surrounding region (ESR). But most of the endosperm cells in cereals synthesize and store reserve proteins and starch until germination occurs. The final structure is the outer endosperm layer (aleurone), where enzymes are released to hydrolyze the stored nutrients in the starchy endosperm to support the hatching embryo (Becraft 2001; Olsen 2001; 2004; Li et al. 2008). In contrast to monocotyledonous in many dicotyledonous species, such as *Arabidopsis thaliana*, most of the endosperm is consumed except of the aleurone layer. Here, the endosperm acts as a temporary storage organ before the nutrients are transported to the developing embryo (Hirner et al. 1998). However, in mature *Arabidopsis* seeds the remaining single-cell aleurone layer plays a crucial role in seed dormancy, germination and seedling nutrition (Penfield et al. 2004; Bethke et al. 2007; Holdsworth et al. 2008).

1.3 The influence of endosperm formation on embryo development

Embryo development is dependent on a well developing and differentiating endosperm. The first evidence for this assumption came from inter-species crosses and inbreeding experiments with *Solanaceous* species and alfalfa. Due to a poorly developing endosperm embryo growth stopped and the seed aborted (Brink and Cooper 1939; Cooper and Brink 1942).

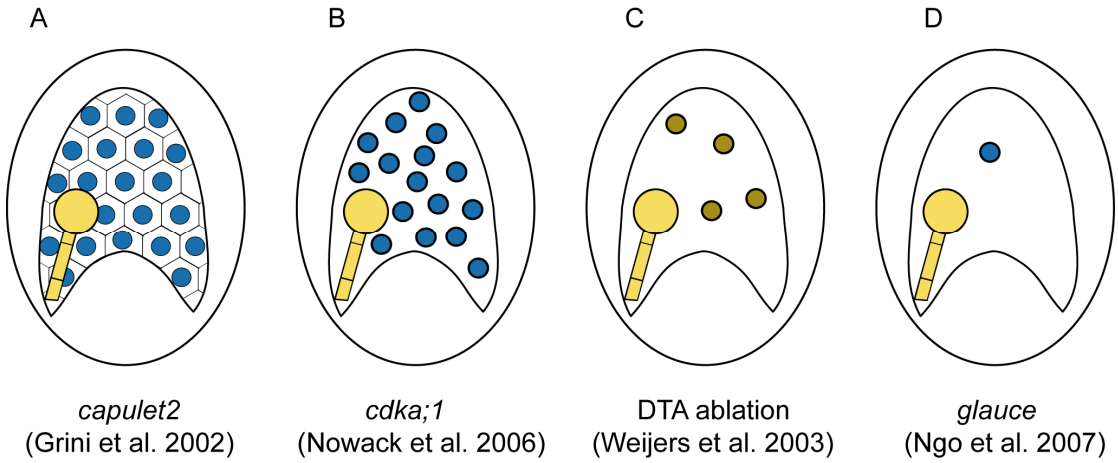

Figure 1: The time around the globular stage of embryo development is assumed to be a major checkpoint in seed development
Different experiments and mutant analyses suggest the developmental period around globular stage as a major checkpoint in seed development. In many cases of early endosperm failure, the embryo continues to develop until the globular stage apparently independent of the amount of endosperm nuclei. (**A**) In *cap2* mutants, endosperm proliferation stops prematurely and occasionally cellularizes. (**B**) In ovules fertilized with *cdka;1* mutant pollen, the fertilization process is accompanied by egg-cell fertilization and failure of karyogamy of the second sperm with the central cell. Here, various numbers of autonomous diploid endosperm nuclei can develop, without any contribution from the paternal genome. (**C**) Early endosperm ablation by DTA expression after the second to third round of free nuclear division. (**D**) In *glc* mutant seeds, the endosperm completely fails to proliferate.

Half a century later, further confirmation for the interaction of endosperm formation and embryo development was observed in early seed development. At this time endosperm-specific DTA (diphtheria toxin A) expression caused a proliferation arrest after two to three rounds of nuclear divisions resulting later in degeneration and causing embryo arrest and ultimately seed abortion (Fig. 1C) (Weijers et al. 2003). However, four to eight endosperm nuclei were enough to sustain embryo development at least for some time. Further evidence for the independency of embryo development was observed in experiments with a female gametophytic *glc* (*glauce*) mutant. In this genetic background just the egg cell was fertilized because the central cell was not able to fuse with the second sperm cell. Interestingly, the

embryo started to develop without any endosperm formation but arrested at globular stage (Fig. 1D) (Ngo et al. 2007).
Likewise, in *cap2* (*capulet*) mutant seeds endosperm proliferation ranged from one to five free nuclear divisions, occasionally involving precocious cellularization together with an embryo arrest at late globular stage in all situations (Fig1A) (Grini et al. 2002). Taken together, these findings suggest the existence of general checkpoints of seed development at the globular stage of embryo development.
One speculation for embryo arrest at the globular stage might be the function of the endosperm to form a sink tissue recruiting nutrients from the mother plant and supplying the developing embryo with nutrients. An abolished, underdeveloped or retarded endosperm formation cannot (properly) fulfill these functions, causing embryo growth to stop and the seed to abort.

1.4 The influence of endosperm formation on integument development

In angiosperms such as *Arabidopsis* the integuments build the seed coat, which protects the embryo and transfers nutrients from the mother plant to the endosperm and embryo (Haughn and Chaudhury 2005; Holdsworth et al. 2008). It is believed that nutrient flow progresses centripetally from the outer through the inner integuments, passing through the apoplast to the endosperm and the embryo on routes that are still not fully understood (Stadler et al. 2005; Morley-Smith et al. 2008). Integuments differentiation and their rapid growth are initiated upon fertilization During subsequent seed development proanthocyanidin flavonoids accumulate in the internal most integument layer called endothelium, where they are oxidized causing the brown colour of the matured seeds (Debeaujon et al. 2001). The two outer integuments accrue starch containing amyloplasts, followed by the deposition of large quantities of pectinaceous mucilage in the outermost cell layer. At the end of seed maturation, all integumental layers die with the exception of the endothelium (Haughn and Chaudhury 2005; Holdsworth et al. 2008).
In recent years several publications underlined the influence of endosperm formation on seed development and particularly on integument differentiation and growth. (Figure 1, Signal E) (Berger et al. 2006). Unfertilized *fertilization independent seed* (*fis*) class mutant ovules showed autonomous endosperm formation accompanied by integument cell elongation and proanthocyanidin accumulation in the endothelium layer, a distinctive characteristic of seed development progression (Ohad et al. 1996;

Grossniklaus et al. 1998; Kohler et al. 2003a; Berger et al. 2006; Ingouff et al. 2006). In cereals the influence of endosperm formation on seed coat growth is also well established. One example is *MN1*, which encodes for an endosperm-specific cell wall invertase. This enzyme metabolizes the incoming sucrose, possibly contributing to the establishment of the endosperm as a sink tissue. Due to the lack of nutrient supply, *mn1* (*miniature1*) mutant in maize or *mn1-like* mutant in barley develop little endosperm and display much smaller seeds than wild-type plants (Felker et al. 1985; Cheng et al. 1996).

A principal role of endosperm during seed development was highlighted in interploidy crosses in maize. An increased paternal genomic contribution (e.g. in 2C x 4C crosses) enhanced endosperm proliferation and enlarged seed size. A higher maternal contribution (e.g. in 4C x 2 C crosses) had the opposite effect (Lin 1982; 1984; Pennington et al. 2008). Similar phenotypes were obtained in interploidy crosses in *Arabidopsis* in which endosperm is not as developed as in maize or other cereals (Scott et al. 1998). Mutant phenotypes resembling dosage imbalances in the endosperm can also be found in iku mutants (Garcia et al. 2003).

1.5 The FIS-complex is a suppressor of endosperm development

Results from seed mutant screens show that a proper endosperm formation appears to be dependent on gene doses effects. Genes such as *MEDEA* (*MEA*) and *PHERES1* (*PHE1*) are mainly regulated by imprinting and are expressed in a parent-of-origin-specific manner (Kohler et al. 2005; Baroux et al. 2006). Genomic imprinting has been shown in insects, mammals and flowering plants (Bushell et al. 2003; Kinoshita 2007; Walia et al. 2009). In plants imprinting is restricted to the endosperm (Lin 1982; 1984; Scott et al. 1998).

Imprinting is based on Histone methylation and demethylation. Histones are proteins, which package 146 base pairs of the DNA superhelical into an octameric contour. These octamers are composed of the core histones H2A, H2B, H3 and H4. The herby formed repetitive structure is called chromatin (Kornberg 1974; Kornberg and Thomas 1974). Chromatin does not only serve as a scaffold for establishment of chromosomes and their distribution during mitosis and meiosis; it also controls gene expression. The amino acids of the histone tails are constantly modified in response to developmental or environmental signals by various combinations of acetylation, methylation, ubiquitinylation and phosphorylation with distinctive outcomes for gene

expression (Turner 2002). Histone methylation is pivotal for the establishment of hetero- and euchromatin, genomic imprinting, X chromosome inactivation and transcriptional regulation and is mitotically inheritable. Histone itself can bind molecules to activate or repress gene expression (Turner 2002). In this context, histone H3 lysine 4 and lysine 36 methylation support transcription initiation and transcript elongation and thus, activate gene expression (Grasser 2005; Volkel and Angrand 2007). On the other hand, repressive histone marks such as histone H3 lysine 9 and lysine 27 are linked with heterochromatin establishment and gene repression (Zhou 2009).

One method of transcriptional regulation including activation or repression is accomplished by histone acetyltransferases (HATs) and histone deacetyltransferase (HADC). To this date, more than 15 members of HATs have been discovered in *Arabidopsis* acetylating all four core histones. Among the proteins involved in histone code assembly and interpretation, the Polycomb proteins have been shown to be one of the major players and were first identified in *Drosophila* (Jürgens, 1985; Lewis, 1978).

Of importance for seed development and imprinting is the FIS-PRC2 complex, composed of at least four core components: the Zinc-finger transcription factor FERTILIZATION-INDEPENDENT SEED 2 (FIS2, the WD40-repeat protein MULTICOPY SUPPRESSOR OF IRA 1 (MSI1), the SET domain protein MEDEA (MEA) and the WD40-repeat protein FERTILIZATION - INDEPENDENT ENDOSPERM (FIE).

MEA has been shown to be a histone methyltransferase that methylates lysine 27 of histone H3 (Gehring et al. 2006). In fertilized mutant seeds the endosperm overproliferates and displays an enlarged embryo, which arrests at late heart stage. Moreover, unfertilized *fis*-class mutant ovules reveal the presence of a multinuclear endosperm (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999; Bartee et al. 2001; Kohler et al. 2003a). One function of the FIS-PRC2 complex might be to stop the gametophytic phase and mediate the switch to zygotic stage and/or to prevent seed development in the absence of a fertilization signal by suppressing endosperm development (Grossniklaus et al. 2001; Johnston et al. 2008).

1.6 Maintenance of DNA methylation marks in *Arabidopsis*

Besides histone modification by the FIS-complex, cytosine methylation is another mechanism to control gene expression. DNA methylation is well known to protect the genome against selfish DNA elements such as transposons (Yoder et al. 1997; Martienssen and Colot 2001). In eukaryotes like *Arabidopsis,* DNA methyltransferases have structural similarities to prokaryotic restriction enzymes, which are involved in degrading non-host DNA (Cheng 1995). Therefore, it is likely that all known cytosine methylation processes in higher organisms evolved from the original task of defending the genome.

In contrast to mammals, plant DNA methylation includes not only symmetrical CG but also CHG (H is either A, T or C) and CHH methylation. To this date, three classes of DNA cytosine methyltransferases, which maintain DNA methylation sites, are known. METHYLTRANSFERASE 1 (MET1) transfers CG methylation sites (Saze et al. 2003), CHROMOMETHYLASE 3 (CMT3) mainly controls CHG sites (Lindroth et al. 2001) and DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2 (DRM1 and 2) are also responsible for CHH sites (Cao and Jacobsen 2002). CMT3, DMR1 and 2 act redundantly to enforce non-CG methylation (Lindroth et al. 2001; Cao and Jacobsen 2002; Chan et al. 2005).

1.7 DNA demethylation in *Arabidopsis*

DNA demethylation can be divided into a passive and an active demethylation process. The passive demethylation process occurs in the absence of MET1 during replication; while cytosines without methyl groups are incorporated in a semi conservative manner into the new DNA strand (Morgan et al. 2005). Conversely, active DNA demethylation occurs via an active dismiss of methyl groups. Evidence for this process was gained from studies of mammalian embryo development. Here, the demethylation of the paternal inherited chromosomes in fertilized egg cells is suggested to be an active process, because it is accomplished before the first replication (Mayer et al. 2000). Furthermore, imprinting in mice is actively released during the development of the primordial germ cells (Hajkova et al. 2008).

The active demethylation process might function by the cleavage between the deoxyribose moiety and the 5-methylcytosine. Later, the base excision repair machinery replaces the methylated with an unmethylated cytosine. Recent genetic

studies have suggested an active DNA demethylation process by the DNA glycosylases DMETER (DME), REPRESSOR OF SILENCING1 (ROS1) and DEMETER LIKE 2 and 3 (DML2 and DML3) (Choi et al. 2002; Gong et al. 2002; Agius et al. 2006; Morales-Ruiz et al. 2006; Penterman et al. 2007b; Ortega-Galisteo et al. 2008). DME is thought to demethylate imprinted genes, while DML proteins remove methylation directed by the RNA-directed DNA methylation (RdDM) pathway against repetitive sequences and transposable elements, and that ROS1 is also able to remove DNA methylation directed by an RNA-DEPENDENT RNA POLYMERASE2 (RDR2) independent RNAi pathway (Choi et al. 2002; Gong et al. 2002; Agius et al. 2006; Gehring et al. 2006; Morales-Ruiz et al. 2006; Penterman et al. 2007b; Ortega-Galisteo et al. 2008).

All four proteins erase symmetrical and asymmetrical methylation residues (Gehring et al. 2006; Morales-Ruiz et al. 2006). The impact of a lacking demethylation system can be best seen in the endosperm of *dme* mutants. Under wild-type conditions the endosperm genome is demethylated, while in *dme* mutants endosperm CG methylation is partially restored (Hsieh et al. 2009). *dme* mutant seeds are maternally gametophytic lethal and abort at late heart stage (Choi et al. 2002).

1.8 *De novo* establishment of methylation marks by siRNAs in *Arabidopsis*

One way to establish de-novo cytosine methylation is by RNAi directed DNA methylation (RdDM) (Matzke and Birchler 2005; Slotkin and Martienssen 2007). Proteins, which belong to the dicer family such as DICER-LIKE 3 (DCL3) and RDR2 cleave dsRNA into 21–30-nucleotide small interfering RNAs (siRNAs) and ARGONAUTE4 (AGO4) incorporate and direct these siRNAs fragments to homologous regions in the genome (Zilberman et al. 2003; Chan et al. 2004; Xie et al. 2004). This process is accompanied by DRM2 together with DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DDM1) at all symmetrical and asymmetrical methylation sites to induce, for example, transcriptional gene silencing (Matzke and Birchler 2005; Vaucheret 2006).

In addition, methylated CHG and CG is connected to H3K9me2, and CHG methylation co-localizes with H3K9me2 by CMT3 and KRYPTONITE (KYP) (Jackson et al. 2002; Tariq et al. 2003; Johnson et al. 2007). Nevertheless, the complex methylation process by siRNA, DRM2 and CMT3 is not fully understood. It is thought

that these proteins or RNA fragments act redundantly together with other factors and locus specific pathways to silence different loci in the genome (Cao and Jacobsen 2002; Chan et al. 2006a; Henderson and Jacobsen 2008). Furthermore, recent studies showed that siRNA are transported between cells, over large distances and influence spatial separated developmental processes by silencing through de-novo methylation and/or posttranscriptional silencing (Dunoyer et al. 2010a; Dunoyer et al. 2010b).

In addition, the expression of a large number of endogenous genes is regulated by CG methylation inside the OPEN READING FRAME (ORF) (Tran et al. 2005; Zhang et al. 2006; Zilberman et al. 2007; Cokus et al. 2008). However, repression of gene expression is also accomplished by promoter methylation, for example in the promoter of the gene *FLOWERING WAGENINGEN* (*FWA*) (Soppe et al. 2000). Here, the *FWA* promoter is silenced by methylation at tandem repeats, which generate siRNA. Furthermore, only methylated promoter sites were able to recruit further RdDM or cause methylation of additional introduced transgenic *FWA* copies. The recruitment of *de novo* methylation may require preexisting chromatin modifications or DNA methylation. Thus, pre-established methylation can enforce further methylation and therefore increase silencing (Soppe et al. 2000; Chan et al. 2006b). Besides gene repression, DNA methylation can also prevent repressor binding resulting in gene activation. For example, maternally inherited *PHERES1* (*PHE1*) is repressed by the FIS-complex, but the methylation of a downstream located region away from the *PHE1* locus enhances *PHE1* expression (Makarevich et al. 2008).

1.9 Maternal *FIS*-class gene activation prior to fertilization and the maintenance of maternal and paternal imprinting after fertilization

A *pMET1:H2B-RFP* reporter line showed that the *MET1* promoter was on in 4 celled and off in the 8 celled embryo sacs in a RBR and MSI dependent manner. Consequently, the maternal genome remains in a hemimethylated status after female gametogenesis. It has been shown that hemimethylation enhance DME activity triggering complete demethylation. Hereby, DME can demethylate the 5′ promoter region and the 3′ *MEA* intergenic subtelomeric repeat of the maternally derived *MEA* allele resulting in activation. In addition, *DME* also demethylates direct repeats of the 5' region of *FWA* and *FIS2* (Kinoshita et al. 2004; Jullien et al. 2006b). Similar results

were observed in isolated central cells in maize (Gutierrez-Marcos et al. 2006; Hermon et al. 2007).

In this context, it is surprising that a hypomethylated paternal genome does not release paternal *MEA* silencing (Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008). Interestingly, if the maternal *MEA* or *FIE* allele is mutant, silencing of the paternal allele is released and *MEA* gets paternally expressed. Molecular data have also underlined these results, which show that a repressive H3 lysine 27 methylation mark on the paternal *MEA* promoter is removed if the maternal MEA protein is absent. Thus, the maternal actively represses the expression of the paternal FIS-complex genes (Gehring et al. 2006).

Maternal FIS-class proteins are not only responsible for silencing their paternal siblings; they are also likely to be involved in the repression of many other genes. One example is the regulation of *PHE1*. The maternal repression of *PHE1* is partly reliant on *MEA* expression in the central cell. Therefore, MEA maintains paternally imprinted genes (*MEA*) silent, whereas it induces the maternal repression of another imprinted gene (*PHE1*) (Kohler et al. 2005; Makarevich et al. 2008).

Thus, imprinted genes such as *MEA* are parentally differentially marked prior to fertilization by DNA methylation and/or histone modification, and early seed formation is mainly under the control of maternally expressed genes. However, the entire molecular pathways and genetic regulations upstream and downstream of the FIS-complex are still not fully understood. Thus, further work is necessary to unravel the molecular networks responsible for a proper seed formation.

1.10 *cdka;1* and *fbl17* single fertilization and their impact on embryo – endosperm interaction

In fertilization experiments *CDKA;1* (*CYCLIN-DEPENDENT KINASE A;1*) and *FBL17* (*FBOX-LIKE PROTEIN 17*) mutant pollen formed just a single sperm cell at anthesis (Iwakawa et al. 2006; Nowack et al. 2006; Kim et al. 2008; Gusti et al. 2009). Surprisingly, pollen containing a single sperm could complete fertilization, but seemed to prefer to fertilize the egg cell. However, further analyses revealed that in many cases *cdka;1* mutant pollen underwent a second male mitotic division during pollen tube growth. As a result, instead of just one single sperm, two sperm like cells arrived at the embryo sac. Nevertheless, fertilization was probably only accomplished in the egg cell, while the karyogamy of the second sperm like cell with the central cell

did not succeed. Here, the male pronucleus was excluded from the central cell (Aw et al. 2010).

Most of the central cells started to proliferate without containing a paternal genome resulting in a developing endosperm. This process could be set off by a positive signal coming from the fertilization event of the egg cell or a developing embryo (Nowack et al. 2006). In light of recent results the fusion without karyogamy of the second sperm like cell with the central cell could be an alternative or additional positive signal for the onset of endosperm proliferation (Aw et al. 2010).

In the so-called single fertilized seed the endosperm proliferation was limited to four to six rounds of free nuclear divisions resulting in a maximum of 64 nuclei, followed by abortion (Fig. 1b) (Nowack et al. 2006). On the contrary, in *feronia*/*sirene* mutants the growing pollen tube reached the egg cell without releasing the two sperm cells. In this mutant background no autonomous endosperm formation was observed, underlining that either the fusion of the sperm cell with the central cell or the fusion with the egg cell triggers endosperm formation (Huck et al. 2003; Rotman et al. 2003; Escobar-Restrepo et al. 2007).

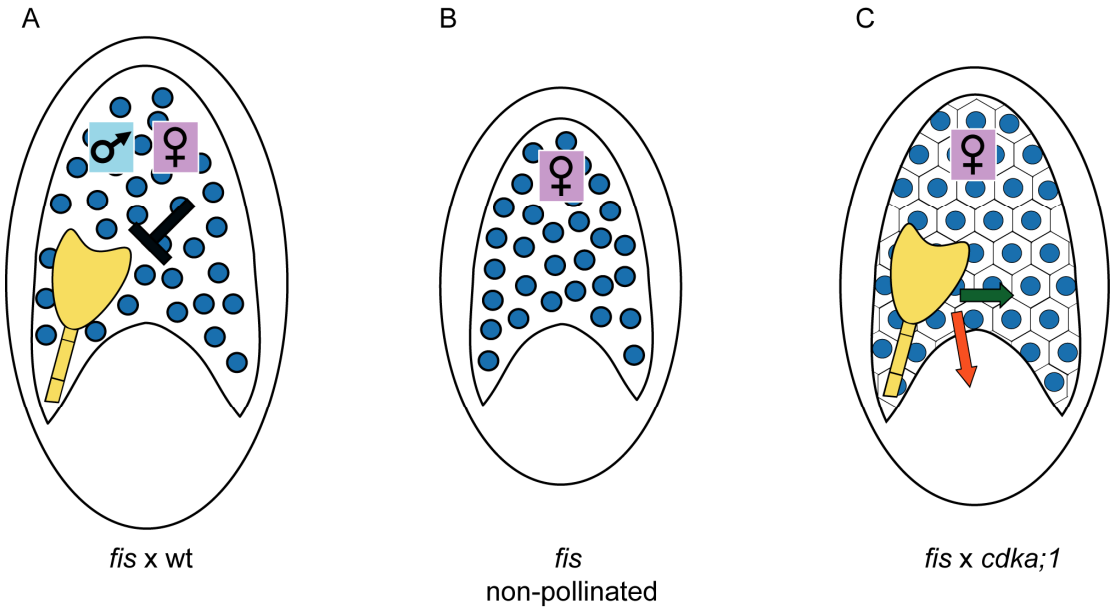

Figure 2: Communication processes during heart stage of embryo development, where the embryo has an impact on endosperm differentiation
(**A**) Around 6 DAP of a *fis*-class mutant ovule by wild-type pollen, the endosperm stops differentiating and the seed aborts with an embryo at late heart stage. (**B**) Autonomous endosperm in unfertilized *fis*-class mutant ovules shows characteristics of early endosperm differentiation. (**C**) *fis*-class mutant ovules fertilized with *cdka;1* mutant pollen complete seed development. The fertilization process is accompanied by egg-cell fertilization and failure of karyogamy of the second sperm with the central cell. Here, a fertilized embryo develops alongside an autonomous maternal homodiploid endosperm. This phenotype shows that embryo development can trigger terminal differentiation of the endosperm (green arrow) and so directly or indirectly sustain seed development and survival (orange arrow).

Beside *cdka;1* pollinated wild-type ovules, unfertilized *fis*-class mutant ovules such as *fie, fis2* and *mea* develop autonomous endosperm (Fig 2B). In fertilized *fis*-class mutant seeds embryo development is disturbed and the endosperm overproliferates resulting in seed abortion (Fig. 2A) (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999).

Thus, in *fis*-class mutant seeds the repression of endosperm formation is released. By fertilizing these seeds with *cdka;1* or *fbl17* mutant pollen differentiation of the endosperm was restored, leading to successful cellularization and viable embryo development, which gave rise to healthy and fertile plants (Figure 2C) (Nowack et al. 2007; Gusti et al. 2009). Since the genetic composition of the unfertilized and single fertilized autonomously developing endosperm in *fis*-class mutant seeds is the same, the presence of the embryo appears to be necessary and sufficient for endosperm differentiation and seed survival. Furthermore, bypassing imprinting can form a homodiploid unfertilized endosperm and cause proper endosperm development without any paternal genomic contribution (Nowack et al. 2007; Gusti et al. 2009).

1.11 Aim of this work

Seeds generated from crosses with *cdka;1* pollen develop endosperm with solely maternal and no paternal contribution. Little is known about the presumptive signal that triggers central cell proliferation without prior fertilization. This signal either arises from the fertilization of the egg cell and/or the sperm-central cell fusion without karyogamy. In this work, the use of *cdka;1* fertilized seeds as a novel tool promises to unravel the interplay between endosperm formation and embryo growth and further, to dissect the genomic basis of these signal transduction pathways. This allows, not only, identifying single genes, responsible for the onset of central cell proliferation, but also determining the impact of the paternal or maternal genome on endosperm formation. In this context, the presented study intends to answer aspects such as allele specific gene expression, epigenetic influence on endosperm development and *de novo* methylation of paternal or maternal genes.

2 RESULTS

2.1 The central cell nuclei in *cdka;1* fertilized seeds display characteristics of genuine endosperm

Given that an unfertilized diploid central cell can proliferate upon *cdka;1* fertilization of the egg cell, it was interesting to investigate, whether, and if so, to what degree the central cell represents a developing endosperm. One characteristic of early endosperm formation is the migration pattern of the proliferating nuclei after fertilization, which was previously described by Boisnard-Lorig (Boisnard-Lorig et al. 2001): Shortly after the first division one nucleus moves to the chalazal pole (opposite the embryo) while the other nuclei are distributed at the micropylar pole (close to the embryo). In this context, a *FIS2-GUS* reporter was chosen to better visualize the migration pattern of the endosperm nuclei. *FIS2* was shown to be solely expressed in the central cell and during the first five free nuclear divisions of the endosperm (Luo et al. 2000).

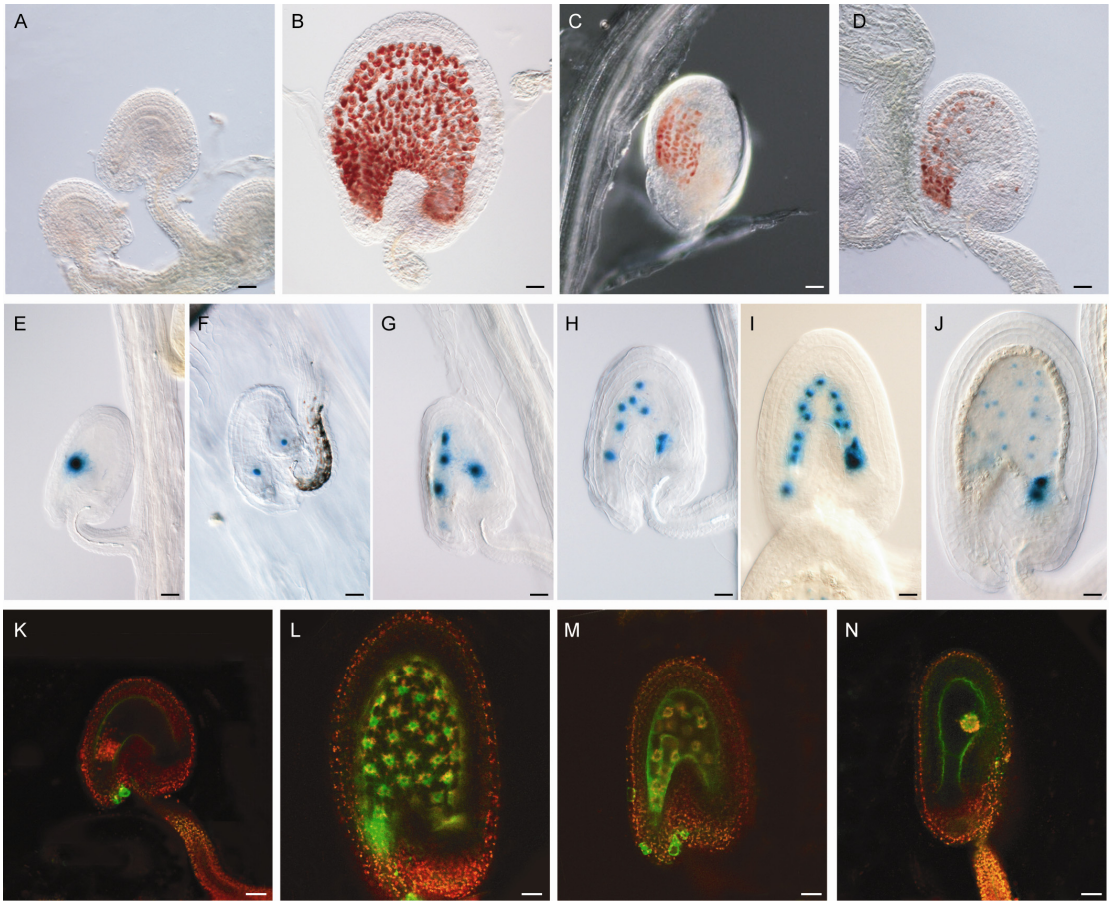

Figure 3: Endosperm characteristics of autonomously proliferating central cell nuclei
DIC light- and Confocal Laser Scanning (CLSM) - micrographs of developing double-fertilized (with wild-type pollen) and single-fertilized (with *cdka;1* pollen) seeds. (**A** - **D**) Detection of proanthocyanidin accumulation by vanillin staining in unfertilized Sha ovules or fertilized seeds at 3 DAP. (**A**) No proanthocyanidin can be detected in unpollinated Sha ovules. (**B**) Sha seeds pollinated with wild-type pollen synthesize proanthocyanidin in the seed integuments indicating activation of an endosperm developmental program. (**C**) Col-0 seeds pollinated with *cdka;1* mutant pollen also initiate proanthocyanidin accumulation. (**D**) Even low or non-proliferating central cell nuclei as seen for instance in Sha seeds pollinated with *cdka,1* are vanillin positive. (**E** - **J**) Histochemical detection of GUS activity in seeds expressing a maternal *FIS2:GUS* construct (in the C24 ecotype) and pollinated with *cdka;1* mutant pollen (**E** - **I**) or with wild-type pollen (**J**). The division pattern and nuclear migration of autonomous developing endosperm in single fertilized seeds is comparable to that observed for double-fertilized seeds wild-type. (**E**) FIS2 GUS expression in central cell nucleus (fused polar nuclei). (**F**) After first division, one nucleus migrates to the chalazal end of the seed. (**G**) Second division. (**H**) Third division. (**I**) Fourth division. (**J**) After fifth division in an ovule fertilized with wild-type pollen. (**K** - **N**) CLSM micrographs of seeds or ovules a maternal KS22 construct at 3DAP, green GFP from the marker construct, red autofluorescence of plastids. (**K**) No GFP could be observed in an unfertilized wild-type C24 central cell. (**L**) Double fertilized wild-type seeds showing expression of the KS22 GFP reporter in the endosperm nuclei. (**M**) The autonomous endosperm in seeds fertilized with *cdka;1* mutant pollen express GFP, even in cases with an undivided central cell nucleus (**N**). All pictures are oriented such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Scale bars are 10 µm.

The detection of the GUS activity in seeds pollinated with *cdka;1* mutant pollen (ES = 2n) displayed the same endosperm migration pattern as found in seeds pollinated with wild-type pollen (ES = 3n) (Fig. 3 E-J, data not shown).

Next, the integument differentiation in seeds fertilized with *cdka;1* mutant pollen was analyzed. Fertilization triggers the accumulation of proanthocyanidin pigments in the endothelium cell layer of the seed coat, which can be detected as a red stain by vanillin assay (Debeaujon et al. 2003). In unfertilized ovules endothelium differentiation is not started and vanillin staining is negative. The same observation was reported for unfertilized *rbr* mutant ovules, showing autonomously proliferating cells. Thus, the *rbr1* female gametophyte likely produces a tissue that does not have a proper endosperm identity (Ingouff et al. 2006). In contrast, *msi1* or *fis2* unfertilized ovules containing autonomous developing endosperm display proanthocyanidin accumulation and differentiation of the seed coat without an embryo (Ingouff et al. 2006). Consequently, a developmental trigger appears to be required to induce the endothelium differentiation process.

More than 60 percent of all seeds of the accession Sha pollinated with *cdka;1* mutant pollen showed just a single nucleus in the central cell (Fig. 6b). However, more than 85 percent of the *cdka;1* fertilized Sha seeds were vanillin positive (Fig. 3d, data not shown).

This shows, that in contrast to unfertilized seeds, seeds fertilized with *cdka;1* pollen demonstrated successful cross talk between endosperm and endothelium (Fig. 3 a-d). Furthermore, given the fact that more than 40 percent of all ovules displayed proanthocyanidin accumulation, it is reasonable to state that endothelium differentiation is independent of endosperm nuclei number and might be triggered by either an embryo and/or a single central cell nucleus with endosperm characteristics. However, the experiment could not rule out if a developing embryo alone could promote seed coat differentiation.

Next, the *KS22:GFP* marker line was tested, which is solely expressed in the endosperm but not in the central cell of unfertilized ovules (Ingouff et al. 2005). Upon *cdka;1* pollination the autonomous dividing endosperm and even a non-dividing central cell accompanied by a fertilized embryo showed a GFP signal. Thus, the unfertilized endosperm and even a non-dividing unfertilized central cell appear to adopt some characteristics of wild-type endosperm (Fig. 3 k-n). Therefore, endosperm differentiation can be uncoupled from proliferation.

2.2 Development of autonomous endosperm varies between different *Arabidopsis* accessions

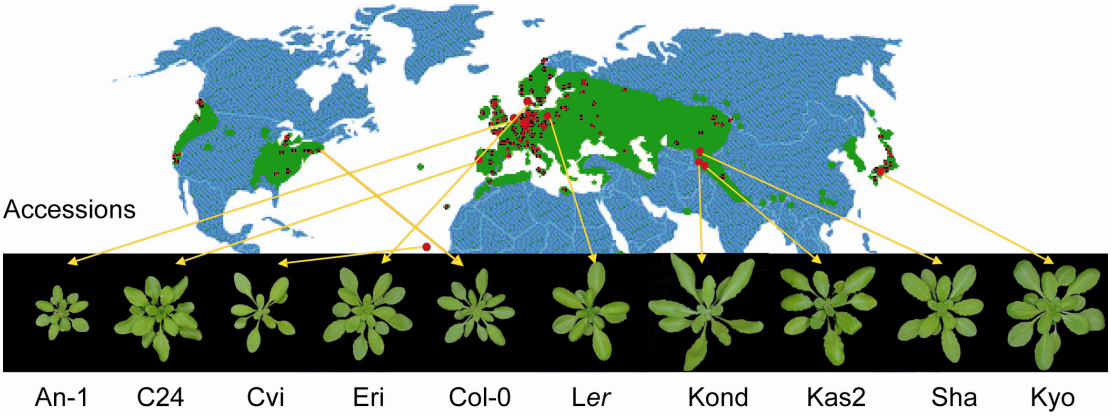

Figure 4: Natural variation in *A. thaliana* ecotypes
A. thaliana accessions are widely spread over the world and habituated in different eco-zones (Koornneef et al. 2004; Nordborg et al. 2005; Bakker et al. 2006; Schmid et al. 2006).

Up to now, hundreds of different accessions of *Arabidopsis thaliana* have been collected, displaying large genetic variation within the species (Fig. 4) (Koornneef et al. 2004; Nordborg et al. 2005; Bakker et al. 2006; Schmid et al. 2006). This natural variation is used as a powerful tool for quantitative genetics to dissect many physiological or developmental processes.

From the existing ecotypes, 14 frequently used accessions were chosen and pollinated using pollen from heterozygous *cdka;1* mutant plants. The seed development 3 DAP was subsequently analyzed. Approximately half of the seeds in these crosses were double fertilized, while in the other half, only the egg cell was successfully fertilized (Iwakawa et al. 2006; Nowack et al. 2006). Consequently, half of the developing seeds in each tested accession displayed wild-type characteristics (Fig. 5 a, data not shown), while the other half showed the above described autonomous proliferation of the central cell along with the development of an embryo that eventually leads to seed abortion (Fig. 5 c-e) (Nowack et al. 2006). Interestingly, a large variation in endosperm nuclei number between the different accessions ranging from one up to 64 nuclei was found.

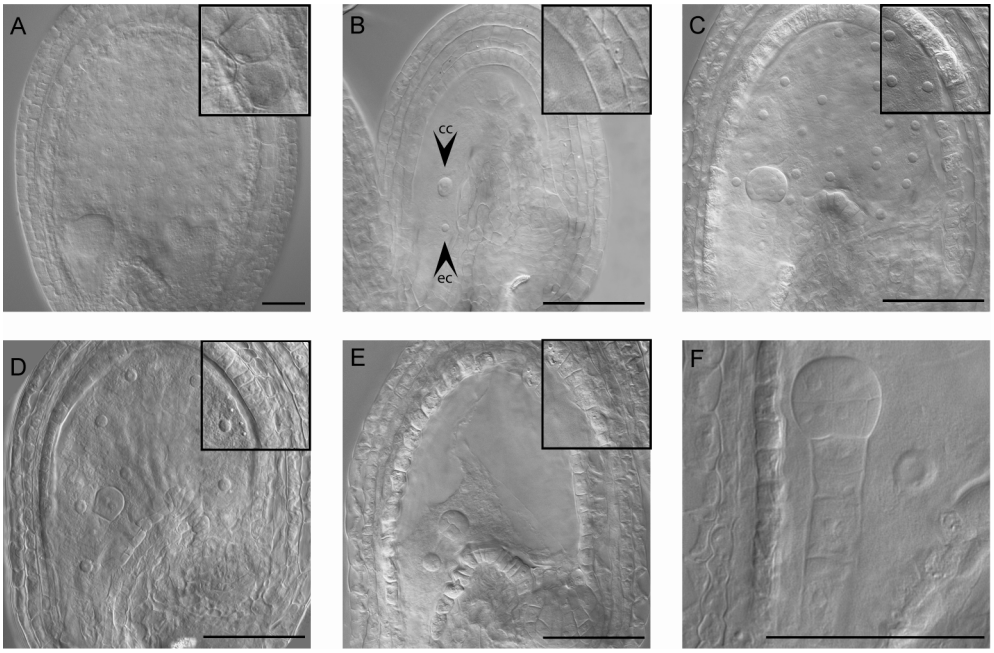

Figure 5: Autonomously proliferating central cell nuclei after single fertilization with *cdka;1* mutant pollen
Light micrograph of cleared whole mount ovules (unfertilized) or seeds stemming from the fertilization with wild-type pollen (double fertilized), or *cdka;1* mutant pollen (successful egg-cell fertilization and lacking karyogamy of the second sperm with the central cell). (**A**) Col-0 seed fertilized with Col-0 wt pollen at 3 DAP. (**B**) Non-fertilized Col-0 ovules at 6 days after emasculation - (ec and cc indicate egg cell and central cell, respectively) (**C**) Col-0 seed fertilized with *cdka;1* mutant pollen at 3 DAP, the central cell nucleus went through 5 rounds of nuclear division and here, 28 nuclei are visible. (**D**) Bay-0 seed fertilized with *cdka;1* mutant pollen at 3 DAP, the central cell nucleus went through 3 rounds of division and 8 nuclei are visible. (**E**) Sha seed fertilized with *cdka;1* mutant pollen at 3 DAP, containing a undivided central cell. (**F**) Close up of a Sha seed fertilized with *cdka;1* mutant pollen showing an undivided central cell nucleus and an 16 celled embryo at 3 DAP. Inlays in panels **A**-**E** show the inner integument layers all at the same magnification. The integument cells strongly enlarge and vacuolize in wild-type fertilized seeds (**A**) whereas in unfertilized ovules (**B**) no cell expansion is detected. Upon single fertilization with *cdka;1* mutant pollen (**C**-**E**) the integuments slightly expand. All pictures are oriented such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Scale bars are 50 µm.

For a better description and quantification of the occurring variation, a fast and robust method was required to present the data. It should also facilitate comparison between the different data sets. Therefore, it was decided to take the mean number of nuclei per developing seed as a measurement of endosperm growth. However, this system displays some disadvantages: First of all, endosperm proliferation is an exponential process and thus, it overemphasizes seeds with high nuclei numbers and generates an increasingly larger standard deviation with increasing nuclei numbers. Therefore, categorization of the data was more difficult. Secondly, experimental problems and counting errors occurred with increasing nuclei numbers due to difficulties while counting the nuclei under the microscope, *e.g.* >30.

Consequently, the measure Endosperm Division Value (EDV) was introduced, defined as the number of anticipated division cycles per aborting seed or in other words, the number of divisions necessary to reach the corresponding nuclei numbers; *e.g.*, 26 nuclei were scored as being in the fifth cycle ($26 \approx 2^5$).

On the other hand, the EDV overestimates the real endosperm nuclei number, in particular past the first three very synchronous division rounds. Afterwards, the mitotic domains change (Boisnard-Lorig et al. 2001). Nevertheless, previous studies support the correctness of this approach, because larger numbers can still be assigned to the correct division cycles 4–8 (Boisnard-Lorig et al. 2001). To support the method, the determined nuclei numbers of the autonomous endosperm were generally approximate to the value for a completed division cycle. The 14 different accessions analyzed displayed EDVs ranging from 0.64 for Wassilewskija (Ws-0) and 3.84 for C24. Figure 6a is a statistical approach to sort accessions that can be separated into groups with an error of 5%. The EDV of accessions within one group are not significantly different. To give an example, Sha and L*er*-1 are significantly different, because they are in different groups, while the EDV of Sha and Est-1 are within the same group, because the EDVs cannot be statistically separated.

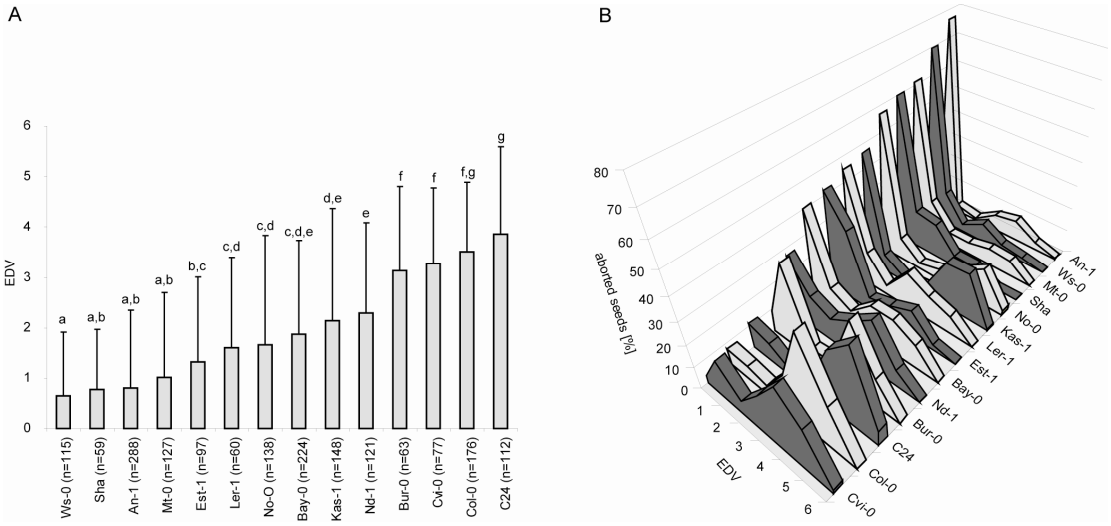

Figure 6: Natural variation in initiation of autonomous endosperm proliferation upon *cdka;1* pollination

(**A**) Endosperm division values (EDVs) for 14 *Arabidopsis* ecotypes. Means are shown with error bars representing the standard deviation. a,b,c,d,e,f,g specify groups of significantly different subsets as indicated by a Student Newman-Keuls test with alpha = 0,5. (**B**) Distribution of autonomously proliferating endosperm nuclei of *cdka;1* pollinated ovules in the *Arabidopsis* accessions An-1, Bay-0, Bur-0, C24, Cvi-0, Col, Est-1, Kas-1, L*er*-1, L*er*-2, Mt-0, Nd-1, WS-0, Sha, No-0. The x -axis indicates EDV, the y-axis shows the number of wild-type ovules pollinated with *cdka,1* mutant pollen in percentage. The diagram shows that the observed natural variation in endosperm proliferation is in the frequency of seeds that start proliferating of endosperm nuclei. If nuclear proliferation has started endosperm usually proceeds through 4 to 5 rounds of division.

2.3 General seed growth parameters contribute only little to autonomous endosperm formation

The natural variation of the autonomous endosperm proliferation could be based on general seed growth parameters such as natural differences in seed growth, *i.e.* high autonomous endosperm formation within an accession could correlate to a larger or heavier seed and vice versa. To check this, first the seeds of the used accessions were weighed to determine seed mass. A large variation in seed mass was measured, ranging from 0.73 mg/50 seeds in An-1 to 1.41 mg/50 seeds in the accession Kas-1 (Fig. 7a). Furthermore, the data was in line with previous studies, which confirms the accuracy of the measurements, for example for the seed masses of L*er*-1 and Cvi-0 (Alonso-Blanco et al. 1999). Beside Kas-1 and Cvi-0, large seed masses were also found for the accessions C24, Nd-1, Est-1, and Bur-0 (Figure 7a).

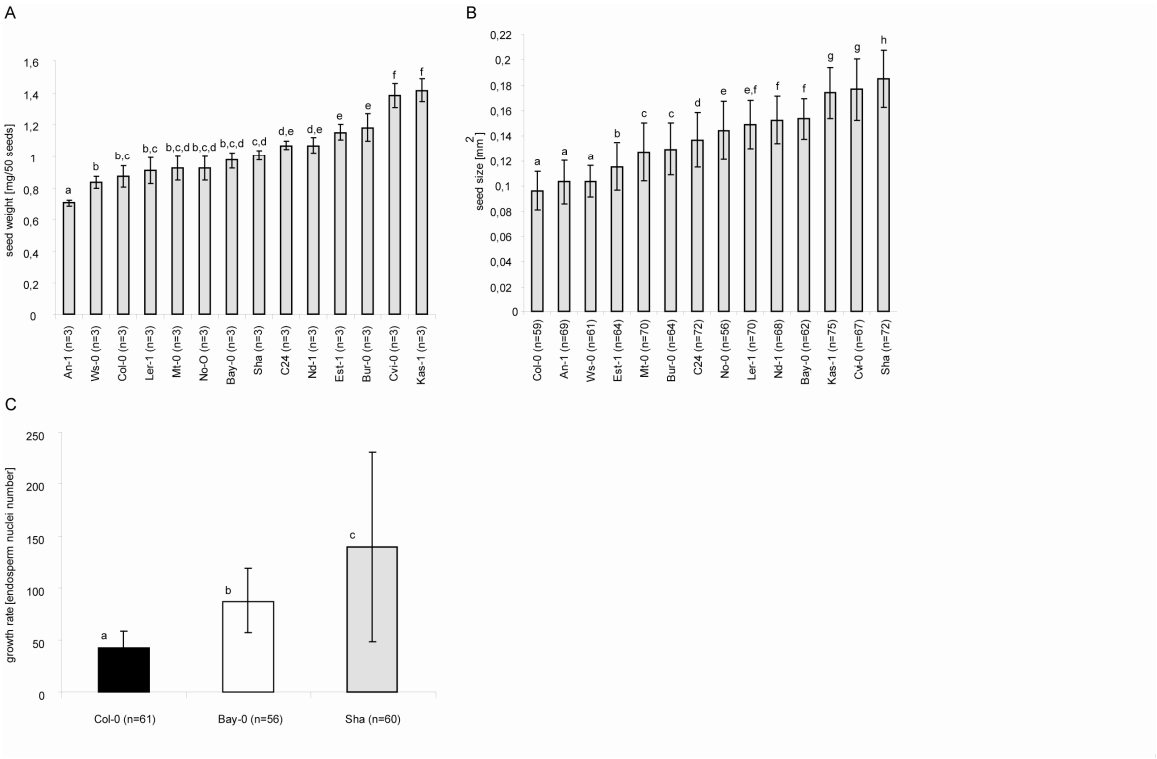

Figure 7: Autonomous endosperm proliferation can be uncoupled from general seed growth aspects
(**A**) Natural variation in the seed mass (mg per 50 seeds) of double-fertilized ovules of the 14 accessions; no strong correlation between high EDVs and heavy seeds could be found (R^2=0.20). (**B**) Natural variation in seed size in mm^2 of double-fertilized seeds; no correlation between high EDV and large seeds could be found (R^2=0.0043). (**C**) Natural variation in endosperm growth rate in nuclei numbers of double-fertilized endosperm at 3 DAP; no strong correlation between high EDV and fast proliferating endosperm could be found (R^2=0.3189). Means are shown with error bars representing standard deviations. a,b,c,… indicate groups of significantly different subsets as shown by a Student Newman-Keuls test with alpha = 0,5.

Disproving the initial assumption, just 20% of the EDV might be explained by seed mass (linear regression R^2=0.20) and the influence of seed size on autonomous endosperm proliferation was even less important (linear regression R^2=0.0043, Fig 6a, Fig 7b).

To assess whether general growth rate might have an effect on EDV, endosperm nuclei in wild-type seeds of three representative accessions were counted at 3 DAP. Interestingly, a variation of wild-type endosperm was observed between the accessions in respect to proliferation rate (Fig. 7c). Nevertheless, the impact of a general growth rate at the beginning of seed formation was not very pronounced either (linear regression R^2=0.3189, Fig 6a, Fig 7c).

Finally, the nuclei numbers counted within an accession were not normally distributed The natural variation found is not based on the ability to form high values of autonomous endosperm but rather on the frequency that the central cell starts proliferating (Fig. 6b). Once a central cell divided it regularly goes up to the fourth or fifth division cycle, approximately between 16 and 32 nuclei, even in an accession with a low EDV.

To summarize, natural variation was detected in the three common seed growth parameters - mass, size and growth rate, also used in previous studies (Krannitz et al. 1991; Alonso-Blanco et al. 1999). However, although the data shows that parameters, such as seed growth influence endosperm proliferation upon fertilization with *cdka;1* mutant pollen, the development of autonomous endosperm relies largely on additional factors.

2.4 Relationship between endosperm and embryo development during seed growth

In *Arabidopsis* and other plants it was reported that first the endosperm nucleus starts proliferating reaching up to 12 to 16 nuclei before the zygote undergoes its first division (Boisnard-Lorig et al. 2001). This might indicate a certain dependence of embryo development upon endosperm formation. Therefore, the impact of an incomplete proliferated endosperm on embryo formation was analyzed.

Embryo development was observed in all *cdka;1* pollinated seeds, even in cases where the central cell did not divide (n = 45) (Fig. 8, Fig. 9). In addition, fertilized *glc* mutant seeds displayed embryo arrest up to globular stage in the absence of endosperm or in seeds showing 2 to 8 nuclei (Fig. 1e). Here, the central cell was not

able to fuse with the second sperm cell (Ngo et al. 2007). Thus, the start of embryo development seems to be independent from endosperm formation and that embryo development might rely only on the fertilization of the egg cell.

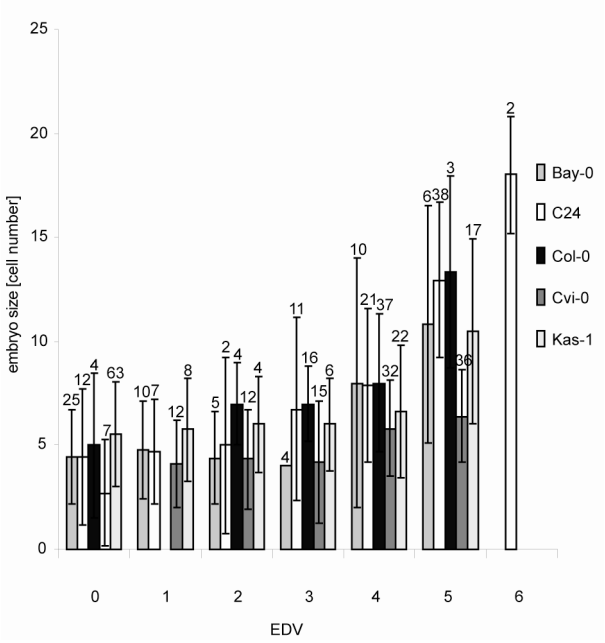

Figure 8: Early correlation of embryo development and endosperm proliferation
Correlation between the size of the autonomously formed endosperm (in EDV) with embryo size (in numbers of cells) at 3 DAP. In all 5 analyzed accessions, endosperm size correlated with embryo size (R^2 (Bay-0) = 0,64; R^2 (C24) = 0,82; R^2 (Col-0) = 0,74; R^2 (Cvi-0) = 0,90; R^2 (Kas-1) = 0,61). Error bars indicate standard deviation.

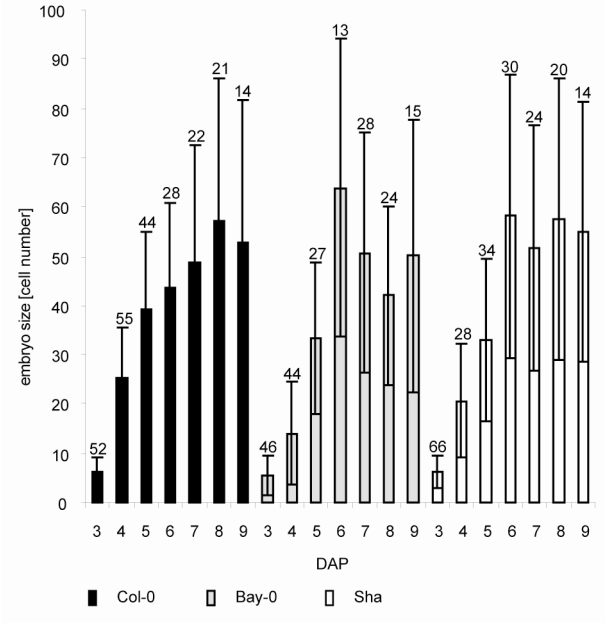

Figure 9: Independence and constraints of embryo development
Time course of embryo development in *cdka;1* pollinated seeds of the 3 accessions, Col-0, Bay-0, Sha, from 3 DAP to 9 DAP. After 3 DAP, no additional divisions in the endosperm were observed, yet embryos in all three accessions continued to grow until 6 to 9 DAP indicating a self-sufficient embryo developmental program. However, embryos only reached a size of 55 to 65 cells (globular stage) showing the limitations of this endosperm-independent growth. Error bars indicate standard deviation.

The autonomous embryo formation raised the question to which extent embryo development is dependent on embryo size. Here, the variation of autonomous endosperm formation within an accession was used as a tool to analyze this dependency. Embryo growth in cell numbers was plotted against endosperm nuclei number in *cdka;1* fertilized seeds at 3 DAP. A strong correlation of embryo cell numbers and endosperm size was observed. The more endosperm nuclei were formed, the higher was the number of embryo cells (Fig. 8). This suggests an early positive feedback interaction between endosperm and embryo growth (R^2 (Bay-0) = 0,64; R^2 (C24) = 0,82; R^2 (Col-0) = 0,74; R^2 (Cvi-0) = 0,90; R^2 (Kas-1) = 0,61). Nevertheless, a more complex interaction cannot be excluded, for example via maternal layers or a positive and dose-dependent effect of developing endosperm on embryo growth.

Moreover, the autonomous endosperm in *cdka;1* fertilized seeds decomposed around 3 DAP. However, the embryo continued to grow independently for about 3 more days reaching an average size of approximately 50 cells at 9 DAP (Fig. 9). This data emphasizes the autonomous developmental program of the embryo, but it also shows the limitations of embryo growth with or without endosperm formation. The findings confirm results reported by Cooper and Brink half a century ago that completion of embryogenesis is dependent on a proper endosperm formation (Cooper and Brink 1942)

2.5 QTL analysis of Sha –Bay RIL population reveals two new loci involved in autonomous endosperm development

An additional explanation for the occurring natural variation in autonomous endosperm formation, apart from the natural variation in seed growth, is a presumptive signal that triggers autonomous endosperm proliferation upon fertilization of the egg cell but failing karyogamy of the second sperm cell with the central cell (Nowack et al. 2006; Aw et al. 2010). The variation could be explained by the strength or the transmission of the signal. On the other hand, variation might exist on the perception side, i.e. in the central cell. Due to the fact that *fis*-class mutants ovules and *cdka;1* mutant pollen can mutually restore their seed viability (Nowack et al. 2007), an obvious hypothesis was that natural variation in the expression or function of *FIS*-class genes might be responsible for the here observed variation in autonomous endosperm proliferation.

A quantification approach was chosen to identify possible loci on the chromosome that might relate to the trait by using an established Recombinant Inbred Line (RIL) population. The two ecotypes forming the RIL population were *Bayreuth-0* (*Bay-0*), originating from southern Germany, and *Shahdara* (*Sha*), from Tajikistan, which differed significantly in their EDV (Fig 6a) (Loudet et al. 2002). From the 165 RILs of the core collection 160 were grown. Each line was pollinated with pollen from heterozygous *cdka;1* mutant plants. Seeds 3 DAP from 4 to 8 siliques of each cross were cleared and the number of endosperm nuclei were determined in the aborting seeds. The large variation of the EDVs among the RIL population differed substantially from the *Bay-0* and *Sha* parents (Fig 6a, data not shown).

Two QTLs were detected, which explained roughly 20 percent of the total variance in EDVs with a heritability of 0.93. The QTLs were identified and localized by composite interval mapping (CIM) (Fig. 10). The first QTL, located on chromosome I (peak at 40,9 cM), linked with the marker *T27K12* (at 43,6cM or 15,9 Mb) was named *KIRKE* (*KIR*). The detected LOD of this QTL was 4,66. The QTL explained 13,7 % of the phenotypic variation in the *Bay-0* x *Sha* population. The alleles of Bay-0 at this position increased the number of endosperm divisions by a factor of 0,64.

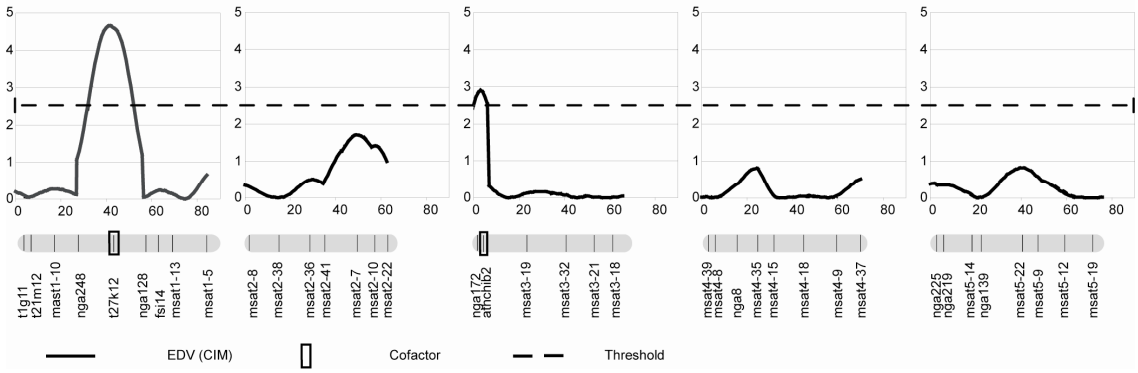

Figure 10: QTL mapping of factors controlling the autonomously proliferation of the central cell after *cdka;1* pollination

LOD traces of QTL mapping (in cM) for EDV in the Bay-Sha RIL population are reported for each of the 5 *Arabidopsis* chromosomes represented by the grey bars on the bottom of each chart (top on the left and bottom on the right). Marker names are indicated on each chromosome at their genetic position (Loudet et al. 2002). Composite interval mapping (CIM; continuous line) is reported. The markers used as cofactors in CIM are surrounded. The threshold of QTL detection (LOD=2.4; hatched line) has been determined using a permutation test (see materials and methods).

The second QTL was mapped to the top of chromosome III around the marker *athcib2* (6,8 cM or 3,96 Mb) and was named *KALYPSO* (*KAL*). In Greek mythology the hero Odysseus had to master the two witches Kirke and Kalypso on his journey (Homer 2005). Both tried to seduce Odysseus and made him stay for several years.

KAL had a LOD of 2,91 and described 8,1 % of the observed phenotypic variation in the *Bay-0* x *Sha* population. Different to *KIR*, the presence of the *Bay-0* alleles at the *KAL* locus decreased the number of divisions. This resulted in an overall higher EDV reaching up to 4,5 in some RILs. Thus, no RIL showed a lower EDV than determined for Sha.

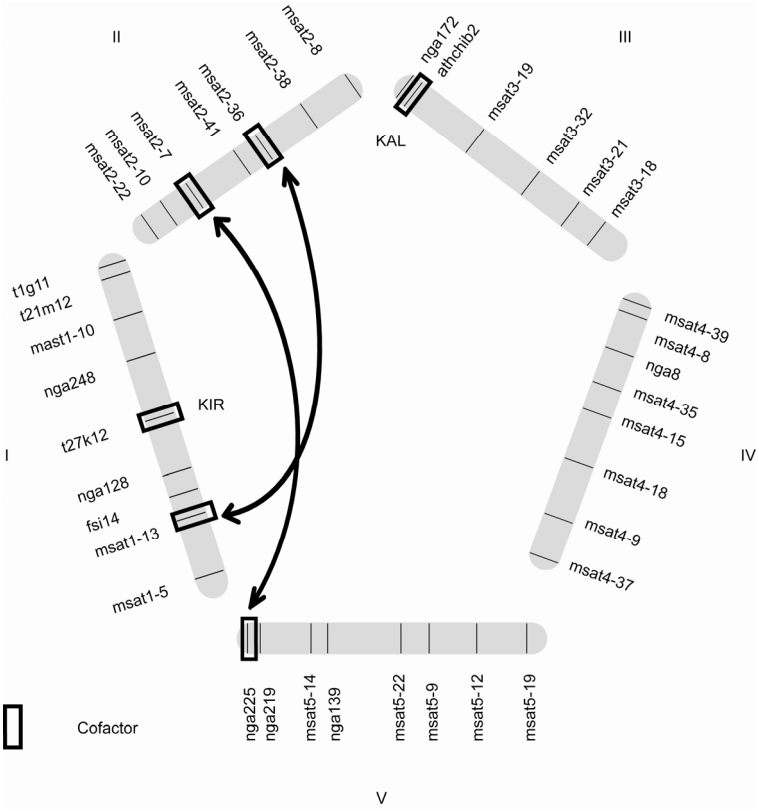

Figure 11: Complex QTL mapping of factors controlling the autonomously proliferation of the central cell after *cdka;1* pollination
LOD traces of complex QTL mapping for EDV in the Bay-Sha RIL population are reported for each of the 5 *Arabidopsis* chromosomes represented by the grey bars. The two complex QTLs are represented by the black arrows. Marker names are indicated on each chromosome at their genetic position (Loudet et al. 2002). The markers used as cofactors in CIM are surrounded. The threshold of QTL detection (LOD=2.4) has been determined using a permutation test (see materials and methods).

Further analysis of the data set revealed no genetic interaction between *KAL* and *KIR*. However, two complex QTLs were detected. The first interaction was between chromosome I linked to marker msat1.13 (70,4 cM) and II linked to marker msat2.36 (26,8 cM) (Fig.11). The second one was positioned between chromosome II marker msat2,7 (42,7 cM) and V marker nga225 (0 cM) (Fig. 11).

The map positions of *KIR*, *KAL* and the two complex QTLs were compared with the location of the *FIS* class genes and genes associated to it in the *Arabidopsis* genome, i.e. *SWN/EZA1* (AT4G02020), *MEA* (AT1G02580), *FIE* (AT3G20740), *FIS2*

(AT2g35670), the not molecularly identified *BORGIA* (*BGA*) located at the vicinity of *FIS2* (Guitton et al. 2004), *RBR* (AT3G12280) and *MSI1* (AT5G58230). Furthermore, the map positions of other genes known to influence seed size were compared; which are: *TTG2* (AT2G37260), *IKU2* (AT3G19700), *MINI3* (AT1G55600), *AP2* (AT4G36920) and *ARF2/MNT* (AT5G62000). However, none of the genes mapped closely to the QTLs. Interestingly*, DME* (AT5G04560) involved in the demethylation and activation of maternal *FIS*-class genes correlated close to the position of marker nga 225 on top of chromosome V and the close relative *ROS1* (AT2G36490) to marker msat 2,7 on chromosome II. The antagonist to *DME, MET1* (AT5G49160) was not located near any QTL.

In summary, neither *KAL* nor *KIR* mapped near any of seed developmental related genes indicating that they represent two new loci involved in endosperm development. However, the gene location of *ROS1* and *DME* were close to the marker positions of complex QTL 2 between chromosome 2 and 5. Thus, by making an educational guess, it was worthwhile for these two genes to undergo further testing.

2.6 *ROS1* and *DME* mutant seeds display a shift towards the onset of central cell division upon *cdka;1* fertilization in Col-0 background

The QTL analysis revealed a complex QTL between chromosome 2 and 5 linked to the markers msat 2,7 and nga 225 co-located close to the genes *ROS1* (AT2G36490) and *DME* (AT5G04560) (Fig. 11). Therefore, *ros1*-3 was crossed with a *dme*-1 mutant allele (GK-252E03-014577) to receive a *ros1*-3*/dme*-1 double mutant line. The *ros1*-3 allele was isolated from Ws-0 heterozygotes (*Arabidopsis* Knockout facility, University of Wisconsin, Madison, WI) and introgressed into the Col-0 background six times (Penterman et al. 2007a).

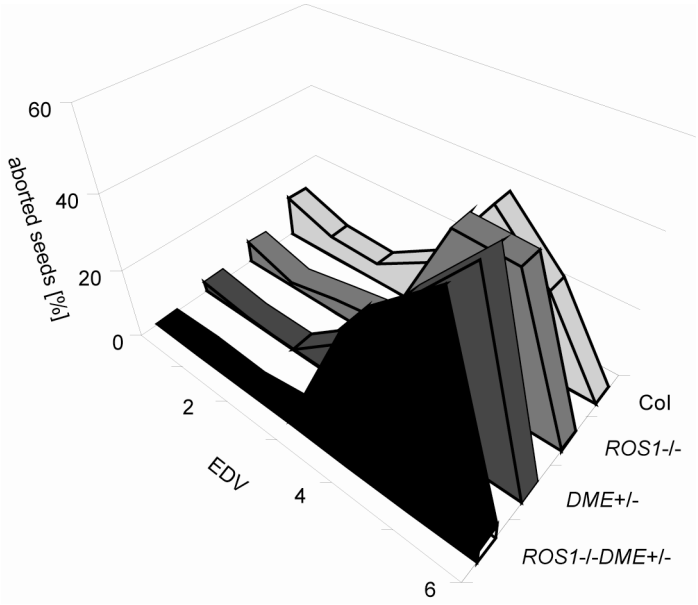

Figure 12: Variation in initiation of autonomous endosperm proliferation upon *cdka;1* pollination in a wild-type and *ros1*-3, *dme*-1 single and *ros1*-3 *dme*-1 double mutant genetic background
Distribution of autonomously proliferating endosperm nuclei of *cdka;1* pollinated ovules in Col-0 wild-type, *ros1*-3/*ros1*-3, *DME*/*dme*-1 and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. The x-axis indicates EDV (Endosperm Division Value), the y-axis shows the number of ovules pollinated with *cdka;1* mutant pollen in percentage. The diagram shows that the frequency of seeds in which endosperm nuclei that start to proliferate increases in *dme* and *ros1* single mutants. Finally in the double mutant *dme-ros*1 all seeds display a proliferating central cell nucleus. In addition, there is a slight tendency to undergo more division cycles in the single mutants and again an additive effect in the double mutant situation.

Since the two candidate genes were found by QTL analysis using the trait EDV, I first tried to discover whether *ROS1* and *DME* influenced endosperm formation and secondly, if both did genetically interact. Therefore, the EDVs of *cdka;1* pollinated *ros1*-3 and *dme*-1 single and double mutant plants were determined. The percentage of ovules pollinated with *cdka;1* mutant pollen were plotted against their EDV. Interestingly, autonomous central cell division was enhanced in comparison to the wild-type Col-0 background (Fig. 12). 11,5% (N=355) of Col-0 ovules aborting seeds contained an undivided central cell, of *ros1*-3/*ros1*-3 plants 6% (N=334), of *DME*/*dme*-1 2,9% (N=385) and of the double mutant *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants 0,25% (N=401). Almost every seed originating from a *ros1*-3/*ros1*-3 *DME*/*dme*-1 plant and fertilized with *cdka;1* mutant pollen showed autonomous central cell division (Fig. 12).

The presented data show not only that *DME* and *ROS1* influence endosperm development but also that the higher EDV in the *ros1 dme* double mutant background (Fig. 12) might indicate a redundancy between *ROS1* and *DME*.

2.7 Fertilized *ros1* mutant seeds exhibit wild-type development

First, a phenotypic characterization was performed and *ros1*-3, *dme*-1 and *ros1*-3 *dme*-1 mutant seeds were cleared and compared with wild-type seed development over a time course of 12 DAP.

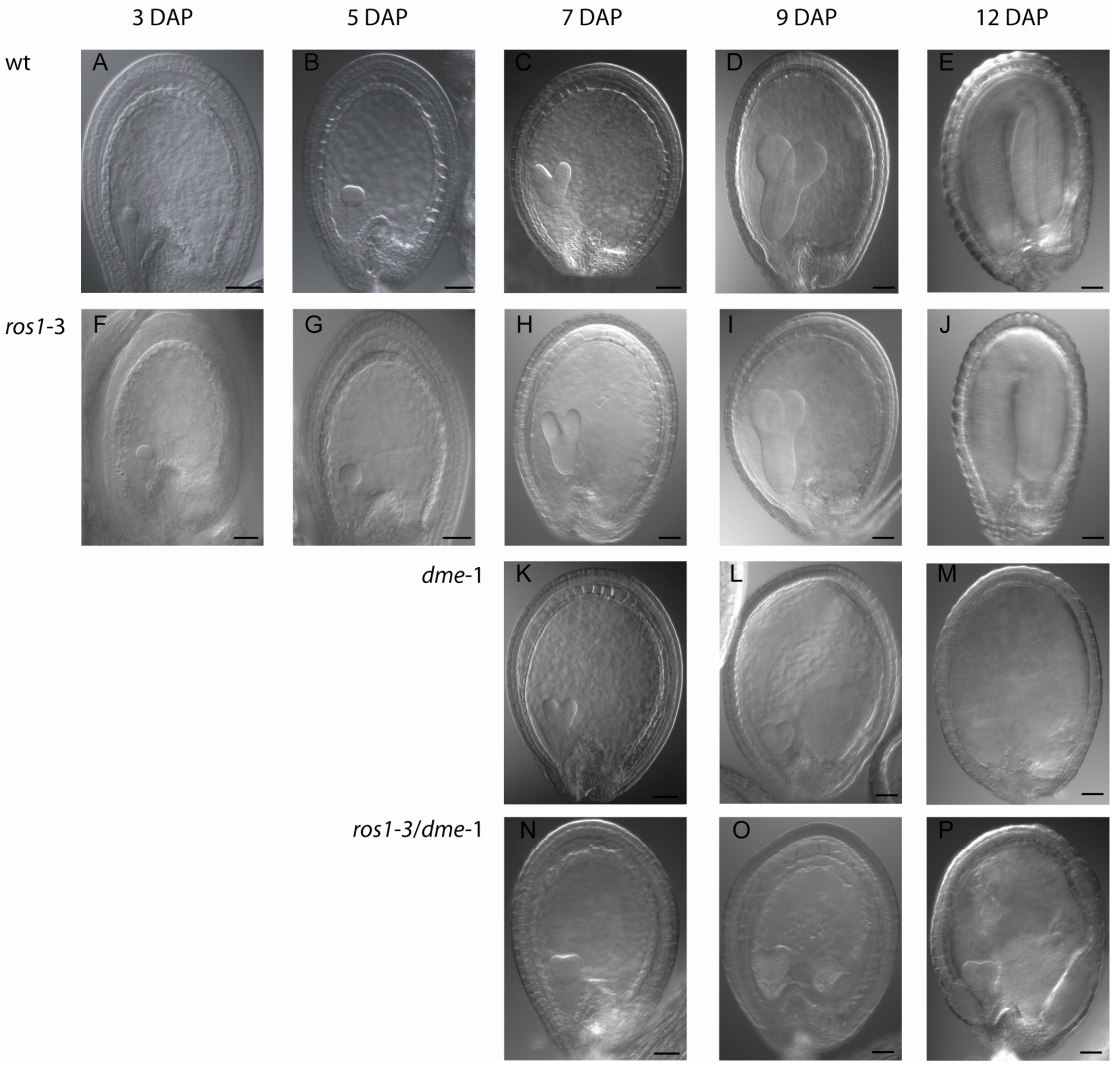

Figure 13: *ros1*-3 *dme*-1 double mutant show seed abortion at heart stage of embryonic development
DIC light micrographs of (**A**-**E**) Col-0 seed pollinated with Col-0 wild-type pollen, showing typical embryo development with globular stage at 3 DAP (**A**) and 5 DAP (**B**), heart at 7 DAP (**C**), torpedo at 9 DAP (**D**) and bend cotelydon stage at 12 DAP (**E**). (**F**-**J**) ros1-3 mutant seeds display same phenotypic characteristics as wild-type (**K**-**M**) *dme*-1 mutant seed pollinated with Col-0 arrested at heart stage at 7 DAP (**K**) and no additional growth at 9 DAP (**L**) or 12 DAP (**M**). Similarly, *ros1*-3/*dme*-1 double mutant seeds pollinated with Col-0 stopped growth at heart stage (**N-P**). In both *dme*-1 and *ros1*-3/*dme*-1 mutants, seeds aborted at 12 DAP with an disintegrating embryo (**M** and **P**) and vanishing endosperm (**L** and **O**). All pictures are placed such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Bars: 50 µm.

ros1-3 mutant seeds developed indistinguishable from wild-type (Fig. 13 f-j), while

seeds inside siliques of *DME*/*dme*-1 plants displayed a characteristic maternal gametophytic abortion rate of around 49% and seeds of *DME*/*dme*-1 *ros1*-3/*ros1*-3 plants around 45%. The *dme*-1 dependent mutant phenotype was in line with previous reports, and was characterized by an enlarged endosperm and an aborting embryo at late heart stage (Fig. 13 k-m) (Choi et al. 2002).

2.8 Unfertilized *ros1* and *ros1-dme* ovules show autonomous endosperm formation with endosperm-like features

One prominent phenotype of *FIS*-class genes is the formation of autonomous endosperm in unfertilized ovules (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999). Two maternal *FIS*-class genes *MEA* and *FIS2* are activated by DME, suggesting a similar phenotype for *dme* mutant ovules as observed for *mea* or *fis2* mutants. Thus, unfertilized ovules were analyzed in wild-type, *ros1*-3/*ros1*-3, *DME*/*dme*-1 and *DME*/*dme*-1 *ros1*-3/*ros1*-3 mutant plants.

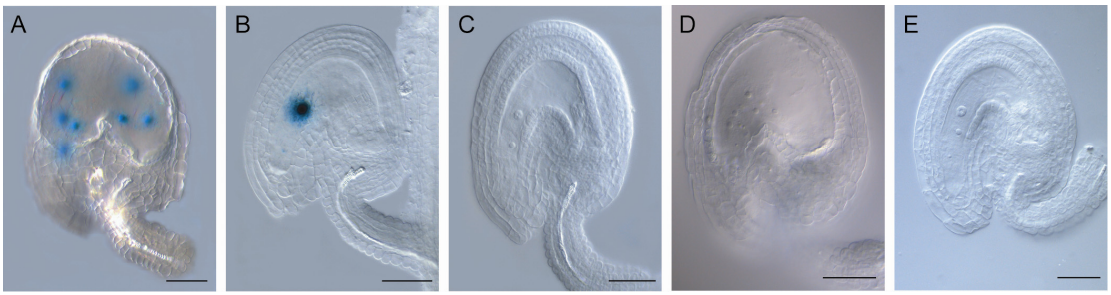

Figure 14: Unfertilized ovules of *ros1*-3 single and *ros1*-3 *dme*-1 double mutant plants display autonomous endosperm proliferation.
(**A**–**E**) DIC light micrographs of 7 DAE unfertilized ovules. (**A**-**C**) Histochemical detection of GUS activity in 7 DAE unfertilized ovules expressing a maternal p*FIS2:GUS* construct (Chaudhury et al., 1997; Luo et al., 2000) (**A**) Unfertilized ovule (7 DAE) from *FIS2::GUS*/*FIS2::GUS ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant shows seven GUS stained nuclei. (**B**) Unfertilized wild-type ovule (in the C24 ecotype) containing a GUS stained central cell. (**C**) 7 DAE unfertilized ovule from a *FIS2::GUS*/*FIS2::GUS ros1*-3/*ros1*-3 *DME*/*dme*-1 plant displaying no GUS staining. (**D** and **E**) Light micrograph of cleared whole mount unfertilized ovules (7DAE). (**D**) Unfertilized ovule from a *ros1*-3/*ros1*-3 *DME*/*dme*-1 plant with eight autonomously proliferated endosperm nuclei. (**E**) Unfertilized wild-type ovules (Col-0) displaying the egg cell and the central cell. All pictures are placed such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Bars: 20 μm.

7 DAE ovules from wild-type and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants were cleared. No autonomous endosperm proliferation could be observed in ovules originated from Col-0 wild-type and *DME*/*dme*-1 mutant plants, which was in line with previous reports (Choi et al. 2002). Autonomous endosperm formation was detected in some

ovules of *ros1*-3 *dme*-1 double mutant plants (Fig 14 d,e). A *FIS2-GUS* reporter was chosen to better visualize the endosperm nuclei in unfertilized ovules. *FIS2* is reported to be solely expressed in the central cell and during the first five free nuclear divisions of the endosperm (Luo et al. 2000). The same mutant phenotype was obtained from mutant ovules carrying the *pFIS2-GUS* construct (Fig. 14 a-c).

Table 1: Unfertilized ovules of *ros1*-3 single and *ros1*-3 *dme*-1 double mutant plants display autonomous endosperm proliferation
Around 2% of all ovules of *ros1*-3/*ros1*-3 mutant plants display autonomously proliferating endosperm. However, in combination with *ros1*-3 the mutant phenotype increases, indicating a redundant action of *ROS1* and *DME*.

A

Genotype of parents	Phenotype (%)		
	CC > 1	CC = 1	N
Col-0	0,00	100,00	740
DME/*dme*-1	0,00	100,00	876
ros1-3/*ros1*-3	1,95	98,00	943
ros1-3/*ros1*-3 *DME*/*dme*-1 (F2)	11,99	88,00	1241

B

Genotype of parents	Phenotype (%)		
	CC > 1	CC = 1	N
pFIS2:*GUS+/+*	0,00	100,00	749
pFIS2:*GUS+/+ DME*/*dme*-1	0,00	100,00	494
pFIS2:*GUS+/+ ros1*-3/*ros1*-3	2,16	97,84	1386
pFIS2:*GUS+/+ ros1*-3/*ros1*-3 *DME*/*dme*-1 (F2)	12,14	87,86	1411

To determine the percentages of ovules with autonomous endosperm formation, the number of ovules originating from *ros1*-3/*ros1*-3, *DME*/*dme*-1 and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants was counted. Ovules from wild-type and *DME*/*dme*-1 7 DAE (N=740) plants showed no autonomous endosperm formation, while *ros1*-3/*ros1*-3 displayed 1,95% (N=943) and *ros1*-3/*ros1*-3 *DME*/*dme*-1 11,99% (N=1241) (Tab. 1 a). Mutant plants carrying a *pFIS2:GUS* construct displayed very similar proportions of autonomous endosperm proliferation as observed in mutants without the construct (Tab. 1 b). 8 nuclei was the highest amount of autonomous endosperm that was detected (Fig 14 Surprisingly the *dme*-1 together with the *ros1*-3 mutant allele increased the *ros1*-3 mutant phenotype, indicating a probable redundancy between *ROS1* and *DME.*

2.9 The autonomously proliferating central cell nuclei in unfertilized ovules from *ros1-dme* mutant plants display characteristics of genuine endosperm

Next, it was tested to what degree the proliferating central cell in unfertilized *ros1*-3 single and *ros1*-3 *dme*-1 double mutant background displays features of a developing endosperm.

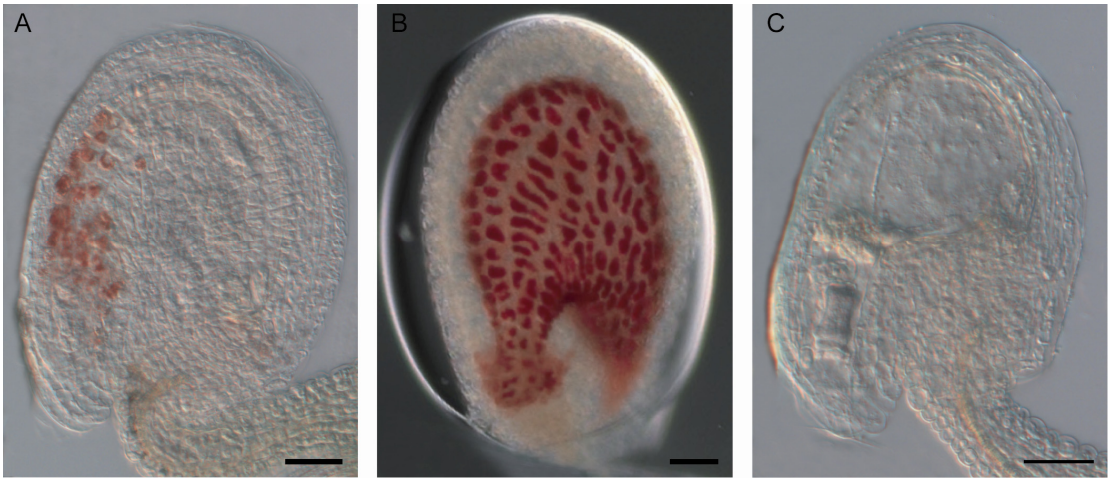

Figure 15: The autonomously proliferating central cell nuclei in unfertilized ovules of *ros1*-3 *dme*-1 mutant plants display characteristics of genuine endosperm
Detection of proanthocyanidin accumulation by vanillin staining in 7 DAE unpollinated ovules and in double fertilized Col-0 wild-type seed 3 DAP. (**A**) Unfertilized ovule originated from *ros1*-3/*ros1*-3 *DME*/dme-1 plants start proanthocyanidin synthesis. (**B**) Double fertilized wild-type seeds accumulate proanthocyanidin in the endothelium. (**C**) No proanthocyanidin can be detected in unfertilized wild-type ovules (Col-0). All pictures are placed such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Bars 50 µm.

Therefore, integument differentiation was analyzed in wild-type fertilized seeds 3 DAP and unfertilized ovules 7 DAE from *ros1*-3/*ros1*-3 *DME*/*dme*-1 double mutant and Col-0 plants (Fig. 15 a-c). In general, fertilization triggers the deposition of proanthocyanidin pigments in the endothelium cell layer of the seed coat, which can be visualized as a red stain using vanillin assay (Fig. 15 b) (Debeaujon et al. 2003; Ungru et al. 2008). Unfertilized wild-type ovules do not set off endothelium differentiation and vanillin staining is negative (Fig. 15 c). In contrast to unfertilized wild-type ovules, some ovules from *ros1*-3/*ros1*-3 *DME*/*dme*-1 double mutant plants were vanillin positive (Fig. 15 a). Thus, the autonomous dividing central cell appears to possess endosperm-like characteristics.

2.10 *ros1-dme* mutants appear to disturb pollen viability and/or the fertilization process

The here used *dme*-1 mutant allele is maternal gametophytic lethal and therefore is not transmitted through the female site (Choi et al. 2004). In addition, *DME* is reported to be not expressed in the male reproductive organs like stamen and pollen. To proof the transmission of the *dme*-1 allele the offspring of self-pollinated *DME*/*dme*-1 and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants and of reciprocal crosses with Col-0 wild-type were genotyped.

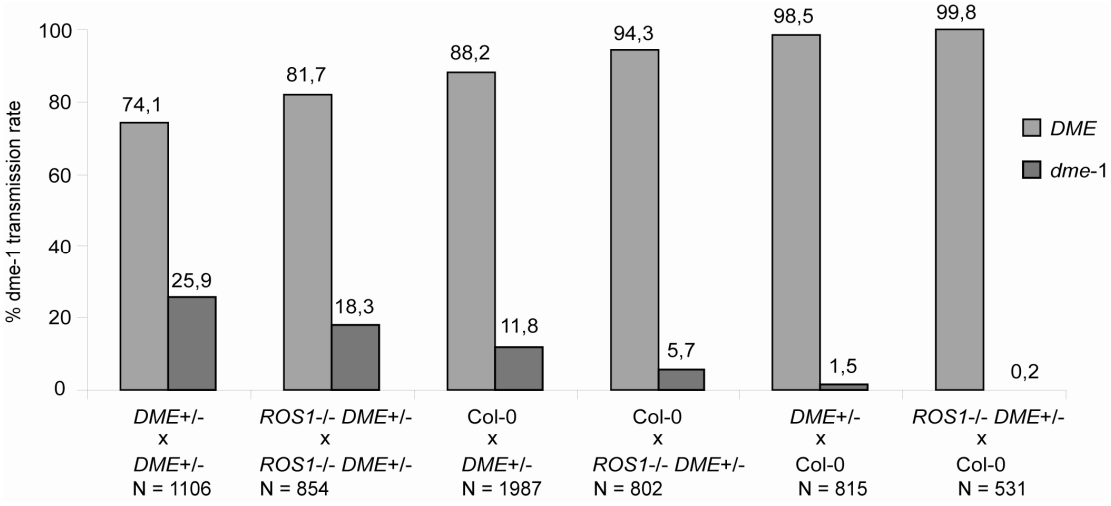

Figure 16: *dme*-1 transmission rate in *dme*-1 single mutant and *ros1*-3 *dme*-1 double mutant plants
The *dme*-1 transmission rate was highest in self-pollinated/fertilized mutant plants, decreasing in Col-0 x mutant and lowest in mutant x Col-0. This indicates that the *ros1*-3 and *dme*-1 mutations had an impact on the *dme*-1 transmission through the paternal gametophyte and the following fertilization process. The x-axis indicates the genotype of the parents and the y-axis the transmission of *dme*-1 in percentages, N = absolute number of plants.

Surprisingly, *dme*-1 transmission was lower than 50% in all crosses (Fig. 16). It was highest in the self-pollinated plants with 25,9% for *DME*/*dme*-1 x *DME*/*dme*-1 and 18,3% for *ros1*-3/*ros1*-3 *DME*/*dme*-1 x *ros1*-3/*ros1*-3 *DME*/dme-1 (Fig. 16). *dme*-1 transmission was 11,8% in Col-0 x *DME*/*dme*-1 and 5,7% Col-0 x *ros1*-3/*ros1*-3 *DME*/*dme*-1, while in the reciprocal crosses *dme*-1 transmission rate was almost completely abolished (Fig. 16). Interestingly, an additional *ros1*-3 mutant allele decreased dme-1 transmission rate in self-pollinated and in the reciprocal crosses with Col-0 wild-type. In addition, no aborting seeds could be detected from crosses, which received a paternal *dme*-1 allele (data not shown). Although *DME* is not reported to be expressed in the paternal reproductive organs, our data suggest an impact of *ROS1* and *DME* on pollen viability, the fertilization process and/or the

communication between male gametophyte and maternal sporophyte, for example during pollen tube guidance.

2.11 Bypassing genomic imprinting allows seed development in *ros1* single and *ros1-dme* double mutant plants fertilized with *cdka;1* mutant pollen

Fertilizing the seed with *cdka;1* mutant pollen, only the egg cell accomplished fertilization, while the central cell undergoes few rounds of free nuclear divisions without fertilization. Nowack et al (2007) bypassed genomic imprinting in developing *fis*-class mutant seeds fertilized with *cdka;1* mutant pollen. Viable seedlings developed without any paternal contribution to the endosperm (*cdka;1* mutant pollen), together with an absent suppression (mutant *fis*-class complex) of endosperm proliferation. As shown above, a relaxed endosperm proliferation was observed in unfertilized ovules coming from *ros1*-3/*ros1*-3 and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants (Fig. 14 a,d,e). To test whether the observed phenotypes were dependent on the *ros1* and *dme* alleles used, the crosses and analyses were repeated with a second *ros1* (*ros1*-4) and *dme* (*dme*-4) allele. *dme*-4 was previously characterized as a weak *dme* mutant allele (Guitton et al. 2004). Double mutants in the following different allelic combinations were used: *ros1*-3 and *dme*-1, *ros1*-4 and *dme*-1 and *ros1*-3 and *dme*-4. They were fertilized with pollen from *CDKA;1*+/- plants.

Table 2: *ros1* single and *ros1 dme* double mutant plants produce viable offspring in crosses with dka;1
Transmission rate of *cdka;1* in crosses with Col-0 wild-type, *ros1*-3/*ros1*-3, *DME*/*dme*-1 and *ros1*-3/*ros1*-3 *DME/dme*-1. The different *dme* and *ros1* mutant alleles suppress the *cdka;1* mutant phenotype. Crosses with the weak *dme*-4 allele show lower *cdka;1* transmission rates. The relative transmission rate is defined as the percentage of viable single fertilized in relation to all viable seeds (viable without aborted seeds), while the percentage of rescued seeds are viable single fertilized seeds in relation to the total seed number of the silique (viable + aborted seeds).

Genotype of cross	Genotype of viable progeny (%)			relative transmission (%) (mut *cdka;1* - Col-0 *cdka;1)*	rescued seeds (%)
	CDKA;1+/-	CDKA;1+/+	N		
Col-0 (control) x *CDKA;1*+/-	3,33	96,67	480		0
ros1-3/*ros1*-3 x *CDKA;1*+/-	5,36	94,63	672	2,02	1,03
DME/*dme*-1 x *CDKA;1*+/-	7,14	92,86	672	3,81	0,99
ros1-3/*ros1*-3 *DME*/*dme*-1 x *CDKA;1*+/-	14,38	85,62	480	11,04	3,10
ros1-4/*ros1*-4 x *CDKA;1*+/-	6,10	93,90	672	2,77	1,42
ros1-4/*ros1*-4 *DME*/*dme*-1 x *CDKA;1*+/-	13,09	86,92	298	9,75	2,70
DME/*dme*-4 x *CDKA;1*+/-	7,94	92,06	768	4,61	1,21
ros1-3/*ros1*-3 *DME*/*dme*-4 x *CDKA;1*+/-	7,29	92,71	960	3,96	1,03

The *cdka;1* transmission rate in the F1 offspring was determined. In addition, the cdka;1 mutant phenotype is not fully penetrant and between 1 and 15% of *cdka;1* mutant pollen contain two instead of just one sperm cells dependent on temperature and growth conditions (Nowack et al. 2006). Therefore, the cross with Col-0 was used as a control to receive the exact number of viable seeds, which were *CDKA;1*+/- but double fertilized. The relative transmission rate was defined as the observed transmission rate in a mutant minus the transmission rate in wild-type. Hereby, it was possible to determine the amount of viable seeds containing a fertilized, diploid embryo and an unfertilized, homoparental diploid endosperm. This procedure was in line with previously reported data and calculation for *cdka;1* pollinated *fis*-class mutants (Nowack et al. 2007). All plants were cultivated at the same time and under the same growth conditions to avoid differences in the proportion of 2 sperm cells to 1 sperm cell in *cdka;1* mutant pollen.

In *DME*/*dme* plants approximately 75% of all seeds died due to both the *cdka;1* single fertilization (early abortion) and the *dme* dependent maternal gametophytic lethality (late abortion). The following equation was used to receive the total percentage of rescued seeds upon *cdka;1* pollination, where ***a*** is the percentage of viable seeds pollinated with wild-type pollen, ***b*** the relative transmission rate of *cdka;1* in percentages and ***x*** the percentage of ovules inside the siliques, which were rescued upon *cdka;1* pollination.

$$x = b * a / (100\% - b)$$

Surprisingly, all mutant backgrounds displayed seed rescue upon *cdka;1* fertilization from 1% in *DME*/*dme*-1 up to 3,1% in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants (Tab. 2). In comparison to the strong *dme*-1 mutant allele, *DME*/*dme*-4 and *ros1*-3/ *ros1*-3 *DME*/*dme*-4 plants showed around 1% rescue, which was the same as for single mutant plants (Tab. 2). In addition, previous work reported that in *dme-4* mutant ovules *FIS2:GUS* reporter expression remained at lower levels than wild-type ovules while in *dme*-2 mutant ovules expression disappeared almost completely (Jullien et al. 2006b). Hence, the reduced *cdka;1* transmission in the offspring of *ros1*-3/ *ros1*-3 *DME*/*dme*-4 plants in comparison with *ros1*-3/ *ros1*-3 *DME*/*dme*-1 was probably due to a more active DME in *dme*-4 mutants.

Interestingly, the percentage of single fertilized viable seeds was lower than the actual percentage of unfertilized ovules displaying autonomous endosperm formation in *ros1*-3/ *ros1*-3 *DME*/*dme*-1 plants, i.e. 12% of all unfertilized ovules showed a

divided central cell, while 3,1 % of all seeds were rescued upon single fertilization (Tab. 1, 2). Thus, only 25% of all seeds, showing autonomous endosperm formation, were rescued upon *cdka;1* pollination. This was in line with previous work of Nowack et al. (2007) in which it was reported that 20% of the offspring of *mea*/*mea* plants fertilized with pollen from *CDKA;1*+/- plants produced a viable seed containing a homodiploid, solely maternally derived endosperm. In comparison, almost 100% of all unfertilized *mea* mutant ovules showed autonomous endosperm formation.

2.12 Unfertilized ovules, displaying autonomous endosperm formation and single fertilized and viable seeds of *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants appear to be *DME* wild-type

As shown above, *dme*-1 in combination with *ros1*-3 or -4 increased *cdka;1* transmission and autonomous endosperm formation (Tab. 1, 2). However, *dme* was previously not reported to cause the formation of autonomous endosperm raising the question whether the ovules (CC > 1) and/or the viable *CDKA;1*+/- offspring were mutant for *DME*.

Since the amount of autonomous endosperm in unfertilized ovules was quite small (12% of all ovules with not more than 8 nuclei each) and the rescued seeds in crosses with *cdka;1* could not be distinguished from wild-type pollinated seeds, it was impossible to laser dissect the autonomous endosperm and genotype subsequently.

Table 3: Unfertilized ovules with autonomous endosperm formation and viable, single fertilized seeds appear to be *DME* wild-type

(**A**) The transmission of the *dme*-1 allele was determined by PCR, by phenotypic analysis and by sulfadiazine selection on ½ MS plates. First of all, the *dme*-1 and the *ros1*-3 alleles are necessary for the suppression of the *cdka;1* mutant phenotype. Nevertheless, the *dme*-1 allele is only transmitted through the maternal gametophyte to ~1 % in wild-type and single fertilized viable seeds. Thus, *dme*-1 is maternally gametopytic lethal. It further indicates a pre meiotic or trans effect of the *dme*-1 heterozygous mutation on the phenotype. (**B**) Autonomous endosperm formation and GUS detection in 7 DAE unfertilized ovules from *ros1*-3, *dme*-1single or double mutant plants (Col-0). Samples were received from *ros1*-3/*ros1*-3, *DME*/*dme*-1, *ROS1*/*ros1*-3 *DME*/*dme*-1 (F2) and *ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant plants expressing a homozygous maternal *FIS2::GUS* construct (Chaudhury et al., 1997; Luo et al., 2000). Wild-type ovules show ~100%, while *DME*/*dme*-1 mutant plants ~50% GUS activity. Ovules displaying autonomous endosperm proliferation are GUS negative, indicating a *DME* wild-type allele. (**C**) Expression analysis of *FWA* (At4g25530), *FIS2* (At2g35670), which are activated by DME prior to fertilization (Jullien et al. 2006b). 2 DAE samples were collected from wild-type pollinated Col-0 wild-type, *DME*/*dme*-1 plants. The values show a down regulation of *FIS2* and *FWA* expression in *DME*/*dme*-1 mutant samples, indicating activation by DME.

A

Genotype of parents	Genotype of viable progeny (%) by PCR				by selection	
	CDKA;1+/-	*CDKA;1*+/+	*DME*/*dme*-1	N	*DME*/*dme*-1	N
DME/*dme*-1 x Col-0					1,47	815
ros1-3/*ros1*-3 *DME*-1+/- x Col-0					0,19	531
DME/*dme*-1 x *CDKA;1*+/-	17,08	94,63	1,00	672	1,00	603
ros1-3/*ros1*-3 *DME*/*dme*-1 x *CDKA;1*+/-	23,50	85,62	1,41	480	1,32	781
Col-0 (control) x *CDKA;1*+/-	11,40	96,67		480		

B

Genotype of parents	Phenotype (%)		*GUS* expression (%)			
	CC > 1	CC = 1	CC > 1	CC = 1	total	N
pFIS2:*GUS*+/+	0,00	100	0,00	100,00	98,80	749
pFIS2:*GUS*+/+ *DME*/*dme*-1	0,00	100	0,00	49,80	49,80	494
pFIS2:*GUS*+/+ *ros1*-3/*ros1*-3	2,16	97,84	2,16	96,84	98,99	1386
pFIS2:*GUS*+/+ *DME*/*dme*-1 *ROS1*/*ros1*-3 (F2)	6,27	93,73	6,27	43,77	50,05	4411
pFIS2:*GUS*+/+ *DME*/*dme*-1 *ros1*-3/*ros1*-3 (F2)	12,14	87,86	12,14	37,88	49,83	1411

C

Genotype of parents	Expression compared to Col-0 in x fold				
	FIS2	*stdev.*	*FWA*	*stdev.*	BR
DME/*dme*-1	0,71	0,21	0,43	0,19	3

Therefore, a more indirect approach was chosen. First the strength of the *dme*-1 allele was characterized. The *pFIS2:GUS* and *FIS2* and *FWA* expression in unfertilized ovules 2 DAE were examined by using GUS staining and qRT-PCR. These genes are known to be regulated by *DME* (Jullien et al. 2006b). *DME*/*dme*-1 plants were taken as the maternal parent. Here, *FIS2* expression was reduced by

0,71 and *FWA* by 0,43 fold (Tab 3 c). Furthermore, GUS expression was reduced by 50,2% in ovules from *pFIS2:GUS*+/+ *DME*/*dme*-1 plants (Tab. 3 b). Altogether, *dme*-1 was considered to be a strong allele because *FIS2* and *FWA* expression were strongly reduced. Thus, ovules without a GUS signal were likely to carry a *dme*-1 allele.

Interestingly, autonomous endosperm formation was always accompanied by a GUS signal, for example 12,14% of all ovules contained more than one endosperm-like nuclei and these ovules were always GUS positive in *pFIS2:GUS*+/+ *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants (Tab. 3 b). In addition, a similar amount (11,99%) of autonomously proliferating ovules were observed in the same mutant background without the *pFIS2*:*GUS* construct (Tab. 1 a). Furthermore, the *dme*-1 transmission rate was less than 1,5% in all crosses using the mutant plant as the maternal and either Col-0 or *CDKA;1*+/- as the paternal parent (Tab. 3 a). Thus, development of a homoparental diploid endosperm appeared to be independent of *dme*-1 co-transmission in autonomously proliferating ovules and viable, single fertilized seeds coming from *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. Given the fact that the transmission rate of *cdka;1* and the amount of autonomously proliferating ovules increased in dependency on *dme*-1 presence, *dme*-1 was not expected to be absent in the single fertilized offspring or in the homodiploid and autonomously proliferating endosperm.

2.13 *ROS1* and *DME* are involved in DNA demethylation during maternal gametophytic development in a MET1 dependent manner

DME and *ROS1* are involved in epigenetic gene regulation. Due to their function the hypothesis was raised that the mutant phenotype was based on a misregulated DNA methylation during or after the three mitotic divisions of the maternal gametes. It was also previously shown that *MET1* expression is down regulated during gametogenesis, resulting in a hemimethylated status of the maternal genome (Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008). A hemimethylated DNA status is pivotal for a proper *DME* activity on a specific 5-methylcytosine, while DME activity is highly reduced, if the target region is fully methylated. For example, DME function is reduced by up to 10 fold in case of an abasic site, sitting on the opposite DNA strand (David-Cordonnier et al. 2001; Weinfeld et al. 2001). Conversely, the inhibition of DME activity was less prominent when the abasic site was shifted by 4 or 7 nucleotides away from the 5-methylcytosine (Gehring et al.

2006). Thus, a down regulated *MET1* expression may be crucial for DME activity to demethylate remaining methylation sites and hereby activate *FIS*-class genes. Conversely, *MET1* is not down regulated during paternal gametogenesis resulting into a methylated DNA (Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008). To test whether our mutant phenotype was based on a hypermethylated status of certain DNA regions, a *met1*-1 allele was crossed into *ros1*-3, *dme*-1 single and double mutant plants. Hereby, MET1 and/or ROS1, DME target genes should be completely demethylated in the mature embryo sac since the methylation sites could not be transferred during the three mitotic divisions of maternal gametogenesis. Hence, ROS1 and DME activity would not be required to erase remaining methylation sites any longer. Consequently, the *ros1*-3 *dme*-1 mutant phenotype could be reversed.

Table 4: Table 4: Relative *cdka;1* transmission in *ros1-3*, *ros1*-4, *dme*-1 and *dme*-4 mutant backgrounds is dependent on MET1

By the detection of the *cdka;1* allele, seeds, originating from the pollination with *cdka;1* mutant pollen, can be differed from seeds pollinated with wild-type pollen (Nowack et al. 2006). The table demonstrates that the *dme* and the *ros1* mutant alleles promote the transmission of the *cdka;1* allele. The maternal parents were genotyped for the *met1* mutant and *MET1* wild-type allele. The table shows that the transmission of the *cdka;1* allele is dependent on the presence of diploid maternal *MET1*.

Genotype of cross	Genotype of viable progeny (%)			relative transmission (%) (mut *cdka;1* - Col-0 *cdka;1)*
	CDKA;1+/-	*CDKA;1*+/+	N	
Col-0 (control) x *CDKA;1*+/-	3,33	96,67	480	
ros1-3/*ros1*-3 x *CDKA;1*+/-	5,36	94,63	672	2,02
DME/*dme*-1 x *CDKA;1*+/-	7,14	92,86	672	3,81
ROS1/*ros1*-3 *DME*/*dme*-1 (F1) x *CDKA;1*+/-	13,89	86,10	864	10,56
ROS1/*ros1*-3 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	8,48	91,52	672	5,15
ros1-3/*ros1*-3 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	14,38	85,62	480	11,04
ros1-4/*ros1*-4 x *CDKA;1*+/-	6,10	93,9	672	2,77
ROS1/*ros1*-4 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	7,59	92,40	672	4,26
ros1-4/*ros1*-4 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	13,09	86,92	298	9,75
DME/*dme*-4 x *CDKA;1*+/-	7,94	92,06	768	4,61
ROS1/*ros1*-3 *DME*/*dme*-4 (F2) x *CDKA;1*+/-	8,93	91,08	672	5,60
ros1-3/*ros1*-3 *DME*/*dme*-4 (F2) x *CDKA;1*+/-	7,29	92,71	960	3,96
MET1/*met1*-1 *ros1*-3/*ros1*-3 x *CDKA;1*+/-	3,72	96,28	672	0,39
met1-1/*met1*-1 *ros1*-3/*ros1*-3 x *CDKA;1*+/-	3,96	96,04	480	0,63
MET1/*met1*-1 *DME*/*dme*-1 x *CDKA;1*+/-	4,17	95,83	480	0,83
MET1-1-/- *DME*/*dme*-1x *CDKA;1*+/-	3,54	96,46	480	0,21
Col-0 (control II) x *CDKA;1*+/-	7,29	92,71	480	
MET1/*met1*-1 *ROS1*/*ros1*-3 *DME*/*dme*-1 (F1) x *CDKA;1*+/-	7,81	92,20	576	0,52
MET1/*met1*-1 *ROS1*/*ros1*-3 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	7,06	92,94	864	-0,23
MET1/*met1*-1 *ros1*-3/*ros1*-3 *DME*/*dme*-1 (F2) x *CDKA;1*+/-	7,29	92,71	480	0,00

The here presented data was in line with previous findings that heterozygous *met1* plants showed hypomethylation in all gametes (Saze et al. 2003). Even one additional *met1* mutant allele was enough to decrease the relative transmission rate of the *cdka;1* mutant allele in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants from 11,04 down to 0,00 (Tab. 4). Furthermore, cleared unfertilized ovules 7 DAE of the double and triple mutant plants with one met1-1 allele displayed no autonomous endosperm formation (pictures not shown). This suggests a haplo-insufficiency in the heterozygous mutants. In general, the aging gradient becomes highest in the reproductive organs. A heterozygous *met1* mutant allele probably caused a gradual loss of methylation sites through plant development. A phenotypical argument for haplo-insufficiency was given by the fact that I and others observed a reduced general plant and leaf size, disturbed floral organ composition and a delay of general plant growth and flowering time (Saze et al. 2003).

Summing up, the relative *cdka;1* transmission and the mutant phenotype were dependent on the presence of a diploid maternal *MET1* genotype before maternal gametogenesis. This supports previous observations that Met1 might not be completely inactive during maternal gametogenesis and an active DME is still needed to erase remaining methylation sites to activate, for example, *FIS*-class genes (Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008).

2.14 The *dme-ros1* mutant phenotype segregates in a non-Mendelian manner

The mutant phenotype appeared to be independent of *dme*-1 co-transmission and dependent on hypermethylation. This rose up the question how the mutant phenotype was inherited in a segregating population.

First, Col-0 plants were fertilized with pollen from a *ros1-3*/*ros1*-3-/-*DME*/*dme*-1 plant. *ROS1*/*ros1*-3-/-*DME*/*dme*-1 (F1), *ROS1*/*ros1*-3-/-*DME*/*dme*-1 (F2) and *ros1-3*/*ros1*-3-/-*DME*/*dme*-1 (F2) plants were obtained by sulfadiazine treatment and confirmed by PCR for the *dme*-1 allele. The F1 heterozygous generation displayed 11,46% of ovules with autonomous endosperm formation and 10,56% *cdka;1* relative transmission rate (Tab. 4, Fig. 17 a, b). The F2 generation with the same genotype *ROS1*/*ros1*-3-/-*DME*/*dme*-1 displayed on average 5,91% seeds with autonomous endosperm formation and 5,15% relative *cdka;1* transmission (Tab. 4, Fig. 17 a, b). However, zooming into individual plants (12 to 14 individuals) revealed that a double

heterozygous mutant displayed either 1-2% or around 11% seeds with autonomous endosperm formation (Tab. 4, Fig. 17 a, b). Double mutant F2 plants homozygous for *ros1*-3 gave again almost the same phenotypic proportions as for the *ros1*-3 heterozygous F1 plants with 11,99% autonomous endosperm formation and 11,04% relative transmission rate (Tab. 4, Fig. 17 a, b). In addition, all F2 individual double mutants either heterozygous or homozygous for *ros1*-3 descended from the same plant.

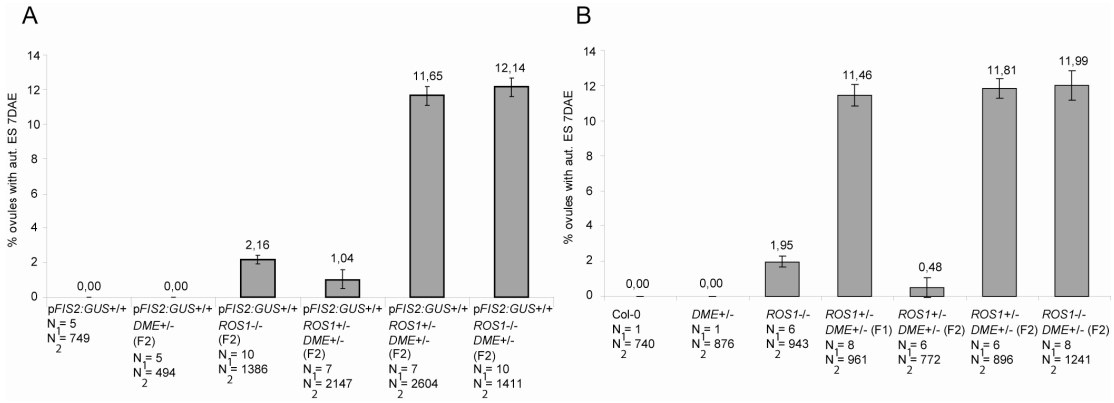

Figure 17: Autonomous endosperm formation in 7 DAE unfertilized ovules in *ros1*-3, *dme*-1 single or double mutant background
(**A)** 7 DAE old unfertilized ovules from *ros1*-3/*ros1*-3, *DME*/*dme*-1, *ROS1*/*ros1*-3 *DME*/*dme*-1 (F2) and *ros1*-3/*ros1*-3 *DME*/*dme*-1- mutant plants expressing a homozygous maternal *FIS2:GUS* construct. (**B**) Detection of autonomous endosperm formation in cleared whole mount unfertilized ovules from *ros1*-3/*ros1*-3, *DME*/*dme*-1, *ROS1*/*ros1*-3 *DME*/*dme*-1 (F1 and F2) and *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. At the x-axis the genotype of the parents is given and the y-axis indicates the percentages of autonomous endosperm formation; N1 = total amount of individual plants, N2 = total amount of ovules. Error bars indicate standard deviation.

The same pattern was observed for the two filial generations and different *ros1*-4 *dme*-1 genotypes, including an average 50% reduction of the relative *cdka;1* transmission rate in the offspring from *ROS1*/*ros1-4 DME*/*dme*-1 F2 plants (Tab. 4).
First of all, the two mutant alleles *ros1*-3 and *dme*-1 could have influenced seed development by a sporophytic effect in a haplo-insufficient manner. In this context, a haplo-insufficient mutant would be expected to show the same phenotypic penetrance in plants of the same zygosity independent of the filial generation. This was not the case. The phenotypic proportions in the *ROS1*/*ros1*-3 *DME*/*dme*-1 mutant plants of the first filial generation were almost the same as in the *ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant plants (~ 12%). Furthermore, *ROS1*/*ros1*-3 *DME*/*dme*-1 displayed two distinct classes with either ~ 12% autonomous endosperm or just very little, i.e.~ 1-2%.
In addition, the mutant phenotype was not reduced by half in *ROS1*/*ros1*-3 *DME*/*dme*-1 compared to *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. Thus, the mendelian

laws were probably not the basis of the phenotypic heredity. Consequently, in *ros1*-3 *dme*-1 double mutant plants one allele of a gene influenced the appearance of the other allele.

2.15 The mutant phenotype is likely to be established by in trans interactions between homologous DNA sequences on different chromosomes

The effect of one allele onto the other one was described for the first time in 1956 when Brink published his work about the heredity of the R-locus in maize (Brink 1956; 1973). Many years later he named it paramutation (Brink 1956; 1973). More recent studies revealed that epigenetic paramutations are involved in trans interactions between homologous DNA sequences on different chromosomes resulting in alterations in gene expression, associated with changes in DNA methylation and chromatin structure. In plants these epigenetic marks remained stable throughout mitosis and meiosis, and are therefore inheritable. Thus, paramutation is the epigenetic transfer of information from one allele of a gene to another to ascertain a state of gene expression that is heritable for generations (Chandler and Stam 2004; Stam and Mittelsten Scheid 2005; Chandler 2007; Cuzin et al. 2008).

In contrast to the definition of an epigenetic paramutation, the mutant phenotype was not inheritable through generations. Conversely, it was reversible in the manner that the F2 generation of the heterozygous *ROS1*/*ros1*-3 *DME*/*dme*-1 plants displayed either a complete mutant phenotype (~12%) or not (~1-2%) in the unfertilized ovules or in the single fertilized progeny. It was rather more likely that the epigenetic identities of certain genes were influenced in a parentally dependent manner, firstly because *MET1* is differentially activated between paternal and maternal gametogenesis (Jullien et al. 2008), secondly the *ROS1* wild-type allele was either coming from the maternal or the paternal gamete after the first filial generation of *ROS1*-3+/-*DME*-1+/- plants, and finally *dme*-1 was 99% inherited by the paternal site. On the one hand, the presence of a *ROS1* or *DME* wild-type allele on the paternal site resulted in a highly reduced phenotype in the ovules of the next filial generation, i.e. that *ROS1*-3*/ros1*-3 *DME*/*dme*-1 F2 plants displayed either a mutant phenotype or not with 50% chance. On the other hand, a *ROS1* allele from the maternal site did not reduce the mutant phenotype, i.e. that the first filial generation from the cross

Col-0 x *ros1*-3/*ros1*-3 *DME*/*dme*-1 showed the same proportion of mutant phenotypes as observed in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. Thus, the methylation sites on an allele had to be established *de novo* consistently throughout each and every generation by the presence of a dominant epiallele coming from the paternal site.

To elucidate a probable inter chromosomal methylation transfer, methylation marks of putative DME target genes such as *PHE1* (*AGL37*), *AGL34* and *AGL36* were quantified in DNA samples of leave material coming from *ros1*-3 *dme*-1 single and double mutants and Col-0 wild-type plants (Kohler et al. 2003b; Kohler et al. 2005; Makarevich et al. 2006; Makarevich et al. 2008; Shirzadi et al. submitted).

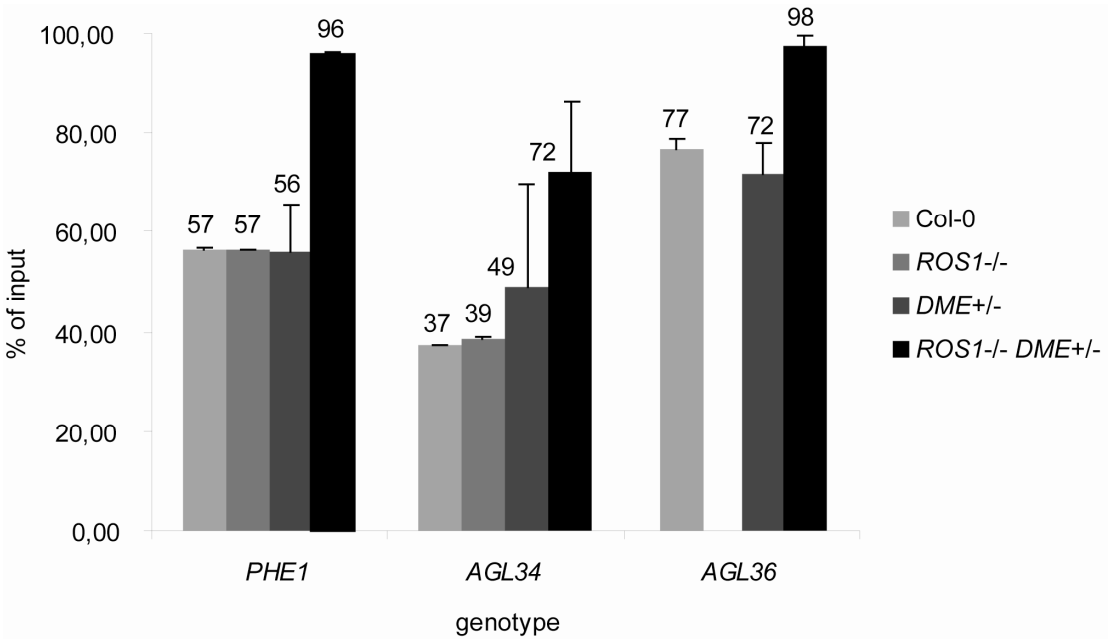

Figure 18: *ros1*-3 *dme*-1 double mutants display almost 100% relative methylation at three loci
Methylation marks of putative *DME* target genes such as *PHE1*, *AGL34* and *AGL36* were quantified to elucidate their epigenetic status and a probable inter chromosomal methylation transfer. The y-bar indicates the % of input or the amount of relative methylation.

The relative methylation of the measured *PHE1* region was around 57% in the wild-type and single mutant plants, but almost 100% in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants. The same pattern was true for *AGL36*. *AGL34* did show an increasing methylation up to 72% in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants but almost doubled compared to wild-type or *ros1*-3, single mutants (37%, 39%) (Fig. 18).
First of all, the highly increased methylation of *PHE1*, *AGL36* and *AGL34* loci in *ros1*-3/*ros1*-3 *DME*/*dme*-1 underlined the function of ROS1 and DME to erase methylation sites from certain target genes. In this context, a reduced *MET1* expression and the

presence of a maternal *DME* wild-type allele in *DME*/*dme*-1 heterozygous plants should cause a reduction of methylation sites of the maternal *PHE1* and/or *AGL36* alleles during maternal gametogenesis and herby reduce their relative methylation levels. However, the relative methylation in *PHE1* and *AGL36* increased to almost 100% in the progeny supporting the theory that a paternal hypermethylated allele (*ros1*/*dme*) influenced the methylation levels of the maternal epiallele (*ros1*/*DME*) during and/or after fertilization. Consequently, a trans methylation transfer between the epialleles might be necessary to explain the heredity of the mutant phenotype and the detected high methylation levels for *PHE1* and *AGL36*.

2.16 *ROS1* reveals a regulative function on endosperm proliferation and/or seed development related gene expression around 6 DAP

To unravel molecular mechanisms possibly underlying the seed phenotypes in homozygous *ros1*-3 single and/or *ros1*-3 *dme*-1 double mutants, the expression of endosperm proliferation and/or seed development related genes was analyzed. cDNA of 3 and 6 DAP seed material from Col-0, *ros1*-3/*ros1*-3, *DME*/*dme*-1, *ros1*-3/*ros1*-3 *DME*/*dme*-1, *MEA*/*mea*72, and *mea55*/*mea*55 mutant plants fertilized with Col-0 wild-type pollen were used for qRT-PCR to test *PHE1*, *DME* , *FWA*, *ROS1*, *MEA*, *FIS2*, *FIE*, *MSI1*, *AGL36*, *MPC* (*MATERNALLY EXPRESSED PAB C-TERMINAL*), *AGL28* and *AGL90*.

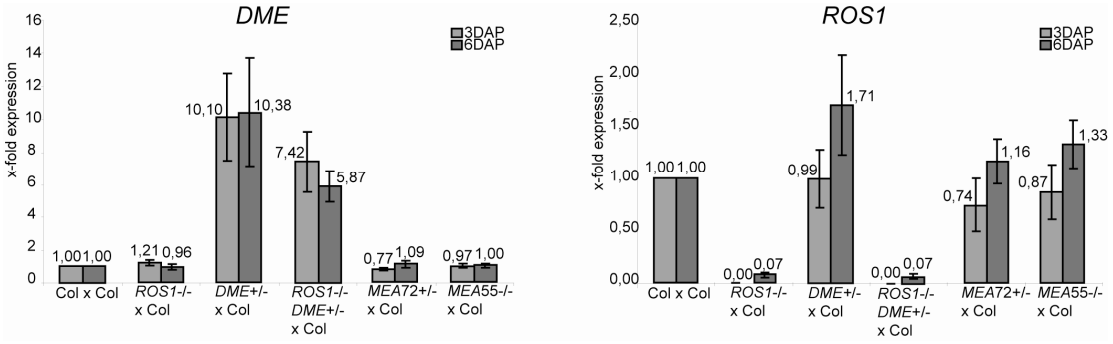

Figure 19: Expression analysis of *DME* and *ROS1*
cDNA of four biological replica of 3 and 6 DAP seed material were used in a Probe based qPCR using Universal Probe Library (UPL) hydrolysis probes (Roche).The *x*-axis indicates different mutant backgrounds analyzed; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

First of all, the expression of *DME* and *ROS1* was analyzed. A simple assumption was that the expression of *DME* in *DME*/*dme*-1 heterozygous mutant plants would be reduced by half. However, *DME* expression increased in *DME*/*dme*-1 and *ros1*-

3/*ros1*-3 *DME*/*dme*-1 mutant seeds 3 DAP and 6 DAP (Fig 19). Currently, the most likely explanation for this expression pattern comes from a t-DNA insertion in intron 2 at the position 2374bp downstream of the 5'UTR region. The insertion was located 15bp downstream of the start of Intron 2. The primers were designed to amplify a region spanning intron number 12 and 13. The Probe was located within the 95 nucleotide amplicon. Amplification started at the position 6151bp and ended at 6513 downstream of the 5'UTR region. Thus, the increased *DME* expression could be explained by a t-DNA derived promoter, which led to the transcription of the part downstream of the t-DNA insertion. Consistently, the same *DME* expression would have been expected in *ros1*-3/*ros1*-3 *DME*/*dme*-1- plants. Previous studies reported that ROS1 removes methylation sites from trans genes (Gong et al. 2002; Penterman et al. 2007a). Thus, the lower expression of *DME* in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants could be explained by elevated methylation levels inside the t-DNA sequence. However, at the current stage other explanations are also possible.
ROS1 gene expression in *ros1*-3/*ros1*-3 mutants crossed with wild-type pollen was completely abolished at 3 DAP and very low at 6 DAP (Fig. 19). This implied that either *ROS1* was only expressed in maternal structures such as the integuments and not in the fertilization products, endosperm or embryo. Alternatively, *ROS1* could be subject to imprinting in such a way that the paternal allele would be silenced

Subsequently, *PHE1* as an imprinted gene was tested. *PHE1* expression was slighted elevated in *ros1*-3/*ros1*-3 and even increased in *dme*-1 and *ros1*-3 *dme*-1 double mutants. It has been previously shown that maternal *dme* mutant gametes carried a partially hypermethylated genome that resulted in the lack of activation of *MEA*, *FIS2* and *FWA* (Gehring et al. 2006). The transcription of the maternally inherited *PHE1* allele is repressed by the FIS2-complex, and *PHE1* becomes upregulated in *mea* mutants (Kohler et al., 2003b; Kohler et al., 2005). Consequently, upregulation of *PHE1* in *DME*/*dme*-1 could be explained by a reduced expression of *MEA* in *DME*/*dme*-1. Furthermore, methylation marks on a region 2,5 kbp downstream of the *PHE1* locus prevents the repression of *PHE1* expression (Makarevich et al. 2008). A maternal *ros1* mutant allele increased methylation amounts and upregulated maternal *PHE1* expression. A combined effect is consistent with the additive levels of overexpression observed in *ros1*-3/*ros1*-3 *DME*/*dme*-1 than in *DME*/*dme*-1 or *ros1*-3/*ros1*-3 seeds.

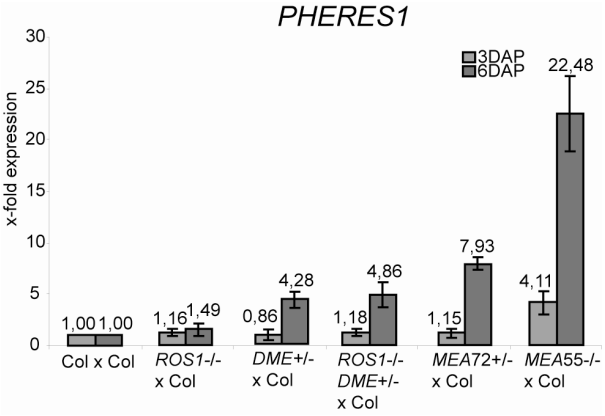

Figure 20: Expression analysis of *PHE1*
cDNA of four biological replica of 3 and 6 DAP seed material were used in a qPCR. The diagram shows that the maternal *PHE1* allele was not activated by DME but silenced by the FIS-class complex at both time points (3 DAP and 6 DAP). The *x*-axis indicates the genotype of the parents; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

However, no upregulation could be detected at the earlier stage 3 DAP (Fig. 20). One possibility is that only the paternal and not the maternal allele becomes hyper-active in a *DME*/*dme*-1, *ros1*-3/*ros1*-3 or *ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant background during early endosperm development. Thus, a detailed, i.e. allele-specific expression analysis is now required to investigate, which parental allele is upregulated.

To test whether ROS1 as a demethylase is involved in the expression of *FIS*-class genes, qRT experiments were performed with the putative *FIS2* components *FIE*, *MSI1*, *MEA* and *FIS2* and *FWA* as a second positive control.

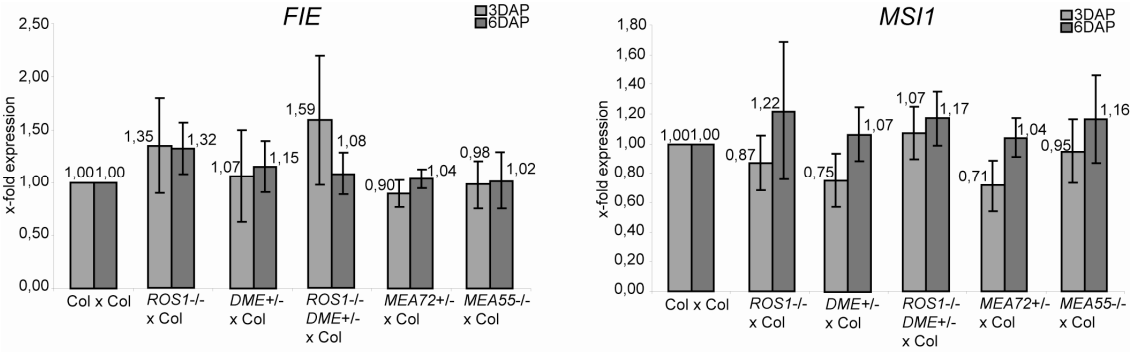

Figure 21: Expression analysis of *FIE* and *MSI1*, two members of the FIS-class complex, which are known to suppress endosperm proliferation
cDNA of four biological replica of 3 and 6 DAP seed material were used in qPCR. The diagram shows that *FIE* or *MSI1* expression is not regulated by DME or MEA. The *x*-axis indicates the genotype of the parents; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

So far, *MSI1* and *FIE* have not been identified to be imprinted or to be under tight transcriptional control of DME or the FIS2 complex itself. Consistently, no obvious effect of *dme*-1 and *ros1*-3 on their expression was observed (Fig. 21).

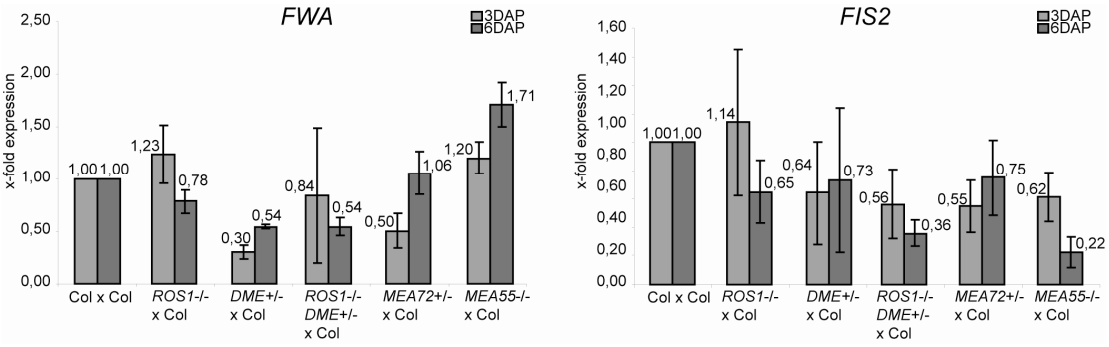

Figure 22: Expression analysis of *FWA* and *FIS2*
cDNA of four biological replica of 3 and 6 DAP seed material were used in qPCR. Early (3 DAP) *FWA* and *FIS2* expression was dependent on the activation by *DME*. The *x*-axis indicates the genotype of the parents; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

FWA and *FIS2* get activated by *DME* in the central cell (Kinoshita et al. 2004; Jullien et al. 2006b). Thus, *FWA* and *FIS2* were used as a positive control. The data exhibit the expected downregulation of *FIS2* and *FWA* in *DME*/*dme*-1 and *ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant material at both timepoints (Fig. 22). Furthermore, both genes display the above explained *ros1* dependent downregulation in 6 DAP material (Fig. 22).

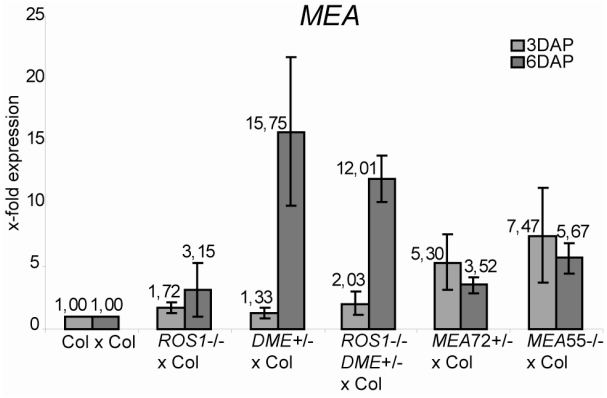

Figure 23: Expression analysis of *MEA*
cDNA of four biological replica of 3 and 6 DAP seed material were used in qRT-PCR. The diagram shows the late silencing of the paternal *MEA* allele by maternal expressed *MEA*. The *x*-axis indicates the genotype of the parents; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

Next, *MEA* expression was analyzed. *MEA* belongs to the imprinted genes, where only the maternal allele is expressed (Kinoshita et al. 1999). Maternal *MEA* expression is regulated in a MET1 and DME dependent manner (Choi et al. 2002; Zilberman et al. 2003; Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008). The paternal allele is enriched in H3K27 chromatin methylation, which is maintained by maternal MEA. Consequently, paternal *MEA* is released when the maternal *MEA* allele is silent (Gehring et al. 2006). This is consistent with the upregulation observed in *ros1*-3/*ros1*-3, *DME*/*dme*-1, *ros1*-3/*ros1*-3 *DME*/*dme*-1 and *mea* mutant seeds at 6 DAP (Fig. 23).

In contrast to *PHE1*, no additive *MEA* overexpression was measured in the *ros1*-3/*ros1*-3 *DME*/*dme*-1 double compared to the single mutants at 6 DAP. Increased maternal methylation levels in *ros1* mutants (Gong et al. 2002; Zhu et al. 2007; Penterman et al. 2007b) probably caused a decreased paternal and maternal *MEA* expression in *ros1-3 dme*-1 double compared to *mea* or *dme*-1 single mutant seeds. However *MEA* upregulation was much lower at 3 DAP, where *MEA* expression is reported to be DME dependent (Gehring et al. 2006). Thus, only the paternal and not the maternal allele becomes upregulated in *DME*/*dme*-1, *ros1*-3/*ros1*-3 or *ros1*-3/*ros1*-3 *DME*/*dme*-1 mutant backgrounds, which resulted in lower upregulation at 3 DAP compared to 6 DAP. To prove this, an allele-specific expression analysis is required to assess which parental allele gets overexpressed.

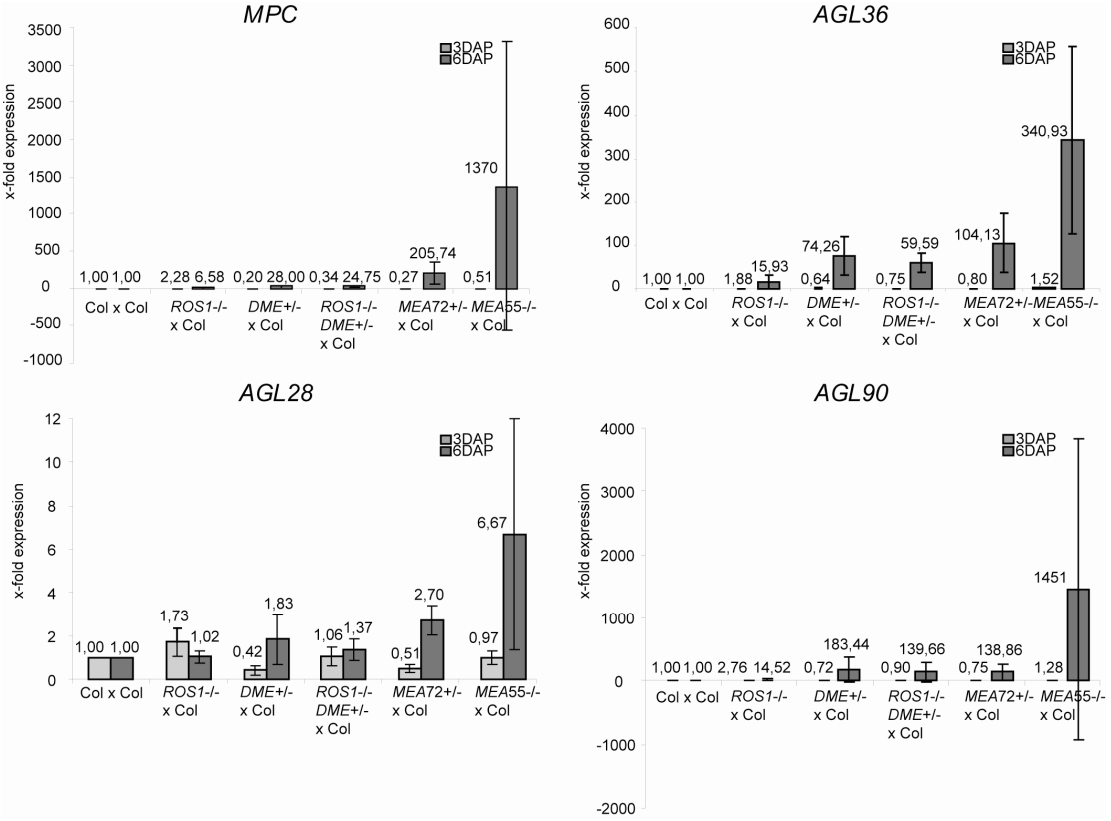

Figure 24:Expression analysis of *MPC*, *AGL36*, *AGL28* and *AGL90*
cDNA of four biological replica of 3 and 6 DAP seed material were in qRT-PCR. In all cases over expression is highest at 6 DAP. All genes tested showed an early (3 DAP) activation by *DME* and a late (6 DAP) suppression by *MEA*. The *x*-axis indicates the genotype of the parents; the *y*-axis shows the expression in x-fold. Error bars indicate standard deviation.

Paul Grini from the University of Oslo used *cdka;1* single pollinated seeds to identify genes which influenced seed development in a parent-of-origin specific manner (Shirzadi et al. submitted). By this approach the expression levels of imprinted genes were altered compared to wild-type seeds. A microarray transcriptional profile showed down regulation of mainly Type-I MADS-box transcription factors of the *AGL* family. Due to the fact that *DME* and *ROS1* were found as probable candidate genes in a QTL analysis based on the same response of seed development upon *cdka;1* single fertilization, the three candidate genes *AGL28*, *36* and *90* were included in qRT studies, suggesting that these genes were probably influenced by demethylation through DME and/or ROS1. *MPC*, a member of imprinted genes, was chosen as a positive control (Tiwari et al. 2008).

At 3 DAP the control *MPC* and all tested *AGL*s were downregulated in *dme*-1 mutant material, suggesting that *DME* was involved in the activation of the maternal alleles similar to *FWA* and *FIS2* (Fig. 22, 24). Conversely, in 6 DAP samples *MPC* and all *AGL*s were surprisingly elevated in *ros1*-3 *dme*-1, *dme*-1, and in *mea* mutant seed

material (Fig. 24). Here, *mea* mutant seed material displayed the highest overexpression, indicating that the *FIS*-complex was a repressor of *MPC* and/or *AGL* expression (Fig. 23).

In summary, the expression profiles for *MEA*, *FIS2*, *FWA*, *FIE* and *MSI1* were in accordance with published observations in *dme* and *mea* mutant material (Kinoshita et al. 2004; Gehring et al. 2006; Jullien et al. 2006b). Furthermore, increased methylation levels in *ros1* mutants (Gong et al. 2002; Zhu et al. 2007; Penterman et al. 2007b) probably caused lower expression levels in *ros1*-3/*ros1*-3 *DME*/*dme*-1 compared to *DME*/*dme*-1 mutant 6 DAP material for almost every gene tested except *FIE*, *MSI1* and *PHE1*. In the case of *PHE1* higher methylation levels may have increased *PHE1* expression, which is in line with a previous report of methylation dependent activation of *PHE1* (Makarevich et al. 2008). Thus, the qRT-data suggest a regulative function for *ROS1* on maternal gene expression around 6 DAP, while *DME* dependent maternal *FIS*-class gene activation is reported to happen prior to fertilization (Choi et al. 2002; Zilberman et al. 2003; Gehring et al. 2006; Jullien et al. 2008; Kinoshita et al. 2008). Nevertheless, an allele-specific expression analysis is required to assess which parental alleles of the genes tested get overexpressed in a mutant background. This is important to unravel the complex regulation of suppression and expression of either maternal or paternal alleles by *DME*, *ROS1* and also by the *FIS*-class genes in the context of imprinting.

3 DISCUSSION

Seed growth requires an intricate interplay between the different tissues that compose a seed. However, little is known about the coordination and integration of these parts into a functional whole. Analysis of seed development appears to be particularly difficult due to the observed coordinated growth behavior; *i.e.*, small seeds contain a reduced endosperm and large seeds a correspondingly larger endosperm (Berger et al. 2006). Here, I have followed a genetic dissection of double fertilization and seed development and exploited the existing natural variation found in *Arabidopsis* to study embryo–endosperm interactions during seed development.

A genetic dissection became possible through the use of *cdka;1* mutant pollen that lead to a successful fertilization of only the egg cell but triggered endosperm development without a contribution of a paternal genome. Indeed, among 14 different accessions tested, a large range of endosperm proliferation was found upon fertilization with *cdka;1* mutant pollen.

Since it was previously found that *cdka;1* mutants can be rescued in combination with *fis*-class mutants, a simple hypothesis would be that natural variation in the imprinting machinery might be responsible for the observed difference in endosperm size upon *cdka;1* pollination (Nowack et al. 2007). In fact, it has been observed that a *mea* mutant in an accession background that was identified to have a high EDV, *i.e.*, Col, displayed greater numbers of autonomously formed endosperm nuclei than a *mea* mutant in an accession with a low EDV, *i.e.*, Ws, while in L*er* intermediate values were obtained for both traits. These data, however, do not substantiate that natural variation in the imprinting machinery might be responsible since the loci identified here do not map to any known *FIS*-class genes.

3.1 **Interdependency of embryo and endosperm development** (Ungru et al. 2008)

The QTL identified in this study did also not correlate to any other known loci affecting seed growth. Moreover, I could demonstrate that factors regulating seed growth account for only a minor effect on the development of autonomous endosperm, pinpointing a new class of seed regulators. At the same time, variation in EDV is largely driven by genetic factors (h^2 = 0.93) but the two QTL identified here

together explain only ~20% of the total variance. Presumably, many minor QTL were presumably not detected due to a lack of statistical power.

Currently, it seems likely that the two QTL identified here might either influence a possible signal transduction cascade between the fertilization of the egg cell and the central cell or the endosperm formation upon sperm – central cell fusion without karyogamie (Nowack et al. 2006; Aw et al. 2010). In any case, the pending molecular identification of these loci offers a chance to obtain molecular insights into the signaling system that coordinates seed development.

Data from this work and previous studies suggest that not only one but also multiple signals are at work and that information has to be exchanged repeatedly between the developing parts of a seed (Fig. 25). Initially, evidence for an immediate early signal from the zygote to the central cell comes from the observation of the autonomous endosperm proliferation observed upon fertilization of the egg cell via fertilization with *cdka;1* mutant pollen (Nowack et al. 2006). However, more detailed analysis of the fertilization process with *cdka;1* mutant pollen revealed that some pollen containing a single sperm cell undergo a second mitotic division during pollen tube growth. Then, the second sperm cell fuses with the central cell but fails karyogamie (Aw et al. 2010). Thus, the first signal, which sets off endosperm proliferation is likely to be caused by the cytoplasmic but not nuclear fusion of a sperm a the central cell .As presented in this study, there likely exists a second interaction phase during early endosperm development, since at least ~3 DAP embryo growth was found to correlate with the amount of endosperm formed (Fig. 25). A similar correlation was also found for the maternal-effect *capulet1* (*cap1*) mutant (Grini et al. 2002). Although the direction of a putative signaling pathway underlying this phenotype cannot be explicitly clarified, it seems likely that the degree of endosperm influences embryo size and not vice versa. Consistent with this directionality is that, in experiments in which the embryo was genetically ablated, no obvious effects on endosperm development were reported (Baroux et al. 2001a; Baroux et al. 2001b).

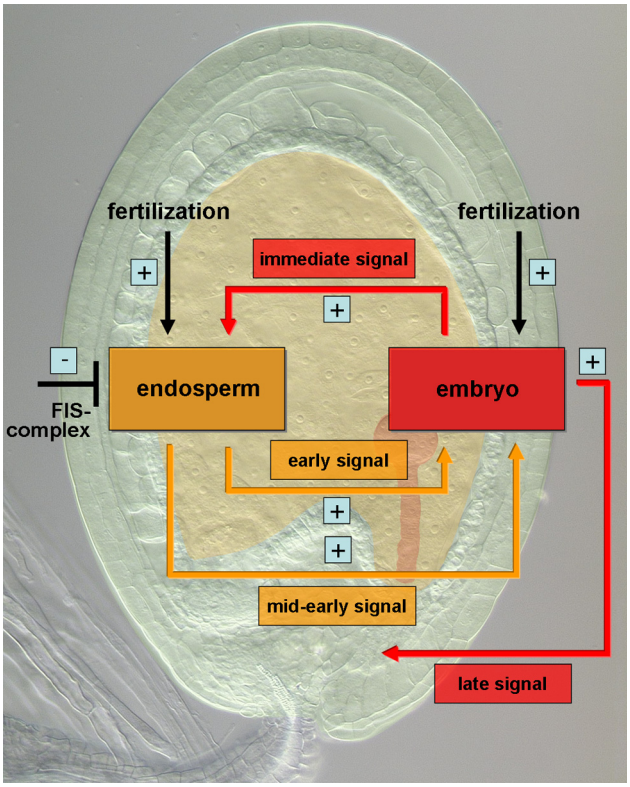

Figure 25: Model of signal transduction pathways and cross-talk between embryo and endosperm (Ungru et al. 2008)
Based on genetic evidence, four different signaling pathways between the embryo and the endosperm are postulated: (i) an immediate early signal originating from the fertilization of the egg cell and/or the cytoplasmic but not nuclear fusion of a sperm with the central cell stimulating endosperm fate adoption of the central cell (ii) an early signal from the endosperm that stimulates embryo development in a quantitative manner (iii) a mid-early action of the endosperm that is required for embryo development past a globular stage (iv) finally a late signal stemming from the embryo and responsible for seed survival. For further explanation see text.

For further embryo development, the endosperm has to reach a critical size as already suggested by Cooper and Brink (1942). It is possible that an endosperm size checkpoint at or beyond 64 nuclei exists since in all accessions pollinated with *cdka;1* mutant pollen the embryo reached roughly the same size of ~50 cells (Fig. 25). Evidence for such a checkpoint also comes from the maternal-effect mutant *capulet2* (*cap2*) in which the embryo developed to roughly the same stage as *cdka;1* fertilized seeds (Grini et al. 2002). Interestingly, such checkpoint behavior was previously not found in plants in which the endosperm was genetically ablated, and Weijers et al. (2003) found that embryogenesis could continue with very little endosperm although these embryos would eventually arrest. The driver line used for these ablation experiments becomes expressed ~2 DAP, and a possible explanation is that this expression is already later than a putative seed size checkpoint. One hypothesis derived from this is that the establishment phase of endosperm might be especially crucial for seed development. A large number of recently identified gametophytic and

seed-specific reporter and driver lines will enable a high-resolution dissection of the requirements of endosperm for embryo and seed development (Stangeland et al. 2005; Tiwari et al. 2006; Steffen et al. 2007). The function of this putative endosperm checkpoint could be complex and could involve, for example, interaction with the mother plant. Cooper and Brink (1940) observed that in interspecies crosses the development of both embryo and endosperm were initiated but endosperm proliferation slowed down, followed by seed abortion. Interestingly, they found that the maternal layer, in particular the nucellar tissue, overproliferated preceding seed degeneration. On the one hand, this overproliferation could reflect a competition between the different seed tissues and an underdeveloped endosperm that might not be able to draw sufficient resource from the mother plant. On the other hand, this overproliferation could be an active execution mechanism of the mother to cut off developing seeds from nutrient supply. In any case, the successful establishment of endosperm as a sink tissue is likely to be a major step in seed development. In addition, there appears to be a late signal coming from the embryo that is crucial for seed survival (Fig. 25). Seeds in unpollinated *fis* mutants with a developing endosperm and no embryo will abort whereas *fis* mutants pollinated with *cdka;1*, which develop autonomous endosperm accompanied by an embryo, can complete embryogenesis and give rise to viable plants (Nowack et al. 2007).

3.2 **Autonomy of embryo and endosperm development** (Ungru et al. 2008)

The observed natural variation also allowed addressing the developmental potential of endosperm and embryo. An unexpected finding was the relatively large degree of embryonic autonomy. Even without any divisions in the endosperm, embryo development was started and on a morphological basis was indistinguishable from wild-type embryo formation. Another possibility is the nutrient uptake via the mother plant through the suspensor. Experiments have shown that the suspensor transports molecules such as sucrose and polyamine to the embryo proper to support embryo development (Yeung 1980; Nagl 1990; Kawashima and Goldberg 2009). Thus, embryo establishment and early growth is dependent on paternal gene contribution and the nutrient uptake through the suspensor, and not on endosperm formation.
However, a single-nucleated endosperm appears to be functional, and differentiation of the central cell into endosperm along with morphological changes of the single-

fertilized seeds was independent of cell divisions. For example, I observed that even in seeds with a single endosperm nucleus differentiation of the endothelium layer was induced. Notably, the accumulation of proanthocyanidins started from the micropylar pole of the seed in the surrounding area of the developing embryo and not in the immediate vicinity of the single endosperm nucleus. Similarly, the accumulation of proanthocyanidins started at the micropylar side in unfertilized seeds of the *fis*-class mutants *msi1*, *fis2*, and *mea* (Ingouff et al. 2006) (R. Shirzadi and P. E. Grini, unpublished results). Thus, it is possible that proanthocyanidin production is characteristic for a rapidly polarizing central cell with a very distinct basal (micropylar) domain. However, no subcellular markers are currently available to follow this putative polarization.

The differentiation of the single central cell nucleus became most evident in the expression of endosperm marker gene KS22. Thus, the developmental potential for endosperm appears to be already programmed into the central cell as a part of the female gametophyte and neither fertilization nor proliferation of this cell is required for the adoption of this fate. However, the fertilization of the egg cell and the generation of subsequent signals appear to be required for a cell fate change. This is consistent with the hypothesis of a gametophytic evolutionary origin of endosperm in flowering plants and with (Strasburger 1900) hypothesis that the fertilization of the central cell is used in higher plants to trigger proliferation.

3.3 *ROS1* and *DME1* are redundantly involved in endosperm formation

The here performed QTL analysis revealed a complex QTL between chromosomes 2 and 5 and the two genes *ROS1* (Chr. 2) and *DME* (Chr.5) lay in or close to the calculated mapping intervalls. DME is reported to be the major activator of the two *FIS*-class genes *FIS2* and *MEA* in the central cell, which suggests that *dme* should show similar mutant phenotypic characteristics to *fis* class mutants (Ohad et al. 1996; Chaudhury et al. 1997; Grossniklaus et al. 1998; Kiyosue et al. 1999; David-Cordonnier et al. 2001; Weinfeld et al. 2001; Choi et al. 2002; Gehring et al. 2006; Nowack et al. 2007; Jullien et al. 2008; Kinoshita et al. 2008). However, unfertilized *dme* ovules did not show autonomous endosperm formation. Interestingly, the phenotypic analysis of a *ros1* mutant allele, a member of *DEMETER LIKE* (*DML*) genes, revealed such mutant *fis* class related phenotypes. Unfertilized *ros1* mutant ovules displayed autonomous endosperm formation accompanied by integument

differentiation and a *cdka;1* related seed rescue. The penetrance of the mutant phenotype such as autonomous endosperm formation and *cdka;1* related seed rescue increased in a *ros1 dme* double mutant background. Furthermore, the percentages of ovules with an undivided central cell upon *cdka;1* pollination gradually decreased from 11,5% in Col-0 wild-type plants to 0,25% in *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants indicating reduced suppression of endosperm proliferation. Thus, the phenotypic analysis of the mutants suggests a probable redundancy between *ROS1* and *DME.* This is consistent with a complex QTL build by *ROS1* and *DME1*. An impact on endosperm formation and seed development has never been reported for *ROS1* and neither in combination with *DME*.

3.4 *DME* and *ROS1* regulate gene expression during different time points of endosperm development

The obtained qRT data supports previous observations that *MEA*, *FIS2* and *FWA* expression is dependent on the activation of DME in the central cell. During endosperm development expression of these genes becomes increasingly DME independent (Kinoshita et al. 2004; Jullien et al. 2006b). In contrast to DME, the impact of ROS1 on gene expression increases during endosperm formation. Here, 6 DAP *MEA* or *FIS2* overexpression levels in *ros1 dme* double mutants were reduced compared to expression levels in *dme* single mutants.

In general, *ros1* mutant plants display a disturbed balance of RdDM and demethylation resulting in increased genomic methylation, which is likely to result in reduced levels of transcription (Gong et al. 2002; Zhu et al. 2007; Penterman et al. 2007b). Conversely, the 6 DAP *PHE1* expression level was highest in *ros1 dme* double mutant plants, which is consistent with a methylation dependent *PHE1* activation (Makarevich et al. 2008).

3.5 *AGL36* reveals a novel regulation mechanism of imprinting in *A. thaliana*

Together with recent work submitted by Shirzadi et al. we showed that *AGL36* is imprinted throughout early seed development by silencing the paternal allele during male gametogenesis in a *MET1*-dependent manner. In the central cell *AGL36* is subject to *DME* dependent activation and expressed during early endosperm development (Fig. 26).

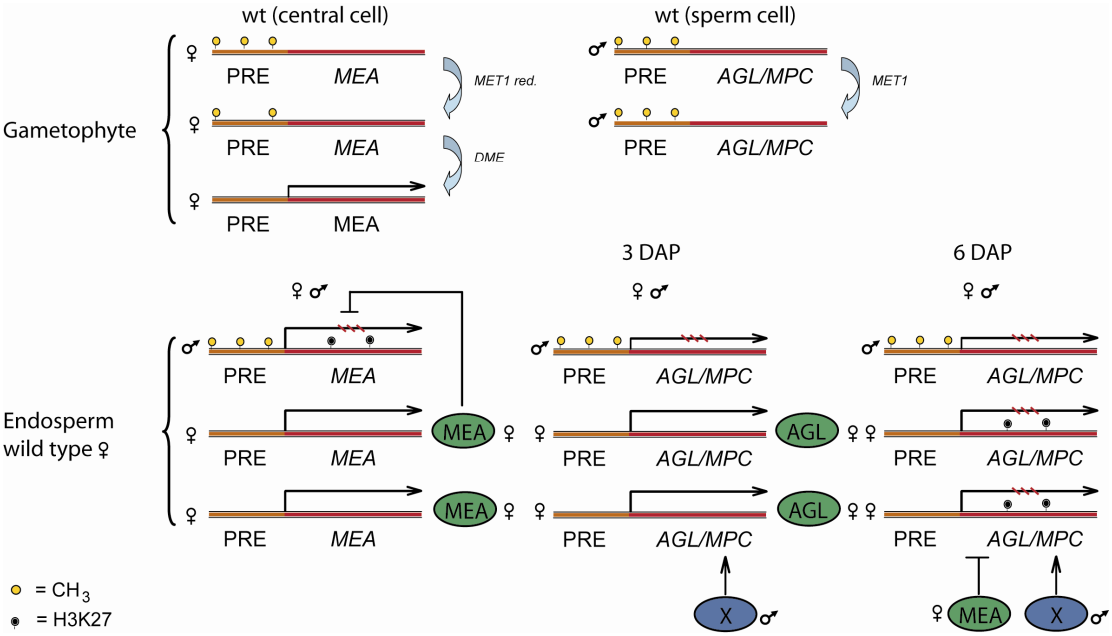

Figure 26: Possible regulation of *AGL28*, *36*, *90* and *MPC*
AGL36 is not paternally expressed and is thus, imprinted. Maternal *AGL36* activation depends on DME and later repression on the FIS-class complex. Demethylation might take place in the promoter region (PRE). Furthermore, *AGL36* was found to be down regulated when the paternal contribution to the endosperm was absent (Shirzadi et al. submitted). Therefore, the maternal *AGL36* allele is directly or indirectly activated by the paternal genome (indicated by a blue round box with an X). *AGL28*, *36*, *90* and *MPC* showed similar expression patterns in *fis* class, *dme* and *ros1* mutants backgrounds, indicating a similar regulation mechanism as hypothesized for *AGL36*.

During development in wild-type plants *AGL36* expression was almost absent at the stage of endosperm cellularization while in *mea* and/or *fis2* mutant seeds the expression was highly increased and persisted throughout seed development (Walia et al. 2009; Shirzadi et al. submitted). Furthermore, an allele specific RT-PCR in *mea*55/*mea*55 mutant seeds fertilized with wild-type pollen revealed that the paternal *AGL36* allele remained silent, while the maternal allele was upregulated. Thus, although only the maternal AGL36 allele is expressed, its expression is negatively

regulated by the FIS-complex. This represents a new regulatory wiring during seed development that has never been described before.

In two independent studies the genome-wide methylation profile of the seed were examined (Gehring et al. 2009; Hsieh et al. 2009). Here, seeds were dissected and cytosine methylation in wild-type embryos was compared to wild-type and *dme* endosperm. This revealed that endosperm development was accompanied by an extensive demethylation of the maternal genome, especially at specific transposon sequences. According to the Zilberman Lab Genome Browser (http://dzlab.pmb.berkeley.edu/browser/ 2010), such demethylation does indeed take place in the promoter and within the gene sequence for *AGL36*, *90*, *28* and *MPC*, suggesting that these genes were maternally activated by DME. Furthermore, *AGL28*, *36*, *90* and *MPC* displayed highly similar expression patterns in *mea*, *ros1*, dme single and *ros1 dme* double mutant backgrounds.

The similar expression pattern and epigenetic features imply regulatory mechanism for *AGL28*, *90* and *MPC* similar to that hypothesized for *AGL36*. Therefore, *AGL90*, *28* and *MPC* are candidate genes to be imprinted in such a way that the paternal allele is silenced, already shown for *MPC* (Tiwari et al. 2008). Furthermore, *AGL28*, *90* and *MPC* might play a regulatory function during seed development. Interestingly, previously reported DME dependent demethylation of a −500 bp region and a 183-bp in an intergenic region (ISR) adjacent to the *MEA* gene and a -220 bp DMR downstream of *FIS2,* also appeared in the Zilberman Lab Genome Browser (Gehring et al. 2006; Jullien et al. 2006b) (http://dzlab.pmb.berkeley.edu/browser/). Conversely, *PHE1*, *MSI1* and *FIE* did not show any DMRs between wild-type and *dme* endosperm, supporting the assumption that these genes were not maternally activated by DME.

3.6 The mutant phenotype in *ros1* and *ros1-dme* mutant plants is linked to hypermethylation

In mammalian sexual reproduction, DNA methylation patterns are rearranged genome wide at two time points during gametogenesis and embryogenesis. In embryos, rearrangement is initiated by a massive wave of DNA demethylation immediately after fertilization, followed by rapid de novo DNA methylation (Reik 2007). Conversely, in plants there is no evidence for such an extensive resetting of DNA methylation patterns during gameto- or embryogenesis. For instance, hypomethylation induced by a met1 or ddm1 mutation were stably inherited for no less than eight generations following outcrossing of the mutant alleles (Johannes et al. 2009; Reinders et al. 2009). In contradiction, Arabidopsis mutants defective in RdDM or active DNA demethylation still exhibited near-normal CG methylation within and across generations (Tran et al. 2005; Zhang et al. 2006; Penterman et al. 2007a; Cokus et al. 2008; Lister et al. 2008). Nevertheless, plants seem to be more prone to the inheritance of DNA methylation defects rather than re-establishing regular methylation levels anew at each generation like in mammals (Richards 2006; Whitelaw and Whitelaw 2008).

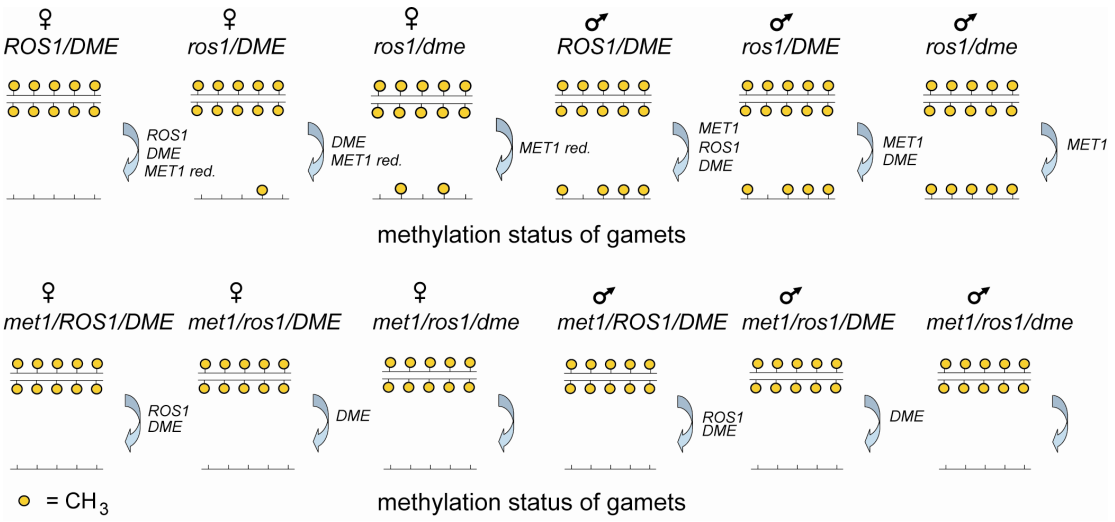

Figure 27: Reversion of the *ros1 dme* mutant phenotype in the offspring from *MET/met1* plants
The first line shows the hypothesized methylation patterns in maternal and paternal wild-type gametes and *ros1*, *dme* single and double mutant gametes. In *ros1* and *dme* mutant gametes demethylation does not take place resulting in DNA hypermethylation. The methylation transfer depends on MET1 activity (David-Cordonnier et al. 2001; Weinfeld et al. 2001; Gehring et al. 2006). Therefore, maternal gametes are likely to be less affected by the *ros1* and *dme* mutant allele than the paternal ones, because MET1 activity is down regulated during maternal gametogensis. All gametes in *met1* heterozygous mutant plants were reported to be hypomethylated (Saze et al. 2003) and the second line of the model displays this situation. Thus, the mutant phenotype is dependent on the presence of a diploid maternal *MET1* genotype before maternal gametogenesis.

On the one hand, in wild-type plants demethylation does take place due to a reduced MET1 activity and an active demethylation process by DME during maternal gametogenesis, which is an argument against the inheritance of hypermethylation. On the other hand, genomic sequences displayed almost 100% relative methylation levels in *ros1*-*dme* double mutants compared to *ros1*, *dme* single mutants, indicating an inheritance of hypermethylation either by the paternal and/or maternal site. Congruently, a *met1* induced hypomethylation reversed the mutant phenotype in all zygosities and allelic combinations of *ros1* and *dme* mutant alleles, suggesting that the mutant phenotype was dependent on hypermethylation (Fig.27). Thus, hypermethylation seems to be inheritable by the next generation, although reduced MET1 activity and a *DME* wild-type allele was present. In addition, a single *met1* mutant allele was enough to cause hypomethylation in all maternal gametes (Saze et al. 2003). The reason for the establishment might be haplo insufficiency throughout plant development and a lacking *de novo* DNA methylation during the vegetative phase and/or maternal gametogenesis.

3.7 The heredity of the mutant phenotype was likely to be established by a trans methylation transfer dependent on a hyper-methylated paternal allele (epiallele)

In this work, the inheritance patterns of the mutant phenotype suggested a paramutation-like phenomenon.

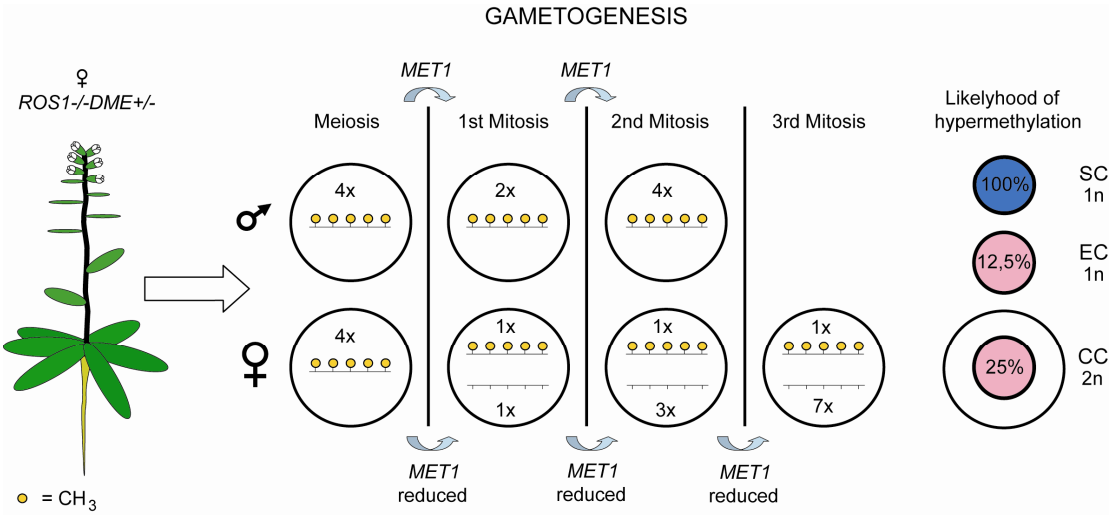

Figure 28: Likelihood of hypermethylation in maternal and paternal gametes from *ROS1-/- DME+/-* plants
The model shows hypothesized methylation patterns in paternal and maternal *ros1*, *dme* single and double mutant gametes. *ros1-dme* mutant plants display a hypermethylated genome. Hypomethylation depends on MET1 activity (David-Cordonnier et al. 2001; Weinfeld et al. 2001; Gehring et al. 2006). In maternal gametes MET1 activity is down regulated. If a hypermethylated allele goes into the three mitotic divisions of maternal gametogenesis, it results into a loss of methylation sites in a semi-conservative manner (Morgan et al. 2005). Therefore, 12,5% of all egg cells (EC) and 25% of all central cells (CC) would contain a hypermethylated allele. Conversely, 100% of all sperm cells (SC) should be hypermethylated, because MET1 is fully active. This model might explain the stable amount of autonomous endosperm proliferation in 12% of all unfertilized gametes. Here, dme mutant ovules do not show the mutant phenotype. Therefore, 12,5% of all gametes were likely to have the predisposition for autonomous endosperm proliferation. Furthermore, these seeds might have been allowed to bypass imprinting and allow successful seed development upon *cdka;1* single fertilization.

In general, paternal gametes go through two and maternal through three mitotic divisions after meiosis. After gametogenesis the paternal gametophyte comprises a three celled pollen containing the homodiploid vegetative and two haploid sperm cells, while the maternal gametophyte contains seven cells including the haploid egg cell and the homodiploid central cell (Fig. 28). In *ros1-dme* double mutants at least the two tested genes were found to be up to 100% hypermethylated compared to genes of single mutants or wild-type plants. In maternal gametes MET1 activity is down regulated (Johnston et al. 2008; Jullien et al. 2008). If such a hypermethylated allele undergoes the three mitotic divisions of maternal gametogenesis, it causes a loss of

methylation sites in a semi-conservative manner (Morgan et al. 2005). Thus, 12,5% of all egg cells (EC = 1n) and 25% of all central cells (CC = 2n) would contain a partly hypermethylated genome (Fig. 28). Conversely, 100% of all sperm cells (SC) should be hypermethylated, because MET1 is fully active (Fig.28).

In addition, the viable offspring of single fertilized *ros1*-3/*ros1*-3 *DME*/*dme*-1 plants was *DME* wild-type. Therefore, 12,5% of the viable offspring contained a hypermethylated allele (25%/2 = 12,5%). Interestingly, the same percentage of unfertilized ovules, displaying autonomous endosperm, was found. These ovules contained presumably a *DME* wild-type allele and probably a hypermethylated genome, too (Fig. 28).

Previous reports showed that fully methylated DNA sequences cause a reduction of DME activity by 10 fold (David-Cordonnier et al. 2001; Weinfeld et al. 2001). A reduced DME activity still decreases methylation levels, resulting in an intermediate status between strong and weak levels of hypermethylation in the central cell. Thus, it is possible that a *DME* wild-type allele together with reduced DME activity in the central cells was necessary to cause autonomous endosperm formation and to bypass imprinting upon *cdka;1* pollination. However, it can currently not be excluded that autonomous endosperm production in *dme* mutant ovules is hindered due to a function of *DME* in other, unknown processes influencing seed development.

3.8 Methylation transfer by siRNA

The inheritance of the mutant phenotype in a segregating population showed that the paternal site was likely to be responsible for the methylation status of the upcoming progeny. This theory is based on the observation that the first filial generation with the genotype *ROS1*+/- *DME*+/- from the cross Col-0 x *ROS1*-/-*DME*+/- displayed in its siliques the same mutant phenotypic proportions as the paternal plant. Conversely, a *ROS1* or *DME* wild-type allele on the paternal site resulted in an reduced phenotype in the ovules of the next filial generation, i.e. that *ROS1*+/-*DME*+/- F2 plants displayed either a high mutant phenotype or a very reduced one by 50% chance. Thus, the methylation sites on the epiallele may be established *de novo* in each generation by a hypermethylated dominant allele coming from the paternal site. Furthermore, it was shown that the probability of a maternal transmitted hypermethylated chromosome via the egg cell was 12,5% (Fig 29). If the heritability of the hypermethylated allele has been dependent on transmission through the

female gametophyte, it would have been diluted throughout generations (F1: 12,5%, F2: 1,6% …). Thus, *de novo* methylation of the maternal genome during or after fertilization is a logical consequence to ensure a stable heredity throughout the mitotic sporophytic divisions until the development of new gametes (Fig. 29).

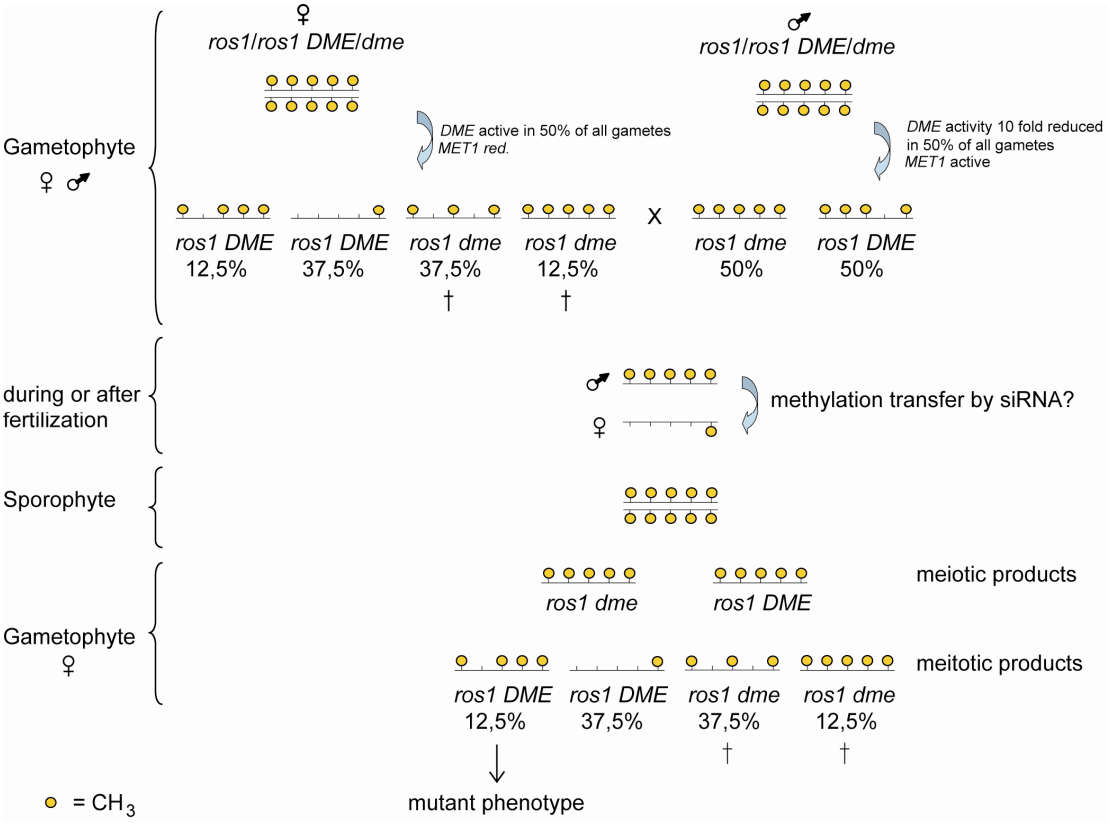

Figure 29: Illustration of methylation transfer from the paternal to the maternal allele during or after fertilization
The model shows the establishment of a hypermethylated progeny by a dominant paternal epiallele. The epigenetic information is likely to be transferred by siRNA during or after fertilization (see text). The maternal gametes of the next generation display the same epigenetic features as the maternal parent generation. Here, 12,5 % of all central cells might develop a mutant phenotype such as autonomous endosperm proliferation due to their epigenetic condition indicated by the yellow dots.

In plants re-methylation occurs by an RNAi-dependent pathway (Johannes et al. 2009; Teixeira et al. 2009). However, several generations are needed to restore the previous methylation status, which is consistent with a gradual transposable element inactivation in maize and transgene silencing in many other plant species throughout generations (Chandler and Stam 2004; Slotkin and Martienssen 2007). It seems that re-methylation is a prominent feature of the reproductive phase, which was also observed for *met1* heterozygous plants, where all gametes displayed hypomethylation, demonstrating haploinsufficiency and a lack of *de novo* methylation during vegetative growth. Furthermore, transposable element silencing was reported to be established by an RNAi dependent pathway in gametes and embryos (Mosher

and Melnyk 2010). However, given the fact that methylation transfer is down regulated during maternal gametogenesis, it is likely that remethylation and resilencing takes place during paternal gametogenesis or pollen development accompanied by a methylation transfer accomplished by MET1. On the maternal site histone *de novo* methylation and silencing of maternal and paternal gene loci were reported as a prominent feature of the FIS-class complex. However, imprinting is limited to the central cell and therefore its DNA methylation status is not transmitted to the next generation.

In this work it is shown, that the paternal genome has to ensure the reestablishment of the methylation status and the mutant phenotype anew in every generation. Nevertheless, I cannot exclude that the central cell also generated siRNAs due to an active and widespread demethylation by a down regulated MET1 and an active DME (Gehring et al. 2009; Hsieh et al. 2009; Mosher et al. 2009). A precondition that the central cell or the endosperm could influence the inheritable change of methylation pattern in the progeny would be a transfer of epigenetic information from the endosperm into the zygote. In fact, this has been proposed as a possible explanation for why *de novo* DNA methylation of introduced *FWA* transgenes required fertilization (Chan et al. 2006b). This requirement for fertilization is also supported by transcriptome data indicating that many components of RdDM are highly expressed in developing seeds. Nonetheless, this pathway of methylation reinforcement remains highly speculative.

However, there exist more information about the paternal contribution to *de novo* methylation. Here, the vegetative nucleus of the pollen was shown to display low levels of DDM1 protein resulting in activation of a transposable element and a high accumulation of siRNAs similar to that observed in ddm1, which were then probably transported by an unknown pathway to the two sperm cells (Fig. 30 a) (Slotkin et al. 2009). Apparently, they induced either RdDM or post-transcriptional silencing (Barthe et al. 1976; Schoft et al. 2009; Slotkin et al. 2009). In addition, sperm cells show a long S phase, the phase of the cell cycle where epigenetic marks were reported to be established via siRNAs (Friedman 1999; Kloc et al. 2008). Similar to other transcripts produced in pollen, siRNAs could remain stable until fertilization occurred, and change the transcriptional and methylation status of the zygote and the endosperm (Fig. 30 c) (Bayer et al. 2009). In addition, the theory that siRNA were responsible for the establishment of 100% relative methylation in *PHE1* and *AGL36* is supported by previous reports demonstrating that ROS1 removes RNAi directed *de novo* DNA methylation (RdDM) marks from promotors of *RD29A:LUC* and *RD29A* as well as of other loci (Gong et al. 2002; Zhu et al. 2007; Penterman et al. 2007b). There is

evidence of a connection between local DNA hypermethylation and overaccumulation of matching siRNAs in *ros1* mutants (Lister et al. 2008). Furthermore, many secondary suppressors such as *rdm3* and/or *rdm4* (*RNA-directed DNA methylation 3 and/or 4*) of *ros1* generated silencing and hypermethylation were part of the RdDM pathway (Zheng et al. 2007; Penterman et al. 2007b; He et al. 2009a; He et al. 2009b). In this context, a hypermethylated vegetative pollen nucleus might have caused siRNA overaccumulation by which epigenetic information was transferred from the paternal to the maternal genome (Fig. 30 c) (Hsieh et al. 2009).

3.9 *ROS1* and *DME* affect paternal – maternal communication during fertilization

Beside the influence of a hypermethylated paternal genome on the epigenetic status of the next generation, hypermethylation also influenced pollen development and/or the paternal fertilization process. Here, the paternal *dme*-1 transmission rate was reduced from the expected 50% to 25,9% in a self-pollinated *dme*-1 mutant. It even decreased in self-pollinated *ros1*-3/*ros1*-3 *DME*/*dme*-1 double mutant plants to 18,3%. It is important to mention that a hypermethylated paternal genome did not disturb seed development after fertilization because no early seed or additional late abortion phenotype was observed in seeds originating from wild-type plants fertilized with pollen from *ros1*-3 *dme*-1 double mutant plants. Furthermore, germination was not disturbed either. Thus, it was more likely that the *ros1*-3-*dme*-1 induced paternal hypermethylation solely affected pollen development or viability through gene silencing.

Interestingly, the *dme*-1 transmission rate decreased in the cross of a hypermethylated paternal (*ros1*-3/*ros1*-3 *DME*/*dme*-1) with a wild-type (Col-0) methylated maternal plant. This raised the interesting question of whether or not the maternal part influenced the success of paternal fertilization. During the fertilization process the pollen lands on the stigma, rehydrates, and starts germination. The pollen tube grows through female tissue until it reaches the ovule, penetrates one of the synergids, and releases the sperm. Thus, pollen tube growth and/or guidance to the egg cell are highly complicated communication processes between the maternal sporophyte and the paternal derived pollen (Higashiyama et al. 2003; Ma 2003; Crawford and Yanofsky 2008). One speculation is that the accumulation of siRNAs in the pollen might not only have affected the epigenetic status of the sperm cells,

central cell and egg cell, but also the communication between the maternal tissues and the pollen during pollen reception, growth and sperm cell release (Fig. 30 a-c). Intercellular siRNA transport does occur even over large distances within the plant and influence spatial separated development processes (Dunoyer et al. 2010a; Dunoyer et al. 2010b). One advantage of recognizing an incoming hypermethylated paternal genome via siRNA concentrations would be to protect the upcoming generation against abnormal methylation patterns. Interestingly, the transmission of the hypermethylated genome was dependent also on the methylation status of the maternal genome. The *dme* transmission rate was higher in self-pollinated mutant plants than in paternal mutant plants crossed with Col-0. Here, not only the paternal siRNA composition but also the maternal siRNA recognition or maternal expression of certain genes important for the interplay between pollen tube growth, guidance or perception might have been disturbed (Fig. 30 b).

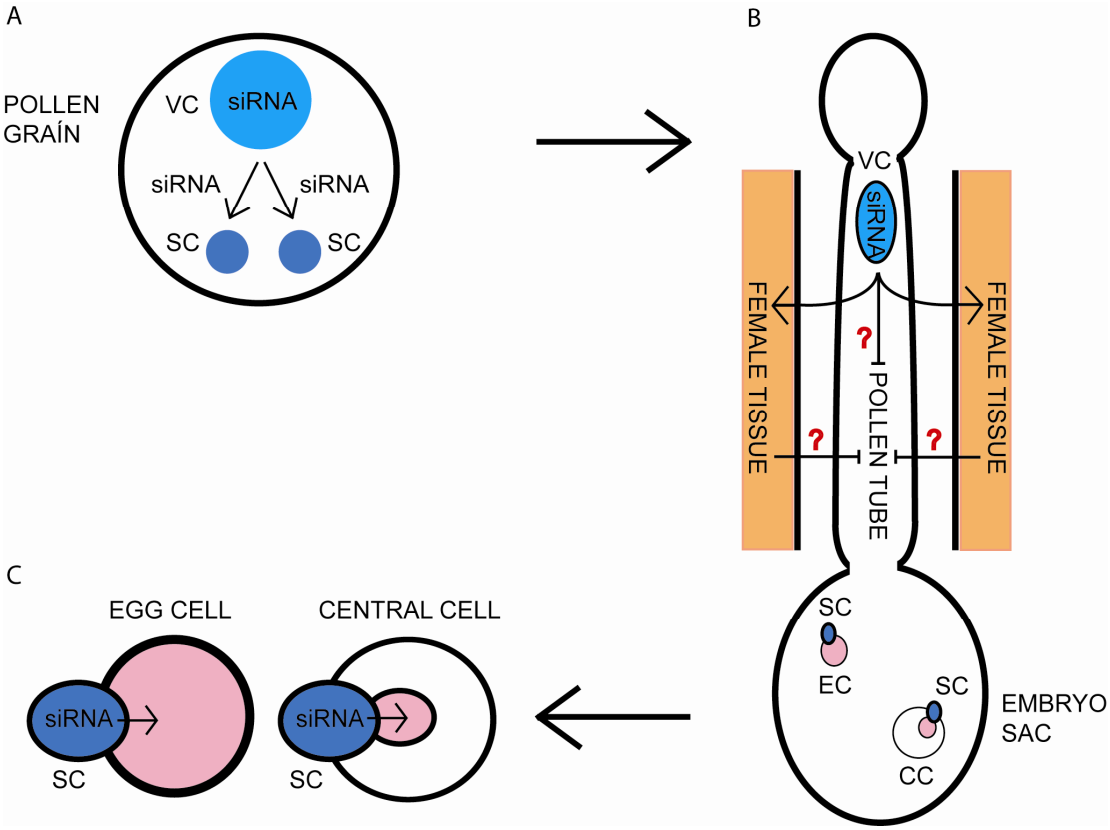

Figure 30: siRNA response during the fertilization process in *ros1 dme* mutant plants
(**A**) Epigenetic information is probably transferred via siRNAs from the vegetative cell (VC) to the two sperm cells (SC) during pollen development or immediately after germination. (**B**) siRNAs might influence general pollen development and/or a proper communication during pollen reception, tube growth and or guidance. (**C**) siRNAs could have been accumulated in the two sperm cells and changed the epigenetic status of the maternal epiallele in the egg cell (EC) and/or central cell (CC) leading to hypermethylation of the endospermatic and/or embryonic genome.

Beside the fact that a maternal inherited *dme*-1 allele caused seed abortion, the paternal *dme*-1 transmission was disturbed, too. This illustrates the pivotal regulatory

functions of *DME* and *ROS1* during maternal and paternal sexual reproduction. Furthermore, paternal *dme*-1 transmission was influenced by the methylation status of the paternal and the maternal genome. The parental epigenetic identity might have influenced general pollen development and/or a proper communication during pollen reception, tube growth and/or guidance (Fig. 30 a-c). Maybe this recognition pathway was triggered via abnormal siRNA concentrations inside the pollen through post transcriptional silencing and *de novo* methylation of paternal (gametophytic) and maternal (sporophytic) genes

4 MATERIALS & METHODS

4.1 Materials

4.1.1 Chemicals and antibiotics

All used chemicals and antibiotics of analytical quality have been used from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Duchefa (Haarlem, Netherlands) and Sigma (Deisenhofen, Germany).

4.1.2 Enzymes, primers and kits

Modifying enzymes were used from Invitrogen (Karlsruhe, Germany), Roche (Mannheim, Germany), MBIfermentas (St.Leon-Rot, Germany) TaKaRa (Otsu, Japan). Primers were generated by Invitrogen (Karlsruhe, Germany. Kits were supplied from Sigma (Deisenhofen, Germany), Roche (Mannheim, Germany) and QIAGEN (Hilden, Germany).

4.1.3 Plant material

Plants were germinated on soil or 1/2 MS medium and grown under standard greenhouse conditions or in a growth room. *Arabidopsis* plants used in this study were of the following accessions: Antwerp-1 (An-1; ABRC22626), Bayreuth-0 (Bay-0; ABRC22633), Burren-0 (Bur-0; ABRC22656), C24 (ABRC22620), Cape Verde Islands-0 (Cvi-0; ABRC22614), Columbia-0 (Col-0; ABRC22625), Estland-1 (Est-1; ABRC22629), Kashmir-1 (Kas-1; ABRC22638), Landsberg *erecta*-1 (L*er*-1; ABRC22618), Martuba-0 (Mt-0; ABRC22642), Niederzenz-1 (Nd-1; ABRC22619), Nossen-0 (No-0; CS1394), Shahdara (Sha; ABRC22652), Wassilewskija-0 (WS-0; ABRC22623). Furthermore, a core set of 165 recombinant inbred lines (RIL) derived from a cross between Bay-0 and Sha was used (Loudet et al. 2002). Throughout this

work a previously characterized *cdka;1-1* allele in the Col-0 genetic background (SALK_106809.34.90.X) was used (Nowack et al. 2006). *ros1*-4 was obtained from SALK (SALK_045303.23.25.x) and *dme*-1 from GabiKat (GK-252E03-014577). (Choi et al. 2002) *dme*-4 was isolated from a gamma ray screen (Guitton et al. 2004) and *ros1–3* was received from Ws-0 heterozygotes (*Arabidopsis* Knockout facility, University of Wisconsin, Madison, WI) and introgressed into the Col-0 background six times (Penterman et al. 2007a). m*et*1 was received from SALK (SALK_07652) and mea55 (SAIL_55_C04) and mea72 (SAIL_724_E07) from SAIL. The endosperm marker line *KS22* was contributed by F. Berger and is in the C24 genetic background (Ingouff et al. 2005). The p*FIS2:GUS* reporter line was kindly provided by A. Chaudhury and is in the C24 genetic background (Luo et al. 2000).

4.2 Methods

4.2.1 Microscopy

Light microscopy was performed with an Axiophot microscope (Zeiss, Heidelberg, Germany) equipped with differential interference contrast (DIC) (Nomarski) optic and AxioVs40 V 4.5.0.0 software. Confocal-laser-scanning microscopy was carried out with LSM 510 META (Zeiss, Heidelberg, Germany) or Leica TCS SP2 AOBS (Leica, Wetzlar, Germany).

4.2.2 Histology

Light microscopy pictures were taken of pistils and siliques of different developmental stages. Dissected siliques were fixed on ice with FAA (10:7:2:1 ethanol:distilled water:acetic acid:formaldehyde [37 %]) for 30 minutes, hydrated in a graded ethanol series to 50 mM $NaP(OH)_4$ buffer, pH 7.2, and mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol. The samples were cleared 1 hour at 4 °C before examination. For vanillin staining, siliques were emasculated, hand pollinated, and harvested between 3 and 6 days after pollination (DAP). The silique walls were removed and dissected seeds were incubated in 6 N HCl solution containing 1% (w/v) vanillin (Sigma-Aldrich) at room temperature for 1 hr.

Siliques were mounted on slides in a drop of vanillin containing acidic solution and directly inspected using an Axioplan 2 Carl Zeiss Microscope. GUS-activity was assayed according to Sessions and Yanofsky (Sessions and Yanofsky, 1999). To allow complete penetration of the X-Gluc-solution plants or parts of plants were vacuum infiltrated in staining buffer (0.2 % Triton X-100, 50 mM $NaPO_4$ pH 7.2, 2 mM potassium-ferrocyanide $K_4Fe(CN)_6$*H2O, 2 mM potassium-ferricyanide $K_3Fe(CN)_6$ containing 2 mM X-Gluc) for 15 to 30 minutes and afterwards incubated at 37 °C over night. Clearing was performed in 70 % ethanol at 37 °C over night. GFP fluorescence in seeds was analyzed by confocal laser scanning microscope as described in Nowack *et al.* (2007).

4.2.3 QTL mapping

For QTL mapping, three to five independent plants per RIL, each with 15–20 siliques, were emasculated and pollinated 3 days later with heterozygous *cdka;1* mutant plants, giving rise, on average, to 100–200 analyzable single-fertilized seeds per RIL. The endosperm division value (EDV; for definition, see text) was determined at 3 DAP. QTL mapping was performed on the mean EDV of each RIL. QTL analysis was done using the software MapQTL 5.0 (van Ooijen 2004). A permutation test using 1000 permutations of the original data resulted in a genomewide 95% LOD threshold of ~2.4. The automatic cofactor selection procedure was applied per chromosome to select markers to be used as cofactors for the composite interval mapping procedure (CIM). Markers most closely linked to QTL that appeared only after each round of CIM mapping were also selected as cofactors. The results of CIM mapping provided the variance explained by each and by all detected QTL as well as their additive allelic effect. The heritability was calculated by dividing the genetic variance by the sum of the genetic and the environmental variance.

4.2.4 Plant growth conditions

Arabidopsis thaliana seeds were germinated on soil or half-strong MS-2 medium containing 0,5 % sucrose and 0,8 % agarose. The seeds, which germinated and/or were selected on agar plates, were sterilized inside a small vacuum container. 20 ml of bleach (DanKlorix by Colgate-Palmolive, Hamburg) were poured in a 50 ml glass beaker together with 2 ml of concentrated HCl. The lid of the vacuum container was closed immediately. The evaporating chlorine sterilized the surface of the seeds within approximately 4 to 12 hours. Sulfadiazin, kanamycin or a hygromycin resistance were selected on MS-2 agar plates containing 5,25 µg/ml sulfadiazine, 50 µg/ml kanamycin or 25 µg/ml hygromycin, respectively. BASTA resistance plants were selected on soil for 10 to 15 days. The seedlings were sprayed with a 0.001 % BASTA solution, the spraying was repeated after 3 to 7 days.Plants were grown between 18 and 25 ºC under standard greenhouse conditions or in culture rooms under long-day conditions with a 16/8 h light/dark cycle at 18 ºC or 20 °C, respectively.

4.2.5 Crossing of plants

At a stage when the flowers were closed and the pollen of the anthers was not ripe the anthers of the acceptor flower were removed completely using very fine forceps. All remaining older and younger flowers were also removed. After two days the stigma of the carpels were pollinated with pollen from the donor plant.

4.2.6 Genomic DNA preparation from plant tissue

High-quality genomic DNA was extracted using CTAB-preparation (Rogers and Bendich 1988). Plant material was grinded using liquid Nitrogen. Then, 200 µl of extraction buffer (2 %(w/v) CTAB, 1,4 M NaCl, 20 mM EDTA, 100 mM Tris/HCl pH 8.0, 0.2 % β-mercaptoethanol) was added and the solution was incubated for 30 min. at 65 °C. After addition of 150 µl Chloroform/Isoamylalcohol (24:1) and careful shaking, the samples were centrifuged for 15 minutes at 4000 rpm. The aqueous phase was transferred into a new tube and mixed with 200 µl isopropanol and

centrifuged for 15 min. at 4000 rpm. The pellet was washed with 70 % Ethanol and dried; afterwards the pellet was re-suspended in 20 µl 20 mM Tris/HCl pH 8.0.
For high trough put PCR based genotyping, genomic plant DNA was isolated using a dirty preparation method after (Berendzen et al. 2005). One or two young leafs were put into a 2 ml well of a 96 deep-well titer plate (Part Number: 267001, Beckman or similar product from VWR). 500 µl of DNA extraction buffer (5 ml 1 M Tris/HCl pH 7.2 + 6 ml 5 M NaCl + 10 g sucrose and adjusted to the final volume of 100 ml with water) together with a small metal bead were added. The titer plate was closed with collection microtube caps from Qiagen (Mat. No.1051163). The plant tissue was ground by shaking for 2 to 3 min at a high frequency in the Mixer Mill MM 301 by Retsch (Haan, Germany) and then centrifuge for 10 sec. to spin down liquid from the microtube caps to avoid cross contamination. 1,5 µl of this solution was used as template for PCR using standard Taq-Polymerase and 10 times PCR buffer
(Tris/HCl pH 8.7: 200 mM / KCl: 500 mM / MgCl2: 20 mM). The DNA preparation could be stored at –20 °C for further use.

4.2.7 Identification of mutant alleles by PCR

Allele-specific PCRs were carried out to determine a wild-type or mutant allele by using the primer combination, which are listed in table 5. The primer combination to amplify a wt allele is the forward and the wt reverse primer and to detect the mutant allele the same forward primer and the mutant reverse primer, respectively.

Table 5: Primer used to amplify a wild-type or mutant allele

primer name	primer sequence (5'-3')	Gene
*cdka;1*_forw	GCGTGGACCGCTTGCTGCAACTCTCTCAGG	cdka;1
*cdka;1*_rev	CAGAAAGGAGATCGACTCCATCGGGATC	
ROS1-3_forw	TGGAAGGGATCCGTCGTGGATTCT	*ros1*-3, *ROS1*
ros1-3_mut_rev	CATTTTATAATAACGCTGCGGACATCTAC	
ROS1-3_wt_rev	CCCGCGACTCTTGATTGTTTCAGCAACTT	
ROS1-4_forw	TCGTCTTTCGATCAAATCCAC	*ros1*-4, *ROS1*
ros1-4_mut_rev	ATTTTGCCGATTTCGGAAC	
ROS1-4_wt_rev	CCAGTTAAGGACAGAACACCG	
DME-1_forw	CACAAGATGTGGAGAGACATAACAGCAG	*dme*-1, *DME*
dme-1_mut_rev.	CCCATTTGGACGTGAATGTAGACAC	
DME-1_wt_rev	CGAAGGAATTTCATTTAAGTTCAGAATGC	

DME-4_forw	CAAACTCGATCAACGAACACTGTATGC	*dme*-4, *DME*; *Eco*RI. digest (wt: 79, 93, 184 bp; mut.: 79, 248 bp.)
DME-4_rev	AGCATAAGCACTGGCAAAGTGTCTGC	
*MET1*_forw	CAGAACAGGTTTCCACCAAGG	*met*-1, *MET*
*met1*_mut_rev	TAGCATCTGAATTTCATAACCAATCTCGATACAC	
*MET1*_wt_rev	TCAATGAGCAGTGGAAGCAAG	
*MEA*55_forw	GAATGGTGAGGCACTAGAATTGAGC	*Mea*55*, MEA*
*mea*55_mut_rev	TAGCATCTGAATTTCATAACCAATCTCGATACAC	
*MEA*55_wt_rev	CCTTCTCTACAGGCGTCCACATAG	

4.2.8 Quantification of methylation marks

To quantify methylation marks digestion with the McrBC enzyme (New England Biolabs), which cuts methylated DNA, was followed by quantitative PCR (McrBC-qPCR) with specific primer pairs. Digestion was carried out using 1 µg of genomic DNA in a total volume of 100 µl. Quantitative PCR was performed on equal amounts (5 ng) of digested and undigested DNA samples for at least three technical replicates, using a Roche LightCycler 480 machine and Roche SYBR green I MasterMix Plus. As control loci unmethylated or chloroplast-sequences and highly methylated heterochromatic loci were used. Primers are listed in table 6.

Table 6: Primer used for quantification of methylation marks

Name	Atg-number	primer sequence (5'-3')	amplicon
demethy3-forw.	At2g39010	GCTTCATGAGCTCCATGCTTAAA	154 bp
demethy3-forw-rev.		TGCATCAAATAACACATACATTGC	
chloro3-forw.	C-20372...21495	CAGCGATGTAATGAAAGTGAAGGTC	194 bp
chloro3-rev.		GCCGTGGATACACTTCTTGATAATG	
methy5-forw.	At5g43800	AAGGGATTACTATCTCGCAAAGCAC	197 bp
methy5-rev.		AATCAGGTCTGGTTGCAGTCAAATA	
AGL34-forw.	At5g26580	TCGTGTTCTTGTGGCCGATACG	60 bp
AGL34-rev.		GCAGTTACCGCAGTTGCATTCG	
AGL36-forw.	At5g26650	CATTCCTTTCGTGGACGGAAACTG	66 bp
AGL36-rev.		ACGGCTGGTAGTTGATTGGATGG	
PHE1-forw.	At1g65330	TCATCCGTAGCCCGTACAACTC	60 bp
PHE1-rev.		CTTCAACGCCTTCCCTTGATGG	

4.2.9 Quantitative PCR

Total RNA was isolated from siliques using the spectrum plant total RNA kit (Sigma) and treated with DNAse (TaKaRa) according to the manufacturer's protocol. The RNA concentration was measured using a Nanodrop ND-1000 instrument. cDNA was synthesized using 3.5 µg of total RNA in a Reverse Transcription (RT) reaction using Superscript III (Invitrogen). A RNase H treatment at 37 °C for 20 minutes was performed to eliminate remaining RNA. The complete solution was used with a QIAquick PCR Purification Kit (Qiagen) to purify and concentrate the cDNA. Then, concentration was determined by applying a Nanodrop ND-1000 instrument. Finally, the cDNA was used in quantitative Real-time PCR (qPCR). QPCR was performed on a Light-cycler LC480 instrument (Roche) according to the manufacturer's protocol. To ensure that the primer combinations did not produce any undesired PCR fragments or primer dimers, a SYBER-GREEN qPCR with melting point analysis was done using the LightCycler 480 SYBR Green I Master Kit (Roche). Probe based qPCR was performed using the analyzed primers and Universal Probe Library (UPL) hydrolysis probes (Roche), which are listed in table 7 All samples and reference controls were measured in four independent biological replicas. The qPCR efficiency was determined in all replicas independently by a dilution series of 100, 50, 20 and 5 ng cDNA per reaction for each experiment. The relative expression ratios was calculated according to a model described by Pfaffl (Pfaffl 2001).

Table 7: Primer and Probes for quantitative real-time PCR

primer name (ATG-number of gene measured_gene_UPL probe_border; R = right border, L = left border)	primer sequence (5'-3')
At3g12110_*ACT11*-77-R	TGTCTTCACCATCTGCCATT
At3g12110_*ACT11*-77-L	CAAAAACTACACACCCGTACCA
At5g04560_*DME*_163_R	GTGCCGAATTCGCTGTTT
At5g04560_*DME*_163_L	TCGTCTCCTTGATGGTATGGA
At2g36490_*ROS1*_127_R	CCGCACACACTCCACACT
At2g36490_*ROS1*_127_L	TGTTCCACCAGATAAAGCAAAA
At5g26650_*AGL36*-160_R	GATCCATCATCTTCTTGGTTCG
At5g26650_*AGL36*-160_L	AGGTGGCTTCAAGGTTTCTG
At3g19350_*MPC*_31_R	TCCTGTGATTTTTGGTGCAA
At3g19350_*MPC*_31_L	CCCAAAGAACAACGAGATTTG

At1g01530_*AGL28*_22_R	TGAGCTAAGTTGAGTTCTGTTGGA
At1g01530_*AGL28*_22_L	GAGAGAACAAGGACGCTGAGA
At5g27960_*AGL90*_116_R	TGTTGTAGAATTCTCGTTGAATCTG
At5g27960_*AGL90*_116_L	ATGTTCTTGCGGGCGATA
At1g01530_*AGL28*_22_R	TGAGCTAAGTTGAGTTCTGTTGGA
At1g01530_*AGL28*_22_L	GAGAGAACAAGGACGCTGAGA
At1g02580_*MEA*_56_R	TGTTGCAATCCTTTGAGCAC
At1g02580_*MEA*_56_L	AAGCACTACACACCATGCACTT
At4g25530_*FWA*_138_R	GCCAATCAAAGGCTGGTAGA
At4g25530_*FWA*_138_L	CGACCTGTCCAATGGGTACT
At2g35670_*FIS2*_111_R	GGGTAGACTTAAAGGTTACGACACA
At2g35670_*FIS2*_111_L	TGGAAGTTGATGAATAACAGTGG
At3g20740_*FIE*_163_R	TCCAAAACTCTTTCATTGACCA
At3g20740_*FIE*_163_L	TCGCTATGAAGTTCTAAGTGTGGA
At5g58230_*MSI1*_120_R	AGCGAGCTCGATTAACCTCA
At5g58230_*MSI1*_120_L	TGGCTGTGCAACTGGAAA
Oligo_dt(18)	TTTTTTTTTTTTTTTTTT

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6 APPENDIX

6.1 ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen - , die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen
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