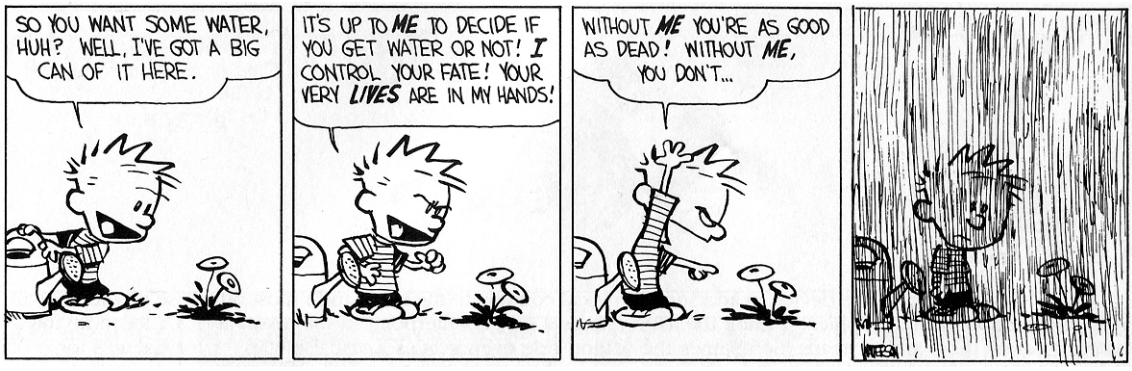

The illusion of (cell cycle) control. (Adapted from Watterson, 1995)

Functional analysis of CDKA;1, the *Arabidopsis thaliana* homologue of the p34cdc2 protein kinase

Inaugural-Dissertation

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CONTENTS

ZUSAMMENFASSUNG

CYCLIN-DEPENDENT KINASEs (Cyclin-abhängige Kinasen, CDKs) sind zentrale Steuerungselemente der Zellzykluskontrolle und homologe CDK-Proteine sind in allen Eukaryonten konserviert. Die vorliegende Promotionsarbeit beschreibt die funktionelle Analyse von CDKA;1, einer bedeutenden CDK in *Arabidopsis thaliana*. CDKA;1 ist im Arabidopsis-Genom mit nur einer Kopie vertreten und nur CDKA;1 ist ein funktionelles Äquivalent der cdc2/CDC28-Kinasen in Hefen.

Ein Screening von zwei T-DNA Insertionsmutanten-Sammlungen ergab die Isolierung von zwei unabhängig entstandenen *cdka;1*-mutanten Pflanzenlinien. Beide Linien erwiesen sich als Nullmutanten und zeigten den gleichen Phänotyp. Eine nähere Untersuchung ergab, dass CDKA;1 für die Zellzykluskontrolle sowohl in der sporophytischen als auch in der gametophytischen Generation von Arabidopsis benötigt wird. Während heterozygote Sporophyten keinerlei Abweichungen in ihrer Entwicklung aufwiesen, waren homozygote Mutanten nicht lebensfähig und starben während der frühen Embryonalentwicklung. Außerdem führte das Fehlen der CDKA;1-Funktion im männlichen Gametophyten (Pollen) zu einer Unterbrechung des Zellzyklus-Programms in der G2-Phase vor der letzten pollenspezifischen Mitose. Durch diesen Zellzyklusdefekt bildete sich reifer *cdka;1*-Pollen mit nur einem statt der üblichen zwei Spermazellen.

Trotz dieses Defekts war *cdka;1*-Pollen lebensfähig und in der Lage, den weiblichen Gametophyten (Embryosack) zu erreichen und zu befruchten. Dadurch, dass *cdka;1*-Pollen nur eine Spermazelle zur Befruchtung beisteuern konnte, erfolgte eine einfache Befruchtung anstelle der für Blütenpflanzen typischen Doppelbefruchtung. Interessanterweise wurde bei dieser einfachen Befruchtung ausschließlich die Eizelle befruchtet, während die Zentralzelle, die sich normalerweise nach der Befruchtung zum Endosperm entwickelt, unbefruchtet blieb.

Nichtsdestotrotz begann nach der Befruchtung der Eizelle nicht nur die Embryonalentwicklung, sondern auch der unbefruchtete Zentralzellkern begann sich zu teilen. Diese Tatsache ließ auf einen Signalmechanismus schließen, der von der befruchteten Eizelle in Gang gesetzt wird und den Zentralzellkern zur Proliferation anregt. Diese autonome Proliferation umfasste allerdings nur maximal fünf mitotische Teilungen, bevor das unbefruchtete Endosperm seine Entwicklung stoppte und anschließend abstarb. Im Folgenden brach auch der Embryo seine Entwicklung ab und der gesamte Samen abortierte in einer frühen Entwicklungsphase. Durch diesen Samenabort kann man dem *cdka;1*-Pollen einen sogenannten paternalen Effekt zuschreiben, da er unabhängig von der genetischen Situation im weiblichen Kreuzungspartner zu einem Absterben des Samens nach der Befruchtung führt.

Um die Endosperm-Entwicklung zu verstärken, wurde *cdka;1*-Pollen auf verschiede *fis*-Mutanten gekreuzt. Diese Mutanten zeichnen sich durch einen Defekt im FIS-Proteinkomplex aus, der über die weibliche Seite vererbt wird und als Polycomb-group-Komplex die genomische Prägung (Imprinting) im Endosperm kontrolliert. In *fis*-Mutanten kommt es ohne Befruchtung zu autonomer Endosperm-Proliferation. Befruchtete *fis*-Mutanten weisen eine starke Überproliferation des Endosperms auf und aufgrund eines maternalen Effekts abortieren ihre Samen zu einem späteren Zeitpunkt der Samenentwicklung.

Wenn *cdka;1*-Pollen zur Bestäubung von *fis*-Mutanten verwendet wurde, entwickelte sich das Endosperm deutlich stärker als im *cdka;1*-bestäubten Wildtyp und viele Samen entwickelten sich über das Stadium des *cdka;1*-bedingten Aborts hinaus. Überraschenderweise wurde aber auch der durch die maternalen *fis*-Allele hervorgerufene Samenabort zum Teil aufgehoben und einige Samenanlagen entwickelten sich zu reifen, lebensfähigen Samen.

Diese Rettung der Samenentwicklung ging mit einer deutlichen Verringerung des Expressionsniveaus des MADS-box Transkriptionsfaktors *PHERES1* im Endosperm einher. *PHERES1* ist ein direktes Ziel der transkriptionellen Repression durch den FIS-Komplex und ist daher in *fis*-Mutanten stark überexprimiert. Die Abschwächung des *PHERES1* Expressionsniveaus im Endosperm der Kreuzung *fis* x *cdka;1* lässt vermuten, dass die Abwesenheit der paternalen Expression, kombiniert mit der maternalen Überexpression, zu einem Normalisierung des Expressionsniveaus von *PHERES1* führte. Möglicherweise sind von dieser Normalisierung auch andere, bisher unbekannte Gene betroffen, deren Expressionsniveau für die Endosperm-Entwicklung von Bedeutung ist.

Zusammengenommen deuten die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass der FIS-Komplex für die Endosperm-Entwicklung nicht essentiell ist. Vielmehr scheint die Funktion der FIS-Proteine darin zu liegen, die Genexpression von maternal und paternal vererbten Genen aufeinander abzustimmen. Darüber hinaus verdeutlichen die hier gewonnenen Erkenntnisse, dass das paternale Genom für die Entwicklung eines funktionellen Endosperms in Arabidopsis nicht benötigt wird, wenn das Imprinting im maternalen Genom durch einen Defekt im FIS-Komplex umgangen wird.

Die Tatsache, dass ein rein maternal vererbtes Endosperm für eine funktionelle Samenentwicklung ausreicht, unterstützt eine Hypothese von Eduard Strasburger aus dem Jahr 1900. Strasburger mutmaßte bereits damals, dass der evolutive Ursprung des Endosperms im weiblichen Gametophyten zu suchen sei und sich die Doppelbefruchtung als Auslöser für die Endosperm-Proliferation entwickelt habe.

ABSTRACT

CYCLIN-DEPENDENT KINASEs (CDKs) are the central gatekeepers of cell cycle progression and conserved in all eukaryotes. In this study, the *Arabidopsis thaliana* master cell cycle regulator CDKA;1 was functionally analyzed. *CDKA;1* is a single gene in Arabidopsis and homologous to the human *Cdk1* and the yeast *cdc2/CDC28*. Screening of two T-DNA insertion mutant collections resulted in the isolation of two independent *cdka;1* null mutant alleles, which displayed the same phenotype. CDKA;1 was found to be required for both the sporophytic and the male gametophytic generations of the flowering plant Arabidopsis. While during sporophyte development, heterozygous mutant plants were unaffected, homozygous *cdka;1* mutants were not viable and died as young embryos. During male gametophyte (pollen) development, the lack of CDKA;1 function caused a cell cycle arrest in the G2 phase prior to the last mitotic division. This cell cycle defect led to *cdka;1* mutant pollen with only one instead of the usual two sperm cells.

Nevertheless, the mutant *cdka;1* pollen was viable and could fertilize the female gametophyte (embryo sac). Because *cdka;1* pollen grains had only one instead of two sperm cells, they only performed single fertilization and thus, disrupted the double fertilization event characteristic of flowering plants. Interestingly, the *cdka;1* mutant single fertilization exclusively targeted the egg cell, leaving the progenitor of the endosperm, the central cell, unfertilized. However, upon *cdka;1* fertilization of the egg cell, not only the embryo started to develop, but the unfertilized central cell nucleus also began to divide. This onset of endosperm development without fertilization revealed a hitherto unrecognized endosperm proliferation signal emitted from the fertilization of the egg cell.

The autonomous endosperm in *cdka;1*-fertilized seeds only underwent up to five nuclear division cycles before it stopped proliferating, followed by an early abortion of the whole seed. Thus, the *cdka;1* mutant belongs to a rare class of paternal effect mutants that cause seed abortion irrespective of the genetic constitution of the female partner.

In order to enhance endosperm proliferation in *cdka;1*-fertilized seeds, *cdka;1* pollen was crossed to various *fis*-class mutants. These mutants are defective in the maternally inherited FIS-complex, a Polycomb-group repressive complex controlling genomic imprinting in the endosperm. In *fis*-class mutants, autonomous endosperm develops in the absence of fertilization. When fertilized, the *fis*-class mutant endosperm over-proliferates and due to a maternal effect these seeds abort later during development.

The endosperm development in *cdka;1*-fertilized *fis*-mutant seeds was substantially enhanced and led to a partial rescue of the *cdka;1*-mediated seed abortion. Unexpectedly, the maternally conferred seed abortion caused by *fis*-class mutants was also partially reversed, producing viable seeds among the *fis*-class x *cdka;1* offspring. This rescue was characterized by a down-regulated expression of the MADS-box transcription factor *PHERES1*, a downstream target of FIS-complex repression which is highly over-expressed in fertilized *fis*-class mutants.

The down-regulation of *PHERES1* in *fis*-class x *cdka;1* endosperm suggests that the lack of paternal expression in combination with the defective gene repression of *fis*-class mutants results in a more balanced gene dosage of *PHERES1* and potentially other genes of which the dosage is pivotal for regular seed development.

These results indicate that the FIS-complex is not essential for endosperm development, but is important to harmonize maternal and paternal gene expression by the control of imprinting in the female genome. Furthermore, these data demonstrate that the paternal genome is not required for functional endosperm development if maternally derived genomic imprinting is bypassed due to mutations in the FIS-complex.

The finding that a solely maternally derived endosperm can sustain seed development supports a hypothesis raised by Eduard Strasburger, who proposed in 1900 that the endosperm of flowering plants is of female gametophytic origin and that central cell fertilization might have evolved as a trigger to start endosperm proliferation.

PUBLICATIONS

Novel Functions of Plant Cyclin-Dependent Kinase Inhibitors, ICK1/KRP1, Can Act Non-Cell-Autonomously and Inhibit Entry into Mitosis

Weinl, C., S. Marquardt, S. J. Kuijt, M. K. Nowack, M. J. Jakoby, M. Hulskamp and A. Schnittger. Plant Cell 17(6): 1704-22 (2005).

- For this paper, I cloned the fusion construct Pro_{GL2}*:GUS:YFP:KRP1*^{109} and generated the corresponding transgenic plant lines

A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis

Nowack, M. K., P. E. Grini, M. J. Jakoby, M. Lafos, C. Koncz and A. Schnittger. Nat Genet 38(1): 63-7 (2006).

- Apart from the analysis of the *mea/fis1* x *cdka;1* crosses and the ultra-structural pollen analysis, which were done by P.E.G. and the cloning of the $Pro_{CDKA;1:}$*CDKA;1* rescue construct, which was done by M.J.J., I did all the work for this paper

T-Loop Phosphorylation of Arabidopsis CDKA;1 Is Required for Its Function and Can Be Partially Substituted by an Aspartate Residue

Dissmeyer, N., M. K. Nowack, S. Pusch, H. Stals, D. Inzé, P. E. Grini and A. Schnittger. Plant Cell (in print)

- For this paper, I performed parts of the mutant analyses of the non-phosphorylatable *CDKA;1*^{T161V} and the phospho-mimicry *CDKA;1*^{T161D} version, including analyses of pollen and embryo development as well as ploidy-analyses by flow cytometry.

Bypassing genomic imprinting allows seed development

Nowack, M. K., R. Shirzadi, N. Dissmeyer, A. Dolf, E. Endl, P. E. Grini and A. Schnittger. (Manuscript under review)

- Apart from major parts of the quantitative PCR and the in situ hybridisations, which were done by R.S. and P.E.G., and the cloning of the $Pro_{CDKA;1:}$*CDKA;1:YFP* rescue construct, which was done by N.D., I did all the work for this paper.

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
ANOVA	Analysis of variance, statistical method
ATP	Adenosinetriphosphate
bp	base pair
C	DNA content of a haploid genome
CAK	*CDK ACTIVATING KINASE*
CDK	*CYCLIN DEPENDENT KINASE*
CDKA;1	*CYCLIN-DEPENDENT KINASE A1*
cDNA	complementary DNA
CDS	coding sequence
CKI	*CYCLIN DEPENDENT KINASE INHIBITOR*
CKS1	*CDC KINASE SUBUNIT 1*
CLF	*CURLY LEAF*
Col	*Arabidopsis thaliana* Columbia accession
CYC	*CYCLIN*
CZE	chalazal endosperm
d.a.g.	days after germination
d.a.p.	days after pollination
DAPI	4',6'-diamidino-2-phenylindole
DME	*DEMETER*
DMSO	Dimethylsulfoxide
DNA	desoxyribonucleic acid
DP	*DIMERIZATION PARTNER*
E(z)	*Enhancer of zeste (Drosophila melanogaster)*
e.g.	*exempli gratia* [Lat.] for example
E2F	*ADENOVIRUS E2 PROMOTOR BINDING FACTOR*
EDTA	ethylenediaminetetraacetic acid
Esc	*Extra sex combs (Drosophila melanogaster)*
et al.	*et alii* / *et aliae* [Lat.] and others
F1, F2, F3	first, second, third... filial generation after a cross
FDA	fluorescein diacetate
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
G1	Gap phase between M phase and S phase
G2	Gap phase between S phase and M phase
gene$^{-/-}$	homozygous mutant of a gene
gene$^{+/-}$	heterozygous mutant of a gene
GFP	green fluorescent protein
GUS	*beta-glucuronidase*
h.a.p.	hours after pollination
H3K27	Lysine residue 27 of the histone H3
i.e.	*id est* [Lat.] that is

ICK/KRP	*INHIBITOR OF CYCLIN-DEPENDENT KINASE / KIP-RELATED PROTEIN (plant CKI)*
Ilgf2	*mammalian Insulin-like growth factor 2*
kb	1000 base pairs
kD	kilo Dalton
LB	T-DNA left border
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession
m	maternally inherited genome
M phase	mitotic phase of the cell cycle
MCE	micropylar endosperm
MEA	*MEDEA*
MET1	*METHYLTRANSFERASE 1*
mRNA	messenger RNA
MSI1	*MULTICOPY SUPPRESSOR OF IRA 1*
n	Number
p	paternally inherited genome
PBS	phosphate bufferd saline buffer
PcG	Polycomb-group
PCR	polymerase chain reaction
PEN	peripheral endosperm
PHE1	*PHERES1*
PMI	pollen-specific mitosis one
PMII	pollen-specific mitosis two
PRC2	Polycomb Repressive Complex 2
Pro_{GENE}	promoter sequence of a *GENE*
QPCR	quantitative Real-time PCR
RB	T-DNA right border
RBR1	*RETINOBLASTOMA RELATED 1* Arabidopsis homologue of the *Retinoblastoma* gene
RNA	ribonucleic acid
rpm	rotations per minute
RT PCR	reverse transcription followed by a polymerase chain reaction
S phase	synthetic phase of the cell cycle
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SET	domain common to cytosine methyltransferases, derived from *Su(var), E(z) and Trithorax*
SSLP	Single Sequence Length Polymorphism
Su(var)	*Suppressor of variegation (Drosophila melanogaster)*
Su(z)	*Suppressor of zeste (Drosophila melanogaster)*
SWN	*SWINGER*
T1, T2, T3	first, second, third transgenic generation after stable plant transformation
T-DNA	transferred DNA
Tris/HCl	buffer containing 2-amino-e-hydroxymethyl-1,3-propanediol and HCl
UTR	untranslated region
UV	ultra-violet light
WEE	*WEE kinase*
wt	wild type
x	crossed to (crosses are always indicated in the order: female x male)
YFP	yellow fluorescent protein

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.

Figure and table index

Figures

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1 INTRODUCTION

“Omnis cellula e cellula”: The fundamental biological dogma that every cell is created by division of a pre-existing cell was formulated some 150 years ago and opposed the idea of spontaneous generation of life (Virchow, 1855). To date, extensive research has provided a detailed molecular understanding of how one cell is derived from another cell and it is now known that all living organisms depend on the duplication of their genetic material and subsequent cell division to reproduce, grow, and develop. These events are tightly coordinated in a highly conserved cellular programme known as the cell cycle.

1.1 Cell cycle control

1.1.1 CDKs: microprocessors at the heart of cell cycle control

The basic mitotic cell cycle is divided in four phases: During the synthetic (S) phase, the DNA is replicated, while during the mitotic (M) phase, the sister chromatids are segregated to the newly forming daughter cells that are afterwards separated by cytokinesis. Between M phase and S phase, there are two gap phases (G1 and G2), in which cells proceed with important physiological functions and eventually prepare for the entry into the next cell cycle phase. There are several modifications of the basic cell cycle theme and one widespread cell cycle mode is an endocycle, in which the M phase is skipped while the DNA continues to be replicated leading to polyploid cells.

Progression through the mitotic cell cycle is controlled at two major checkpoints, the G1/S transition and the G2/M transition. The molecular machinery that controls progression through these checkpoints is highly conserved in all eukaryotes investigated so far (Inze and De Veylder, 2006). Its core consists of CYCLIN-DEPENDENT KINASEs (CDKs). CDKs serve as information processors that integrate intracellular and extracellular signals to ensure the appropriate progress of the cell cycle (Morgan, 1997). Upon favourable conditions, e.g. the presence of nutrients or mitogens, cells advance in the cell cycle, while in response to negative cues such as DNA damage, cells arrest at a checkpoint (**Fig. 1-1 a**).

Information is conferred to the CDKs by a complex molecular machinery that tightly controls the patterns of CDK catalytic activity throughout the cell cycle. CDK activity depends on the association with subunits; most importantly with CYCLINs (CYC) the oscillating concentrations of which create the basic cell-cycle dependent activity patterns

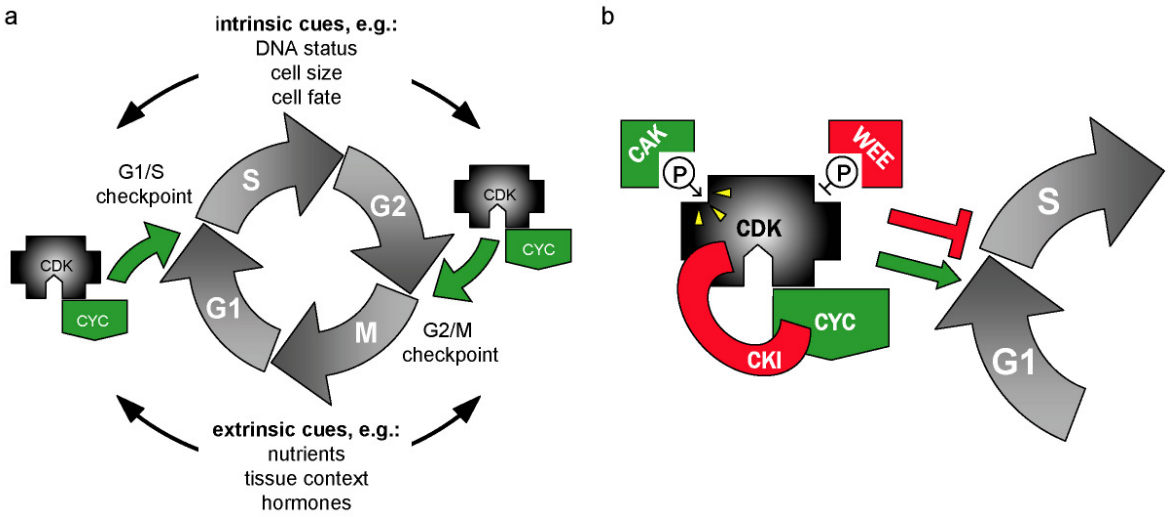

Figure 1-1. CDKs are the core of eukaryotic cell cycle control. (a), CDK/CYC complexes trigger the progression through the cell cycle at two major checkpoints, the transition from G1 to S phase and the transition from G2 to M phase. CDKs act as processors of multiple signalling pathways conferring intrinsic and extrinsic cues to the cell cycle machinery. **(b)**, CDK activity is controlled by multiple mechanisms, including association with activating (CYC) or inhibiting (CKI) subunits. CDK activity is further controlled by activating or inactivating phosphorylations effected by CDK-ACTIVATING KINASES (CAK) or WEE kinases, respectively. The action of CDK activators (green) eventually causes cell cycle progression, while CDK inhibitors (red) can effect a cell cycle arrest at certain checkpoints.

of CDKs. CYCLINs enhance the CDK/CYC substrate specificity and specific CYCLINs bind to CDKs in different cell cycle phases (Morgan, 1997).

Next to CYCLINs, other interactors modify the activity of the CDK/CYC complex: CYCLIN-DEPENDENT KINASE INHIBITORS (CKIs) are able to block CDK/CYC kinase function when cell cycle progression needs to be stopped or modified (**Fig. 1-1 b)** (Sherr and Roberts, 1999).

Additionally, CDK/CYC activity is controlled by a regulatory network of protein kinases and antagonistic protein phosphatases causing CDK/CYC activation or inactivation by phosphorylation or dephosphorylation (Morgan, 1997). These regulatory mechanisms help to fine-tune the intrinsic activity patterns of CDK/CYC complexes (Pomerening et al., 2003) and represent an additional pathway to feed external signals into the cell cycle control (**Fig. 1-1 b)** (De Schutter et al., 2007).

Once fully activated, CDK/CYC complexes phosphorylate a vast number of target proteins that directly or indirectly prepare the cell for the entry into a new cell cycle phase (Ubersax et al., 2003).

1.1.2 CDKs and cell cycle control in plants

In plants, as in other eukaryotes, the conserved CDK/CYC core cell cycle machinery controls progression through the cell cycle. Many features of CDK/CYC activity control are conserved in plants although some of the regulation is realized in plant-specific ways (Boudolf et al., 2006; Inze and De Veylder, 2006).

In contrast to unicellular eukaryotes such as yeasts, in which a single CDK controls the progression through all cell cycle phases, in multicellular organisms like animals and plants, small families of CDKs have evolved. The CDK family of the model plant *Arabidopsis thaliana* consists of twelve members including one A-type, four B-type, two C-type, three D-type, one E-type and one F-type CDK (Vandepoele et al., 2002). Only the single A-type CDK in Arabidopsis, CDKA;1, contains the conserved PSTAIRE motif and is able to complement the *cdc2* mutant in fission yeast (*Schizosaccharomyces pombe*) and the *cdc28* mutant in budding yeast (*Saccharomyces cerevisiae*) (Ferreira et al., 1991; Hirayama et al., 1991; Porceddu et al., 1999). Therefore CDKA;1 is likely to be an important plant cell cycle regulator. The exact function of the other CDKs is not entirely understood. CDKBs become active at the G2-M transition and thus, might be involved in the control of mitosis (Boudolf et al., 2004a; Boudolf et al., 2004b; Inze and De Veylder, 2006)

The observation that CDKA;1 protein levels remain constant throughout the cell cycle (Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001) and that CDKA;1 activity can be detected at both checkpoints (Hemerly et al., 1995; Porceddu et al., 2001; Joubes et al., 2004) lead to the assumption that CDKA;1 is participating in all cell cycle transitions. Consistently, *CDKA;1* is expressed in all tissues that show cell division or else are competent to divide. This expression pattern suggested a general role of CDKA;1 in the establishment of proliferative competence (Martinez et al., 1992; Hemerly et al., 1995).

While over-expression of native *CDKA;1* did not alter the cell cycle nor plant development, misexpression of a dominant negative $CDKA;1^{DN}$ version caused lethality, suggesting an essential role of CDKA;1 in Arabidopsis cell cycle control (Hemerly et al., 1995).

In tobacco (*Nicotiana tabaccum*), the same dominant-negative construct had no effects on plant viability, but strongly decreased cell division rates. However, G1/G2 ratios were unaltered, indicating the participation of CDKA;1 at both cell cycle checkpoints (Hemerly et al., 1995). In maize (*Zea mays*) endosperm, expression of a *CDKA;1*^{DN} version reduced endoreplication, arguing for a role of CDKA;1 in the control of G1/S transition (Leiva-Neto et al., 2004).

Other experimental approaches to study the function of CDKA;1 were made by misexpression of CDKA;1 inhibitors called *INHIBITORS OF CDK/KIP-RELATED PROTEINS* (*ICK/KRP*s). ICK/KRPs bind to CDKA;1 and block its kinase activity (De Veylder et al., 2001). In short, these experiments demonstrated that lowered CDKA;1 kinase activity leads to an overall reduction of cell division resulting in smaller plants (Wang et al., 2000; De Veylder et al., 2001). Furthermore, while high levels of ICKs/KRPs block CDKA;1 activity at both G1/S and G2/M transition, moderate levels specifically target the G2/M-specific CDKA;1/CYC complexes. Taken together, CDKA;1 appears to function as a master cell cycle regulator essential for both the G1/S and the G2/M transition in plants.

1.2 Plant development

1.2.1 General features of plant cell division and development

In both plants and animals, the control of cell division is crucial to the correct realization of the genetically programmed body plan. However, in contrast to animals, the plant body develops mainly post-embryonically, producing its biomass out of small clusters of pluripotent stem cells called meristems. While the shoot meristem typically produces the above-ground organs of a plant, such as shoots, leaves, and the reproductive organs, the root meristems located in the root tips builds up the root system. The iterative development of life-long sustained populations of stems cells leads to an enormous potential of morphological plasticity in plant development.

However, fully differentiated cells also show a remarkable pluripotency: In root pericycle cells, for instance, re-activation of the cell cycle leads to the formation of a new meristem that subsequently forms a new side root (Casimiro et al., 2003).

Furthermore, plant cells are surrounded by a rigid cell wall and tightly connected to neighbouring cells in the tissue, which means that cell migration is largely impossible and other means to build up the body structure have been implemented. Therefore, cell division ratios and the orientation of cell division planes are of great importance for the plant body architecture (Hemerly et al., 2000).

1.2.2 The plant life cycle

A major feature characteristic for plants is their two-phase life cycle of alternating generations of sporophytes and gametophytes (**Fig. 1-2**). In flowering plants (angiosperms), the predominant generation is the typically diploid sporophyte. This generation builds up the main plant body with roots, shoots, leaves and flowers, but it does not undergo sexual reproduction. Instead, meiotic divisions in specialized floral tissues lead to the formation of haploid microspores and megaspores. These spores are the starting point for the second, haploid generation, called gametophyte. The purpose of the gametophytes is to produce the male and the female gametes and to bring them together during sexual reproduction. In Arabidopsis, a typical angiosperm, the gametophytic generation is reduced to minute few-celled organisms which are embedded in the maternal tissue and completely dependent from the mother sporophyte.

Microspores are produced in the anthers of a flower and undergo two cell cycle rounds to complete their development into mature male gametophytes or pollen grains. During pollen-mitosis one (PMI), the microspore undergoes an unequal division to form the large vegetative cell and the small generative cell. Subsequently, the pollen-mitosis two (PMII) divides the generative cell in two sperm cells (McCormick, 2004).

Megaspores usually undergo three cell cycle rounds to produce the female gametophyte or embryo sac containing eight nuclei. Subsequent cellularization forms the mature seven-celled embryo sac, which includes the two gametes, the egg cell and the homodiploid central cell, as well as the accessory synergids and the antipodal cells. The mature embryo sac is surrounded by several layers of maternally derived integuments. The entity of embryo sac and the integuments is called the ovule, and this structure will develop into a seed after successful fertilization (Drews and Yadegari, 2002; Yadegari and Drews, 2004).

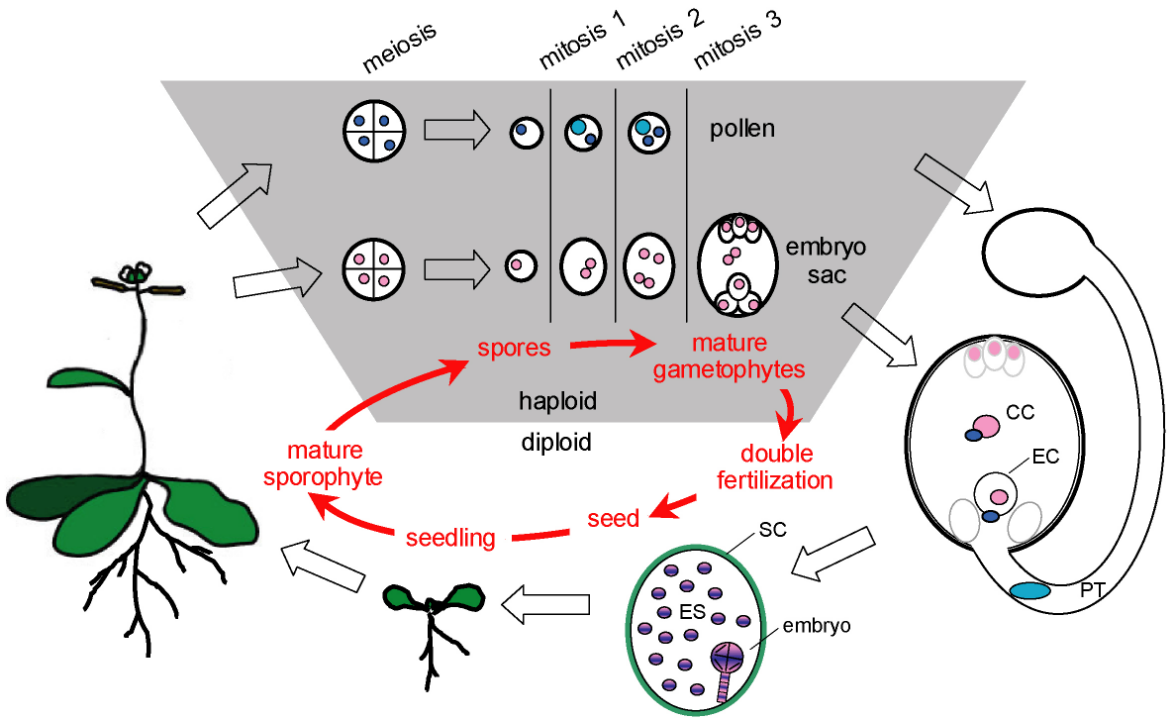

Figure 1-2. The plant life cycle. Plants have a two-phase life cycle of alternating generations. The typically diploid sporophyte produces haploid spores through meiosis. The spores develop into the haploid gametophytes. Microspores undergo two mitotic cycles to form the mature pollen that contains two sperm cells (dark blue). Megaspores produce a seven-celled gametophyte by three mitotic divisions. The female gametes are the egg cell (EC) and the central cell (CC), respectively. After the pollen tube (PT) has transported the two sperm cells to the embryo sac, double fertilization occurs: One of the sperm cells fuses with the egg cell to form the diploid embryo, while the other sperm cell fuses with the homodiploid central cell to give rise to the triploid endosperm (ES). Embryo and endosperm are surrounded by the maternally derived seed coat (SC). After completion of embryo development, the seedling is released from the seed and develops into the new sporophyte. (Adapted from Berger et al. 2006).

1.2.3 Double fertilization

For the fertilization process in flowering plants, pollen is released and has to be transported to the stigma, a specialized receptive tissue formed by the maternal sporophyte. In contact with the stigma cells, the pollen germinates and forms a pollen tube which penetrates the maternal sporophytic tissue and grows towards an ovule. After penetration of the ovule, the pollen tube releases the two sperm cells. In a process called double fertilization, one of the sperm cells fertilizes the haploid egg cell to form the embryo while the second sperm cell fertilizes the homodiploid central cell to give raise to the triploid endosperm (Faure et al., 2002).

Successful double fertilization requires a sequence of signalling events, starting from stigma-pollen interaction followed by guidance of the pollen tube to the ovules (Higashiyama et al., 2003), and terminating with signalling that accompanies the actual fertilization process (Berger et al., 2006).

1.2.4 The endosperm: an integrator of seed development

Successful double fertilization leads to seed and fruit development. The seed containing the embryo and a fertilized endosperm has exclusively evolved in the angiosperms. Seeds are located within the carpel tissue which after pollination will develop into the fruit.

The seed itself is made up of three basic units representing three different organisms: the embryo is the new sporophyte generated by the fusion of the egg cell and the sperm cell; the endosperm is the fertilization product of a second sperm cell and the homodiploid central cell; and the seed coat which is produced by the mother sporophyte.

In order to form one functionally integrated whole, these three organisms have to tightly coordinate their growth and development. Proliferation of the endosperm and the embryo has to be balanced and the seed integuments have to grow accordingly. One has to postulate the existence of repeated signalling events coordinating the development of these components during seed development (Berger et al., 2006).

During seed development the embryo grows and develops to the mature seedling undergoing specific developmental stages of morphogenesis (**Fig. 1-3 a-f**): At first the zygote develops into a globular embryo with a filiform suspensor. With the subsequent onset of embryonic leaf formation, the embryo acquires a heart shape. The heart stage embryo gains in length during the torpedo stage and finally bends in the mature seed (Mansfield, 1994; Jurgens et al., 1995). The mature embryo consists of an embryonic root, shoot and a pair of embryonic leaves as well as two embryonic meristems. These meristems will establish the postembyonic plant body, later supported by secondary meristems.

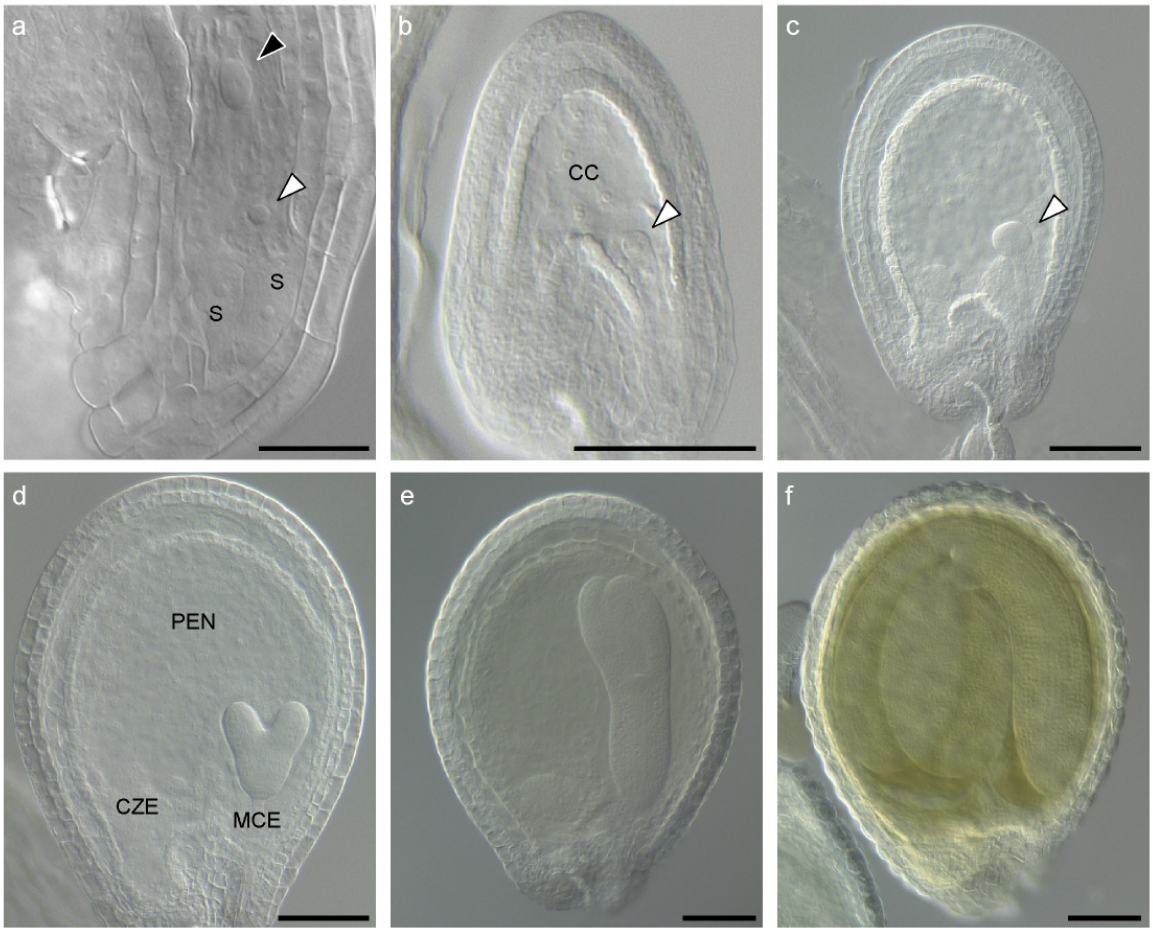

Figure 1-3. Seed development in *Arabidopsis thaliana*. (a-f), Seed development from before fertilization to seed maturation. **(a)**, Mature female gametophyte prior to fertilization. Visible are the egg cell (white arrowhead), the central cell nucleus (black arrowhead) and the two synergid cells (S). **(b)**, 2 days after pollination (d.a.p.), the two-celled proembryo (arrowhead) is accompanied by the early endosperm syncytium in the central cell (CC). **(c)**, 4 d.a.p., globular-stage embryo and syncytial endosperm. **(d)**, 6 d.a.p. heart-stage embryo, at this point the endosperm starts to cellularize around the embryo. **(e)**, 8 d.a.p., torpedo-stage embryo, with completely cellularized endosperm. **(f)**, 12 d.a.p., embryo with bent embryonic leaves near maturity, filling the major part of the seed. **(g)**, Seed with a globular stage embryo (arrowhead) and the endosperm differentiated in the three domains central peripheral endosperm (PEN), chalazal endosperm (CZE), and miroyplar endosperm (MCE). Scale bars are 20 µm in a, and 100 µm in b-f.

Growing alongside the embryo in the seed, the endosperm also develops according to a well defined though fundamentally different programme (**Fig. 1-3 a-f**) (Mansfield, 1994; Boisnard-Lorig et al., 2001). Endosperm development is characterized by four phases: syncytium, cellularization, differentiation and death (Berger, 1999). First, mitotic cycles of the fertilized central cell in the absence of cytokinesis form a syncytial endosperm containing several hundreds nuclei that fill the central cell. The endosperm nuclei are

organized in mitotic domains that display different rates of proliferation. Morphologically, three domains are recognized: The micropylar endosperm (MCE) surrounds the embryo at the anterior pole of the seed, the peripheral endosperm (PEN) lines the inside of the seed integuments and the chalazal endosperm (CZE) has dense cytoplasm and occupies the posterior pole of the seed (Brown et al., 1999).

In the seed stage characterized by a heart-shaped embryo, the syncytial phase of the endosperm ends and cellularization sets in (Sorensen et al., 2002). In Arabidopsis, the cellularized endosperm gets mostly consumed by the growing embryo and dies after seed germination (Berger, 1999).

The endosperm has been connected with nutrient acquisition from the mother plant and is thought to be a nurse tissue for the developing embryo (Hirner et al., 1998; Berger, 2003).

Furthermore, the endosperm has been described as a central integrator of signals during seed development (Berger et al., 2006). There is evidence of reciprocal signalling between the seed integuments and the endosperm, taking influence on the final size of the seed. On the one hand, the seed integuments can influence endosperm proliferation as shown in sporophytic mutations leading to reduced or enhanced growth of endosperm and embryo (Ray et al., 1996; Garcia et al., 2005; Schruff et al., 2006). Conversely, enhanced or reduced endosperm growth promotes or inhibits growth of the seed integuments (Garcia et al., 2005; Luo et al., 2005).

Data so far suggest both a maternal sporophytic and a zygotic control of seed development, the latter apparently mediated via the endosperm. In comparison to the endosperm, the embryo seems to play less of a role during communication in seed development (Berger et al., 2006).

1.3 Imprinting and he role of FIS-class genes during seed development

1.3.1 The FIS-PRC2

One important class of genes controlling the development of endosperm in Arabidopsis are the *FIS*-class genes. FIS-class proteins form a Polycomb-group (PcG) complex homologous to the Polycomb Repressive Complexes (PRCs) of animals (Chanvivattana et al., 2004). In mammals, the PRC2 is a complex involved, for instance, in the inactivation

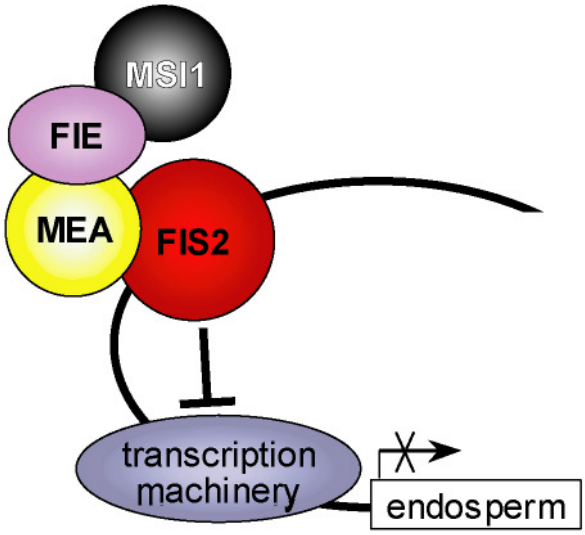

Figure 1-4. The Arabidopsis FIS-PRC2. The FIS-PRC2 consists at least of four core proteins, MEA, FIS2, FIE, and MSI1. The FIS-PRC2 is exclusively active in the female gametophyte and in the endosperm to control the expression status of imprinted genes via maintenance of H3K27 methylation. The repressive influence of the FIS-PRC2 on the expression of endosperm genes is thought to restrict endosperm proliferation.
(Adapted from Kinoshita et al. 2001).

of the X-chromosome (Wang et al., 2001). It contains subunits conferring a H2K27-specific histone methyltransferase activity targeting lysine 27 in the basic tail domain of histone H3 (hereafter referred to as H3K27) (Cao et al., 2002).

In plants, there are several PRC2s built up by members of small gene families that show homologies to their animal PRC2 counterparts. Of importance for seed development and imprinting is the FIS-PRC2, composed of at least four core components: MEDEA (MEA), FERTILIZATION INDEPENDENT SEED 2 (FIS2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (**Fig. 1-4**) (Guitton and Berger, 2005a). MEA is a SET-domain protein homologous to the Drosophila Enhancer of zeste E(z), which has been shown to confer histone lysine methyltransferase activity to the PRC2 (Cao et al., 2002; Muller et al., 2002). In the Arabidopsis FIS-PRC2, MEA interacts with FIE, the homologue of the Drosophila Extra sex combs (Esc) in vitro and in vivo (Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000; Bracha-Drori et al., 2004). FIS2 is another member of the FIS-PRC2, and is the homologue of Su(z)12, a zinc finger protein which is important for the association of PRC2 with a selected set of target genes (Guitton and Berger, 2005a). Finally, MSI1 has also been identified as part of the FIS-PRC2 (Kohler et al., 2003a). MSI1 is a histone-binding protein homologous to the Drosophila p55.

Mutants of the Arabidopsis FIS-complex components have first been identified a decade ago. Their loss-of-function phenotypes are quite similar and display two effects:

First, lack of FIS-class proteins confers a failure of cell cycle arrest in the mature female gametophyte, leading to autonomous endosperm proliferation without fertilization inside embryo-less seed-like structures (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus et al., 1998; Kohler et al., 2003a). An exception is the *msi1* mutant, which shows occasional development of non-viable parthenogenetic embryos (Guitton and Berger, 2005b).

Second, after fertilization, maternally inherited mutant *fis*-class alleles lead to heterochronically altered endosperm development and subsequent seed abortion (Kiyosue et al., 1999; Guitton et al., 2004; Ingouff et al., 2005). In *fis*-mutant endosperms, mitotic domains are missing or ill-defined. The *fis*-class endosperms are delayed in differentiation, leading to over-proliferation and lack of cellularization and differentiation (Grossniklaus et al., 1998; Kiyosue et al., 1999). The molecular nature of this pleiotropic phenotype is not understood to date (Guitton and Berger, 2005a).

1.3.2 Imprinting in angiosperm seed development

Investigations of gene expression patterns suggested that some *FIS*-class genes (*MEA*, eventually also *FIS2* and *FIE*) are exclusively expressed from their maternal alleles in the endosperm, while their paternal alleles are silenced (Kinoshita et al., 1999; Luo et al., 2000; Yadegari et al., 2000).

Asymmetric paternal and maternal expression patterns are characteristic for the fertilization products of mammals and plants; this phenomenon has been described as genomic imprinting.

Up to now, almost 100 imprinted genes have been identified in mammals (Morison et al., 2005). As a consequence of imprinting, gene functions derived from both the maternal and the paternal genome are required for normal embryo development in mammals. Embryos with either only maternal or only paternal genomes show aberrant development and eventually die (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984).

Like mammals, flowering plants have been found to imprint certain genes resulting in a parent-of-origin dependent expression during seed development (Gehring et al., 2004; Autran et al., 2005; Guitton and Berger, 2005a; Kohler and Grossniklaus, 2005; Scott and Spielman, 2006). The experimental evidence so far restricts imprinting in plants to the endosperm and has first been observed as a functional non-equivalency of the parental

genomes in seeds with either maternal or paternal genomic excess (Lin, 1982, 1984; Scott et al., 1998). However, in contrast to mammals, only a handful of imprinted genes have been discovered so far.

Meanwhile, imprinting in both mammals and plants has been implicated with two distinct, yet interconnected, molecular mechanisms controlling gene expression patterns: cytosine methylation of the DNA and lysine methylation on histone H3 tails (Chan et al., 2005).

Recent experimental evidence in Arabidopsis suggests that both, maintenance of DNA methylation as well as maintenance of histone methylation are important for the cellular memory of the expression status of imprinted genes.

Maintenance of DNA methylation largely depends on the activity of METHYL TRANSFERASE1 (MET1) and of DECREASE IN DNA METHYLATION1 (DDM1) (Vielle-Calzada et al., 1999; Finnegan et al., 2000), while the imprinting through H3K27 histone methylation has been implicated with the function of various PRC2s.

Interestingly, the gene locus of the FIS-PRC2 component MEA has been shown to be targeted by PRC2-dependent imprinting itself (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006). *MEA* is kept in a silent state during vegetative development by PRC2s containing SET-domain proteins other than MEA, e.g. CURLY LEAF (CLF) or SWINGER (SWN). Additionally, *MEA* repression might involve DNA methylation maintained by MET1 (Xiao et al., 2003). *MEA* silencing is maintained during male gametophyte development, while the *MEA* allele in the female gametophyte gets activated through the action of the DNA glycosylase DEMETER (DME) (Choi et al., 2004). The mode of DME action remains a matter of debate, yet it results in hypomethylation of the *MEA* gene region as well as a loss of *MEA* silencing by the PRC2 in the female gametophyte. After fertilization, the PRC2 containing the maternally activated MEA represses various target genes through maintenance of H3K27 methylation, among them the paternally derived *MEA* allele (Gehring et al., 2006; Jullien et al., 2006).

Taken together, PRC2s are important to maintain the silent state of target genes by maintenance of H2K27 methylation. If this maintenance is severed, PRC2 target genes get ectopically activated. The activity of FIS-PRC2 is crucial for regular seed development and FIS-PRC2 targets are likely to be important regulators of seed development.

However, apart from *MEA* itself, so far only a few potential target genes of the FIS-complex have been isolated. The gene of the type 1 MADS-box transcription factor

PHERES1 (*PHE1*) is the most intensively studied among them. It has been shown that the FIS-complex represses transcription of the maternally inherited *PHE1* gene by direct association with the *PHE1* promoter region (Kohler et al., 2003b; Kohler et al., 2005) and that H3K27 histone methylation on the *PHE1* locus is partly dependent on FIS-complex activity (Makarevich et al., 2006).

In the *mea*-mutant background that specifically alters the histone methyltransferase activity of the MEA protein, *PHE1* histone methylation is reduced and *PHE1* expression in the endosperm is strongly up-regulated (Makarevich et al., 2006). The *PHE1* up-regulation during seed development, in turn, is causally connected with the seed-abortion phenotype of *mea* mutants: *PHE1* down-regulation by a *PHE*-antisense construct resulted in a partial rescue of *mea* seed abortion (Kohler et al., 2003b). Interestingly, seeds with reduced *PHE1* levels tended to grow larger than wild-type seeds, suggesting a role for *PHE1* as an enhancer of seed growth (Scott and Spielman, 2006).

1.4 Aim of the study

The aim of the present study was the isolation and characterization of *cdka;1* mutants in Arabidopsis.

So far, CDKA;1 function in plant cell cycle and plant development has been studied by dominant-negative versions of CDKA;1 or over-expression of CDKA;1 inhibitors, both of which represent indirect approaches to assess CDKA;1 function in planta. Thus, a mutant approach promised potentially new insights into the role of CDKA;1 in plant cell cycle control and development, in particular during the gametophytic life phase that has not been studied in detail in previous experiments.

Furthermore, a functional *cdka;1* knock-out could provide an interesting tool for cell cycle manipulation in planta. Based on the current data, CDKA;1 plays a major role in Arabidopsis cell cycle control and thus could be an attractive target protein for experiments aiming to interfere with cell cycle progression in various developmental contexts. In this respect, the present study intended to establish and characterize the *cdka;1* mutant as a base for future research in the plant cell cycle field and in plant development.

2 RESULTS

2.1 Isolation and molecular characterization of a *cdka;1* mutant

In a reverse genetics approach, two *Arabidopsis thaliana* T-DNA mutant collections were screened for insertions within the *CDKA;1* gene. In each of the collections, one putative *cdka;1* mutant line was identified. Seeds of both lines were obtained and the mutant plants were subjected to a detailed mutant analysis.

2.1.1 Isolation of two independent *cdka;1* mutant lines

One of the *cdka;1* mutant lines originated from the mutant collection of the SiGNAL collection (SALK institute in La Jolla, California). It carried a T-DNA insertion in the fifth intron of *CDKA;1* (**Fig. 2-1a**). This putative *cdka;1* mutant allele was termed *cdka;1-1*. A second mutant line, *cdka;1-2*, was isolated from the Koncz collection (Csaba Koncz, Max Planck Institute for Plant Breeding Research in Cologne, Germany). This second mutant allele carried a T-DNA insertion in the fourth intron of *CDKA;1* (**Fig. 2-1a**). From both lines, only heterozygous *cdka;1* mutants could be isolated.

Sequencing of PCR products from the T-DNA borders to the adjacent genomic DNA was used to determine the exact positions of the T-DNA insertions in the coding region of *CDKA;1*.

In *cdka;1-1*, exon five was disrupted by the T-DNA insertion. The T-DNA insertion occurred as an inverted tandem repeat so that left border sequence was present at both the 5'- and the 3'-transition between genomic and T-DNA sequences. While the 5'-border showed a simple transition from genomic DNA to vector sequence, at the 3'-border there were some additional base pairs of unknown origin and a repeated fragment from the T-DNA left border sequence (**Fig. 2-1b**).

In *cdka;1-2*, the T-DNA insertion was located close to the end of exon four. Similar to *cdka;1-1*, *cdka;1-2* carried a T-DNA in an inverted tandem repeat pattern. In both the 5'- and the 3'-transitions between genomic DNA and vector left border sequence, there were short DNA stretches of unknown origin (**Fig. 2-1c**). The border sequences of both alleles were deposited at GenBank and have the accession numbers DQ156166 and DQ156167 for *cdka;1-1*, and DQ156168 and DQ158862 for *cdka;1-2*.

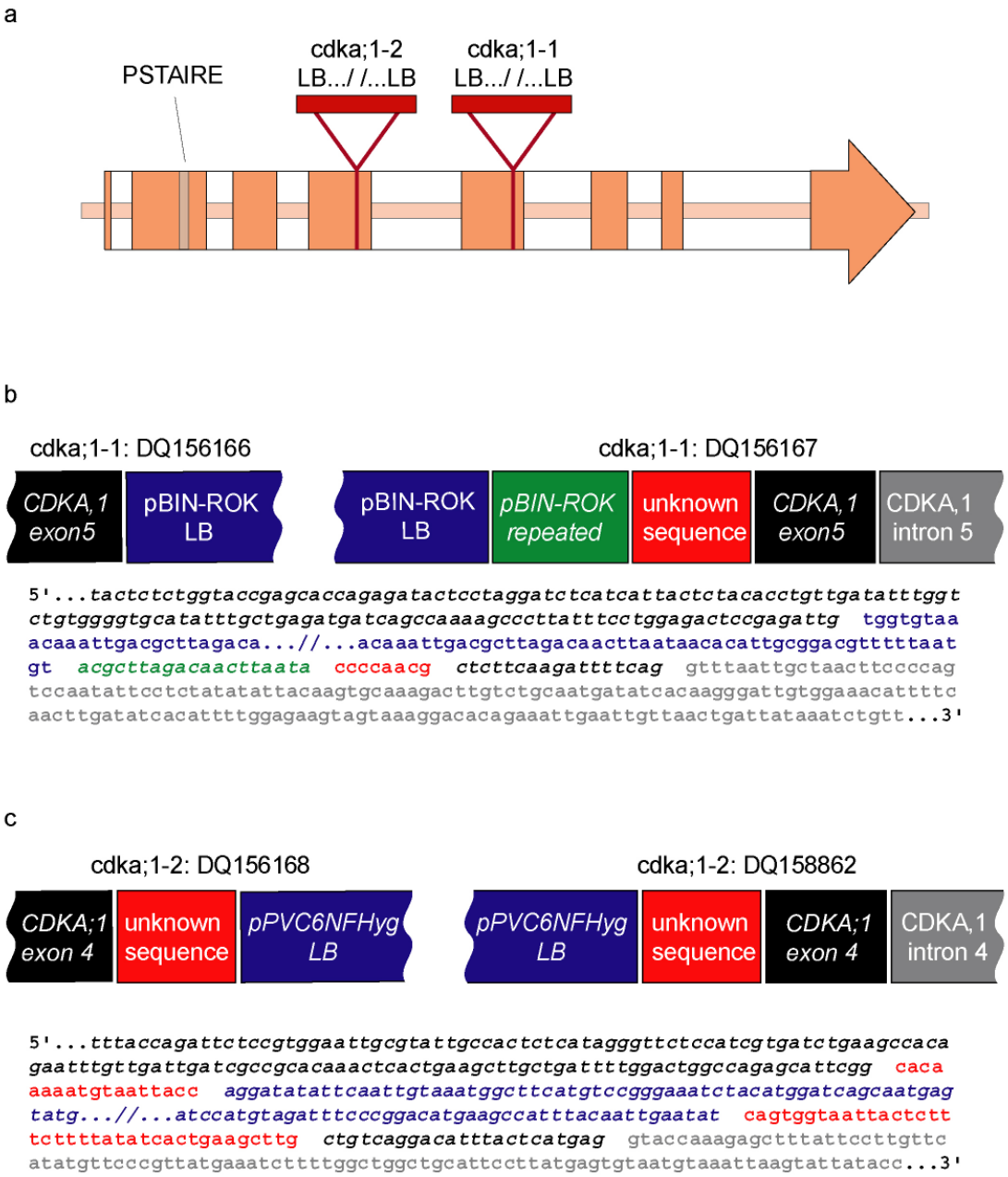

Figure 2-1. T-DNA insertion mutants of the Arabidopsis *CDKA;1*. (a) In *cdka;1-1* and *cdka;1-2*, the T-DNA disrupted the reading frame in the fifth and forth exon, respectively, downstream of the highly conserved PSTAIRE domain necessary for CDK. **(b-c)** The transitions from Arabidopsis genomic DNA to T-DNA inserts in *cdka;1-1* and *cdka;1-2* were sequenced to determine the detailed effects of the T-DNA integration. In both alleles, the T-DNA was inserted in an inverse tandem-repeat fashion, causing the left borders (LB) each to be orientated towards the genomic DNA on both sides of the insertion. Furthermore, in both alleles, the T-DNA insertion caused a deletion of 5 base pairs of genomic sequence. The sequence information was submitted to GenBank, the accession codes are indicated above the corresponding sites. **(b)** In the *cdka;1-1* allele, the T-DNA derived from the vector *pBIN-ROK* was inserted close to the end of exon five. While the 5'-transition is blunt, the transition from the T-DNA to the 3'-*cdka;1* fragment showed a repeated T-DNA fragment and a short strech of sequence of unknown origin. **(c)** In *cdka;1-2*, the T-DNA derived from the vector *pPVC6NFHyg* disrupted the sequence of *CDKA;1* close to the 3'-end of exon four. The T-DNA insert was flanked on both sides by short sequence stretches of unknown origin. Abbreviations are: DQ, GenBank code; LB, T-DNA left border sequence.

2.1.2 *cdka;1-1* is a null allele

To test whether the isolated mutants contained *cdka;1* null alleles, transcripts from both the wild type and the mutant *cdka;1-1* allele were analyzed by '3'-Rapid Amplification of cDNA Ends' (3'-RACE).

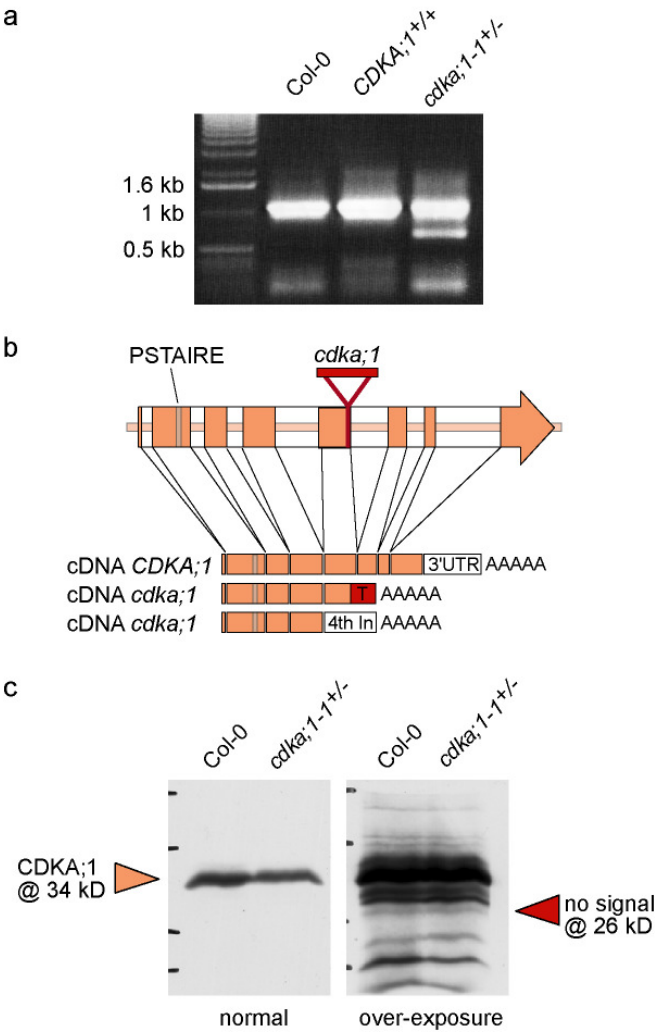

Figure 2-2. The *cdka;1-1* is a null allele. **(a)** Agarose gel separating PCR products of the 3'-Rapid Amplification of cDNA Ends (3'-RACE). Note the additional band in *cdka;1*$^{+/-}$ running at approximately 600 base pairs. 3'-RACE was performed to determine whether the disrupted *cdka;1* alleles were transcribed. **(b)** Sequencing of the 3'-RACE products revealed that the upper bands in a represented wild-type cDNA of *CDKA;1* and that the lower band contained different variants of truncated *CDKA;1* cDNAs. Among these were cDNAs containing parts of the T-DNA insertion and splicing variants that ended at various locations in the forth intron. **(c)** Western blot detecting CDKA;1 protein in plant extracts of wild-type plants (Col) and *cdka;1*$^{+/-}$ mutants. Even after overexposure of the film no additional signal of a truncated cdka;1 mutant protein (expected at 26 kD) was detected in *cdka;1*$^{+/-}$. Thus, in *cdka;1-1*, all functional features of a CDK are deleted, i.e. ATP binding pocket, catalytic cleft, and the CYCLIN binding domain PSTAIRE. Abbreviations are: *CDKA;1*$^{+/+}$, wild-type offspring of *cdka;1*$^{+/-}$; 3'-UTR, 3'-untranslated region; Col, Columbia accession wild type; kb, kilo base pairs; kD, kilo Dalton; In, intron; T, T-DNA.

In both Col wild-type plants and wild-type siblings of the *cdka;1-1* mutant plants, only full-length *CDKA;1* transcript was found. On the contrary, heterozygous *cdka;1*$^{+/-}$ mutants were shown to produce an additional transcript of smaller size (**Fig. 2-2a**). Subcloning and subsequent sequencing revealed that this smaller band was composed of different truncated mutant versions of *cdka;1* cDNA. Some versions contained the transitions from *CDKA;1* sequence to vector sequence that were already known from sequencing of the insertion loci. Others were splicing variants that contained a part of intron four. In any case, mutant mRNA transcripts terminated shortly after the transition to either the T-DNA sequence or intron 4 sequence (**Fig. 2-2b**).

Due to the presence of truncated mRNA versions in the *cdka;1-1* mutant it was conceivable that the T-DNA insertions resulted in the production of a truncated CDKA;1 protein. As such a shortened CDKA;1 version might have residual activity or alternatively could cause dominant negative effects, CDKA;1 protein levels were analyzed by Western blotting and subsequent detection with an antibody that recognized the PSTAIRE-domain in the amino-terminal sheet of the CDKA;1 protein (**Fig. 2-1a**).

In wild-type Col plants and in wild-type siblings of the heterozygous *cdka;1-1*$^{+/-}$ mutants, only the wild-type CDKA;1 protein with a size of 34 kD could be detected. In blotted protein extracts of heterozygous *cdka;1*$^{+/-}$ mutant plants, I found only one band of the same size as the wild-type protein. Even after prolonged exposure of the film, no additional band of the expected size of 26 kD for a truncated CDKA;1 variant could be detected (**Fig. 2-2c**). Thus, *cdka;1-1* is a null mutant at the protein level.

2.2 Analysis of the cdka;1 mutant phenotype

2.2.1 There are no homozygous *cdka;1* mutants

Consistent with the fact that the isolated *cdka;1* mutant alleles did not lead to the expression of any functional CDKA;1, and with the assumption that CDKA;1 is essential for the cell cycle regulation of Arabidopsis, I never found homozygous *cdka;1*$^{-/-}$ mutants in segregating populations. In over 200 F1 plants of a heterozygous *cdka;1*$^{+/-}$ parent, PCR based genotyping could only identify heterozygous mutants or wild-type plants.

This suggested that CDKA;1 is indispensable for either gametophyte or sporophyte development, or both. If one of the gametophytes was affected, the mutant allele could not be passed on to the next sporophytic generation (gametophytic effect). Alternatively, homozygous sporophytes would die during embryogenesis (sporophytic effect).

2.2.2 *cdka;1* represents a paternal effect mutant

Heterozygous *cdka;1*$^{+/-}$ mutants did not differ from the wild type in their vegetative development. In siliques of heterozygous mutants, however, I found a substantial number of seeds aborted early in embryo development (**Fig. 2-3 a-b**).

As the fraction of aborted seeds was significantly higher than one quarter and close to one half (**Table 2-1**), it was likely that the mutant *cdka;1* allele caused a failure in the development of one of the gametophytes. Moreover, since male gametophytic mutants are usually out-competed by pollen that received the wild-type allele, this phenotype seemed to

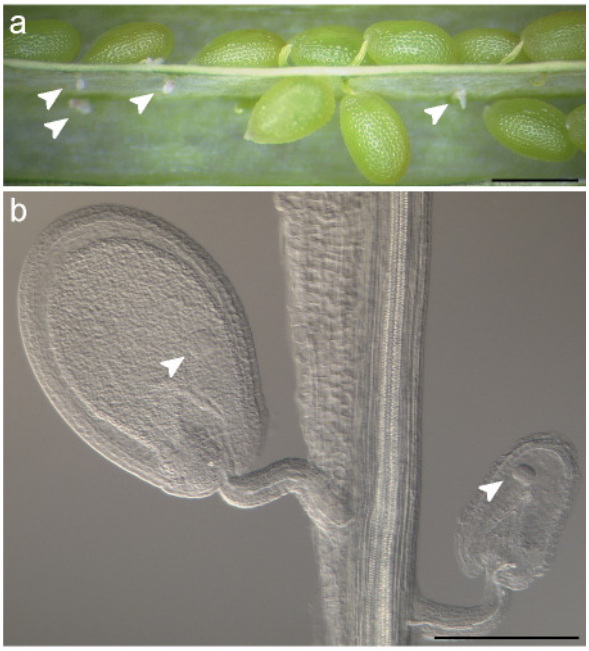

Figure 2-3. Seed abortion in *cdka;1*$^{+/-}$ mutants. (a) Siliques of heterozygous *cdka;1*$^{+/-}$ mutant plants with early aborted seeds (arrowheads) in comparison with normal seeds nearing maturity. **(b)** Differential interference contrast (DIC) micrograph of cleared whole mount seed preparations of a heterozygous *cdka;1*$^{+/-}$ mutant. Left a normally developed seed containing a globular stage embryo (arrowhead), right a typical *cdka;1* aborted seed with an embryo arrested in early globular stage (arrowhead). Scale bars: 500 µm in **a**, 200 µm in **b**.

classify *cdka;1* as a female gametophytic mutant. However, reciprocal crosses between *cdka;1*$^{+/-}$ mutants and wild-type plants revealed that the mutant pollen caused seed abortion, whereas *cdka;1*$^{+/-}$ plants as female partners gave rise to normally developing seeds (**Table 2-1**).

Table 2-1. Percentage of aborted seeds in *cdka;1* mutant lines

Parental genotypes (female x male)	normal	aborted	undeveloped	n	expected aborted (within 95% confidence limits)
Col x Col	95.8%	2.3%	1.9%	406	n.a.
cdka;1-1$^{+/-}$ selfed	51.3%	46.7%	1%	452	43.6 < 47.5%[a] < 52.4
cdka;1-1$^{+/-}$ x Col	96.9%	0.4%	2.6%	602	4.2 < 6.0%[a] < 8.5
Col x *cdka;1-1*$^{+/-}$	57.8%	42.2%	0.5%	472	34.8 < 39.0%[a] < 43.4
cdka;1-2$^{+/-}$ selfed	51.6%	47%	1.4%	230	41.0 < 47.5%[b] < 55.0
resc *cdka;1*$^{+/-}$ selfed	54.2%	44.9%	0.9%	128	38.1 < 47.5%[b] < 58.0
resc *CDKA;1*$^{+/+}$ selfed	98.5%	0%	1.5%	134	0.0 < 0.0%[c] < 3.8
resc *cdka;1:yfp*$^{+/-}$ selfed	54%	46%	n.d.	821	n.d.
resc *CDKA;1:YFP*$^{+/+}$ selfed	96%	4%	n.d.	225	n.d.

[a]expected abortion as determined by transmission rate. [b]expected abortion as in *cdka;1-1*$^{+/-}$. [c]expected abortion in wild type. n, number of seeds scored; Col, wild type Columbia accession; n.a., not applicable; n.d., not determined; resc *cdka;1*$^{+/-}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1* construct in heterozygous condition; resc *CDKA;1*$^{+/+}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1* construct in homozygous condition; resc *cdka;1:yfp*$^{+/-}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1:YFP* construct in heterozygous condition; resc *CDKA;1:YFP*$^{+/+}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1:YFP* construct in homozygous condition.

Consistent with this dominant effect of *cdka;1* mutant pollen on seed development I found that the transmission of the mutant *cdka;1* allele through the male side was strongly reduced: less than 20 percent of the F1 plants received their mutant *cdka;1* allele through the paternal side (**Table 2-2**).

Supporting the reciprocal crosses, the mutant allele was passed on through the female gametophyte to the F1 at a rate of close to 50 percent, as expected for an unbiased transmission (**Table 2-2**).

Table 2-2. Transmission of the *cdka;1-1* allele

Parental genotypes (female x male)	Genotype of F1 plants		n	TE[a]	defect[b]
	cdka;1$^{+/-}$	*CDKA;1*$^{+/+}$			
cdka;1-1$^{+/-}$ x *cdka;1-1*$^{+/-}$	48.6%	51.4%	208	n.a.	n.a.
cdka;1-1$^{+/-}$ x Col	46.6%	53.4%	131	0.87	6.4%
Col x *cdka;1-1*$^{+/-}$	17.4%	82.6%	132	0.21	39.4%

[a]TE transmission efficiency = number of mutant plants / number of wild-type plants as determined by PCR. [b]defective gametophytes in *cdka;1*$^{+/-}$ plants = ½(1 – TE) x 100%. n.a., not applicable; n, number of F1 plants scored; Col, wild type Columbia accession.

2.2.3 The pollen phenotype of *cdka;1*$^{+/-}$ mutants

In the wild type, microspores are generated by meiosis of microspore mother cells. The haploid and unicellular microspores undergo two rounds of mitosis before completing pollen development (**Fig. 2-4 a-e**). In heterozygous *cdka;1*$^{+/-}$ mutants, I observed no difference between wild-type and *cdka;1* mutant pollen development until the second pollen mitosis. About 40 percent of pollen at anthesis have failed to undergo the PMII, resulting in pollen with one vegetative and only one single other cell, hereafter referred to as *cdka;1* pollen (**Fig. 2-4f, Table 2-3**).

In the wild type, sperm cell differentiation includes nuclear DNA condensation (**Fig. 2-4 d, e**). The single sperm cell-like cell *of cdka;1* pollen appeared slightly larger and less condensed than sperm cell nuclei of wild-type pollen in DAPI staining (**Fig. 2-4 e, f**).

In order to investigate the nature of this single mutant cell, I compared pollen ultra-thin-sections under a transmission electron microscope in collaboration with Paul E. Grini from the University of Oslo (**Fig. 2-4 g-m**). We found that the single cell in *cdka;1* pollen differed from the sperm cell counterpart in mature wild-type pollen in having a less compact nucleus

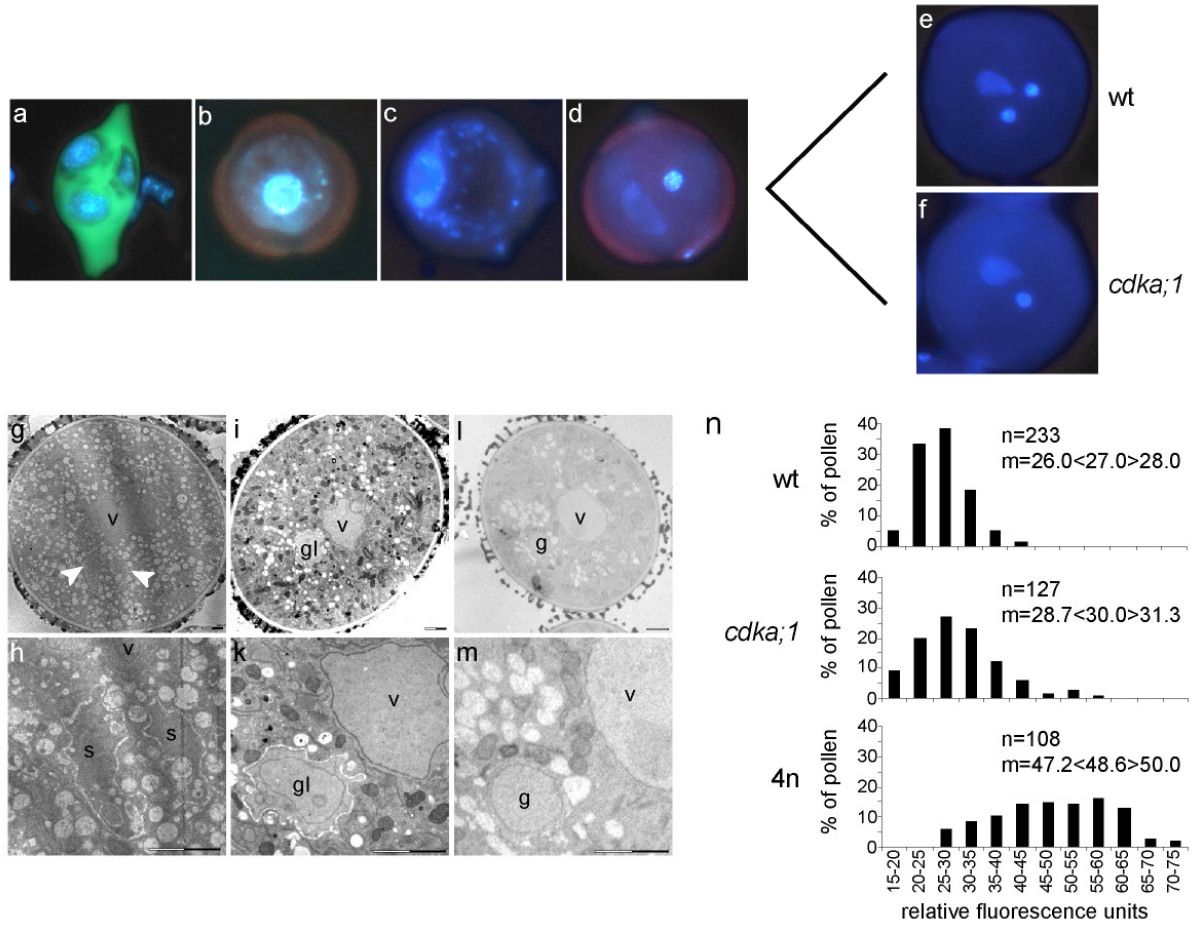

Figure 2-4. Phenotype of *cdka;1* mutant pollen. (a-f) 4',6-Diamidino-2-phenylindole (DAPI) staining of wild-type and *cdka;1* mutant pollen during gametophyte development. **(a)** Tetrad comprising four haploid microspores (counter-stained with aniline blue). **(b)** One-celled microspore. **(c)** Vacuolized one-celled microspore. **(d)** Two-celled pollen grain. **(e)** Mature wild-type pollen comprising one large vegetative and two small sperm cells. **(f)** Mature *cdka;1* mutant pollen with a vegetative and only one other cell. **(g-m)** Transmission electron micrographs of wild-type and *cdka;1* mutant pollen. **(g)** Mature three-celled wild-type pollen, arrow heads indicate the two sperm cells. **(h)** Close up of **g**, showing the two characteristic sperm cells. **(i)** Mature *cdka;1* mutant pollen with one vegetative and only one generative-like cell. **(k)** Close-up of **i**, showing the vegetative cell nucleus and the single generative-like mutant cell. **(l)** Two-celled wild-type pollen. **(m)** Close-up of **l**, showing the generative cell. **(n)** DNA measurements of sperm nuclei DNA content of wild type (wt) and tetraploid plants (4n) in comparison to the *cdka;1* mutant with only one generative-like cell at anther dehiscence. Scale bars: 2 µm. Abbreviations are: g, generative cell nucleus; gl, generative-like mutant nucleus; s, sperm cell nucleus; v, vegetative cell nucleus; wt, wild type; 4n, tetraploid pollen; n, number of sperm nuclei measured; m, mean value of relative fluorescence units within the 95 percent confidence interval determined by ANOVA.

(**Fig. 2-4 i, k**). On the whole, the ultrastructure of the single mutant cell resembled more that of the sperm cell precursor, the generative cell (**Fig. 2-4 l, m**). The structures of the vegetative cells in mutant and wild-type pollen, however, did not differ from each other (**Fig. 2-4 h-k**). This suggests that the sperm cell development of *cdka;1* pollen is retarded or arrested, whereas the vegetative cell differentiates as in wild type.

To determine in detail at what cell cycle stage *cdka;1* pollen got arrested, I measured the DNA content of *cdka;1* pollen at anther dehiscence. In cdka;1 pollen, the single, generative cell-like cell had a slightly but significantly higher DNA content than sperm cells of wild type

Table 2-3. Phenotype of *cdka;1* pollen at anther dehiscence

	normal (2 sperm cells)	aberrant (1generative-like cell)	n	expected aberrant (within 95% confidence limits)
Col	98%	2%	1380	n.a.
cdka;1-1$^{+/-}$	58%	42%	2195	36.0% < 39%[a] < 42.1%
cdka;1-2$^{+/-}$	60%	40%	282	34.8% < 39%[b] < 43.4%
resc *cdka;1*$^{+/-}$	61%	39%	200	34.8% < 39%[b] < 43.4%
resc *CDKA;1*$^{+/+}$	98%	2%	200	0.7% < 1.5%[c] < 5.1%
resc *cdka;1:yfp*$^{+/-}$	54%	46%	279	n.d.
resc *CDKA;1:YFP*$^{+/+}$	99%	1%	392	n.d.

[a]as determined by transmission rate. [b]as *cdka;1-1*$^{+/-}$. [c]as in wild type. n, number of pollen grains scored; n.a., not applicable; n.d., not determined; Col, wild type Columbia accession; resc *cdka;1*$^{+/-}$, resc *CDKA;1*$^{+/+}$, resc *cdka;1:yfp*$^{+/-}$, resc *CDKA;1:YFP*$^{+/+}$, as defined in table 2-1.

pollen at anther dehiscence. At this stage, wild-type pollen had halfway completed the final S phase and displayed a DNA content of about 1.5 C (Friedman 1999) (**Fig. 2-4m**). Conversely, my measurements showed a clear difference between the DNA content of the *cdka;1* generative cell-like cell and tetraploid pollen sperm cells with a presumed DNA content of 3 C at anther dehiscence (**Fig. 2-4m**). These findings point to an arrest of *cdka;1* pollen before PMII but after the preceding S phase so that the single cells in mutant pollen arrest at the with a DNA content of 2 C, just as wild-type sperm cells do prior to fertilization.

2.2.4 The homozygous *cdka;1*$^{-/-}$ mutants

The two-cell phenotype of *cdka;1* pollen is not fully penetrant: less than half the pollen of a heterozygous *cdka;1*$^{+/-}$ mutant lack a second sperm cell. Thus, there is an – albeit strongly reduced – transmission of the *cdka;1* allele through the male side.

Derived from the transmission rate of the *cdka;1* allele, there should be around eight percent of homozygous *cdka;1*$^{-/-}$ mutants segregating in the offspring of a heterozygous mutant. However, the complete lack of homozygous mutants in over 200 F1 plants tested suggested that the lack of CDKA;1 was fatal to the developing embryo.

To test this hypothesis, I compared seed abortion in *cdka;1*$^{+/-}$ selfed plants and wild type plants pollinated with pollen from *cdka;1*$^{+/-}$ plants. The rate of seed abortion in selfed *cdka;1*$^{+/-}$ mutants (47 percent) was slightly higher than the rate found in wild type x *cdka;1*$^{+/-}$ crosses (42 percent) (**Table 2-1**). Possibly, the higher rate of seed abortion in *cdka;1*$^{+/-}$ selfed siliques represented a combination of seeds dying of the fatal paternal effect of *cdka;1* pollen

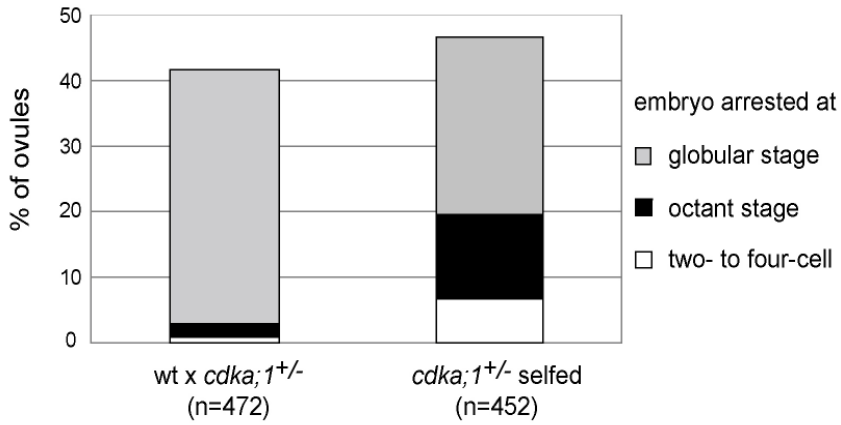

Figure 2-5. Homozygous *cdka;1* mutants abort early in embryo development. Diagram comparing the stages of embryo arrest in wild-type plants pollinated with *cdka;1* pollen to selfed *cdka;1*$^{+/-}$ plants. In selfed *cdka;1*$^{+/-}$ plants, in which one quarter of homozygous *cdka;1*$^{-/-}$ offspring is expected, a higher proportion of earlier embryo arrest (two- to four-celled and octant embryo) was found, consistent with a requirement of CDKA;1 for cell cycle progression.

and of seeds with embryos that aborted because they were homozygous for *cdka;1*.

In a comparison of *cdka;1*$^{+/-}$ selfed and wild type x *cdka;1*$^{+/-}$ five days after pollination (d.a.p.), the majority of aborted embryos in the cross developed until the globular stage, whereas a considerable portion of embryos in *cdka;1*$^{+/-}$ selfed plants arrested well before reaching the globular stage (**Fig. 2-5**).

Consistent with the absence of homozygous *cdka;1*$^{-/-}$ mutant seedlings from a segregating population, it seems likely that the lack of CDKA;1 in homozygous embryos causes a cell cycle arrest after one to a few cell divisions

2.3 Complementation assays

2.3.1 Expression of the *CDKA;1* cDNA from a 2 kb *CDKA;1* promoter fragment can rescue the *cdka;1* mutant

To secure the fact that alone the lack of a functional copy of *CDKA;1* caused the cell cycle arrest observed in *cdka;1* mutant pollen, I cloned a 2000 bp region upstream of the *CDKA;1* start codon as a promoter fragment ($Pro_{CDKA;1}$). This fragment was combined with the cDNA of *CDKA;1* and the construct $Pro_{CDKA;1}$*:CDKA;1* was stably transformed into heterozygous *cdka;1*$^{+/-}$ mutants.

From the transgenic T1 plants the heterozygous *cdka;1*$^{+/-}$ mutants were selected according to their genotype. Among the T2 offspring of these plants, I checked for transgenic plants that were homozygous for the T-DNA insertion causing the *cdka;1* mutation.

And indeed I found homozygous *cdka;1*$^{-/-}$ mutants rescued by the $Pro_{CDKA;1}$*:CDKA;1* construct. When these homozygous mutants carried the $Pro_{CDKA;1}$*:CDKA;1* insertion in a heterozygous fashion, they mimicked the pollen phenotype and the embryo abortion of the heterozygous *cdka;1*$^{+/-}$ mutants (**Tables 2-1, 2-3**). Furthermore, homozygous mutants with two $Pro_{CDKA;1}$*:CDKA;1* transgenic alleles displayed a full restoration of the wild-type conditions (**Tables 2-1, 2-3**).

2.3.2 Complementation by $Pro_{CDKA;1}$*:CDKA;1:YFP*

The fact of a full rescue of the *cdka;1* mutant by $Pro_{CDKA;1}$*:CDKA;1* opened the possibility of introducing a labelled version of the *CDKA;1* gene and thereby marking and tracking every functional *CDKA;1* molecule *in planta*. We chose the YELLOW FLUORESCENT PROTEIN (YFP), fused its coding sequence to the cDNA of *CDKA;1* and expressed it from the $Pro_{CDKA;1}$ promoter fragment (done by Nico Dissmeyer).

Just as $Pro_{CDKA;1}$*:CDKA;1*, the construct $Pro_{CDKA;1}$*:CDKA;1:YFP* was also able to fully complement the homozygous *cdka;1*$^{-/-}$ mutant if present in two transgenic allelescopies. With just one allele present, it mimicked the phenotype of the heterozygous *cdka;1*$^{+/-}$ mutant (**Tables 2-1, 2-3**).

This had the advantage that in a population segregating for the transgene, the CDKA;1 protein was labelled with YFP, and thus the absence of YFP could be equated with the absence of CDKA;1.

2.3.3 CDKA;1:YFP dynamics in pollen development

The *cdka;1* mutants complemented with the *Pro$_{CDKA;1}$:CDKA;1:YFP* construct offered the opportunity to follow the dynamics of a functional CDKA;1:YFP fusion protein during pollen development.

In homozygous transgenic plants after meiosis of the microspore mother cells (**Fig. 2-6a**), each of the generated microspores got a functional *Pro$_{CDKA;1}$:CDKA;1:YFP* allele (**Fig. 2-6b**). Thus, all the microspores and developing pollen grains showed YFP fluorescence in the cytoplasm and in the nucleus (**Fig. 2-6c**). In later development, concomitant with the exit of the vegetative cell from the active cell cycle, the YFP signal in the vegetative cell nucleus weakened while a strong signal persisted in the sperm cells (**Fig. 2-6d**).

Heterozygous transgenic plants did not differ from homozygous plants in their YFP expression in the premeiotic microspore mother cells (**Fig. 2-6e**). But even after meiosis, when individual microspores segregating for the *Pro$_{CDKA;1}$:CDKA;1:YFP* transgene were present, all microspores showed YFP fluorescence, albeit only half of them received the transgene (**Fig. 2-6f**). However, later in pollen development, ,in about half of the pollen, the YFP signal diminished and towards the end of pollen development vanished almost completely from about half the pollen grains (**Fig. 2-6 g, h**). Counterstaining with the DNA dye DAPI revealed that all pollen lacking a strong YFP-signal showed the typical two-cell phenotype of *cdka;1* mutant pollen (n=279) (**Fig. 2-6 i-k**). These results suggest that the CDKA;1:YFP fusion protein or its mRNA, or both, are carried over from the premeiotic microspore mother cells to mutant pollen lacking a functional *CDKA;1:YFP* transgene. This carry-over might account for why *cdka;1* mutant pollen are at all capable of progressing through at least the first, and in some cases even the second, pollen mitosis without a functional *CDKA;1* allele.

Carry-over on the female side could further help the female gametophyte to complete its development without a functional *CDKA;1* allele and might also explain why homozygous *cdka;1* zygotes still are able to undergo one or a few cell divisions forming an early embryo before they abort.

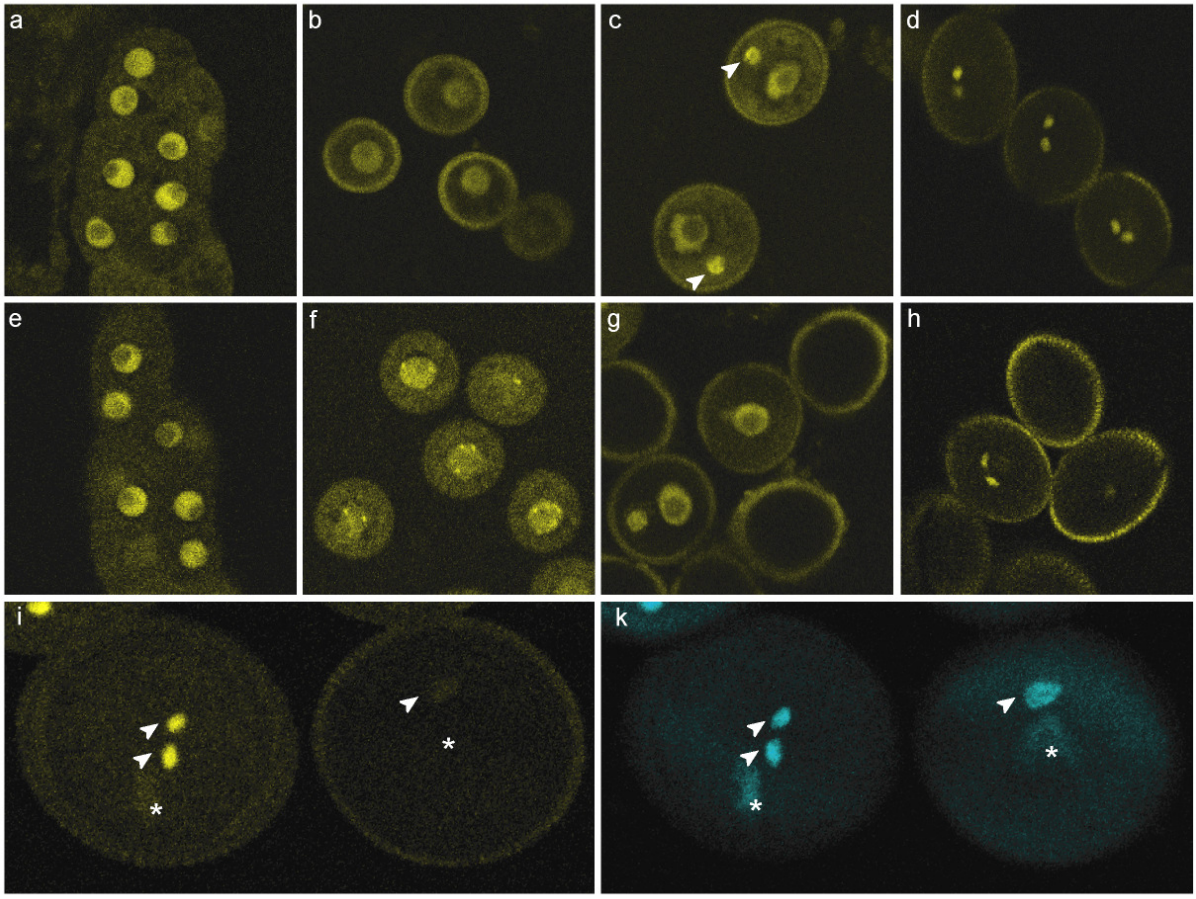

Figure 2-6. CDKA;1:YFP dynamics during pollen development. Homozygous *cdka;1*$^{-/-}$ mutants fully complemented by a *Pro*$_{CDKA;1}$*:CDKA;1:YFP* construct. In the complemented *cdka;1*$^{-/-}$ mutants, the dynamics of the functional CDKA;1:YFP fusion protein were traced by recording the yellow fluorescence. **(a-d)** Fluorescence micrographs of pollen development in a *cdka;1*$^{-/-}$ mutant carrying *Pro*$_{CDKA;1}$*:CDKA;1:YFP* in a homozygous fashion, thus allowing wild-type pollen development. **(a)** Microspore mother cells before meiosis. **(b)** One-celled microspores after meiosis and release from the tetrad. There was a strong YFP signal in the nucleus and a weaker signal in the cytoplasm. Note the autofluorescence of the pollen cell wall. **(c)** Two-celled pollen grains after pollen mitosis one (PMI). The small generative cell (arrowhead) was embedded within the lumen of the big vegetative cell and showed strong YFP fluorescence. **(d)** Mature three-celled pollen after pollen mitosis two (PMII). The generative cell had divided once more to form the two brightly fluorescent sperm cells. The generative cell had exited the cell cycle, mirrored by the disappearance of CDKA;1:YFP. **(e-k)** Fluorescence micrographs of pollen development in a *cdka;1*$^{-/-}$ mutant carrying *Pro*$_{CDKA;1}$*:CDKA;1:YFP* in a heterozygous fashion, thus copying the heterozygous *cdka;1*$^{+/-}$ mutant. **(e)** Microspore mother cells before meiosis. All cells were heterozygous for *CDKA;1:YFP*, and all showed YFP fluorescence. **(f)** One-celled microspores after meiosis. Although half of the microspores did not contain a *CDKA;1:YFP* transgene, all of them showed a strong YFP signal, arguing for carry-over of maternal mRNA or protein. **(g)** Two-celled pollen after PMI. Around and after PMI, the YFP signal started to get weaker in about half of the pollen grains, mirroring the segregation of the transgene. **(h)** In mature pollen grains, only a faint fluorescence was visible in half of the pollen grains, while the other half showed the same CDKA;1:YFP localization as in the homozygous complementation. **(i)** Two mature pollen grains in the heterozygous complementation, the left pollen grain showing a strong YFP fluorescence in the sperm cells (arrowheads) and a weak one in the nucleus of the vegetative cell (asterisk), the right pollen grain showing only one spot of weak YFP fluorescence (arrowhead). **(k)** The same pollen grains as in **i**, stained with DAPI to revealed the location and number of nuclei. The left pollen showing wild-type architecture with two sperm cells while the right pollen grain from *cdka;1* mutant was with only one generative-like cell.

2.4 The secondary phenotype of *cdka;1* mutants

2.4.1 *cdka;1* pollen is viable and able to germinate in vitro

In contrast to most other male gametophyte mutants described so far, *cdka;1* pollen seemed to have some dominant effect on seed development: The frequency of the two-celled phenotype in pollen of heterozygous *cdka;1*$^{+/-}$ mutants and of seed abortion caused by this pollen when used to fertilize a wild-type plant (both about 40 percent) (**Tables 2-1,- 2-3**), suggests a direct connection between pollen phenotype and seed abortion.

To test whether *cdka;1* pollen was still alive at anther dehiscence, I performed a viability test using fluorecein diacetate (FDA). The number of fluorescing and thus living pollen grains did not differ between pollen from *cdka;1*$^{+/-}$ mutants and from wild type (**Fig. 2-7 a-b**). Furthermore, two-celled *cdka;1* pollen was able to germinate in vitro and to transport the single generative cell-like cell through the pollen tube (**Fig. 2-7 c-f**).

Taken together, these findings suggest that mutant *cdka;1* pollen might be able to fertilize the female gametophyte and subsequently influence seed development. This classifies the *cdka;1* mutant as the first paternal effect mutant to be described in plants.

Given the viability of *cdka;1* pollen and its ability to germinate, it was conceivable that *cdka;1* pollen might be able to fertilize the female gametophyte. But even if one expected the single generative cell-like cell in *cdka;1* pollen to act as a functional gamete, the lack of the second sperm cell required for double fertilization should leave either the egg cell or the central cell unfertilized.

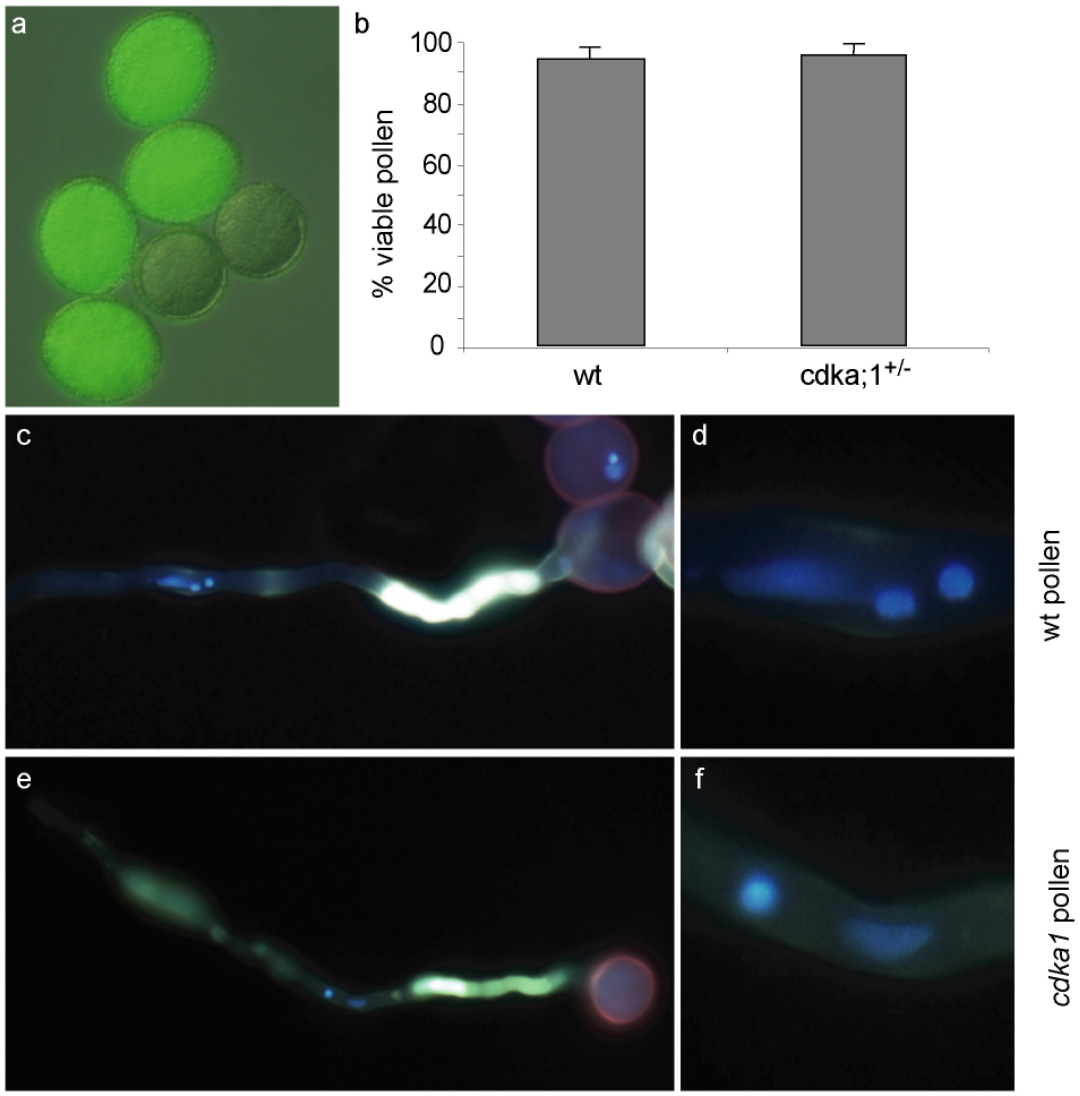

Figure 2-7. Viability and in vitro germination ability of *cdka;1* mutant pollen. (a) Fluorescence micrograph of mature pollen grains. Fluorescein diacetate (FDA) staining of *cdka;1* pollen did not show any differences in viability compared to wild-type pollen. Viable pollen grains showed bright green fluorescence, non-viable ones showed little or no fluorescence. **(b)** Quantification of viable pollen of wild type and heterozygous *cdka;1*$^{+/-}$ mutant plants, standard deviation of three counts are given, (n=100 per experiment and per genotype). **(c-f)** In vitro pollen germination. **(c-d)** Wild-type pollen. **(c)** One large vegetative and two small sperm nuclei were transported through the pollen tube. **(d)** Close-up of **b**. **(e-f)** *cdka;1* pollen. **(e)** Both vegetative nucleus and the single generative-like nucleus were transported through the pollen tube. **(f)** Close-up of **e**. Scale bars, 50 µm. Abbreviation: wt, wild type.

2.4.2 *cdka;1* pollen causes a single fertilization and exclusively fertilizes the egg cell

To test the hypothesis that fertilization with *cdka;1* pollen results in a single fertilization, we stably transformed heterozygous *cdka;1*$^{+/-}$ mutants with a fertilization reporter construct. The idea behind a fertilization reporter was to use a promoter that activates a reporter gene shortly after fertilization in both fertilization products, the embryo and the endosperm. If pollen from

a transgenic plant carrying such a construct is used to fertilize a wild-type plant, the reporter should only appear in the successfully fertilized structures that received a male genome.

For the fertilization reporter, I used the promoter fragment $Pro_{CDKA;1}$ described above and fused it to the *BETA-GLUCURONIDASE* (*GUS*) gene from *Escherichia coli*. The $Pro_{CDKA;1}$ conferred gene activity in both embryo and endosperm as early as 36 hours after pollination (h.a.p.).

Using wild-type pollen carrying the $Pro_{CDKA;1}$*:GUS* construct, blue staining was detectable in embryo and endosperm 36 h.a.p. in 98 percent of cases (n=83, **Fig. 2-8a**). However, when pollen of a *cdka;1*$^{+/-}$ mutant with the reporter construct was applied to wild-type plants, in 34 percent of cases (n=59), only the embryo showed blue staining (**Fig. 2-8 b-c**).

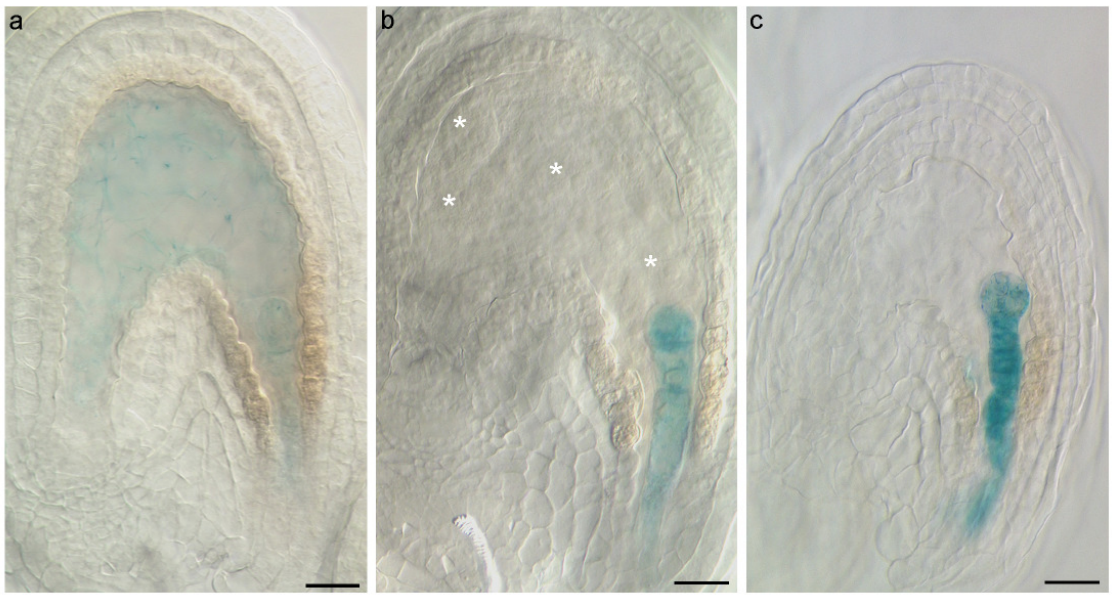

Figure 2-8. Seeds expressing the fertilization reporter $Pro_{CDKA;1}$*:GUS*. **(a)** Wild-type seed 36 h.a.p. with pollen carrying a $Pro_{CDKA;1}$*:GUS* fusion construct, showing blue GUS-staining in both fertilization products, the embryo and the endosperm. **(b)** Wild-type seed 36 h.a.p. with *cdka;1* mutant pollen carrying a $Pro_{CDKA;1}$*:GUS* fusion construct. Note blue staining exclusively in the developing embryo, as found in 34 percent of seeds displaying GUS-staining (n=59). Asterisks mark endosperm nuclei. **(c)** Wild-type seed 72 h.a.p. with *cdka;1* mutant pollen carrying a *proCDKA;1:GUS* fusion construct. Aborting seed with still showing GUS activity in the embryo.

These results allow the following interpretations: First, the *cdka;1* pollen and its single generative-like cell are indeed able to fertilize, and thus the single cell in *cdka;1* pollen acquires the function of a gamete. Second, the egg cell and not the central cell is the preferred

target of *cdka;1* fertilization since the selective GUS staining always occurred in the embryo, but not in the endosperm.

2.4.3 Development of unfertilized endosperm in wt x *cdka;1* seeds

The selective single fertilization of the egg cell by *cdka;1* pollen initiated seed development that showed a characteristic mutant phenotype in comparison to the wild type. In the wild type, after fertilization of the female gametophyte (**Fig. 2-9a**) regular seed development was started (**Fig. 2-9 b-e**): First, the zygote did not divide while the fertilized central cell nucleus underwent several rounds of nuclear divisions to form a syncytial endosperm (**Fig. 2-9b**). Around 36 h.a.p., the zygote divided for the first time, while the endosperm of 96 percent of the seeds (n=230) had already 32 or more nuclei (**Fig. 2-9c**). In the following hours the embryo established its basic architecture through a succession of highly coordinated cell divisions (**Fig. 2-9 d-e**). Meanwhile, the syncytial endosperm filled the whole of the central cell and differentiated in several domains, namely the micropylar (around the embryo), central peripheral, and chalazal domains (**Fig. 2-9 d-e**).

As wild type pollen, *cdka;1* pollen was capable of initiating seed development (**Fig. 2-9 f-i**). When pollen of a heterozygous *cdka;1*$^{+/-}$ mutant (in which some 40 percent had only a single generative-like cell) was used to fertilize wild-type plants, embryos formed and developed normally in almost all ovules (**Fig. 2-9 f-g**), until their arrest at the globular stage in about 38 percent of cases (**Fig. 2-9h**). Eventually, these seeds ceased to grow and started to decay (**Fig. 2-9i**).

Notably, in 92 percent of all *cdka;1*-fertilized seeds (n=374), not only the fertilized embryo initiated development, but also the unfertilized central cell nucleus started to divide and to form an endosperm-like structure composed of free syncytial nuclei (**Fig. 2-9f**). However, the division rate of this syncytium did not keep up with the wild-type endosperm: 24 percent of the wild type x *cdka;1*$^{+/-}$ seeds contained only 4-16 nuclei 36 h.a.p. (n=374, **Fig. 2-9g**). Slightly later, around 48 h.a.p., the endosperm-like nuclei in wild type x *cdka;1*$^{+/-}$ seeds started to decompose (**Fig. 2-9h**) soon followed by that of the embryo and the seed coat (**Fig. 2-9i**).

These results raise fundamentally new implications for the concept of early seed development in sexually reproducing flowering plants: The exclusive fertilization of the egg cell is

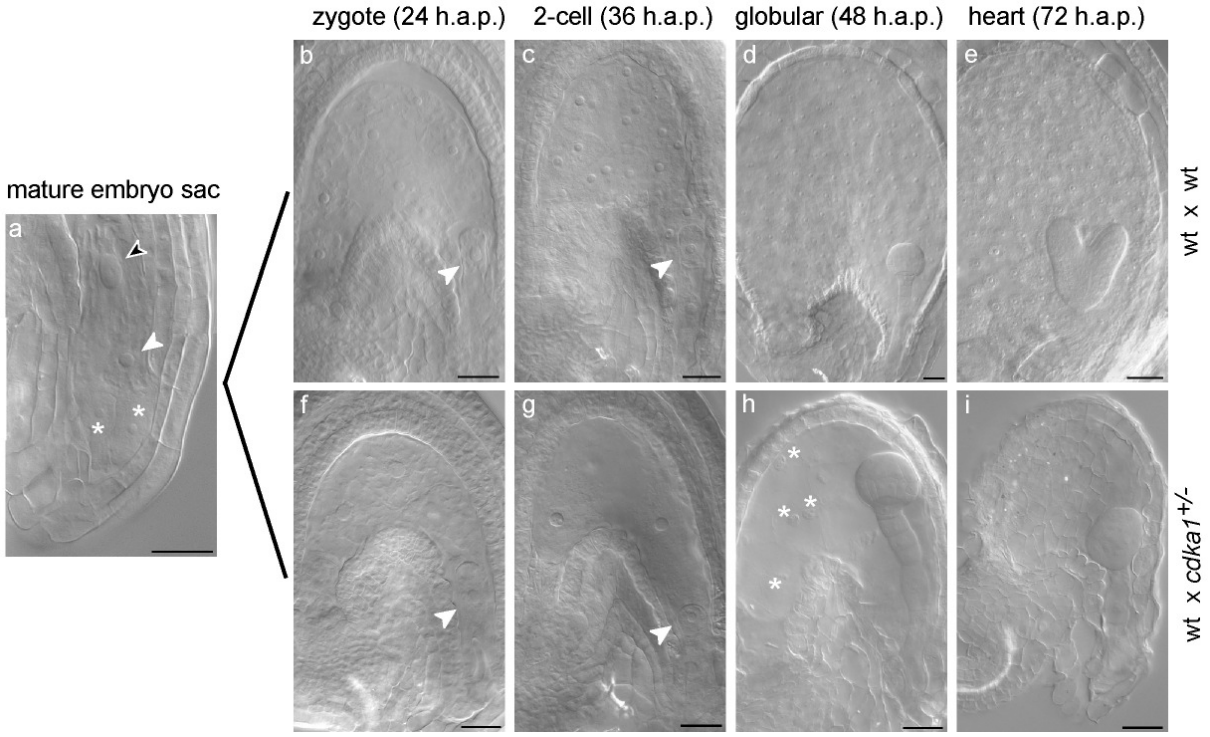

Figure 2-9. Seed development in wild-type plants fertilized with *cdka;1*$^{+/-}$ mutant pollen. (a) Wild-type mature embryo sac immediately before fertilization with synergid cells (asterisks), egg cell (white arrowhead) and central cell nucleus (dark arrowhead). **(b-e)** Wild-type embryo development during the first 72 h.a.p. **(b)** 24 h.a.p., zygote (arrowhead) with endosperm which has undergone three to four rounds of nuclear divisions. **(c)** 36 h.a.p., two-celled embryo (arrowhead); at this stage, 96 percent of the seeds contained endosperm with 32 or more nuclei (n=230). **(d)** 48 h.a.p., globular stage embryo with syncytial endosperm nuclei evenly distributed over the central cell. **(e)** 72 h.a.p., heart-stage embryo, the endosperm started to cellularize. **(f-i)** Seed development in wild-type plants pollinated with *cdka;1* mutant pollen. No evidence for aneuploidy and aborted mitoses, i.e. enlarged cells with multiple nuclei or irregular mitotic figures, were found. **(f)** 24 h.a.p., zygote (arrowhead) and central cell with 4 large endosperm nuclei. **(g)** 36 h.a.p., two-celled embryo surrounded by retarded endosperm displaying only 4-16 nuclei (24 percent of all seeds, n=374). **(h)** Seed 48 h.a.p., globular stage embryo and remnants of endosperm nuclei and cytoplasm in the central cell (asterisks). **(i)** 72 h.a.p., the embryo and the surrounding sporophytic tissue started to decay.

sufficient to allow seed development and double fertilization is not strictly necessary. Therefore, there must be a so far unrecognized signalling pathway that directs a positive proliferation signal to the central cell after the fertilization of the egg cell.

2.5 Combination of the *cdka;1* mutant with the *medea* mutant

2.5.1 wild-type x *cdka;1* seeds abort

Why do the seeds generated by *cdka;1* pollen arrest? There are at least two possibilities: Either the cell cycle arrest during *cdka;1* pollen development produces a single gamete which

can fertilize but remains deficient in some other way, leading to an imperfect embryo development that eventually caused its abortion; or the "single fertilization" by *cdka;1* pollen, although able to initiate proliferation of the unfertilized central cell nucleus, is not sufficient to produce a functional equivalent to the normal, fertilized endosperm.

The observations that the mutant *cdka;1* single gamete was able to fertilize the egg cell, and that the early embryo development in *cdka;1*-fertilized seeds did not differ from the wild-type gamete, argues against the deficiency of the single mutant *cdka;1* gamete.

Conversely, while the embryo developed normally during early seed development, the proliferation of the unfertilized central cell occurred much slower and less profusely than the proliferation of the fertilized endosperm. Thus, it seems probable that the seed abortion in wild type x *cdka;1* seeds is mainly due to the retarded and underdeveloped unfertilized endosperm.

2.5.2 *mea*$^{-/-}$ *x cdka;1*$^{+/-}$: a new class of developing seeds

To test this hypothesis, I used the Arabidopsis *medea* mutant (*mea*), which is defective in a PRC2 controlling endosperm proliferation: The endosperm of unfertilized ovules of *medea* mutant develops autonomously, i.e. independent of fertilization, whereas after fertilization the endosperm over-proliferates and the seeds die (Chaudhury et al., 1997; Grossniklaus et al., 1998).

mea$^{-/-}$ mutant plants were pollinated with *cdka;1*$^{+/-}$ pollen and, as a control, with Columbia wild-type (Col) pollen. As a further control, wild-type Landsberg *erecta* plants (L*er*, the accession background of the *mea* mutant) were fertilized with *cdka;1*$^{+/-}$ pollen.

While *mea*$^{-/-}$ x Col wild-type seeds developed normally until the embryo reached early heart stage (**Fig. 2-10a**), around 40 percent of L*er* x *cdka;1*$^{+/-}$ seeds were arrested and subsequently aborted with an embryo in the early globular stage (**Fig. 2-10b, Table 2-4**). In *mea*$^{-/-}$ *x cdka;1*$^{+/-}$ seeds, however, there was a new, intermediate class of developing seeds: They

Table 2-4. Seed development in *mea*$^{-/-}$ x *cdka;1-1*$^{+/-}$ plants

Parental genotype (female x male)	normal[a]	undeveloped	autonomous endosperm	aborted	n
L*er* x *cdka;1-1*$^{+/-}$	49%	7%	0%	43%	203
mea$^{-/-}$ x *cdka;1-1*$^{+/-}$	68%	5%	4%	22%	222
mea$^{-/-}$ x Col	89%	5%	1%	5%	336

[a]embryos at globular stage. L*er*, wild type Landsberg *erecta* accession; Col, Columbia accession.

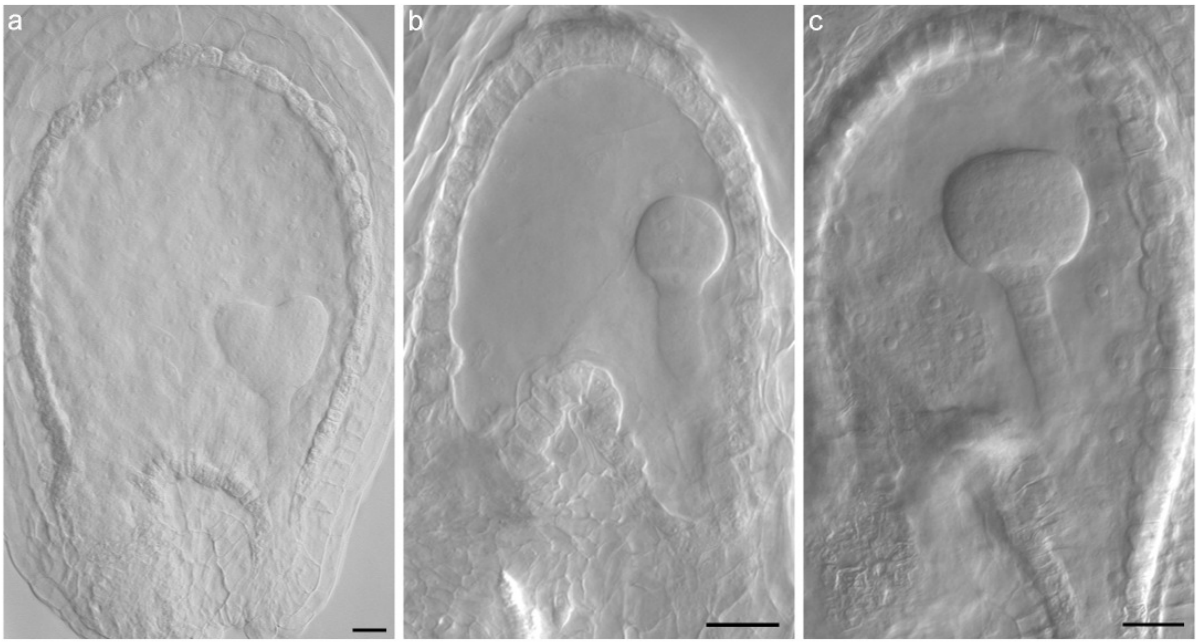

Figure 2-10. Seed development in *mea*$^{-/-}$ mutant plants fertilized with *cdka;1*$^{+/-}$ mutant pollen. **(a)** Seed of a homozygous *mea*$^{-/-}$ mutant after pollination with Col wild-type pollen. Seed with a heart-staged embryo and slightly overproliferated chalazal endosperm. **(b)** Seed of a L*er* wild-type plant after pollination with *cdka;1* mutant pollen showed the same early abortion phenotype as Col wild type after pollination with *cdka;1* pollen. **(c)** Seed of a homozygous *mea*$^{-/-}$ mutant after pollination with *cdka;1* pollen. Note the proliferating endosperm and the advanced stage of embryo development in comparison to the seed depicted in b which is arrested with a globular stage embryo.

were much smaller than the wild type but showed a partial rescue of the abortion conferred by *cdka;1*. In these seeds, the embryos developed much further and there was substantially more endosperm (**Fig. 2-10c**). The fraction of *mea*$^{-/-}$ *x cdka;1*$^{+/-}$ embryos that still aborted at early globular stage was reduced by 50 percent (**Table 2-4**).

These findings suggest that indeed the underdeveloped endosperm in wild type x *cdka;1* seeds is at least one major factor responsible for the early embryo abortion. By raising the quantity of endosperm nuclei using the *fis*-class mutant *mea* as the female partner, it was possible to obtain a considerable rescue rate of the *cdka;1* embryo abortion phenotype.

Notably, the onset of autonomous endosperm proliferation in unfertilized *mea*$^{-/-}$ ovules was delayed and rather rare compared to endosperm proliferation in fertilized seeds. Under the growth conditions in our laboratory, endosperm started to develop autonomously in only 11 percent of all *mea*$^{-/-}$ ovules when inspected five days after the unpollinated flowers had reached maturity (**Table 2-5**). On the contrary, in *mea*$^{-/-}$ mutants pollinated with pollen of a *cdka;1*$^{+/-}$ mutant (in which about 40 percent of the pollen grains confer a single fertilization of the egg cell), endosperm developed in 92 percent of all seeds five days after pollination of mature flowers (**Table 2-5**). This result indicates that the presumed proliferation signal emitted by the fertilization of the egg cell promotes and accelerates endosperm development

in a *mea*$^{-/-}$ mutant background. Furthermore, this suggests that the onset of autonomous endosperm development in *mea*$^{-/-}$ mutants may be retarded owing to the lack of an instructive signal from a fertilized egg cell.

Finally, these results demonstrate that there are two major signalling pathways controlling early seed development, the positive signal from the fertilization of the egg cell and the repression of central cell proliferation embodied by the action of the *FIS*-class genes that instruct early seed development.

Table 2-5. Endosperm development in mea$^{-/-}$ x *cdka;1*$^{+/-}$ plants

Parental genotype (female x male)	developed endosperm	undeveloped endosperm	non-analyzable	n
mea$^{-/-}$ unfertilized	11%	74%	15%	153
mea$^{-/-}$ x *cdka;1*$^{+/-}$	92%	7%	1%	60

2.5.3 *mea x cdka;1* seeds can undergo complete development and develop into normal F1 plants

To study the *mea*$^{-/-}$ *x cdka;1*$^{+/-}$ rescue in more detail, I followed the embryo development from 2 d.a.p. up to 12 d.a.p..

In wild-type seeds (L*er* x Col), this time span covers embryo development from zygote to a nearly mature embryo. In the same period, the endosperm proliferates, differentiates and is finally mostly consumed by the growing embryo.

To follow the fate of ovules fertilized with cdka;1 mutant pollen, I used the *Pro$_{CDKA;1}$:CDKA;1:YFP* lines described in section 2.3.2.

I selected homozygous *cdka;1*$^{-/-}$ mutants that were heterozygously complemented by the *Pro$_{CDKA;1}$:CDKA;1:YFP* transgene to mimic heterozygous *cdka;1*$^{+/-}$ mutants, in which about 40 percent of the pollen displayed a mutant phenotype. From here on, I will refer to these plants as *cdka;1:yfp*$^{+/-}$. To mimic the wild type, I selected homozygous *cdka;1*$^{-/-}$ mutants that were homozygously complemented by the *Pro$_{CDKA;1}$:CDKA;1:YFP* transgene, hereafter referred to as *CDKA;1:YFP*$^{+/+}$.

The wild type-like pollen segregating from *cdka;1:yfp*$^{+/-}$ plants and its progeny are marked by a YFP signal (**Fig. 2-6 i-k, Fig. 2-11a**). Conversely, *cdka;1* mutant-like pollen and its fertilization products do not show any YFP signal (**Fig. 2-6 i-k, Fig. 2-11b**).

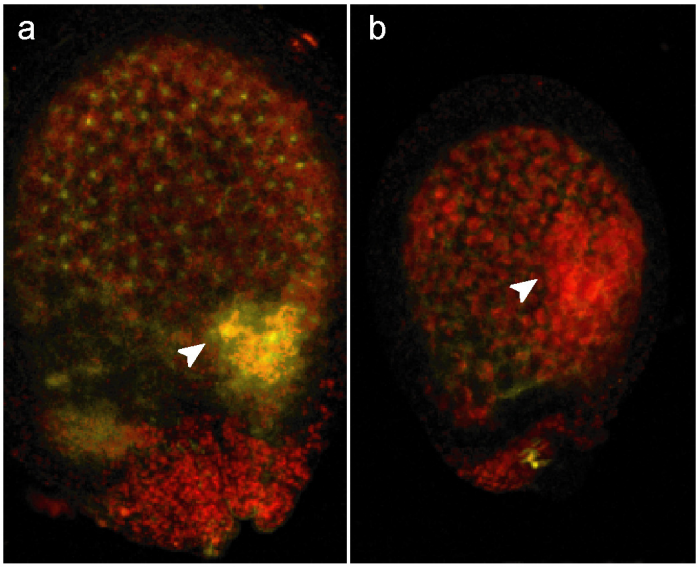

Figure 2-11. Expression of the *Pro$_{CDKA;1}$:CDKA;1:YFP* transgene after fertilization to the *mea*$^{-/-}$ mutant. Projections of Z-series of confocal sections displaying YFP fluorescence and red autofluorescence. **(a)** In normal sized seeds that almost invariably aborted due to the maternally-inherited *mea* allele, both the arrested heart stage embryo (arrowhead) and the endosperm were marked by YFP, indicating the fertilization by wild-type like YFP positive pollen. **(g)** In small seeds showing a rescue of the *mea*-mediated embryo abortion, the YFP signal was absent in the embryo (arrowhead), thereby marking these seeds as products of fertilization by YFP-negative *cdka;1* mutant pollen.

While L*er* x *cdka;1:yfp*$^{+/-}$ seeds that expressed the *CDKA;1:YFP* construct developed like wild-type seeds (**Fig. 2-12 a-d**), YFP-negative seeds aborted around 3 d.a.p. Consistent with earlier data about the *cdka;1* mutant obtained with crosses to Col wild-type plants, these YFP-negative seeds contained an early globular stage embryo surrounded by an underdeveloped endosperm (**Fig. 2-12 e-h**).

As a control, I also investigated seed development in *mea*$^{-/-}$ *x CDKA;1:YFP*$^{+/+}$ seeds which mimicked *mea*$^{-/-}$ x Col crosses. Like all *fis*-class mutants, fertilized *mea*$^{-/-}$ seeds initially developed like the wild type, but were blocked in embryo development around early heart stage and aborted with an overproliferated endosperm (**Fig. 2-12 i-m**). *fis*-class mutants are typical maternal effect mutants: The seed abortion phenotype only occurs if the mutant allele is inherited via the female gametophyte and even a wild-type pollen cannot rescue it (Autran et al., 2005).

When analysing seeds from the cross of the homozygous *mea*$^{-/-}$ mutant with heterozygous *cdka;1:yfp*$^{+/-}$, I found that more than 20 percent of all seeds overcame the point of abortion caused by *cdka;1* fertilization (**Fig. 2-12 n, o**). The same result was obtained when using a *fis* mutant in combination with heterozygous *cdka;1*$^{+/-}$ mutant (without the *CDK:YFP* transgene, compare) (**Fig. 2-10**).

Strikingly, in later stages of seed development about 20 percent (n=134 at 12 d.a.p.) of the *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$, seeds bypassed the *mea*-block around 6 to 7 d.a.p. and developed into mature seeds (**Fig. 2-12 p-q**). These seeds showed a distinctive phenotype: From 4 to 5 d.a.p.

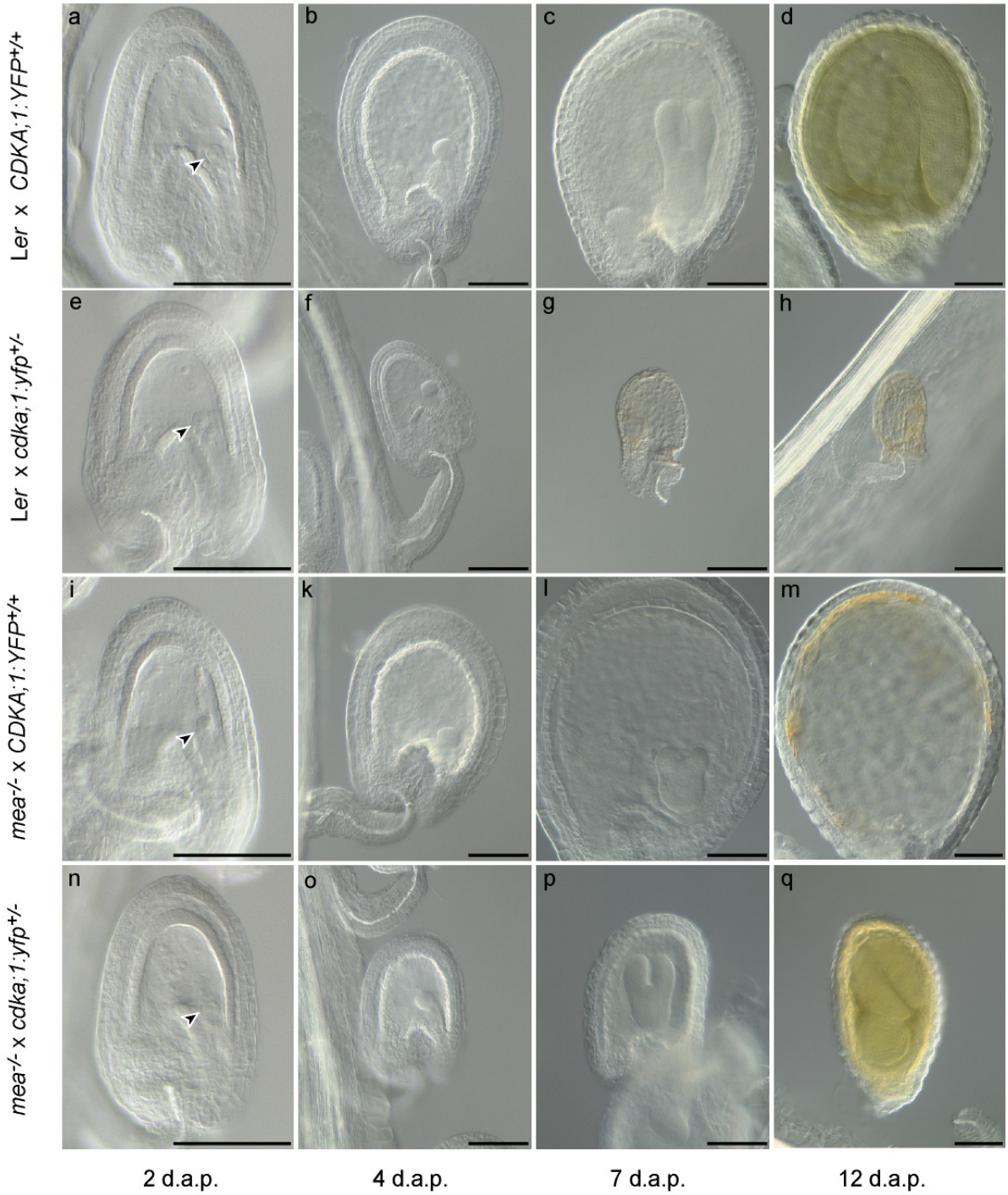

Figure 2-12. Rescue of *mea*-conferred embryo abortion after pollination with *cdka;1* mutant pollen. (a-d) Seed development in L*er* x *CDKA;1:YFP*$^{+/+}$. **(a)** 2 d.a.p., seed with a two-celled embryo (arrowhead) surrounded by a number of endosperm nuclei. **(b)** 4 d.a.p., globular stage embryo with syncytial endosperm nuclei. **(c)** 7 d.a.p., seed with torpedo stage embryo. **(d)** 12 d.a.p., the seeds approached maturity and the embryo occupied most of the space in the seed. **(e-h)** Seed development in L*er* x *cdka;1:yfp*$^{+/-}$, YFP-. **(e)** 2 d.a.p., a seed with a two-celled embryo (arrowhead) and a reduced number of endosperm nuclei. **(f)** 4 d.a.p., seed development was arrested at preglobular embryo stage, the endosperm was decaying. **(g)** and **(h),** 7 d.a.p. and 12 d.a.p., respectively, the aborted seed decayed. **(i-m)** Seed development in *mea*$^{-/-}$ x *CDKA;1:YFP*$^{+/+}$. **(i)** 2 d.a.p., seed with a two-celled embryo (arrowhead) surrounded by a number of endosperm nuclei, no deviation from the wild type is visible. **(k)** 4 d.a.p., globular stage embryo with syncytial endosperm nuclei, similar to wild type. **(l)** 7 d.a.p., seed with an abnormal heart stage embryo typical for *mea*-conferred embryo abortion. **(m)** 12 d.a.p., the embryo was arrested at the heart stage, the seeds were decaying and eventually collapsed. **(n-q)** Seed development in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$, YFP-

Figure legend continued on the next page.

on, the rescue seeds were markedly smaller than the wild type, but the embryos within them were almost as big as that of the wild type. The amount of endosperm was greatly reduced. From the torpedo stage on, also the embryos fell behind in growth (**Fig. 2-12 p-q**). Nevertheless, most of these embryos continued to develop according to their programme, although some failed to bend their cotyledons and remained stretched out in the mature seed. Nevertheless, at the end of seed development, around 17 percent of all seeds appeared viable, i.e. they were light brown and plump, unlike *mea*-aborted seeds which looked dark and shrivelled by the time they reach maturity. By contrast, in *mea* x wild type control crosses, less than one percent of viable-looking seeds was observed (**Table 2-6**).

Table 2-6 Viable seeds in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$

	viable seeds	dead seeds	n
mea$^{-/-}$ x Col	<1%	>99%	1526
mea$^{-/-}$ x *cdka;1*$^{+/-}$	17%	83%	1753

Table 2-7 Flowering time in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1-plants

genotype of parents (female x male)	mean (d.a.g)	stdev	n	subset[a]
L*er* self	31	1.4	27	1
L*er* x Col	35	1.3	28	2
L*er* x *CDKA;1:YFP*$^{+/+}$	35	1.5	24	2 and 3
mea$^{-/-}$ x *CDKA;1:YFP*$^{+/+}$	36	3.6	27	2 and 3
mea$^{-/-}$ self	36	3.9	27	2 and 3
mea$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ (YFP+)	37	3.8	28	3
mea$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ (YFP-)	41	4.2	28	4

[a]homogeneous subsets as determined by a Student-Newman-Keuls test. d.a.g., days after germination; stdev, standard deviation; n, number of F1 plants scored. *CDKA;1:YFP*$^{+/+}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1:YFP* construct in homozygous condition; *cdka;1:yfp*$^{+/-}$, homozygous *cdka;1-1*$^{-/-}$mutant plants, complemented with a *proCDKA;1:CDKA;1:YFP* construct in heterozygous condition, YFP+, offspring with YFP signal; YFP-, offspring without YFP signal.

← **Figure 2-12. continued: (n)** 2 d.a.p., seed with a two-celled embryo (arrowhead) surrounded by a number of endosperm nuclei, no deviation from the wild type morphology. **(o)** 4 d.a.p., globular stage embryo with syncytial endosperm nuclei, some of the seeds lag behind in size development. **(p)** 7 d.a.p., about 25 % of the seeds (n=112) are smaller than wild type but the embryo development in these small seeds passes the threshold of *mea* abortion **(q)** 12 d.a.p., about 10% of the seeds (n=134) contain small, yet ordinarily shaped embryos. Abbreviations are: YFP-, absence of the *CDKA;1:YFP* transgene in the seeds. d.a.p., days after pollination. Scale bars are 100 µm.

The ripe *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ seeds that appeared viable were significantly smaller than the seeds of wild-type and of control crosses like *mea*$^{-/-}$ x wild type (**Fig. 2-13a**). On agar plates around 90 percent of these small, viable-looking seeds germinated. Although the germination rate of viable *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ seeds was lower than that of L*er* x Col seeds, it showed that most of the small rescued seeds were viable and able to geminate (**Fig. 2-13b**).

2.5.4 Post-embryo development of *mea*$^{-/-}$ x *cdka,1*$^{+/-}$ F1 plants

In a series of experiments, I investigated some post-embryonic features of the F1 plants stemming from the small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds.

When put on vertical agar plates, root growth could be assessed during the first few days of growth. The small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds gave rise to small embryos with shorter roots than the ones of the L*er* x Col wild-type control. During the first days of growth, although the *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ roots showed a slightly enhanced growth rate, the wild-type roots stayed clearly longer (**Fig. 2-13 c-e**).

The difference in plant size persisted throughout early vegetative development. Typical *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ plants and the corresponding controls at 28 d.a.g. are depicted in **Fig. 2-13 f-l**. The small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ plants, continued growing vegetatively while the controls had already undergone transition to reproductive development and also flowered significantly later than the controls (**Table 2-7**).

After the plants died, I recorded the dry weight of the plant shoots as a measure of their final size (**Fig. 2-13m**). The shoot biomass of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ plants was not significantly different from the ones of the *mea*$^{-/-}$ x Col control crosses.

These results indicate that the small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds give rise to smaller seedlings, but that despite their slowed development, they will have reached the same final size as plants generated by control crosses when full-grown.

2.5.5 *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ results in a mutual rescue

To test to what extent the increase in viable seeds in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses was associated with a pollination by the *cdka;1* mutant pollen, I determined the genotype of the corresponding F1 seedlings. I found that more than 90 percent of the small rescue seeds in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ did not carry a *CDKA;1:YFP* transgene and therefore had received its paternal genome from a *cdka;1* mutant-like pollen. When crossed to the wild type, pollen of a heterozygous *cdka;1:yfp*$^{+/-}$ mutant produced only six percent of F1 plants which did not carry the *CDKA;1:YFP* transgene (**Table 2-8**).

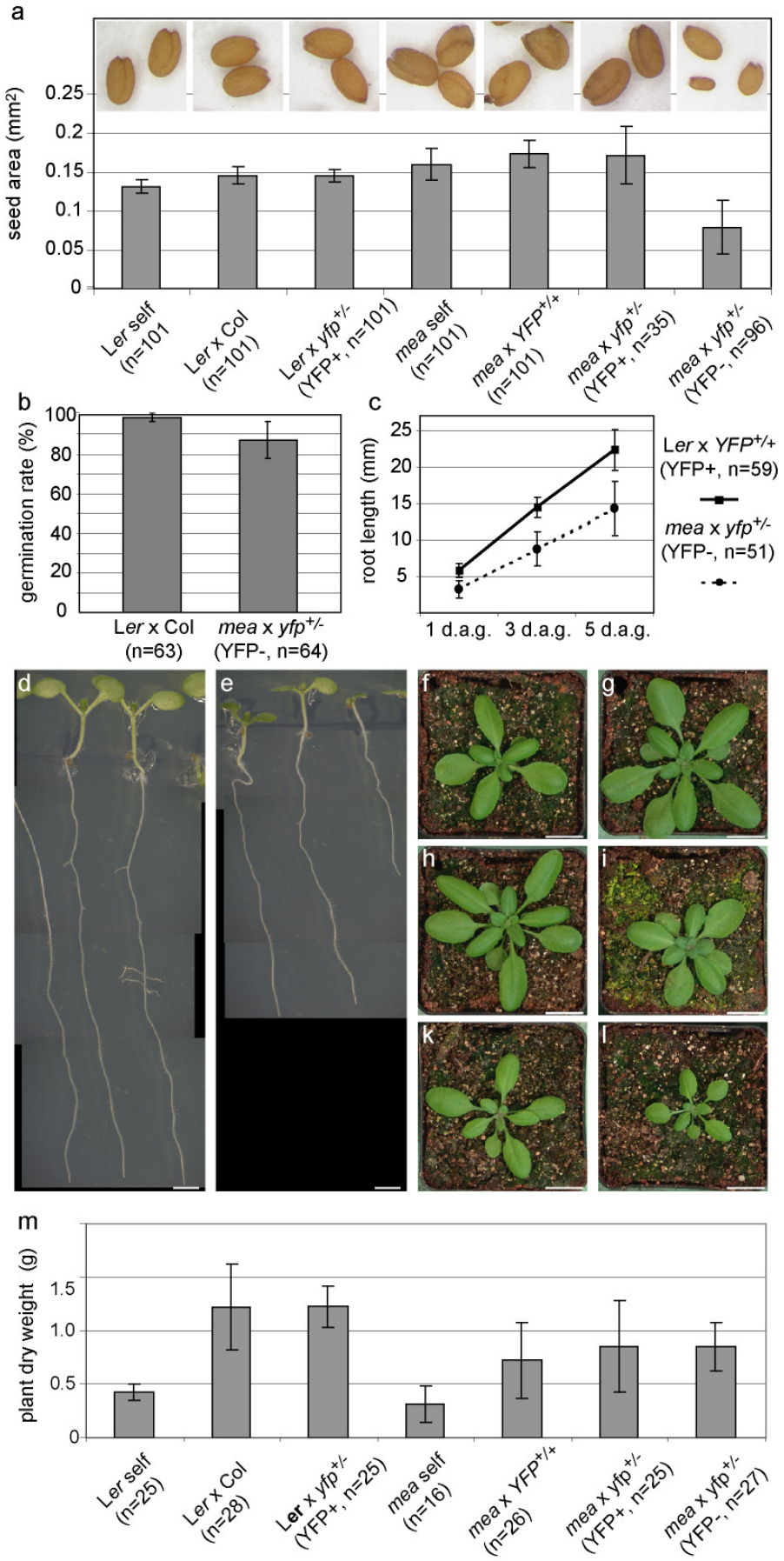

Figure 2-13. Post-embryo development of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 plants, the description is given on the next page.

Table 2-8. Transmission frequencies of the *cdka;1* mutant allele

Genotype of cross	Genotype of progeny			association factor[a]	n
	cdka;1$^{+/-}$	*CDKA;1*$^{+/+}$	*fis-class*$^{+/-}$		
Col x *cdka;1:yfp*$^{+/-}$	6%	94%	n.a.	n.a.	412
mea $^{-/-}$ x *cdka;1:yfp*$^{+/-}$	92%	8%	100%	92	186
Col x *cdka;1*$^{+/-}$	10%	90%	n.a.	n.a.	120
mea$^{-/-}$ x *cdka;1*$^{+/-}$	92%	8%	100%	92	142
fis2-4$^{+/-}$ x *cdka;1*$^{+/-}$	39%	61%	n.d.	n.d.	144
fie11$^{+/-}$ x *cdka;1*$^{+/-}$	36%	64%	18%	100	138

[a]association factor: number of F1 plants carrying both a *fis*-class mutant allele and a *cdka;1* mutant allele divided by the total number of plants carrying a *fis*-class mutant allele in percent. n, number of F1 plants scored; *fis*$^{+/-}$-class: heterozygous mutants for *mea*, *fis2*, or *fie*. *cdka;1:yfp*$^{+/-}$, homozygous *cdka;1*$^{-/-}$ mutant complemented with a heterozygous *Pro*$_{CDKA;1}$*:CDKA;1:YFP*$^{+/-}$ transgene; n.a., not applicable; n.d., not determined.

← **Figure 2-13. Post-embryo development of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 plants (a)** Seed size of *mea*$^{-/-}$ *x cdka;1:yfp*$^{+/-}$ F1 seeds. Seeds of the *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ cross that are YFP-negative were significantly smaller than seeds of the same cross that carry YFP. The bars show the mean values, error bars represent the standard deviation. Scale, all pictures cover a width of 1000 µm **(b)** Germination of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 seeds. F1 seeds of L*er* x Col and *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses were selected according to the criterion whether they were viable-looking, i.e. light brown and plump, or not. Viable-looking seeds were tested for germination on agarose plates. In L*er* x Col, 99 % of the seeds germinated, while in *mea*$^{-/-}$ *x cdka;1:yfp*$^{+/-}$, around 88 % of the small rescue seeds germinated. Error bars represent the standard deviation between three independent crosses. **(c)** Root growth rate of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 plants. F1 seedlings of L*er* x Col and *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses were put on vertical agarose. Root growth was measured at 1, 3, and 5 days after germination (d.a.g). Late germinating seedlings were not taken into account. YFP negative seedlings from *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses derived from small rescue seeds had shorter roots than the L*er* x Col control at 1 d.a.g.. **(d-e)** Typical F1 plants of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ and control crosses at 7 d.a.g. on agarose plates. **(d)** L*er* x Col. **(e)** The roots of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ were still markedly shorter than the ones of L*er* x Col plants. **(f-l)** F1 plants of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ and control crosses 28 d.a.g. **(f)** L*er* self. **(g)** L*er* x Col. **(h)** L*er* x *cdka;1:yfp*$^{+/-}$, YFP positive. **(i)** *mea*$^{-/-}$ self. **(k)** *mea*$^{-/-}$ x Col. **(l)** *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$, YFP negative. The YFP negative *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ plants were markedly smaller than the control plants. **(m)** Final biomass of the *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 offspring. Dry weight of the shoots without the rosette leaves of *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 plants and the control groups were measured after the plants had died and dried out. Plants grown from the small YFP-negative *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ seeds finally reached the same size as the ones that carrying YFP and the corresponding wild-type controls. Note that the crosses between different Arabidopsis accessions tended to accumulate more biomass, probably due to heterosis effects. Note also the size difference between F1 of a L*er* mother to the F1 plants of a *mea*$^{-/-}$ mother. The bars show the mean values, error bars represent the standard deviation. Scale bars are 1 cm. Abreviations are: *mea*$^{-/-}$, homozygous *medea* mutant;YFP+, *mea*$^{-/-}$ *x cdka;1:yfp*$^{+/-}$ F1 seeds with a YFP signal, therefore representing seeds created by wild-type-like pollen ; YFP-, *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ F1 without YFP signal, therefore representing seeds created by *cdka;1* mutant-like pollen; n, number of plants or number of seeds measured.

This result suggested that the combination of *cdka;1* mutant pollen and *mea* mutant autonomous endosperm did not only lead to a rescue of *cdka;1*-mediated seed abortion, but also of the seed abortion conferred by the mutant *mea* allele – representing a mutual rescue situation in the double mutant seed.

As almost all of the F1 seedlings in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses were products of a fertilization with the *cdka;1* mutant pollen and most of this pollen contained only one gamete causing a single fertilization event, it seemed most probable that the mutual rescue in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses was connected to the exclusive fertilization of the egg cell that left the central cell unfertilized. This gave rise to the hypothesis that the endosperms in the small rescue seeds are homodiploid, and only of maternal, i.e. female gametophytic origin.

2.5.6 Flow cytometry

To test whether the endosperm in the small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds is homodiploid rather than triploid, I analysed the DNA profile of these seeds by flow cytometry.

In contrast to sporophytic tissue like leaves for which a ploidy analysis revealed only diploid species of nuclei (i.e. 2 C, 4 C and the endoreplicated nuclei of 8 C and 16 C DNA content), in wild type seeds, additional peaks of triploid endosperm (3 C, 6 C) were observed (**Fig. 2-14 a-b**). The endosperm peaks in the preparations of wild-type seeds at 6 d.a.p. were very prominent, according to the high amount of endosperm contained in wild-type seeds at this developmental stage.

Even in much younger wild-type seeds which contained only little endosperm (about as much as the small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds), there were clear triploid peaks, confirming the sensitivity of our detection method (**Fig. 2-14c**).

In the small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds, however, I could only observe peaks of diploid tissue, suggesting that the endosperm in these seeds, like the seed coat and the embryo, was diploid and therefore probably unfertilized (**Fig. 2-14d**).

2.5.7 SSLP based paternity test

To further test the hypothesis that an unfertilized, diploid endosperm developed in the *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue, I designed a paternity test based on DNA sequence polymorphisms that should detect the presence or absence of genetic material from different Arabidopsis ecotypes (accessions). Single Sequence Length Polymorphisms (SSLPs) between Arabidopsis accessions are frequently used to differentiate genetic material of different accessions.

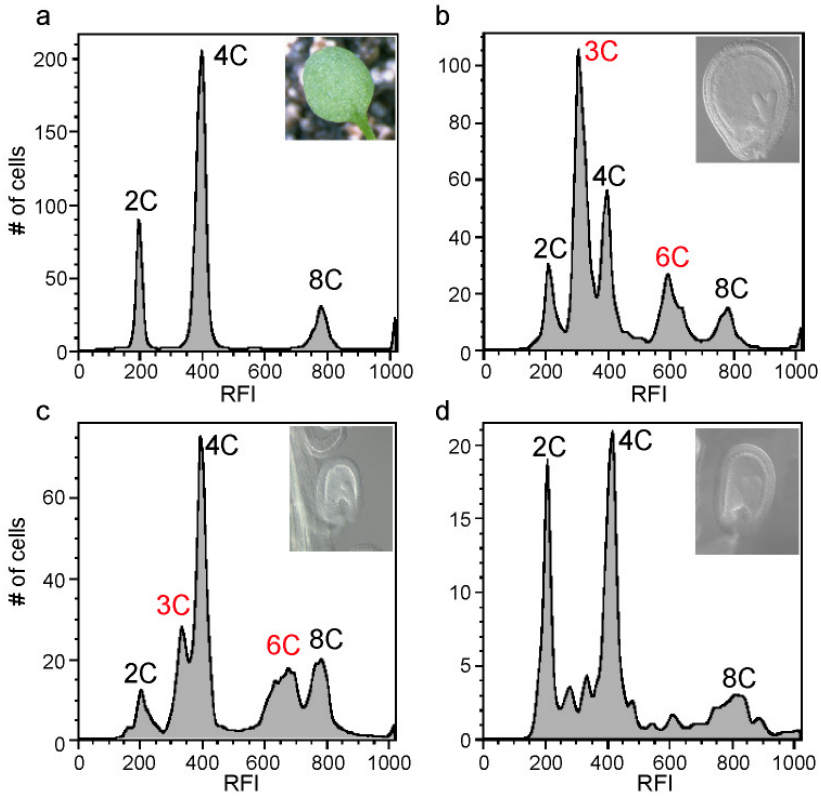

Figure 2-14. Flow cytometry of *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds. Flow cytometry ploidy analysis of leaves and seeds. **(a)** A typical DNA profile generated by diploid sporophytic tissue, in this case a leaf. There were diploid cells before (2 C) and after (4 C) mitosis, and endoreplicating cells (8 C, 16 C). **(b)** DNA profile of a wild type seed at 6 d.a.p., in addition to the peaks of the diploid seed coat and embryo (2 C, 4 C, 8 C), there were peaks representing the triploid endosperm (3 C, 6 C). **(c)** DNA profile of a young wild type seed at 2 d.a.p., even the limited amount of triploid endosperm produced clear triploid peaks (3 C, 6 C). **(d)** DNA profile of the typical small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue seeds at 6 d.a.p., no peaks indicative of triploid tissue were detected.

The small *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ rescue seeds offered the advantage that the *mea* mutant is in a different accession (Landsberg *erecta*, L*er*) than the *cdka;1* mutant (Columbia, Col).

Any fertilization product between these two accessions should contain both DNA polymorphisms which are detectable in a PCR as products of different sizes.

First, I pollinated L*er* plants with Col pollen and harvested the seeds 6 d.a.p. These seeds were fixed and embedded in paraffin, subsequently cut in 11 µm thick sections using a microtome and mounted on plastic-coated slides used for laser dissection microscopy (LDM). By LDM, I separately isolated tissue from the embryos and the endosperms of individual seeds (**Fig. 2-15 a-c**) and used it as a template in an SSLP-PCR.

And indeed the PCR produced both the L*er*- and the Col-specific bands in almost all L*er* x Col embryos and endosperms tested. For seeds of *mea*$^{-/-}$ x Col crosses, I obtained similar results. However, while small rescue seeds from *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses showed

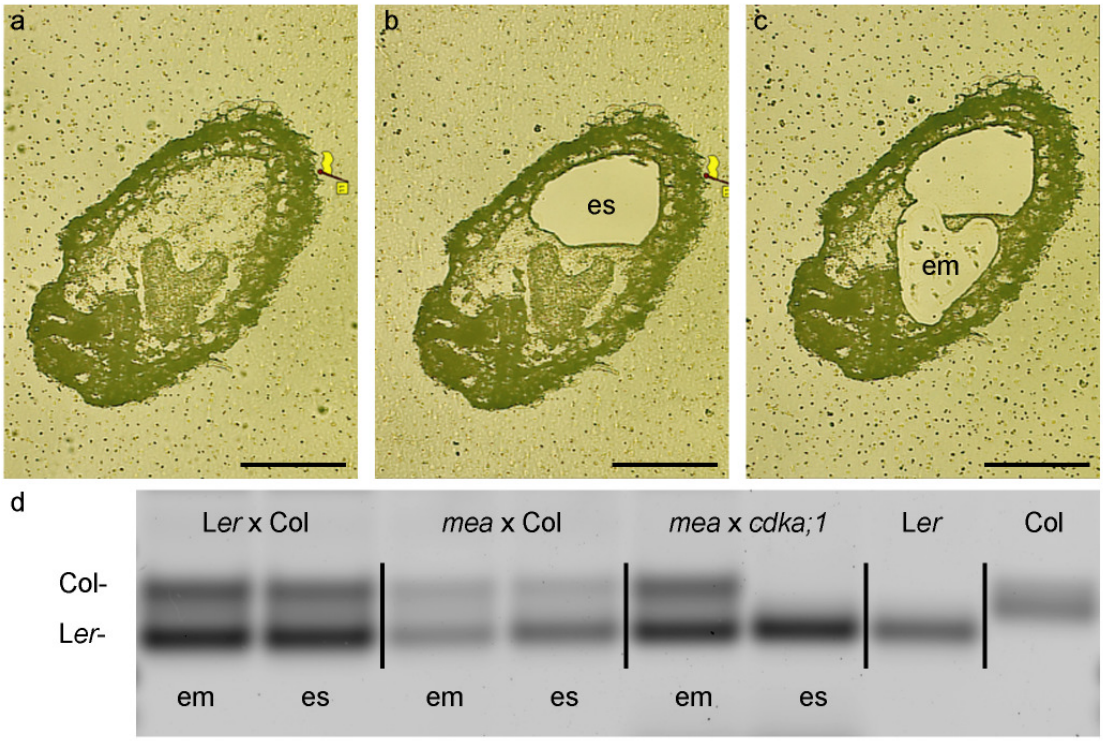

Figure 2-15. Laser microdissection of small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue seeds at 6 d.a.p. **(a-c)** Section of a typical small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue seed at 6 d.a.p. mounted on a plastic coated slide for laser microdissection. **(a)** Section before laser dissection. **(b)** The same section after collecting the peripheral endosperm. **(c)** The same section after collecting the heart-shaped embryo. **(d)** Agarose gel showing the amplification products of accession-specific PCR detecting simple sequence length polymorphisms with the marker NGA6. The L*er* genome was represented by a smaller (low running) band while the Col genome produced a larger (high running) fragment. 90% of the embryos and endosperms of L*er* x Col seeds (n=16), and of *mea*$^{-/-}$ x Col seeds (n=19), displayed both the maternal L*er*-specific band and the paternal Col-specific band. In contrast, only the embryos in the small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds displayed both parental bands while 90% of the seeds tested (n=16) contained endosperms exclusively producing a maternal band. Abbreviations: em, embryo; es, endosperm.

Table 2-9. Results of the laser-mircrodissection PCR

	embryo				endosperm			
	L*er*	Col	L*er* + Col	n	L*er*	Col	L*er* + Col	n
L*er* x Col	6%	0%	94%	16	0%	0%	100%	14
mea$^{-/-}$ x Col	7%	7%	86%	14	11%	0%	89%	19
mea$^{-/-}$ x *cdka;1*$^{+/-}$	6%	0%	94%	17	94%	0%	6%	16

both polymorphisms in almost all the embryos, they exclusively produced a singular L*er*-specific product in more than 90 percent of all endosperms tested (**Fig. 2-15d, Table 2-9**).

The absence of a Col-specific PCR product in the small rescue seeds in *mea*$^{-/-}$ x *cdka;1:yfp*$^{+/-}$ crosses indicates that these seeds are produced by single fertilization with *cdka;1*-mutant pollen. Due to a positive proliferation signal from egg cell fertilization plus the lack of

repression of central cell proliferation in the *mea* mutant, an unfertilized and homodiploid endosperm developed in these seeds.

This finding demonstrates that Arabidopsis seeds can develop with an unfertilized endosperm of exclusively female gametophytic origin and that the paternal allele is dispensable for the development of a functional endosperm.

2.6 Diploid, unfertilized endosperm possesses wild-type characteristics

2.6.1 Marker lines for endosperm differentiation and development

To study the characteristics of the developing endosperm in the *mea* x *cdka;1* crosses, a variety of endosperm stage-specific reporter lines lines were used (Ingouff et al., 2005). The developmental differentiation of the endosperm is mirrored by the activation of distinctive promoters and their genes. If these promoters drive reporter genes such as the *GUS* gene or the *GREEN FLUORESCENT PROTEIN (GFP)* gene in enhancer trap lines, the appearance of the respective reporter can be a specific indicator for a certain developmental phase.

I used enhancer trap lines that are characteristic for early endosperm development and get switched off in later stages (juvenile phase reporters). On the other hand I also looked at mature phase reporters that only get expressed in later stages of endosperm development.

The developing endosperm of self-fertilized *fis*-class mutants (including the *mea* mutant) or *fis*-class mutants crossed with wild-type pollen shows a heterochronic defect, i.e. it never reaches maturity. In these mutants, juvenile reporters are ectopically expressed in late phases of endosperm development while maturity reporters are never activated (Ingouff et al., 2005).

As a juvenile phase reporter I used *KS22*: In the wild-type endosperm this reporter gets turned off when the embryo reaches the heart stage (**Fig. 2-16a**) while in self-fertilized $fie^{+/-}$ seeds, the GFP reporter persisted in the endosperm surrounding arrested *fie*-embryos until the seeds were aborted (**Fig. 2-16b**). In $fie^{+/-}$ x $cdka;1^{+/-}$ crosses, the small rescue seeds contain an endosperm that behaved like wild type: The GFP signal vanished around the early heart stage of the embryo (**Fig. 2-16c**).

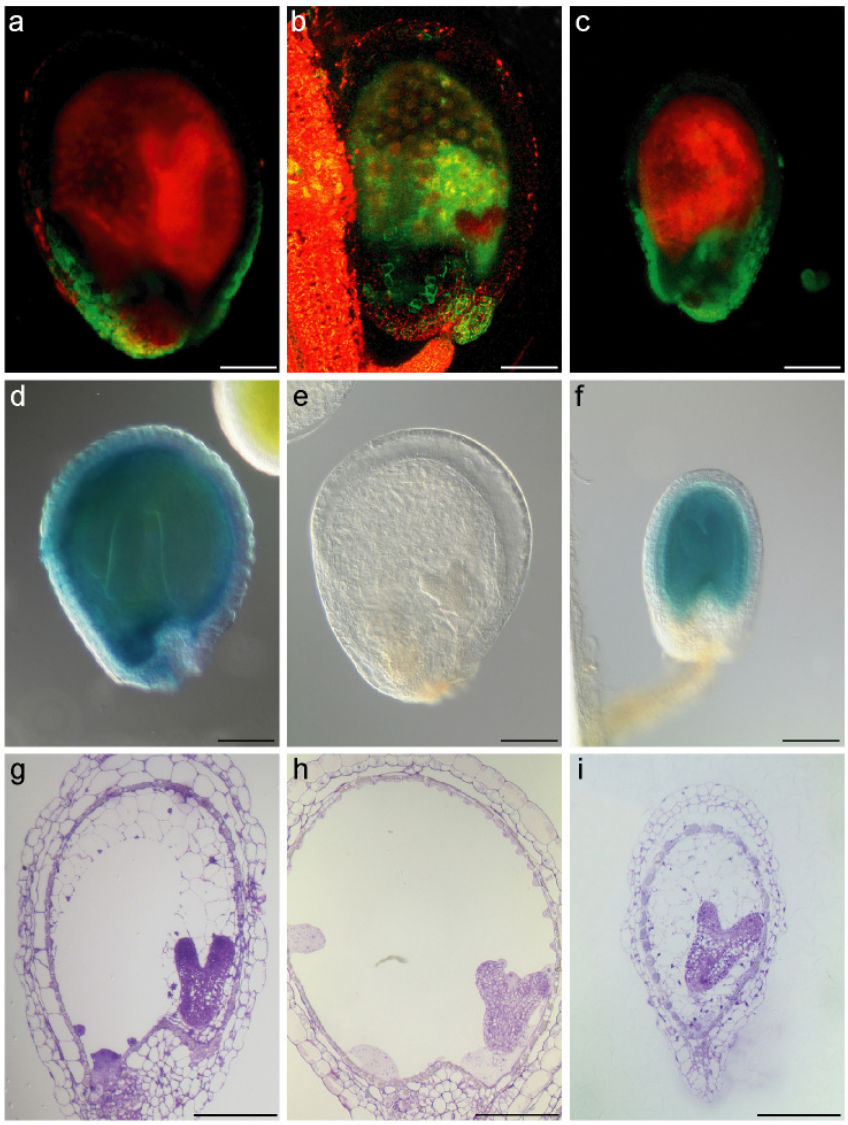

Figure 2-16. Endosperm differentiation of a uniparental, diploid endosperm in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds proceeds as in wild type. (a-c) Projections of Z-series of confocal sections displaying KS22 GFP fluorescence and red autofluorescence. **(a)** In wild type seeds, KS22 was only detected in the young, uncellularized endosperm, whereas **(b)** in *fie-11*-mutant seeds, GFP expression persisted and marked the aborting *fie*-mutant seeds. **(c)** In *fie-11*$^{+/-}$ x *cdka;1*$^{+/-}$ seeds, a class of small seeds was detected that surpassed the threshold of *fie*-mediated embryo abortion. As in wild type, these seeds stopped to express GFP as they matured. **(d-f)** Diphase interference contrast micrographs showing activity of the G222 GUS reporter. **(d)** In the maturing wild-type seed, *G222* was expressed from 6 d.a.p. on. **(e)** In aborting *mea* seeds, however, the reporter construct was not expressed. **(f)** In small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds, the *G222* reporter construct is expressed as in wild type. **(g-i)** Micrographs of semi-thin sections of seeds at 6 d.a.p. stained with a contrasting solution showed endosperm cellularization in wild type and *mea* seeds. **(g)** In wild-type seeds, the endosperm stopped dividing and started to form cell walls around the late heart stage embryo. **(h)** In aborting *mea* seeds no cellularization of the endosperm was detected. **(i)** In small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds, the endosperm underwent cellularization. Abbreviations are *mea*$^{-/-}$, homozygous *medea* mutant; *cdka;1*$^{+/-}$, heterozygous *cdka;1* mutant; eb, embryo; es, endosperm; RFU, relative fluorescence units; *fis*, *fis*-class mutants; Scale bars are 100 µm.

To monitor late endosperm development, I used the *GUS*-marker G222, which has an initiation of expression coinciding with the beginning of endosperm cellularization around the embryo and persists throughout later stages of seed development in the wild type (**Fig. 2-16d**). In half of the *mea*$^{+/-}$-mutant seeds, G222 was not expressed, documenting the lack of differentiation in the *mea* endosperm (**Fig. 2-16e**). In contrast, the G222 gets strongly expressed in the small *mea*$^{+/-}$ x *cdka;1*$^{+/-}$ rescue seeds (**Fig. 2-16f**).

2.6.2 Endosperm cellularization

Another important feature of endosperm differentiation is the stop of nuclear proliferation and the subsequent formation of cell walls. In the wild type, this endosperm cellularization starts around the heart stage embryo and successively cell wall formation spreads throughout the endosperm reaching the chalazal pole last (**Fig. 2-16g**). In the heterochronically-altered endosperm development of *mea* mutants, endosperm cellularization did not take place (**Fig. 2-16h**). In the small *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue seeds, however, endosperm cellularization occurred as in the wild type (**Fig. 2-16i**).

Taken together, these results indicate that the homodiploid, maternally derived endosperm in *fis-class* x *cdka;1*$^{+/-}$ rescue seeds develops and differentiates just as the triploid, fertilized endosperm in wild-type seeds as judged by the above mentioned criteria.

2.6.3 Gene expression is balanced in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds

Up to date, it still remains unclear what exactly causes the *fis*-class mutant seeds to abort. As the endosperm in these mutants tends to overproliferate and fails to differentiate into a mature, cellularized endosperm, it has been speculated that FIS-class proteins function to restrict endosperm growth and are crucial for endosperm differentiation (Scott and Spielman, 2006).

In the search of targets of the FIS-class protein complex, so far only a few genes have been identified; most prominent among them is *PHERES1* (*PHE1*) (Kohler et al., 2003b). The maternal copy of *PHE1* has previously been found to be down-regulated in developing seeds compared to the paternal copy (Kohler et al., 2005). In *fis*-class mutant seeds, the maternal repression is defective, leading to a strongly up-regulated *PHE1* expression level in the fertilized *fis*-class endosperm. The up-regulation of *PHE1* was causally connected with *fis*-class seed abortion, as down-regulation of *PHE1* by expression of a *PHE1*-antisense construct partly restored seed viability in *mea* mutants (Köhler 2003).

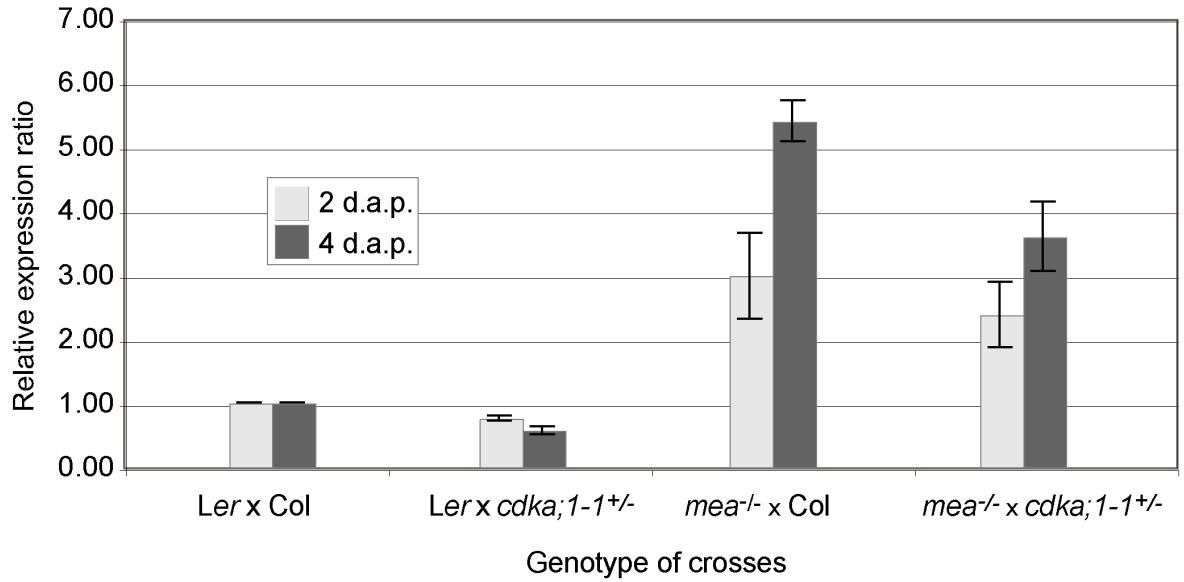

Figure 2-17. Quantitative real-time PCR monitoring *PHE1* expression in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds. Total RNA from siliques was isolated from the indicated crosses at two days after pollination (2 d.a.p., light grey bars) and 4 d.a.p. (dark grey bars) and cDNA synthesized by reverse transcription. Expression levels of *PHE1* were measured by real-time PCR and normalized in each experiment to the expression level of an internal *ACTIN2* reference. Three independent biological replica were performed for each data set and repeated four times. All biological replica showed similar results. The figure presents data from replica number 1. In the *mea*$^{-/-}$ -mutant background, the *PHE1* expression is strongly upregulated, this upregulation is more pronounced at 4 d.a.p. than at 2 d.a.p. On the contrary, in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds the *PHE1* upregulation at 4 d.a.p. is significantly reduced.

It is conceivable that the rescue found in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue seeds was also caused by a re-balancing of expression levels PHE1 and possibly other, yet unidentified genes. In this case, however, the balancing would be achieved by the lack of paternal PHE1 expression due to the missing central cell fertilization in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$.

To investigate this hypothesis, I monitored PHE1 expression levels in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds by real-time PCR in collaboration with Paul E. Grini and Reza Shirzadi from the University of Oslo. We used whole siliques at 2 and 4 d.a.p. of homozygous *mea* mutants fertilized with *cdka;1*$^{+/-}$-mutant pollen.

In accordance with earlier publications (Kohler et al., 2003b), we found that the *PHE1* expression levels in *mea*$^{-/-}$ x Col seeds were strongly up-regulated in comparison to the wild-type control L*er* x Col (**Fig. 2-17**). This difference was more pronounced at 4 than at 2 d.a.p.

In L*er* x *cdka;1*$^{+/-}$ seeds, the *PHE1* expression was slightly down-regulated compared to the expression wild type L*er* x Col seeds.

Most importantly, we found a significant down-regulation of *PHE1* levels in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds compared to expression in *mea*$^{-/-}$ x Col seeds (**Fig. 2-17**). Especially at 4 d.a.p. the difference in expression became quite prominent.

Although the *PHE1* expression in *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ seeds was not reduced to wild-type levels, the relative down-regulation compared to *PHE1* expression in *mea*$^{-/-}$ x Col demonstrated a relation between *PHE1* expression and successful seed development.

These results suggest that a balancing of expression levels of *PHE1* and/or further genes might be the mechanistic cause underlying the *mea*$^{-/-}$ x *cdka;1*$^{+/-}$ rescue. The data of the present study furthermore demonstrate, that the *MEA* gene is required for the balance of *PHE1* expression in the wild type, but is not necessary if *PHE1* and/or further genes are down-regulated by other means, e.g. the lack of the paternal contribution to the endosperm as realized by *cdka;1*-mutant pollen.

3 DISCUSSION

3.1 Cell cycle arrest - the primary *cdka;1* phenotype

3.1.1 CDKA;1 function is essential for Arabidopsis development

The present study is the first functional analysis of the cell cycle master regulator CDKA;1 in plants which uses a reverse genetics approach. Two independent *cdka;1* mutant lines were found to carry T-DNA insertions which disrupt the coding sequence of *CDKA;1* and produce null mutants.

In plants, the development of the two alternating generations, the sporophyte and the gametophyte, rely on cell division and CDKA;1 function is essential for both. In segregating *cdka;1* populations, no homozygous *cdka;1* mutants (sporophytes) were present. Heterozygous *cdka;1* mutants develop normally throughout their vegetative life, but about half of their seeds abort early. A closer examination of the embryos inside the aborting seeds revealed roughly one quarter of very early arrested embryos, while the embryos in the other aborting seeds continued to develop until early globular stage. These numbers suggest that the lack of CDKA;1 causes a combination of lethal effects in both the gametophytic and the sporophytic generation in Arabidopsis.

3.1.2 CDKA;1 function is indispensable for sporophyte development

In unicellular eukaryotes such as yeasts, a single CDK controls progression through the cell cycle. A number of yeast *cdk* mutants are temperature-sensitive and upon the shift to restrictive temperature, the cells invariably arrest the cell cycle programme and stop to proliferate (Simchen, 1978).

In contrast to the unicellular yeasts, multicellular organisms possess small families of related CDKs that take over specific functions in cell cycle control (Morgan, 1997; Vandepoele et al., 2002). While some of these CDKs appear to function in a redundant manner, the cdc2 homologues are essential and mutations in these genes confer lethality during early embryonic development in mammals and Drosophila (Stern et al., 1993; Sherr and Roberts, 2004; Bashir and Pagano, 2005; Martin et al., 2005). Thus, cdc2 function in animals seems to be universally required and irreplaceable for the respective organism.

As in animals, in Arabidopsis a small CDK family is present. Among its 12 members, a single homologue of the yeast cdc2 kinase, CDKA;1 is present. The early embryonic arrest of homozygous *cdka;1* mutants suggests a major role for CDKA;1 during sporophyte cell cycle

control that cannot be compensated for by other CDKs. This finding is in accordance with the apparently lethal effect of over-expressing the dominant negative *CDKA;1*^{DN} versions in Arabidopsis (Hemerly et al., 1995).

The analysis of *cdka;1* knock-out mutants presented here demonstrates that the essential role of CDKA;1 in Arabidopsis cell cycle control, corresponding to the function of its homologues in mouse and Drosophila.

3.1.3 Male, but not female *cdka;1* mutant gametophytes arrest development

Analysis of the male gametophyte in heterozygous *cdka;1* mutants revealed that about 40 percent of the pollen arrests prior to pollen-mitosis II (PMII) resulting in mature *cdka;1* pollen containing only one instead of the usual two sperm cells.

Similar to the situation in the *cdka;1* mutant embryo sac, during *cdka;1* pollen development there seems to be a certain capacity to control mitotic progression in the absence of a functional *CDKA;1* allele: All *cdka;1* pollen progresses through pollen mitosis I (PMI), and some even manage to complete development after passing through PMII. This can be explained by the redundant functions of other CDKs that are able to replace CDKA;1 function specifically during pollen development. Pollen transcriptome analyses show that besides *CDKA;1*, two *CDKB*s and other *CDK*s are also expressed (Pina et al., 2005). However, a number of findings argue for the alternative explanation that the cell cycle arrest prior to PMII could be caused by a carry-over of maternal CDKA;1 protein or mRNA. Unlike many other cell cycle proteins, CDKA;1 is stable throughout the cell cycle (Magyar et al., 1997; Porceddu et al., 1999; Sorrell et al., 2001) and persisting protein might allow a certain degree of cell cycle progression even in the absence of a functional *CDKA;1* gene. The results of the present study corroborate this hypothesis: In homozygous *cdka;1* mutants complemented by a heterozygous *Pro*$_{CDKA;1}$*:CDKA;1:YFP* transgene, the YFP signal is detectable in all microspores after meiosis, although only half of them inherit the *CDKA;1:YFP* construct. Only after the first pollen mitosis do *CDKA;1:YFP* levels start to decline in half of the pollen grains. At maturity, the YFP signal becomes nearly undetectable in *cdka;1* mutant-like pollen. A low or absent YFP signal in these pollen grains coincides with the cell cycle arrest phenotype of *cdka;1* mutant pollen.

Furthermore, it has been shown that genes required for transcription and protein synthesis are under-represented in the pollen transcriptome. This suggests that the cytoplasm of microspore mother cells might have a store of mRNAs and proteins before meiosis separates the microspores (Honys and Twell, 2003).

Another line of evidence comes from pollen-specific over-expression of the CDKA;1 inhibitor ICK1/KRP1 during pollen development in the close relative of Arabidopsis, *Brassica napus* (Zhou et al., 2002). ICK1/KRP1 over-expression produces pollen arresting at the one-cell, two-cell, and three-cell stages suggesting a requirement for CDKA activity as early as PMI. It is plausible that the constitutive expression of the CDKA;1 inhibitor causes variable levels of CDKA;1 activity from early pollen development onwards, while the slow and constant decline of maternal protein in the *cdka;1* mutant mainly affects later stages of the pollen cell cycle programme.

In contrast to pollen, the female gametophyte appear to be unaffected in the heterozygous *cdka;1* mutants although completion of female gametophyte development requires progression through three full mitotic cycles.

However, the megaspore is a large cell and similarly to pollen it seems reasonable that a carry-over of maternal CDKA;1 could drive all cell cycle rounds. It is known that especially the megaspore cells and their descendants are actively provisioned with a large stock of maternal mRNA and protein. In yet unfertilized maize egg cells for instance, large quantities of ribosomal-proteins, histone, and cyclin mRNAs are stockpiled (Dresselhaus et al., 1999). Future experiments will be required to unambiguously determine whether CDKA;1 controls also early stages of gametophytic divisions.

3.1.4 *cdka;1* mutant pollen arrests in G2 phase

DNA content measurements of the single gametes in *cdka;1* pollen suggests that the cell cycle arrest is effected prior to the second pollen-specific M phase, but after the preceding S phase. Therefore *cdka;1* pollen is probably arrested in G2.

This finding was unexpected as so far, no orthologues of the mammalian G1/S-specific *CDK4* and *CDK6* genes have been found in plants. Thus it is generally assumed that CDKA;1 is the only CDK active in the G1 and S phases in plant cells, whereas the entry into mitosis may be controlled by additional CDKs (Inze and De Veylder, 2006). In *cdka;1* pollen, B-type CDKs are present and their activity at the G2/M transition might have been able to compensate for the lack of CDKA;1 and trigger the progression through M phase (Pina et al., 2005). However, this was not the case in *cdka;1* pollen, suggesting that the CDKBs are either generally not able to compensate for CDKA;1 function at the G2/M transition or that they are unable to do so specifically during PMII.

Nevertheless, it has been shown that during the cell cycle different levels of CDK activity are required and that in mammalian cell cultures, CDK1 activity at the G2/M transition is many

times that of CDK1 and CDK2 at the G1/S transition (Bashir and Pagano, 2005). In plants, CDKA;1/CYC complexes from proliferating leaf tissues also exhibit a higher kinase activity than CDKA;1/CYC complexes from endoreplicating tissue, indicating that a relatively low CDK activity is sufficient to drive cells through S phase (Verkest et al., 2005b). Correspondingly, a strong over-expression of the CDKA;1 inhibitors ICKs/KRPs in Arabidopsis leads to inhibition of both, entry into M phase and entry into S phase while mild over-expression results in residual CDKA;1 activity blocking G2/M transition but allowing entry into S phase (Verkest et al., 2005a; Weinl et al., 2005).

Taken together, it is likely that entry into S phase requires less CDKA;1 activity than entry into M phase. This cell cycle arrest in the G2 phase has major implications for the secondary phenotype of the *cdka;1* mutant that concerns fertilization of the female gametophyte.

3.2 Single fertilization - the secondary *cdka;1* phenotype

3.2.1 *cdka;1* pollen produces a fertile gamete

Analyses of *cdka;1* pollen revealed that it has no difference in viability to wild-type pollen. Furthermore, *cdka;1* mutant pollen can germinate in vitro. Crosses of wild-type plants with pollen from a *cdka;1* mutant carrying a $Pro_{CDKA;1}$*:GUS* fertilization reporter gene demonstrated that *cdka;1* mutant pollen with only one sperm cell can fertilize the female gametophyte.

The ability of *cdka;1* pollen to fertilize the female gametophyte represents an exception since most mutants defective in male gametophyte development are non-fertile (McCormick, 2004). Among them, however, there are only few that also affect the progression of the pollen cell cycle. The *duo* mutants (*duo1* and *duo2*), for instance, also fail to progress through PMII. Both mutants arrest their cell cycle before the PMII resulting in mature pollen grains with only two cells, similar to the *cdka;1* mutant. But unlike the *cdka;1* mutant, the *duo* mutants do not arrest in G2: *duo1* pollen fails to undergo mitosis, but enters a new S phase so that its single gamete-like cell at anthesis has a DNA content of about 2,5C. On the contrary, *duo2* pollen enters PMII, but is arrested in the metaphase-to-anaphase transition (Durbarry et al., 2005). This means that *cdka;1* pollen and *duo* pollen have similar phenotypes but different effects on fertilization: *duo1*-pollen tubes are able to enter the female gametophyte and release their gamete-like cells but not to fertilize the female gametes, causing abortion of unfertilized wild-type ovules (Rotman et al., 2005) and Nicolas Rotman, personal communication).

Contrarily, *cdka;1* mutant pollen is mainly arrested at the CDKA;1-dependent checkpoint from G2 to M phase. It has been show only recently that Arabidopsis and other plants, in contrast to most other eukaryotes, fuse their gametes while in the G2 phase (Friedman, 1999; Durbarry et al., 2005; Rotman et al., 2005; Tian, 2005). After PMII, Arabidopsis wild-type sperm cells enter a long S phase that continues during pollen tube growth and leads to sperm cells with a DNA content of 2C just prior to fertilization (Friedman, 1999).

The *cdka;1* mutant cell cycle arrest in the G2 phase results in a single gamete-like cell that matches Arabidopsis wild-type sperm cells in DNA content and thus fulfilled a crucial requirement for the accomplishment of fertilization. It is remarkable that *cdka;1* mutant gamete-like cells, regardless of their cell cycle arrest undergo the same functional differentiation as maturing sperm cells. However, although cell cycle control can be intimately linked with the cell differentiation programme (Schnittger et al., 2003), it has been shown that this does not necessarily need to be so (Hemerly et al., 1995; Weinl et al., 2005).

3.2.2 Preferential fertilization of the egg cell

During fertilization effected by *cdka;1* mutant pollen in Arabidopsis, the usual double fertilization is disrupted as *cdka;1* pollen delivers only one sperm cells instead of two. During the *cdka;1* single fertilization the single male *cdka;1* gamete exclusively fuses with the egg cell, leaving the central cell unfertilized. This demonstrated an unexpected hierarchy of fertilization events in the embryo sac. Three different scenarios might account for this hierarchy:

First, if the two sperm cells of Arabidopsis pollen differ from each other in some yet unrecognized way, one of them could be predestined to fuse with the egg cell. In this case, the single *cdka;1* gamete would acquire the identity of the sperm cell designed for egg cell fertilization. This in turn would suggest that also the second mitotic division is unequal by generating two individual sperms.

In plant species other than Arabidopsis, preferential fertilization of the sperm cells has already been documented. In Plumbago, for instance, one of the sperm cells is rich in plastids and fuses in more than 95 percent of all cases with the egg cell to form the embryo (Russell, 1985). In maize, the generative cell has been reported to divide into two aneuploid sperm cells, one of which gets one extra set of B-chromosomes. The sperm cells with B-chromosomes are more likely to fuse with the egg cell than the ones without, although both kinds of sperm cell could successfully fuse with egg cells in vitro (Faure et al., 2003; Weterings and Russell, 2004).

However, in Arabidopsis, neither sperm cell dimorphism nor molecular marker hinting to a differential specification of the sperm cells has been described so far.

A second scenario involves indirect, for instance biophysical, constraints of the fertilization process: The pollen tube enters in one of the female gametophytic synergid cells and ruptures, releasing the sperm cells (Rotman et al., 2003). From this point on, the cytoskeleton of the female gametophyte takes care of transporting the sperm cells. Two actin "coronas" are formed from the middle of the penetrated synergid, carrying one of the sperm cells to the egg cell and the other one to the central cell (Weterings and Russell, 2004). It is possible that due to the morphology of the female gametophyte, the actin mesh aiming for the egg cell is more successful in recruiting a sperm cell than the actin corona leading to the central cell, guiding the first sperm cell to the egg cell and the one remaining towards the central cell

Finally, a third alternative considers an active signalling process between the egg cell and the sperm cells that might lead to preferential egg cell fertilization. However, no evidence for such a signal exists to date. Similar to the signal guiding the pollen tube to the micropyle of the ovules (Higashiyama et al., 2003), such an egg cell signal would have to cease the moment the first sperm cell fuses with the egg cell in order to avoid a second fusion of a sperm cell with an already fertilized egg cell.

Currently, it is difficult to assess if one of the mentioned mechanism, or a combination of them, ensures preferential egg cell fertilization. In the general context of reproduction biology, egg cell fertilization is of course preferable to central cell fertilization, as only the embryo, but not the endosperm, will develop into a new sporophyte generation capable of further reproduction. It is thus conceivable that natural selection resulted in one or more strategies to ensure the preferential fertilization of the egg cell. In the case of the *cdka;1* single fertilization, these strategies might help to ensure the formation of a fertilized embryo rather than a fertilized endosperm, a first step towards successful sexual reproduction.

3.2.3 A positive signal from the zygote starts endosperm proliferation

Signalling has to occur repeatedly during seed initiation and development, in order to assure the proper initiation, maintenance and termination of seed developmental processes. Experimental evidence so far suggests a central role of the endosperm as the major integrator of seed development (Berger et al., 2006).

After double fertilization, the seed consisting of the embryo, the endosperm and the maternally contributed nucellus and seed integuments starts to develop. First, the block inhibiting precocious egg cell and central cell proliferation has to be removed to allow

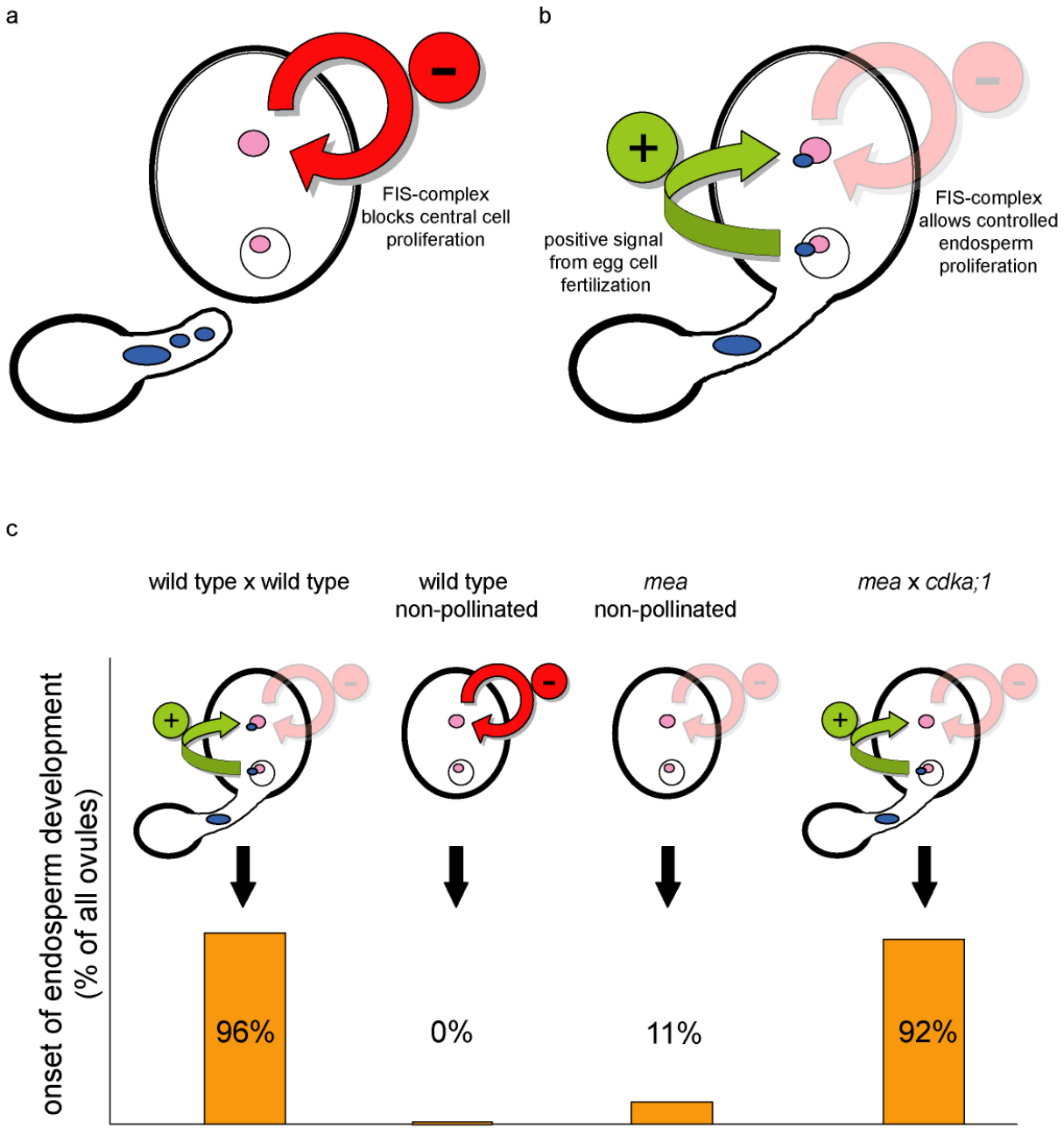

Figure 3-1. The crosstalk between embryo and endosperm during early seed development. **(a)** Prior to fertilization in wild type, the active FIS-PRC2 blocks precocious proliferation of the central cell, the progenitor of the endosperm. **(b)** The fusion of a sperm cell with the central cell nucleus lifts the proliferation block conferred by the FIS-PRC2. In addition, proliferation and likely differentiation of the developing endosperm is triggered by a non-cell autonomous signal coming from the fertilization of the egg cell. **(c)** Interaction between the positive signal and the lift of the repressive signal control early seed development. After double fertilization in the wild type, endosperm proliferation starts in almost every seed. In unfertilized wild-type ovules, the active FIS-PRC2 efficiently blocks central cell proliferation and also no positive signal is emitted from the fertilization of the egg cell. In unfertilized *mea* ovules, the FIS-PRC2 block of central cell proliferation is defective, and autonomous endosperm develops. However, no positive signal is provided and the percentage of seeds with an autonomously developing endosperm is low. After fertilization of *mea* by *cdka;1* pollen, endosperm starts to develop in almost all seeds similar to a double fertilization of wild-type plants.

proliferation of embryo and endosperm cells (Faure, 2001; Guitton and Berger, 2005b). This may require either specific signals that are generated during fertilization or a specific contribution of the paternal genome. It has been hypothesized that the parental genome remains largely inactive during early stages of embryo development leaving this stage of seed development under maternal control (Vielle-Calzada et al., 2000). Although early-expressed paternal genes have been identified (Weijers et al., 2001; Hejatko et al., 2003), no gene function hinting to a paternally derived signal for the initiation of seed development has been found so far (Dresselhaus, 2006).

The single fertilization by *cdka;1* mutant pollen revealed the existence of a positive signal from the fertilized egg cell that is sufficient to start proliferation of the central cell independent of central cell fertilization. Other mutants, e.g. *feronia*/*sirène* and *duo*, display pollen tube entry into the female gametophyte with or without rupture of the pollen tube and release of the sperm cells (Huck et al., 2003; Rotman et al., 2003; Nicolas Rotman, personal communication). This suggests that indeed successful fertilization of the egg cell, but not pollen tube penetration or rupture, is sufficient to emit the proliferation signal to the central cell.

Notably, the positive signal revealed by the *cdka;1* mutant demonstrates a previously unrecognized degree of embryonic control over early seed development in Arabidopsis (**Fig. 3-1 a-c**).

3.2.4 The nature of the proliferation signal

The nature of the positive proliferation signal revealed by *cdka;1* single fertilization is not known. It is conceivable that the signal might be incorporated by a signalling protein or a plant hormone that is produced and emitted by the egg cell upon fertilization. There are many changes in the egg cell metabolism after fertilization, which can be explained by the switch from the inactive egg cell state to the active zygotic phase (Sprunck et al., 2005; Dresselhaus et al., 2006). These changes include the production of a number of putative signal molecules (Hennig et al., 2004; Sprunck et al., 2005).

Furthermore, the onset of phytohormone production including gibberellins, cytokinins and auxins has been assigned signalling processes in seed and fruit development (Garcia-Martinez et al., 1997; Fos et al., 2000; Fos et al., 2001). Fertilization-independent fruit development in Arabidopsis can be initiated by application of exogenous auxins, gibberellins and cytokinins (Vivian-Smith and Koltunow, 1999). or by expression of auxin biosynthesis genes in ovaries and ovules (Rotino et al., 1997). It might be possible that one or several phytohormones

involved in these processes also play a role in the signalling pathway between the fertilized egg cell and the central cell in *cdka;1* mutants.

Cytokinins for instance are known to be potent activators of cell cycle acting via D-type cyclins (Riou-Khamlichi et al., 1999). Possibly, cytokinins get emitted from the egg cell after fertilization and sensed by the central cell via the histidine-kinase phospho-relay pathway (Kiba et al., 2005). Enhancement or attenuation of the cytokinin response might be two ways to test the involvement of cytokinins in the positive signal.

Another important link between phytohormone signalling and initiation of cell cycle activity relates to auxin-dependent pathways. In tobacco cell cultures, exogenously applied auxin increases the abundance and stability of E2FB, an important transcriptional activator of many S phase related genes. According to their function, over-expression of E2FB and its DIMERIZATION PARTNER A (DPA) could induce cell proliferation in the absence of auxin, stimulating both G1/S- and G2/M-transition (Magyar et al., 2005). Interestingly, a negative regulator of the E2FB/DPA pathway, RBR1, is responsible for the proliferation arrest of the mature female gametophyte awaiting fertilization, and *rbr1* mutants show ectopic proliferation of cells in the female gametophyte prior to and independent of fertilization (Ebel et al., 2004). Combination of *cdka;1* mutants with E2FB/DPA over-expressing plants could help to determine the role of auxin in early signalling processes in Arabidopsis seeds.

3.3 The mutual rescue of *fis*-class mutants with *cdka;1* pollen

3.3.1 wt x *cdka;1* seeds abort with an underdeveloped endosperm

Seeds generated by *cdka;1* pollen abort during early development with an endosperm undergoing only one to five rounds of nuclear division and an embryo developed as far as the early globular stage. Notably, the arrest of endosperm proliferation always precedes the arrest of embryo development which leads to the hypothesis that the underdeveloped unfertilized endosperm causes embryo arrest and seed abortion.

3.3.2 *mea* x *cdka;1* seeds are viable but smaller than wild type seeds

To test this hypothesis, I exploited the *medea* (*mea*) mutant as female partner to receive *cdka;1* mutant pollen. As all *fis*-class mutants, *mea* develops autonomous endosperm in the complete absence of fertilization (Chaudhury et al., 1997). Thus, the *mea* mutant offered a useful tool to enhance endosperm proliferation after single fertilization by *cdka;1* pollen.

Indeed, when *mea* mutant female gametophytes are fertilized with *cdka;1* mutant pollen, much more endosperm develops as in wild type x *cdka;1* seeds. Embryo growth is also clearly enhanced, emphasizing that the under-development of the unfertilized endosperm is the primary cause of seed abortion in wild-type ovules fertilized by *cdka;1* pollen.

Unexpectedly, *mea* x *cdka;1* seeds do not only develop past the stage of paternal *cdka;1*-mediated embryo arrest, but some seeds also develop past the seed abortion normally mediated by a maternally inherited *mea* allele (Grossniklaus et al., 1998). Roughly 20 percent of the *mea* x *cdka;1* seeds develop until maturity and form viable seeds implying that by combination of *mea* mutant female gametophytes and *cdka;1* mutant male gametophytes a mutual rescue of both, the *mea*-mediated and the *cdka;1*-mediated seed abortion, can be obtained. Unlike *mea* seeds fertilized by wild-type pollen, which contain an over-proliferated endosperm incapable of final differentiation (Ingouff et al., 2005), the *mea* seeds fertilized with *cdka;1* pollen display a reversal of this phenotype: In comparison to wild-type seeds, *mea* x *cdka;1* rescue seeds are markedly smaller, and they contain a smaller embryo and a less extensively proliferated endosperm that completely cellularizes.

3.3.3 wt x *cdka;1* and *mea* x *cdka;1* copy a maternal excess phenotype

In Arabidopsis and many other plants, normal seed development relies on a 2m:1p ratio of maternal to paternal genomes in the endosperm. In interploidy crosses, this ratio is altered, resulting in characteristic seed phenotypes (Lin, 1982, 1984; Scott et al., 1998; Adams et al., 2000). If the pollen donor is of higher ploidy than the female partner, seeds with a paternal excess phenotype develop. They are larger than the wild type, the endosperm over-proliferates and is delayed in differentiation. While in Arabidopsis, a doubling of the paternal genomes (2m:2p) in the endosperm is still tolerated and leads to abnormally large, but viable seeds, a tripling of the paternal contribution (2m:3p) causes arrest of embryo development and subsequent seed abortion (Scott et al., 1998).

On the contrary, complementary crosses with female plants of higher ploidy produces seeds with a maternal excess phenotype: Seeds with double the amount of maternal genomes (4m:1p) in the endosperm are smaller than the wild type, the endosperm does not proliferate as much and differentiates earlier, yet seeds are viable. However, if hexaploid plants are pollinated with diploid pollen resulting in seeds with a 6m:1p ratio in the endosperm, seeds grow even less, develop only very little endosperm and abort when the embryos has reached the globular to early heart stage (Scott et al., 1998).

The seed phenotypes observed in both, wt x *cdka;1* and *mea* x *cdka;1* seeds are reminiscent of the outcome of interploidy crosses with a high amount of maternal genomic excess. While wild type x *cdka;1* seeds are even more underdeveloped and abort even earlier than seeds with a 6m:1p genomic ratio, *mea* x *cdka;1* seeds mimic the phenotype of a milder maternal excess (4m:1p), including seed viability. It is possible that the same molecular mechanisms which result in characteristic maternal excess phenotypes are also responsible for the phenotypes of wild-type or *mea* ovules crossed with *cdka;1* pollen.

3.3.4 Imprinting and the kinship theory of seed development

The outcome of interploidy crosses in Arabidopsis and maize are interpreted due to a non-equivalent expression of genes depending upon their parent of origin. This phenomenon is known as genomic imprinting and has been described so far only in flowering plants and mammals (Berger, 2004; Guitton and Berger, 2005a; Scott and Spielman, 2006).

Mechanistically, imprinting is achieved by a complex molecular machinery that includes DNA methylation and histone modification to modify the expression status of imprinted genes or gene complexes depending upon their parent of origin (Gehring et al., 2004; Autran et al., 2005; Guitton and Berger, 2005a; Scott and Spielman, 2006).

In an attempt to understand why genomic imprinting convergently evolved in two so distantly related taxa than mammals and flowering plants, Haig and Westoby developed a genetic model termed parental conflict theory or kinship theory (Haig and Westoby, 1989, 1991; Haig, 2000).

According to the kinship theory, imprinting evolved in mammals and flowering plants because they both share the „placental habit“, i.e. the embryo develops in and at the expense of the mother. Hence, that the zygotic gene expression programme is already activated inside the mother and during the time of nutrient transfer to the embryo which in turn allows zygotic genes to gain influence over nutrient acquisition from the mother. As the zygotic gene products are encoded by genes inherited by the maternal side and the paternal side, nutrient acquisition can become a matter of conflict between maternal and paternal interests.

The kinship theory states that divergent maternal and paternal interests emerge from the different degrees of kinship between the offspring and their mothers and fathers. While mothers are always equally related to all of their offspring, fathers often have to face competition and therefore not all the offspring in one mother comes from one father. Hence, in order to maximize their reproductive success, mothers and fathers have to apply different strategies: While fathers need to maximize the nutrient flow to the individual embryo that

certainly bears their genes, mothers have to restrict this nutrient flow and distribute it equally to all of her offspring. According to the kinship theory, the divergent interests of mothers and fathers are incorporated by imprinted genomes to such an extent that genes that promote nutrient acquisition and embryo growth are expressed from the paternally inherited genome while they are silenced in the maternally derived genome. On the contrary, genes that restrict embryo growth should be complementary expressed (Haig and Westoby, 1991).

In mammals, experimental data largely support the predictions made by the kinship theory. Growth factors like *Ilgf2* are expressed from the paternally derived allele while the maternally derived allele is silenced (Haig, 2004). Experiments with flowering plants also revealed some imprinted genes that can be interpreted in the sense of the kinship theory (Mora-Garcia and Goodrich, 2000; Baroux et al., 2002; Scott and Spielman, 2006).

Interestingly, these experiments revealed that the MEDEA and other FIS-class proteins are part of the imprinting machinery active in Arabidopsis and that at the same time MEDEA is a target of imprinting itself (Baroux et al., 2006; Gehring et al., 2006; Jullien et al., 2006). Furthermore, although the molecular details of FIS-complex actions are still largely unknown, the mode FIS-complex function fits to the predictions made by the kinship theory: The FIS-complex directly or indirectly restricts endosperm proliferation and therefore serves the maternal interests – and accordingly, FIS-complex components are maternally expressed while they are paternally silenced (Scott and Spielman, 2006).

3.3.5 *mea* x *cdka;1*: paternalization meets maternalization

After fertilization, all *fis*-class mutants (i.e. *mea*, *fis2*, *fie* and *msi1*) show an over-proliferating, non-differentiating endosperm similar to the paternal excess phenotype in interploidy crosses (Scott et al., 1998; Scott and Spielman, 2006). As the actual ratio of 2m:1p genomes is unchanged, this has been interpreted as an effect of ectopic activation of usually silenced genes in the maternal genome due to the loss of repressive FIS-complex functions. This loss of repression results in the expression of maternal and paternal genes from the *fis*-mutant maternal genome, leading to a paternalization of the maternal expression pattern. Such an expression has been described as having a virtual maternal to paternal genome ratio of 2m:3p, which parallels the one of interploidy crosses with a tripled paternal input. In both, *fis*-class mutants and interploidy crosses, this situation leads to seed abortion (Scott and Spielman, 2006).

On the contrary, single fertilization with *cdka;1* mutant pollen leads to an effective change of the maternal to paternal genome ratio to 2m:0p in the endosperm. Thus, the expression pattern

of unfertilized *cdka;1* endosperm is maternalized in comparison to the fertilized wild-type endosperm.

Following this interpretation, the *mea* x *cdka;1* endosperm is paternalized due to the defective FIS-complex and maternalized because of the lack of a paternally-derived genome. This situation could lead to a balance of gene expression patterns that facilitate the mutual rescue of both the *mea*- and the *cdka;1*-mediated seed abortion phenotype.

3.3.6 *PHERES1* – the final dosage is decisive

To test the gene dosage balance hypothesis of the *mea* x *cdka;1* rescue, we measured expression levels of *PHERES1* (*PHE1*) in *mea* siliques fertilized with pollen from wild-type or *cdka;1*$^{+/-}$ plants. As a direct target of FIS-complex repression, *PHE1* is strongly up-regulated in *mea* mutants when compared to the wild type (Kohler et al., 2003b; Kohler et al., 2005). In *mea* x *cdka;1*$^{+/-}$ siliques, however, *PHE1* expression levels are significantly reduced compared to the expression in *mea* x wild type.

PHE1 expression levels, however, do not decline to wild-type levels, which may be due to various reasons: On one hand, only 40 percent of the *mea* x *cdka;1*$^{+/-}$ seeds receive a single fertilization, and thus, in only less than half of the seeds, the paternal contribution to the total *PHE1* levels is expected to be missing. Furthermore, it has been shown that the up-regulation of *PHE1* levels in *mea* x wild type is predominantly caused by the up-regulation of the two maternal copies, whereas the paternal *PHE1* levels remain unchanged (Kohler et al., 2005). Due to these circumstances, loss of paternal expression can not completely compensate for the up-regulation of maternal expression.

Taken together, it seems likely that the observed down-regulation of *PHE1* levels in *mea* x *cdka;1* siliques is due to the missing paternal *PHE1* expression in about 40 percent of the seeds. This suggests that the *PHE1* expression exclusively from the derepressed maternal loci in the autonomous endosperm is sufficient to allow for the development of a fully functional endosperm. Therefore, it is likely that the final dosage of PHE1 rather than its maternal or paternal expression pattern is important for regular seed development.

It is an appealing hypothesis that the whole imprinting machinery operating on the expression of genes like *PHE1* exists to integrate the expression patterns of maternal and paternal genes. The resulting finely fine-tuned balance of gene dosage may be crucial to normal seed development. Experimental systems that change this gene dosage balance, either by manipulating the parental genome dosage or by interfering with the imprinting machinery, lead to common phenotypes indicative of paternal or maternal excess. Notably, the paternal

excess phenotype can be reversed by defects in the paternal imprinting machinery and vice versa, indicating that imprinting and genome dosage control the same pathways controlling seed development (Scott 1998, Luo 2000, Adams 2000, Vinkenoog 2000).

Finally, it may be possible that a complete removal of all imprinting marks results in a "neutralized" basic expression pattern sufficient to organize endosperm development without biparental genome contribution. On the way to this end, the *mea* x *cdka;1* seeds represent an intermediate step, as functional endosperm development is possible, yet shows signs of maternal excess. Further reduction of imprinting can be obtained by interfering with DNA methylation patterns (Vinkenoog et al., 2000). If hypomethylated *mea* ovules fertilized by *cdka;1* pollen showed a reduction of maternal excess, this would hint to the existence of residual imprinting in the unfertilized *mea* endosperm.

In this context, it is tempting to speculate that the FIS-complex has only been recruited to the endosperm with the evolution of double fertilization in early angiosperms. FIS-related PRC2 have been shown to control the vernalization response and transition from vegetative to reproductive development in Arabidopsis (Guitton and Berger, 2005a). Only with the evolution of the placental habit and the gain of influence of paternally-derived genomes over resource allocation from the mother to the embryo, did it become necessary to control and to harmonize maternal and paternal contribution to endosperm development by imprinting. Furthermore, Polycomb-repressive complexes are also important players of genomic imprinting in mammals (Guitton and Berger, 2005a; Scott and Spielman, 2006). The implementation of the evolutionary conserved PRC2 machinery for analogous tasks in mammals and angiosperms, two taxa that independently evolved the "placental habit" and genomic imprinting, is a prime example of convergent evolution and the recruitment of pre-existing molecular machineries to newly arising tasks.

3.3.7 *mea* x *cdka;1* – two perspectives on the rescue

There are two not mutually exclusive scenarios that might explain the *mea* x *cdka;1* rescue. The first scenario could be termed "The endosperm perspective", while the second puts more emphasis on the importance of the embryo and could therefore be called "The embryo perspective". Both are not contradictory but rather complementary and might help to perceive the complex interactions that lead to the rescue of seed development in *mea* x *cdka;1* crosses.

1. "The endosperm perspective". In this scenario, the *mea* x *cdka;1* rescue is perceived as the ultimate step of a series of experiments that aim to reduce the paternal input in the *mea* seed (**Fig. 3-2**).

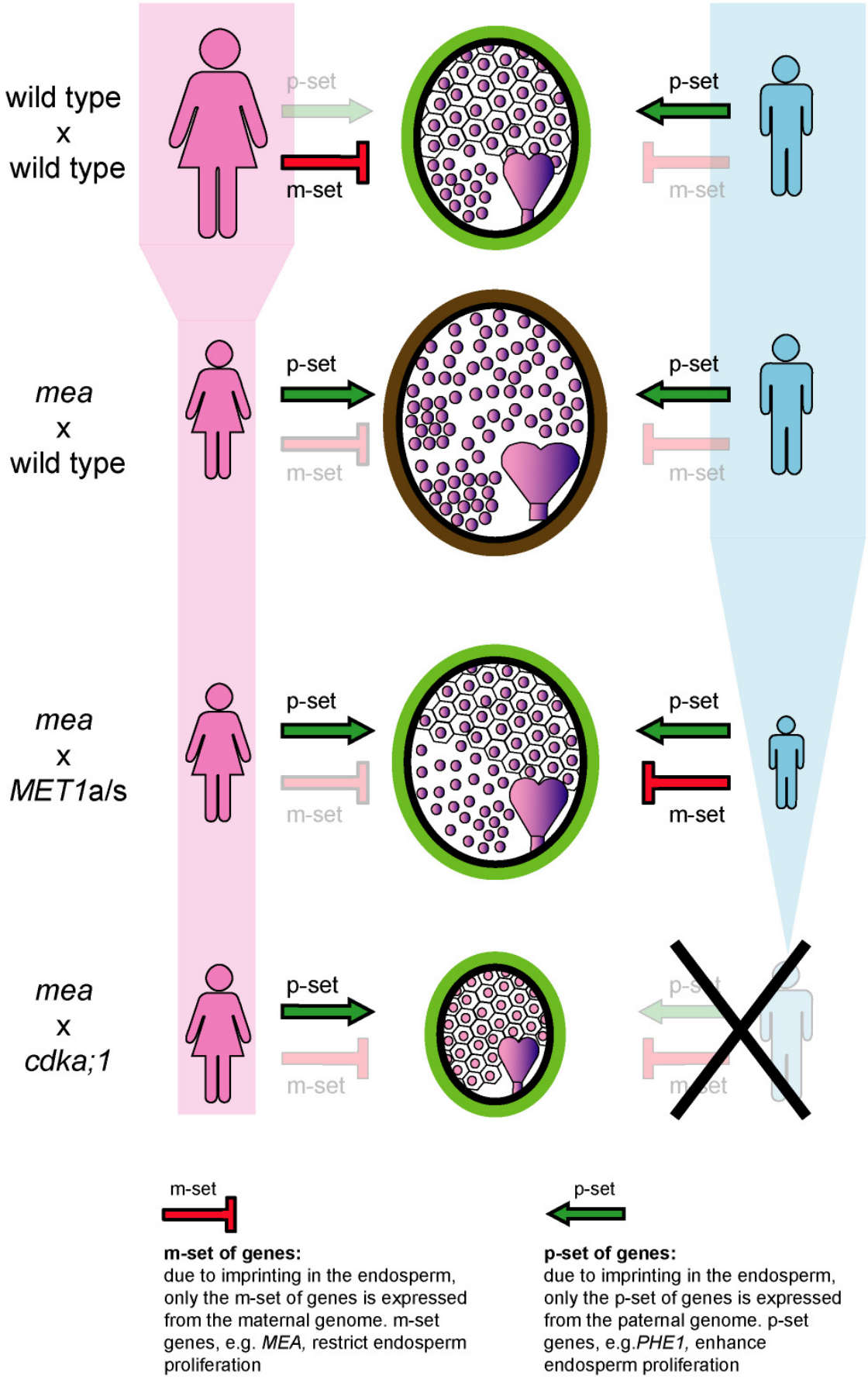

Figure 3-2. The endosperm perspective, the description is given on the next page.

Due to genomic imprinting in wild-type endosperm, maternal and paternal genomes are non-equivalently expressed. The maternal genome expresses a set of maternal genes (m-set), which restricts endosperm growth and is silenced in the paternal genome. Vice versa, a set of paternal genes (p-set) that enhances endosperm growth is expressed by the paternal, but silenced in the maternal genome (Scott and Spielman, 2006).

In *mea* seeds fertilized with wild-type pollen, the normally silenced maternal p-set of genes is reactivated, leading to a surplus of p-set gene products, e.g. PHE1, in the endosperm (Kohler et al., 2005). Thus, the relative influence of p-set genes gets increases, producing paternalized *mea* seeds that eventually abort.

In experiments that aimed to reduce the paternal influence in *mea* seeds to normalize seed development, *mea* mutants were fertilized with pollen carrying globally hypomethylated DNA due to defective DNA methylation maintenance factors (Adams et al., 2000; Luo et al., 2000). These crosses result in a rescue of *mea* seed abortion, likely due to derepressed m-set genes in the hypomethylated paternally derived genome. These inhibitors must act either downstream of the FIS-complex or in an independent pathway, as hypomethylated *mea* mutant pollen can also rescue the maternally conferred *mea* abortion (Luo et al., 2000).

Finally, the combination of the *mea* mutant with *cdka;1* pollen reduces the paternal input to zero, as there is no paternally derived genome contributing to the endosperm at all. Although both, the *mea* fertilization with hypomethylated pollen and *mea* fertilization with *cdka;1* pollen, result in rescue of *mea*-mediated seed abortion, the underlying functions of these rescues are complementary: While the hypomethylated paternal genome may display a gain-of-function phenotype of m-set genes, the missing paternal genome in *cdka;1* clearly acts as a loss-of-function of paternally derived p-set genes. This difference

← **Figure 3-2. The endosperm perspective.** In wild type, a 2m:1p ratio of maternal to paternal genomes in the endosperm is crucial for seed development (symbolized by the larger female versus smaller male icon). Due to genomic imprinting in the endosperm, a certain set of genes is only expressed from the maternal genome (m-set) while other genes are only active in the paternal genome (p-set) (Scott 2006). According to the kinship theory (Haig 1991), the m-set of genes restricts growth of the endosperm while the p-set of genes enhances growth (symbolized by the red bar and green arrow, respectively). In fertilized *mea* seeds, the imprinting function of the PRC2 is defective and thus, the maternal repressive influence on seed development is reduced. As a result, p-set genes, e.g. *PHE1* are misexpressed from the maternal genome, leading to a paternalized seed in which the endosperm over-proliferates and eventually aborts. Contrarily, in *mea* seeds fertilized with hypomethylated pollen carrying a *MET1*-antisense (*MET1*a/s) construct, the paternal endosperm-promoting influence is reduced, as the m-set of genes is activated. This maternalization restores seed viability in *mea* x *MET1*a/s crosses, although some seeds still show signs of paternal excess (Adams 2000, Luo 2000). In *mea* x *cdka;1* seeds, the paternal contribution to the endosperm is completely missing. The viability of *mea* x *cdka;1* seeds demonstrates, that the paternalized *mea* endosperm alone is sufficient for seed development.

is also mirrored in the different quality of the two *mea* rescues: while hypomethylated pollen results in oversized *mea* rescue seeds indicative of paternal excess (Luo et al., 2000), the *mea* x *cdka;1* seeds are undersized, showing signs of maternal excess.

2. "The embryo perspective": This second perspective of the *mea* x *cdka;1* rescue takes into account that *mea* confers autonomous seed development independent of fertilization (Chaudhury et al., 1997). Lack of the FIS-complex leads to a derepression of the central cell arrest prior to fertilization. Autonomous endosperm in some of the *fis*-class mutants shows some developmental characteristics of wild-type endosperm, including expression of the endosperm-specific marker KS117 in *msi1* mutants and the onset of cellularization in *mea* and *fis2* (Chaudhury et al., 1997; Ingouff et al., 2006). Nevertheless, autonomous endosperm proliferation does not reach the vigour of normal endosperm development and autonomous seeds abort after some time (Chaudhury et al., 1997).

   Fertilization with *cdka;1* mutant pollen leads to the development of a sexually produced embryo in combination with an unfertilized autonomous *mea* endosperm. Just as autonomous endosperm, *mea* x *cdka;1* endosperm does not proliferate as much as fertilized endosperm and differentiates up to cellularization.

   Nevertheless, the presence of a sexually produced embryo induces some major changes in the development of the autonomous endosperm: In contrast to unfertilized *mea* mutants which show a low rate of autonomous endosperm proliferation, in *mea* x *cdka;1* seeds the rate of endosperm onset increases dramatically. The promotion of autonomous endosperm development in *mea* x *cdka;1* seeds can be interpreted as the result of a positive signal emitted by the fertilization of the egg cell or by the zygote (**Fig. 3-1**).

   More important still, the presence of the developing embryo change the fate of autonomous seeds during later seed development: Whereas all autonomous seeds are bound to abort after a few days of development, in *mea* x *cdka;1* seeds with autonomous endosperm, a considerable number of seeds reach maturity and form viable seeds. This sustained seed development is likely to be promoted by the presence of a developing embryo. Repeated signalling between embryo and endosperm or embryo and mother plant could promote lasting development in *mea* x *cdka;1* seeds (**Fig. 3-3**).

   Taken together, the combination of *mea* mutant and *cdka;1* mutant can be interpreted as the creation of a chimeric seed in which a sexually produced embryo was put in a seed with autonomous, unfertilized endosperm. This shows on one hand that autonomous maternally derived endosperm can be fully functional in sustaining embryo development.

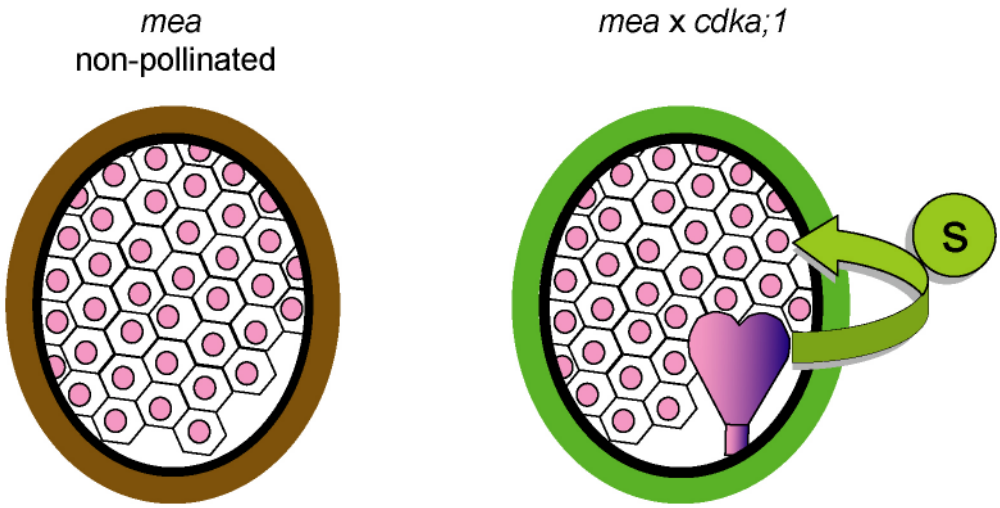

Figure 3-3. The embryo perspective. In unfertilized *mea* ovules, autonomous endosperm develops independent of fertilization. At least in some *fis*-class mutants (such as *mea*), this autonomous endosperm shows characteristics of a fertilized endosperm, e.g. the onset of cellularization. However, unfertilized seed-like structures in *mea* mutants do not mature and abort after a few days of development (brown seed coat). Contrarily, *mea* ovules fertilized by *cdka;1* single fertilization can survive and mature (green seed coat), although the unfertilized endosperm was not fertilized as in unfertilized *mea* mutants. This pinpoints to the embryo as the origin of a signal (s) that sustains seed development, either via a direct or indirect communication with the endosperm and/or the mother plant.

On the other hand, *mea* x *cdka;1* seeds give evidence that the presence of a developing embryo is decisive for sustained seed development in Arabidospis.

The chimeric *mea* x *cdka;1* seeds further show that the paternal genome and paternally derived expression is dispensable for seed development, if the typical maternal expression pattern is disturbed by the lack of the FIS-complex. Thus, seeds of *mea* x *cdka;1*, *fis2* x *cdka;1*, and *fie* x *cdka;1* are the ultimate proof that the FIS-complex is not needed for the development of a functional endosperm. This result corroborates speculations based on earlier experiments showing that some components of the FIS-complex are not needed for seed development (Adams et al., 2000; Luo et al., 2000).

3.4 Reflections on the evolutionary origin of the endosperm in angiosperms

The *cdka;1* mutant pollen with its single sperm cell causing a single fertilization in *Arabidopsis thaliana* is the first in vivo dissection of double fertilization in angiosperms and evokes some speculations about the nature and the origin of the endosperm in flowering plants.

The debate about the origin of the endosperm in angiosperms is as old as the discovery of double fertilization by Nawaschin at the end of the nineteenth century (Nawaschin, 1898).
While there is little doubt about the transition of the many-celled female gametophyte of non-flowering seed plants (gymnosperms) to the highly reduced angiosperm female gametophyte through neothenic reduction of gametophyte proliferation, the origin of a fertilized triploid endosperm as one defining feature of angiosperms remains a matter of dispute (Friedman, 2001; Friedman and Williams, 2004).
In short, there are three hypotheses concerning the nature and the evolutionary origin of the triploid fertilized endosperm of today's angiosperms – and all these hypotheses exist since more than a century:
The endosperm could be a sterilized homologue of a second embryo that evolved to fulfil the altruistic function of feeding the main embryo (Sargant, 1900; Friedman, 1995). Alternatively, it could be a sexualised version of the embryo-feeding prothallium typical for gymnosperms (Strasburger, 1900; Thomas, 1907). A third possibility is that the fertilized endosperm is a functional structure that arose de novo during angiosperm evolution and is, in fact, not homologous with any previously existing component of plant sexual reproduction (Friedman and Williams, 2004).
These hypotheses include different scenarios for the sexual reproduction in the hypothetical angiosperm ancestor:
In the first scenario, double fertilization evolved before the reduction of the prothallium to produce two instead of one embryo, one of which gradually replaced the embryo-nourishing function of the prothallium. This means that the central cell would be homologous to a second egg cell and that the altruistic embryo acquired a fundamentally new developmental programme very different from his original one (Friedman, 2001).
In an alternative scenario, the angiosperm ancestors might have first reduced the prothallium to a single cell (homologous to the modern central cell), that, triggered by egg cell fertilization, started to develop in a prothallium with nourishing function, homologous to the structure found in gymnosperms. Only later, the central cell might have evolved to receive the second sperm cell and to produce a fertilized structure with analogous function to the former prothallium.
The first scenario is mainly founded on examples of double fertilization in gymnosperms producing supernumerary embryos, of which only one survives (Friedman, 1992; Carmichael and Friedman, 1995). But it remains unclear whether these phenomena mirror the situation given in the ancestors of angiosperms at the beginning of double fertilization. Notably, there

does not seem to be a close phylogenetic relation between angiosperms and the gymnosperms known to possess double fertilization (Friedman and Williams, 2004).

The findings of the present study, on the contrary, argue for the second scenario: Single fertilization by *cdka;1* mutant pollen demonstrates that an unfertilized, solely maternally derived endosperm in the modern flowering plant *Arabidopsis thaliana* can develop the same basic characteristics and the same function as a fertilized endosperm.

These results support the hypothesis raised by Eduard Strasburger that the endosperm of flowering plants is a homologue of the female gametophyte of gymnosperms and that the fertilization of the central cell is used as a trigger to start endosperm development (Strasburger, 1900).

The transition from a highly proliferated female gametophyte in gymnosperms to the few-celled embryo sac in angiosperms can be interpreted as advantageous during the selective process of evolution: First, the costs of reproduction are lowered as the investment of nutrient transfer depends largely on successful fertilization. While gymnosperms mostly form large female gametophytes full of storage products (Maheshwari and Singh, 1967), angiosperms invest little in unfertilized ovules. Second, the coordination of fertilization and seed development allows much faster reproduction in angiosperms, so that time between sexually reproducing generations can be reduced to a minimum of months or weeks in angiosperms, while such a rapid reproduction is unknown for gymnosperms. The combination of these putative selection advantages might be one reason for the enormous adaptive radiation of the flowering plants since the evolution of double fertilization approximately 130 million years ago.

3.5 Outlook: The *cdka;1* mutant as a tool investigate plant development

Consistent with previous work, this analysis has revealed a central role of CDKA;1 in cell cycle regulation and development in plants. The finding that *cdka;1* mutants can be rescued by expressing the *CDKA;1* cDNA from a 2000 bp 5´-promotor fragment, now offers the possibility to easily express different rescue constructs to ask specific questions about CDK function during the cell cycle. Moreover, using CDK as a tool to modulate cell cycle progression, also general questions about the cross talk between cell proliferation, cell growth and plant development can be addressed. For instance, *CDKA;1* expression could be restricted in time and space, and at a protein level, conditional or weak *CDKA;1* alleles could be generated.

One way to produce conditional *CDKA;1* alleles is to use the CRE-*lox* deletion system that has already been successfully applied to cell cycle regulators (Serralbo et al., 2006). This experimental setup allows excising transgenic constructs that are stably integrated into the plant genome. The system depends on the recombination of two flanking *lox*-sites mediated by a CRE recombinase, the expression of which effects an irreversible loss of the transgenic construct. In combination with a *CDKA;1* rescue construct, the induction of CRE-*lox* excision will allow to create *cdka;1* loss-of-function mosaics in plant systems otherwise rescued by the *CDKA;1* construct. This approach has the potential to answer basic questions of plant development: How will meristematic cells react if they loose their competence to divide? Will single cells that are rendered unable to divide affect tissue growth and organ development as the "cellular theory" of plant growth suggests? Or will plant tissues be able to sense and compensate for deficient cells as proposed by the "organismal theory"(Beemster et al., 2006)? Furthermore, by choosing specific promoters, CRE expression could be precisely targeted to various cells, tissues or organs. On the one hand, these experiments could provide information about the role of CDKA;1 in special cell cycle modes such as unequal cell division or endoreplication. On the other hand, the effects of *cdka;1*-mediated cell cycle arrest on various developmental programmes could be investigated, leading to a deeper understanding of the connections between cell cycle and development in plants.

4 MATERIALS & METHODS

4.1 Materials

4.1.1 Chemicals and antibiotics

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

4.1.2 Enzymes, primers and kits

Restriction enzymes were used from MBI-fermentas (St.Leon-Rot, Germany) and New England Biolabs (Frankfurt/Main, Germany). Modifying enzymes were used from MBI-fermentas (St.Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Roche (Mannheim, Germany), usb (Cleveland, USA), Qbiogene (Heidelberg, Germany), TaKaRa (Otsu, Japan). Primers were generated by Metabion (München, Germany), Invitrogen (Karlsruhe, Germany), MWG (Ebersberg). Kits were supplied from peqlab (Erlangen, Germany), Roche (Mannheim, Germany) and QIAGEN (Hilden, Germany).

4.1.3 Cloning vectors and constructs

The following vectors and constructs were used in this work:

- *pGEM-T* easy (Promega) for the cloning of the promoter region of *CDKA;1*
- *pDONR 201* vector (Invitrogen) was used as a donor in gateway based clonings
- *pAM-PAT-GW* as a binary gateway target vector containing a *CaMV 35S* promoter cassette and BASTA resistance (GenBank accession AY027531)
- *pAM-PAT-GW* as a binary target vector containing the promoter region of *CDKA;1* instead ot the *CaMV 35S* promoter cassette

4.1.4 Plant material

Arabidopsis plants used in this study were derived from the Columbia (Col) and the Landsberg *erecta* (L*er*) accessions. The *cdka;1-1* (SALK_106809.34.90.X) was obtained from the SALK T-DNA insertion collection (http://signal.salk.edu).). The *cdka;1-2* allele (Koncz_51209) was obtained from Koncz collection (Rios et al., 2002). Seeds of the mutant *fis1/mea* and the *fis2* allele were obtained from A. Chaudhury and are in the Landsberg *erecta* accession (Chaudhury et al., 1997). Lines KS117 and KS22 in $fie^{+/-}$ background and the

enhancer trap GUS line G222 in *fis1/mea* background were contributed by Frédéric Berger (Ingouff et al., 2005). All genotypes were determined by PCR, by resistance to BASTA, or by presence of YFP.

4.1.5 Bacterial strains

For standard cloning the *Escherichia coli* strains DH5alpha was used, the DB3;1 strain, which is resistant to the *ccdB* gene, was used for the Gateway Entry, Donor and Destination vectors. For plant transformation *Agrobacterium tumefaciens* strain GV3101 was used. For all gateway vector based plant transformation GV3101+pMP90RK was used.

4.2 Methods

4.2.1 Plant work

Plant growth conditions

Arabidopsis thaliana seeds were germinated on soil or half-strong MS-2 medium containing 0,5 % sucrose and 0,8 % agarose. Plants were grown between 18 and 25 ºC under standard greenhouse conditions or in culture rooms or Percival growth chambers under long-day conditions with a 16/8 h light/dark cycle at 18 ºC or 20 °C, respectively.

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.

Plant transformation

Plants were transformed according to the "floral dip" method (Clough and Bent, 1998). To gain strong plants, these were allowed to grow at 18 °C until the first flowers appeared at stalks of approximately 10 cm in length. Four days before plant transformation a 5 ml Agrobacterium preculture was incubated for two days at 28 °C. This preculture was used to inoculate the final 500 ml culture which was then incubated again for two days at 28 °C. Before transformation 5 % sucrose and 0.05 % Silwet L-77 were added to the culture. Plants

were dipped in this solution for approximately 20 seconds and then horizontally placed in a moist chamber. The plants were transferred to the greenhouse on the following day.

Seed surface sterilization

The surface of the seeds was sterilized by a five min incubation in 95 % ethanol followed by a 10 min incubation in a 20 % Klorix solution (containing 0.1 % Triton X-100). Afterwards the seeds were washed two to three times with 0.01 % Triton X-100 solution and than plated under the clean bench on MS-Agar plates (1 % Murashige-Skoog salts (MS), 1 % sucrose, 0.7 % agar, pH 5.7).

Alternatively the seeds were sterilized in a small vacuum container. In this container, 20 ml of bleach (DanKlorix by Colgate-Palmolive, Hamburg) were placed in a 50 ml glass beaker. 2 ml of concentrated hydrogen chloride were added to the bleach and the lid of the vacuum container was closed immediately afterwards. The chlorine that was produced by this reaction was used to sterilize the surface of the seeds for approximately 4 hours to 12 hours. The seeds were then plated as indicated above.

Selection of transformants

The seeds of transgenic plants carrying in their T-DNA a kanamycin or a hygromycin resistance were selected on half strong MS-Agar plates with 50 µg/ml kanamycin or 25 µg/ml hygromycin, respectively. Transgenic plants containing the BASTA resistance were grown 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.

4.2.2 Microscopy and cytological methods

Microscopy

Light microscopy was performed with an Axiophot microscope (Zeiss, Heidelberg, Germany) equipped with differential interference contrast (Nomarski) and epifluorescence optics. The DISKUS software package (Carl H. Hilgers-Technisches Büro, Königswinter, Germany; version 4.30.19) was used to quantify the fluorescence intensity of DAPI stained pollen to determine nuclear DNA content. Confocal-laser-scanning microscopy was performed with Leica TCS SP2 AOBS (Leica, Wetzlar, Germany) or LSM 510 META (Zeiss, Heidelberg, Germany).

LR-White embedding and semi-thin sectioning of seeds

For the analysis of endosperm cellularization, seeds were fixed and embedded in LR-White plastic resin after a modified protocol from Lukowitz et.al. (Lukowitz et al., 1996). Semi-thin sections (0.7 µm) of were prepared with a Reichert Ultracut R microtome and mounted in Canada balsam.

Whole-Mount preparation of seeds

Pistils and siliques of different developmental stages were prepared as described previously (Grini et al., 2002). 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 $NaPOH_4$ buffer, pH 7.2, and mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol. The specimens were cleared 1 hour at 4 °C before inspection. Light microscopy was performed with a Zeiss Axiophot microscope using Differential Interference Contrast (DIC) optics.

GUS staining

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 $K_4Fe(CN)_6*H_2O$, 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.

Pollen preparation for fluorescence analysis

Anthers were prepared from flowers of different developmental stages and put in a droplet (15 µl) of DAPI working solution (watery solution containing 2.5 µg/ml of 4',6-Diamidino-2-phenylindole (DAPI), 0.01 % Tween, 5 % DMSO, 50 mM PBS buffer [pH 7.2]) on a microscopy slide. The anther was then covered with a covering glass (18 mm x 18 mm) and slightly squashed to free the pollen or microspores. The slides were then placed in the dark until the rim of the covering glass was dry, and then sealed with nail polish. After 4 hours to 12 hours of incubation in the dark at 4 °C, the preparations were checked for DAPI fluorescence then check under a fluorescence microscope with a UV-filter.

Pollen DNA measurements

Mature pollen at the stage of anther dehiscence was stained with a DAPI solution (2.5 µg/ml DAPI in 50 mM PBS pH 7.2 with 0.01 % Tween20 and 5 % DMSO) for one hour. The DAPI fluorescence intensity was quantified and background fluorescence was subtracted using the DISKUS software package (Carl H. Hilgers – Technisches Büro, version 4.30.19). The values obtained were normalized against wild type and statistically analyzed by Analysis of Variance between Groups (ANOVA) using the STATISTICA software package.

Pollen viability assay

For pollen viability staining, mature pollen at the stage of anther dehiscence was transferred onto microscope slides covered with a thin film of germination medium (0.4 mM $CaCl_2$, 0.4 mM Boric Acid, 0.5 % Agarose, 10 % Sucrose in distilled water, pH adjusted to 7). The pollen was allowed to rehydrate in a moist chamber at 4 °C for 2 hours. Subsequently, fluorescein diacetate (FDA) staining solution (2 mg of FDA in 1 ml acetone as stock solution, which was added drop by drop to 10 ml of a 0,5 M sucrose solution) was applied to the pollen. After 10 minutes of incubation at room temperature, the samples were observed under a fluorescence microscope with a FITC filter.

Pollen in vitro germination assay

For pollen in vitro germination assay, mature pollen at the stage of anther dehiscence was transferred to slides with germination medium (0.4 mM $CaCl_2$, 0.4 mM Boric Acid, 0.5 % Agarose, 10 % Sucrose in distilled water, pH adjusted to 7). Germination occurred in a moist chamber at 25 °C in constant light overnight. Samples were stained with a mixture of DAPI solution (2.5 µg/ml) and aniline blue solution (0.004 % w/v) in 50 mM PBS with 0.01 % Tween20 and 5 % DMSO for 1 hour and viewed under a fluorescence microscope using an UV-filter.

Flow cytometry for seed tissue ploidy analysis

For Flow cytometry analysis, rosette leaves were chopped up finely with a razor blade in nuclear extraction buffer (CyStain UV-precise kit by Partec GmbH, Muenster, Germany). Seeds were collected in 400 µl nuclear extraction buffer in a 2 ml test tube and lightly squashed with a plastic pistil. After 15 min incubation on ice in the dark, all preparations were filtered through a 30 µm nylon mesh and stained with nuclear staining solution (CyStain UV-precise kit by Partec GmbH, Muenster, Germany) containing 4',6-Diamidino-2-phenylindole

(DAPI). After incubation of 15 min to 1 hour, flow cytometry was performed on a three laser LSRII analytical flow cytometer (BD Biosciences, Heidelberg, Germany) using the 405 nm solid state laser for excitation and a 440/40 nm band pass filter for recording of DAPI fluorescence. The ploidy level, represented by the mean peak position in a DAPI fluorescence intensity histogram, was calibrated against the 2C nuclear DNA content peak derived from a preparation of young rosette leaves. Doublets were excluded from the analysis by gating on single nuclei in a DAPI-width versus DAPI-area display according to Wersto et al. (Wersto et al., 2001) Data were presented using Flowjo analysis software (Tree Star, Inc., OR, USA).

4.2.3 Molecular-biological methods

All primers and probes are listed in Table 4-1 at the end of the Materials and Methods section.

Genomic DNA preparation from plant tissue I

To gain high-quality genomic DNA, the CTAB-preparation was used (Rogers and Bendich, 1988). Plant material (single rosette or cauline leave) was grinded and 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 % b-mercaptoethanol) was added and incubated for 30 minutes at 65 °C. After addition of 150 µl Chloroform/Isoamylalcohol (24:1) and careful shaking, the probes 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 resolved in 20 µl 20 mM Tris/HCl pH 8.0.

Genomic DNA preparation from plant tissue II

For PCR based genotyping, genomic plant DNA was isolated following a modified protocol from Berendzen (Berendzen 2005). A small amount of young plant material (e.g. a cotelydon) was put in a 2 ml reaction tube and 300 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) was added. The plant tissue was ground by adding a metal bead and shaking the reaction tubes for 1 min at a high frequencey in the Mixer Mill MM 301 by Retsch (Haan, Germany). 1 µl of this solution (no centrifugation required) was used as template for PCR using standard Taq-Polymerase and the following 10 times PCR buffer: Tris/HCl pH 8.7:

200 mM / KCl: 500 mM / $MgCl_2$: 20 mM. The DNA preparation could be stored at –20 °C for further use.

Plasmid DNA preparation from bacteria

Plasmid preparation was performed using a column pEQ-LAB Plasmid Miniprep KitI (PEQLAB Biotechnology GmbH, Erlangen) according to the manufacturer's protocol.

DNA-manipulation

DNA manipulation and cloning were carried out according to Sambrock et al.(Sambrook et al., 1989) or Ausubel (Ausubel, 1994), using standard procedures. All PCR-amplified fragments were sequenced prior to further investigation.

PCR-Primers and constructs were designed using the VectorNTI-suite 7.1 software (Invitrogen, Karlsruhe).

Cloning of complementation and reporter constructs

For the rescue construct a region of 2 kb 5' upstream of the CDKA;1 start codon together with the CDKA;1 cDNA was used. Alternatively, the same promoter region was used to rescue the cdka;1 mutant with a fusion construct consistent of the CDKA;1 cDNA and the sequence of the yellow fluorescent protein (YFP). To obtain a CDKA;1 promoter reporter construct, again the same 5´ region was fused to the ß-glucuronidase gene (GUS).

Identification of *cdka;1* mutants by PCR

Allele-specific PCRs were carried out to determine the T-DNA insertion sites using the primers J504 (left border T-DNA primer for *cdka;1-1*) and hook1 (left border T-DNA primer for *cdka;1-2*) in combination with CDKA;1-specific primers N034 and N035. To identify homozygous knockout plants rescued by a proCDKA;1:CDKA;1 or a proCDKA;1:CDKA;1:YFP construct, the primers N048 and N049 were used.

3' Rapid Amplification of cDNA Ends (3' RACE)

3' RACE (Rapid Amplification of cDNA Ends) was performed to determine whether the disrupted cdka;1 alleles still were transcribed. RNA was prepared with the RNeasy Mini kit (Quiagen). Reverse transcription was performed with Super-Script II RNase H reverse transcriptase (Invitrogen). Using the transcribed cDNAs as template, two rounds of PCR were

performed, both using CDSIII-NotI as unspecific cDNA primer, and N039 and N040 as first and nested CDKA;1 specific primer, respectively.

Quantitative PCR

Total RNA was isolated from siliques using the RNeasy Plant Minikit (QIAGEN) and treated with DNAse (TaKaRa) according to the manufacturer's protocol. The RNA concentration was measured twice using a Nanodrop ND-1000 instrument and 3.5 g of total RNA was used to synthesize cDNA by Reverse Transcription using Superscript III (Invitrogen). After RNase H treatment at 37 °C for 20 minutes, a 1:1 dilution of the synthesized 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 performed using the LightCycler 480 SYBR Green I Master Kit (Roche). Probe based qPCR with these primers was performed using Universal Probe Library (UPL) hydrolysis probes (Roche) UPL probe #147, cat.no. 04694333001 (PHE1) and UPL probe #102, cat.no. 04692209001 (ACTIN2) and the LightCycler 480 Probes Master Kit (Roche). All samples and reference controls were performed in three independent biological replica and repeated four times. The qPCR efficiency was determined independently in all replica and duplicates by series of 10-fold dilutions for each experiment. Calculation of relative expression ratios was performed according to a model described by Pfaffl et al. (Pfaffl, 2001).

Paternity test of embryo and endosperm

Arabidopsis Ler plants and mea mutants (in L*er* background) were pollinated with pollen from Col plants and cdka;1 mutants (in Col background). 6 days after pollination, the seeds were fixed in ethanol/acetic acid (3:1) and embedded in Paraplast+ (Kendall Healthcare Products, Mansfield, Massachusetts, USA) following a standard procedure for plant tissue preparation. The embedded seeds were dissected using a Rotationsmikrotom 1512 microtome (Leitz, Wetzlar, Germany). The sections (11 µm thick) were fixed on plastic-coated MembraneSlides (P.A.L.M., Bernried, Germany) and stored at 4 °C. Before further handling, the embedding medium was removed using xylole. After rehydration in an ethanol series (100 %; 96 %; 70 %; 50 % ethanol in water; finally pure water for 2 minutes each), the slides were dried and used for laser dissection microscopy. For this, the seed sections were analysed and dissected with a MircoBeam laser dissection microscope (P.A.L.M., Bernried, Germany)

and fragments of embryos or endosperms of individual seeds were separately collected and stored at –20 °C. Subsequently, the samples were sonicated using an ultrasonic water bath (Bransonic 42, Branson, Danbury, CT, USA) for 6 times 30 seconds and applied as template for an accession-specific polymerase chain reaction (PCR) using a marker (NGA6) to detect simple sequence length polymorphisms between L*er* and Col. The PCR consisted of 55 cycles of product amplification and was performed with LA Taq polymerase (TaKaRa, Shiga, Japan).

4.2.4 Biochemical methods

Protein extraction and Western blotting

For protein extraction, inflorescences of 4-5 weeks old plants were harvested, directly frozen in liquid nitrogen and homogenized in Laemmli buffer. The total amount of extracted protein was determined using Bradford reagent. Protein extract from wild-type and cdka;$1^{+/-}$ heterozygous plants were applied onto a 15 % SDS PAGE and blotted on Hybond ECL membrane. Protein gel blots were incubated overnight with anti-PSTAIRE polyclonal antibody (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany), which recognized the PSTAIRE domain residing from amino acid 45 to 51 in the CDKA;1 protein; as a secondary antibody a goat anti-rabbit antibody conjugated with horseradish-peroxidase (1:5000) was used. For detection, the SuperSignal West Pico kit (Pierce, Rockford, Illinois, USA) and BioMax Light films (Kodak) were used. To control equal loading and transfer of the probes, the membranes were stained with Ponceau S.

Table 4-1. Primers and probes

name	primer sequences (5'-3')	target site
N023_S	GGGAAGATAGAAGGGAAGAGAGAGGTAG	promoter of CDKA;1
N024_AS	CAATTCCTGAATAATAAAGCTGAAG	promoter of CDKA;1
N034_S	CCAGATTCTCCGTGGAATTGCG	wild-type CDKA;1, exon 4
N035_AS	GGAGATCGACTCCATCGGGATC	wild-type CDKA;1, exon 7
N039_S	GGATCAGTACGAGAAAGTTGAGAAGATTGG	CDKA;1 cDNA specific primer for 3'RACE
N040_S	CAGGCTAGAGCAGGAGGATGAAGGTG	CDKA;1 cDNA specific primer for 3'RACE
N048_S	CAGATCTCTTCCTGGTTATTCACA	wild-type CDKA;1, intron 4
N049_AS	TGTACAAGCGAATAAAGACATTTGA	wild-type CDKA;1, intron 6
ND10_S	AACACAAGTTTGTACAAAAAAGCAGGCTTCAACAATGGATCAGTACGAGAAAG	Gateway attB1-recombination site and 5' start of the CDKA;1 CDS
ND14_AS	CTTGCTCACCATAGGCATGCCTCCAAGATCCT	fusion primer: CDKA;1 core plus YFP overlap
ND15_S	GGAGGCATGCCTATGGTGAGCAAGGGCGAGG	fusion primer : YFP core plus CDKA ;1 overlap
ND13_AS	AACACCACTTTGTACAAGAAAGCTGGGTCTTACTTGTACAGCTCGTCC	end of the YFP cDNA plus Gateway attB2-recombination site
CDSIII-NotI	ATTCTAGAGGCCGAGGCGGCCGCCATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	unspecific cDNA primer for 3'RACE
hook1	CTACACTGAATTGGTAGCTCAAACTGTC	left border T-DNA primer for cdka;1-2 (Koncz binary vector pPVC6NFHyg)
J504	GCGTGGACCGCTTGCTGCAACTCTCTCAGG	left border T-DNA primer for cdka;1-1 (Salk binary vector pBIN-ROK)
ACT_2_102_R	CGCTCTTTCTTTCCAAGCTC	ACTIN2 (At3g18780)right primer for quantitative RT-PCR
ACT_2_102_L	CCGGTACCATTGTCACACAC	ACTIN2 (At3g18780) left primer for quantitative RT-PCR
ACT_2_102 Amplicon	CGCTCTTTCTTTCCAAGCTCATAAAAAATGGCTGAGGCTGATGATATTCAACCAATCGTGTGTGACAATGGTACCGG	77 nt amplicon of ACT_2_102 left and right primers for quantitative RT-PCR. The UPL#102 probe sequence is highlighted in bold italics.
NGA6 upstream primer	ATGGAGAAGCTTACACTGATC	simple sequence polymorphism
NGA6 downstream primer	TGGATTTCTTCCTCTCTTCAC	simple sequence polymorphism
PHE1_147_R	CGTAGCCCGTACAACTCGAT	PHERES1 right primer for quantitative RT-PCR

name	primer sequences (5'-3')	target site
PHE1_147_L	CATCACTTCTTCAACGC CTTC	PHERES1 left primer for quantitative RT-PCR
PHE1_147 Amplicon	CGTAGCCCGTACAACTC GATCCAGGAGCCTTGGC CATCAAGGGAAGGCGTT GAAGAAGTGATG	63 nt amplicon of PHE1_147 left and right primers for quantitative RT-PCR. The UPL#147 probe sequence is highlighted in bold italics.
Fie-11_F	ATTGGCTCACCACACTT AGAACTTCATAGC	forward RFLP-primer to recognize the fie-11 allele (digest Bsp1286I; wt: 293bp+325bp, fie-11: 618bp)
Fie-11_R	TGTACAATTGTCTCGGA GATGGTGCC	reverse RFLP-primer to recognize the fie-11 allele (digest Bsp1286I; wt: 293bp+325bp, fie-11: 618bp)

5 REFERENCES

Adams, S., R. Vinkenoog, M. Spielman, H. G. Dickinson and R. J. Scott (2000). "Parent-of-origin effects on seed development in Arabidopsis thaliana require DNA methylation." Development **127**(11): 2493-502.

Ausubel, F. M. (1994). Current protocols in molecular biology. New York, John Wiley & Sons, Inc.

Autran, D., W. Huanca-Mamani and J. P. Vielle-Calzada (2005). "Genomic imprinting in plants: the epigenetic version of an Oedipus complex." Curr Opin Plant Biol **8**(1): 19-25.

Baroux, C., V. Gagliardini, D. R. Page and U. Grossniklaus (2006). "Dynamic regulatory interactions of Polycomb group genes: MEDEA autoregulation is required for imprinted gene expression in Arabidopsis." Genes Dev **20**(9): 1081-6.

Baroux, C., C. Spillane and U. Grossniklaus (2002). "Genomic imprinting during seed development." Adv Genet **46**: 165-214.

Barton, S. C., M. A. Surani and M. L. Norris (1984). "Role of paternal and maternal genomes in mouse development." Nature **311**(5984): 374-6.

Bashir, T. and M. Pagano (2005). "Cdk1: the dominant sibling of Cdk2." Nat Cell Biol **7**(8): 779-81.

Beemster, G. T., S. Vercruysse, L. De Veylder, M. Kuiper and D. Inze (2006). "The Arabidopsis leaf as a model system for investigating the role of cell cycle regulation in organ growth." J Plant Res **119**(1): 43-50.

Berger, F. (1999). "Endosperm development." Curr Opin Plant Biol **2**(1): 28-32.

Berger, F. (2003). "Endosperm: the crossroad of seed development." Curr Opin Plant Biol **6**(1): 42-50.

Berger, F. (2004). "Plant sciences. Imprinting--a green variation." Science **303**(5657): 483-5.

Berger, F., P. E. Grini and A. Schnittger (2006). "Endosperm: an integrator of seed growth and development." Curr Opin Plant Biol **9**(6): 664-70.

Boisnard-Lorig, C., A. Colon-Carmona, M. Bauch, S. Hodge, P. Doerner, E. Bancharel, C. Dumas, J. Haseloff and F. Berger (2001). "Dynamic analyses of the expression of the HISTONE::YFP fusion protein in arabidopsis show that syncytial endosperm is divided in mitotic domains." Plant Cell **13**(3): 495-509.

Boudolf, V., R. Barroco, A. Engler Jde, A. Verkest, T. Beeckman, M. Naudts, D. Inze and L. De Veylder (2004a). "B1-type cyclin-dependent kinases are essential for the formation of stomatal complexes in Arabidopsis thaliana." Plant Cell **16**(4): 945-55.

Boudolf, V., D. Inze and L. De Veylder (2006). "What if higher plants lack a CDC25 phosphatase?" Trends Plant Sci **11**(10): 474-9.

Boudolf, V., K. Vlieghe, G. T. Beemster, Z. Magyar, J. A. Torres Acosta, S. Maes, E. Van Der Schueren, D. Inze and L. De Veylder (2004b). "The plant-specific cyclin-dependent kinase CDKB1;1 and transcription factor E2Fa-DPa control the balance of mitotically dividing and endoreduplicating cells in Arabidopsis." Plant Cell **16**(10): 2683-92.

Bracha-Drori, K., K. Shichrur, A. Katz, M. Oliva, R. Angelovici, S. Yalovsky and N. Ohad (2004). "Detection of protein-protein interactions in plants using bimolecular fluorescence complementation." Plant J **40**(3): 419-27.

Brown, R. C., B. E. Lemmon, H. Nguyen and O. A. Olsen (1999). "Development of endosperm in Arabidopsis thaliana." Sex Plant Reprod **12**: 32-42.

Cao, R., L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones and Y. Zhang (2002). "Role of histone H3 lysine 27 methylation in Polycomb-group silencing." Science **298**(5595): 1039-43.

Carmichael, J. S. and W. E. Friedman (1995). "Double Fertilization in Gnetum gnemon: The Relationship between the Cell Cycle and Sexual Reproduction." Plant Cell **7**(12): 1975-1988.

Casimiro, I., T. Beeckman, N. Graham, R. Bhalerao, H. Zhang, P. Casero, G. Sandberg and M. J. Bennett (2003). "Dissecting Arabidopsis lateral root development." Trends Plant Sci **8**(4): 165-71.

Chan, S. W., I. R. Henderson and S. E. Jacobsen (2005). "Gardening the genome: DNA methylation in Arabidopsis thaliana." Nat Rev Genet **6**(5): 351-60.

Chanvivattana, Y., A. Bishopp, D. Schubert, C. Stock, Y. H. Moon, Z. R. Sung and J. Goodrich (2004). "Interaction of Polycomb-group proteins controlling flowering in Arabidopsis." Development **131**(21): 5263-76.

Chaudhury, A. M., L. Ming, C. Miller, S. Craig, E. S. Dennis and W. J. Peacock (1997). "Fertilization-independent seed development in Arabidopsis thaliana." Proc Natl Acad Sci U S A **94**(8): 4223-8.

Choi, Y., J. J. Harada, R. B. Goldberg and R. L. Fischer (2004). "An invariant aspartic acid in the DNA glycosylase domain of DEMETER is necessary for transcriptional activation of the imprinted MEDEA gene." Proc Natl Acad Sci U S A **101**(19): 7481-6.

Clough, S. J. and A. F. Bent (1998). "Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana." Plant J **16**(6): 735-43.

De Schutter, K., J. Joubes, T. Cools, A. Verkest, F. Corellou, E. Babiychuk, E. Van Der Schueren, T. Beeckman, S. Kushnir, D. Inze and L. De Veylder (2007). "Arabidopsis WEE1 Kinase Controls Cell Cycle Arrest in Response to Activation of the DNA Integrity Checkpoint." Plant Cell.

De Veylder, L., T. Beeckman, G. T. Beemster, L. Krols, F. Terras, I. Landrieu, E. van der Schueren, S. Maes, M. Naudts and D. Inze (2001). "Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis." Plant Cell **13**(7): 1653-68.

Dresselhaus, T. (2006). "Cell-cell communication during double fertilization." Curr Opin Plant Biol **9**(1): 41-7.

Dresselhaus, T., S. Cordts and H. Lorz (1999). "A transcript encoding translation initiation factor eIF-5A is stored in unfertilized egg cells of maize." Plant Mol Biol **39**(5): 1063-71.

Dresselhaus, T., K. O. Srilunchang, D. Leljak-Levanic, D. N. Schreiber and P. Garg (2006). "The fertilization-induced DNA replication factor MCM6 of maize shuttles between cytoplasm and nucleus, and is essential for plant growth and development." Plant Physiol **140**(2): 512-27.

Drews, G. N. and R. Yadegari (2002). "Development and function of the angiosperm female gametophyte." Annu Rev Genet **36**: 99-124.

Durbarry, A., I. Vizir and D. Twell (2005). "Male Germ Line Development in Arabidopsis. duo pollen Mutants Reveal Gametophytic Regulators of Generative Cell Cycle Progression." Plant Physiol **137**(1): 297-307.

Ebel, C., L. Mariconti and W. Gruissem (2004). "Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte." Nature **429**(6993): 776-80.

Faure, J. E. (2001). "Double fertilization in flowering plants: discovery, study methods and mechanisms." C R Acad Sci III **324**(6): 551-8.

Faure, J. E., N. Rotman, P. Fortune and C. Dumas (2002). "Fertilization in Arabidopsis thaliana wild type: developmental stages and time course." Plant J **30**(4): 481-8.

Faure, J. E., M. L. Rusche, A. Thomas, P. Keim, C. Dumas, H. L. Mogensen, M. Rougier and A. Chaboud (2003). "Double fertilization in maize: the two male gametes from a pollen grain have the ability to fuse with egg cells." Plant J **33**(6): 1051-62.

Ferreira, P. C., A. S. Hemerly, R. Villarroel, M. Van Montagu and D. Inze (1991). "The Arabidopsis functional homolog of the p34cdc2 protein kinase." Plant Cell **3**(5): 531-40.

Finnegan, E. J., W. J. Peacock and E. S. Dennis (2000). "DNA methylation, a key regulator of plant development and other processes." Curr Opin Genet Dev **10**(2): 217-23.

Fos, M., F. Nuez and J. L. Garcia-Martinez (2000). "The gene pat-2, which induces natural parthenocarpy, alters the gibberellin content in unpollinated tomato ovaries." Plant Physiol **122**(2): 471-80.

Fos, M., K. Proano, F. Nuez and J. L. Garcia-Martinez (2001). "Role of gibberellins in parthenocarpic fruit development induced by the genetic system pat-3/pat-4 in tomato." Physiol Plant **111**(4): 545-550.

Friedman, W. E. (1992). "Evidence of a pre-angiosperm origin of endosperm: Implications for the evolution of flowering plants." Science **255**: 336-339.

Friedman, W. E. (1995). "Organismal duplication, inclusive fitness theory, and altruism: understanding the evolution of endosperm and the angiosperm reproductive syndrome." Proc Natl Acad Sci U S A **92**(9): 3913-7.

Friedman, W. E. (1999). "Expression of the cell cycle in sperm of Arabidopsis: implications for understanding patterns of gametogenesis and fertilization in plants and other eukaryotes." Development **126**(5): 1065-75.

Friedman, W. E. (2001). "Comparative embryology of basal angiosperms." Curr Opin Plant Biol **4**(1): 14-20.

Friedman, W. E. and J. H. Williams (2004). "Developmental evolution of the sexual process in ancient flowering plant lineages." Plant Cell **16 Suppl**: S119-32.

Garcia, D., J. N. Fitz Gerald and F. Berger (2005). "Maternal Control of Integument Cell Elongation and Zygotic Control of Endosperm Growth Are Coordinated to Determine Seed Size in Arabidopsis." Plant Cell **17**(1): 52-60.

Garcia-Martinez, J. L., I. Lopez-Diaz, M. J. Sanchez-Beltran, A. L. Phillips, D. A. Ward, P. Gaskin and P. Hedden (1997). "Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development." Plant Mol Biol **33**(6): 1073-84.

Gehring, M., Y. Choi and R. L. Fischer (2004). "Imprinting and seed development." Plant Cell **16 Suppl**: S203-13.

Gehring, M., J. H. Huh, T. F. Hsieh, J. Penterman, Y. Choi, J. J. Harada, R. B. Goldberg and R. L. Fischer (2006). "DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation." Cell **124**(3): 495-506.

Grini, P. E., G. Jurgens and M. Hulskamp (2002). "Embryo and endosperm development is disrupted in the female gametophytic capulet mutants of Arabidopsis." Genetics **162**(4): 1911-25.

Grossniklaus, U., J. P. Vielle-Calzada, M. A. Hoeppner and W. B. Gagliano (1998). "Maternal control of embryogenesis by MEDEA, a polycomb group gene in Arabidopsis." Science **280**(5362): 446-50.

Guitton, A. E. and F. Berger (2005a). "Control of reproduction by Polycomb Group complexes in animals and plants." Int J Dev Biol **49**(5-6): 707-16.

Guitton, A. E. and F. Berger (2005b). "Loss of function of MULTICOPY SUPPRESSOR OF IRA 1 produces nonviable parthenogenetic embryos in Arabidopsis." Curr Biol **15**(8): 750-4.

Guitton, A. E., D. R. Page, P. Chambrier, C. Lionnet, J. E. Faure, U. Grossniklaus and F. Berger (2004). "Identification of new members of Fertilisation Independent Seed Polycomb Group pathway involved in the control of seed development in Arabidopsis thaliana." Development **131**(12): 2971-81.

Haig, D. (2000). "The kinship theory of genomic imprinting." Annu. Rev. Ecol. Syst. **31**: 9-32.

Haig, D. (2004). "The (dual) origin of epigenetics." Cold Spring Harb Symp Quant Biol **69**: 67-70.

Haig, D. and M. Westoby (1989). "Parent-specific gene expression and the triploid endosperm." Am. Nat. **134**: 147-55.

Haig, D. and M. Westoby (1991). "Genomic imprinting in endosperm:its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis." Phil Trans R Soc Lond B **333**: 1-13.

Hejatko, J., M. Pernisova, T. Eneva, K. Palme and B. Brzobohaty (2003). "The putative sensor histidine kinase CKI1 is involved in female gametophyte development in Arabidopsis." Mol Genet Genomics **269**(4): 443-53.

Hemerly, A., A. Engler Jde, C. Bergounioux, M. Van Montagu, G. Engler, D. Inze and P. Ferreira (1995). "Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development." Embo J **14**(16): 3925-36.

Hemerly, A. S., P. C. Ferreira, M. Van Montagu, G. Engler and D. Inze (2000). "Cell division events are essential for embryo patterning and morphogenesis: studies on dominant-negative cdc2aAt mutants of arabidopsis." Plant J **23**(1): 123-30.

Hennig, L., W. Gruissem, U. Grossniklaus and C. Kohler (2004). "Transcriptional programs of early reproductive stages in Arabidopsis." Plant Physiol **135**(3): 1765-75.

Higashiyama, T., H. Kuroiwa and T. Kuroiwa (2003). "Pollen-tube guidance: beacons from the female gametophyte." Curr Opin Plant Biol **6**(1): 36-41.

Hirayama, T., Y. Imajuku, T. Anai, M. Matsui and A. Oka (1991). "Identification of two cell-cycle-controlling cdc2 gene homologs in Arabidopsis thaliana." Gene **105**(2): 159-65.

Hirner, B., W. N. Fischer, D. Rentsch, M. Kwart and W. B. Frommer (1998). "Developmental control of H+/amino acid permease gene expression during seed development of Arabidopsis." Plant J **14**(5): 535-44.

Honys, D. and D. Twell (2003). "Comparative analysis of the Arabidopsis pollen transcriptome." Plant Physiol **132**(2): 640-52.

Huck, N., J. M. Moore, M. Federer and U. Grossniklaus (2003). "The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception." Development **130**(10): 2149-59.

Ingouff, M., J. Haseloff and F. Berger (2005). "Polycomb group genes control developmental timing of endosperm." Plant J **42**(5): 663-74.

Ingouff, M., P. E. Jullien and F. Berger (2006). "The female gametophyte and the endosperm control cell proliferation and differentiation of the seed coat in Arabidopsis." Plant Cell **18**(12): 3491-501.

Inze, D. and L. De Veylder (2006). "Cell Cycle Regulation in Plant Development." Annu Rev Genet.

Joubes, J., K. De Schutter, A. Verkest, D. Inze and L. De Veylder (2004). "Conditional, recombinase-mediated expression of genes in plant cell cultures." Plant J **37**(6): 889-96.

Jullien, P. E., A. Katz, M. Oliva, N. Ohad and F. Berger (2006). "Polycomb Group Complexes Self-Regulate Imprinting of the Polycomb Group Gene MEDEA in Arabidopsis." Curr Biol **16**(5): 486-92.

Jurgens, G., U. Mayer, M. Busch, W. Lukowitz and T. Laux (1995). "Pattern formation in the Arabidopsis embryo: a genetic perspective." Philos Trans R Soc Lond B Biol Sci **350**(1331): 19-25.

Kiba, T., T. Naitou, N. Koizumi, T. Yamashino, H. Sakakibara and T. Mizuno (2005). "Combinatorial microarray analysis revealing arabidopsis genes implicated in

cytokinin responses through the His->Asp Phosphorelay circuitry." Plant Cell Physiol **46**(2): 339-55.

Kinoshita, T., R. Yadegari, J. J. Harada, R. B. Goldberg and R. L. Fischer (1999). "Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm." Plant Cell **11**(10): 1945-52.

Kiyosue, T., N. Ohad, R. Yadegari, M. Hannon, J. Dinneny, D. Wells, A. Katz, L. Margossian, J. J. Harada, R. B. Goldberg and R. L. Fischer (1999). "Control of fertilization-independent endosperm development by the MEDEA polycomb gene in Arabidopsis." Proc Natl Acad Sci U S A **96**(7): 4186-91.

Kohler, C. and U. Grossniklaus (2005). "Seed development and genomic imprinting in plants." Prog Mol Subcell Biol **38**: 237-62.

Kohler, C., L. Hennig, R. Bouveret, J. Gheyselinck, U. Grossniklaus and W. Gruissem (2003a). "Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development." Embo J **22**(18): 4804-14.

Kohler, C., L. Hennig, C. Spillane, S. Pien, W. Gruissem and U. Grossniklaus (2003b). "The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1." Genes Dev **17**(12): 1540-53.

Kohler, C., D. R. Page, V. Gagliardini and U. Grossniklaus (2005). "The Arabidopsis thaliana MEDEA Polycomb group protein controls expression of PHERES1 by parental imprinting." Nat Genet **37**(1): 28-30.

Leiva-Neto, J. T., G. Grafi, P. A. Sabelli, R. A. Dante, Y. M. Woo, S. Maddock, W. J. Gordon-Kamm and B. A. Larkins (2004). "A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm." Plant Cell **16**(7): 1854-69.

Lin, B. Y. (1982). "Association of Endosperm Reduction with Parental Imprinting in Maize." Genetics **100**(3): 475-486.

Lin, B. Y. (1984). "Ploidy Barrier to Endosperm Development in Maize." Genetics **107**(1): 103-115.

Lukowitz, W., U. Mayer and G. Jurgens (1996). "Cytokinesis in the Arabidopsis embryo involves the syntaxin-related KNOLLE gene product." Cell **84**(1): 61-71.

Luo, M., P. Bilodeau, E. S. Dennis, W. J. Peacock and A. Chaudhury (2000). "Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing Arabidopsis seeds." Proc Natl Acad Sci U S A **97**(19): 10637-42.

Luo, M., E. S. Dennis, F. Berger, W. J. Peacock and A. Chaudhury (2005). "MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis." Proc Natl Acad Sci U S A **102**(48): 17531-6.

Magyar, Z., L. De Veylder, A. Atanassova, L. Bako, D. Inze and L. Bogre (2005). "The role of the arabidopsis E2FB transcription factor in regulating auxin-dependent cell division." Plant Cell **17**(9): 2527-41.

Magyar, Z., T. Meszaros, P. Miskolczi, M. Deak, A. Feher, S. Brown, E. Kondorosi, A. Athanasiadis, S. Pongor, M. Bilgin, L. Bako, C. Koncz and D. Dudits (1997). "Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells." Plant Cell **9**(2): 223-35.

Maheshwari, P. and H. Singh (1967). "The female gametophyte of gymnosperms." Biological Review **42**: 88-130.

Makarevich, G., O. Leroy, U. Akinci, D. Schubert, O. Clarenz, J. Goodrich, U. Grossniklaus and C. Kohler (2006). "Different Polycomb group complexes regulate common target genes in Arabidopsis." EMBO Rep **7**(9): 947-52.

Mansfield, S. G. (1994). Arabidopsis embryo development. Arabidopsis: An Atlas of Morphology and Development. J. Bowman**:** 367-383.

Martin, A., J. Odajima, S. L. Hunt, P. Dubus, S. Ortega, M. Malumbres and M. Barbacid (2005). "Cdk2 is dispensable for cell cycle inhibition and tumor suppression mediated by p27(Kip1) and p21(Cip1)." Cancer Cell **7**(6): 591-8.

Martinez, M. C., J. E. Jorgensen, M. A. Lawton, C. J. Lamb and P. W. Doerner (1992). "Spatial pattern of cdc2 expression in relation to meristem activity and cell proliferation during plant development." Proc Natl Acad Sci U S A **89**(16): 7360-4.

McCormick, S. (2004). "Control of male gametophyte development." Plant Cell **16 Suppl**: S142-53.

McGrath, J. and D. Solter (1984). "Completion of mouse embryogenesis requires both the maternal and paternal genomes." Cell **37**(1): 179-83.

Mora-Garcia, S. and J. Goodrich (2000). "Seeds of conflict." Curr Biol **10**(2): R71-4.

Morgan, D. O. (1997). "Cyclin-dependent kinases: engines, clocks, and microprocessors." Annu Rev Cell Dev Biol **13**: 261-91.

Morison, I. M., J. P. Ramsay and H. G. Spencer (2005). "A census of mammalian imprinting." Trends Genet **21**(8): 457-65.

Muller, J., C. M. Hart, N. J. Francis, M. L. Vargas, A. Sengupta, B. Wild, E. L. Miller, M. B. O'Connor, R. E. Kingston and J. A. Simon (2002). "Histone methyltransferase activity of a Drosophila Polycomb group repressor complex." Cell **111**(2): 197-208.

Nawaschin, S. G. (1898). "Resultate einer Revision der Befruchtungsvorgänge bei *Lilium martagon* und *Fritillaria tenulla*." Bulletin de l'Academie des Sciences de Saint Petersbourg **9**: 377-382.

Ohad, N., L. Margossian, Y. C. Hsu, C. Williams, P. Repetti and R. L. Fischer (1996). "A mutation that allows endosperm development without fertilization." Proc Natl Acad Sci U S A **93**(11): 5319-24.

Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.

Pina, C., F. Pinto, J. A. Feijo and J. D. Becker (2005). "Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation." Plant Physiol **138**(2): 744-56.

Pomerening, J. R., E. D. Sontag and J. E. Ferrell, Jr. (2003). "Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2." Nat Cell Biol **5**(4): 346-51.

Porceddu, A., L. De Veylder, J. Hayles, M. Van Montagu, D. Inze and V. Mironov (1999). "Mutational analysis of two Arabidopsis thaliana cyclin-dependent kinases in fission yeast." FEBS Lett **446**(1): 182-8.

Porceddu, A., H. Stals, J. P. Reichheld, G. Segers, L. De Veylder, R. P. Barroco, P. Casteels, M. Van Montagu, D. Inze and V. Mironov (2001). "A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants." J Biol Chem **276**(39): 36354-60.

Ray, S., T. Golden and A. Ray (1996). "Maternal effects of the short integument mutation on embryo development in Arabidopsis." Dev Biol **180**(1): 365-9.

Rios, G., A. Lossow, B. Hertel, F. Breuer, S. Schaefer, M. Broich, T. Kleinow, J. Jasik, J. Winter, A. Ferrando, R. Farras, M. Panicot, R. Henriques, J. B. Mariaux, A. Oberschall, G. Molnar, K. Berendzen, V. Shukla, M. Lafos, Z. Koncz, G. P. Redei, J. Schell and C. Koncz (2002). "Rapid identification of Arabidopsis insertion mutants by non-radioactive detection of T-DNA tagged genes." Plant J **32**(2): 243-53.

Riou-Khamlichi, C., R. Huntley, A. Jacqmard and J. A. Murray (1999). "Cytokinin activation of Arabidopsis cell division through a D-type cyclin." Science **283**(5407): 1541-4.

Rogers, S. O. and A. J. Bendich (1988). Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. B. Gelvin, R. A. Schilperoort and D. P. S. Verma. Dordrecht, Kluwer Academic Publishers**:** A6/1-A6/11.

Rotino, G. L., E. Perri, M. Zottini, H. Sommer and A. Spena (1997). "Genetic engineering of parthenocarpic plants." Nat Biotechnol **15**(13): 1398-401.

Rotman, N., A. Durbarry, A. Wardle, W. C. Yang, A. Chaboud, J. E. Faure, F. Berger and D. Twell (2005). "A Novel Class of MYB Factors Controls Sperm-Cell Formation in Plants." Curr Biol **15**(3): 244-248.

Rotman, N., F. Rozier, L. Boavida, C. Dumas, F. Berger and J. E. Faure (2003). "Female control of male gamete delivery during fertilization in Arabidopsis thaliana." Curr Biol **13**(5): 432-6.

Russell, S. D. (1985). "Preferential fertilization in Plumbago: Ultrastructural evidence for gamete-level recognition in an angiosperm." Proc Natl Acad Sci U S A **82**(18): 6129-6132.

Sambrook, J., E. Fritsch and T. Maniatis (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Sargant, E. (1900). "Recent work on the results of fertilization in angiosperms." Ann. Bot. **14**: 689–712.

Schnittger, A., C. Weinl, D. Bouyer, U. Schobinger and M. Hulskamp (2003). "Misexpression of the cyclin-dependent kinase inhibitor ICK1/KRP1 in single-celled Arabidopsis trichomes reduces endoreduplication and cell size and induces cell death." Plant Cell **15**(2): 303-15.

Schruff, M. C., M. Spielman, S. Tiwari, S. Adams, N. Fenby and R. J. Scott (2006). "The AUXIN RESPONSE FACTOR 2 gene of Arabidopsis links auxin signalling, cell division, and the size of seeds and other organs." Development **133**(2): 251-61.

Scott, R. J. and M. Spielman (2006). "Genomic imprinting in plants and mammals: how life history constrains convergence." Cytogenet Genome Res **113**(1-4): 53-67.

Scott, R. J., M. Spielman, J. Bailey and H. G. Dickinson (1998). "Parent-of-origin effects on seed development in Arabidopsis thaliana." Development **125**(17): 3329-41.

Serralbo, O., J. M. Perez-Perez, R. Heidstra and B. Scheres (2006). "Non-cell-autonomous rescue of anaphase-promoting complex function revealed by mosaic analysis of HOBBIT, an Arabidopsis CDC27 homolog." Proc Natl Acad Sci U S A **103**(35): 13250-5.

Sessions, A. and M. F. Yanofsky (1999). "Dorsoventral patterning in plants." Genes Dev **13**(9): 1051-4.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-12.

Sherr, C. J. and J. M. Roberts (2004). "Living with or without cyclins and cyclin-dependent kinases." Genes Dev **18**(22): 2699-711.

Simchen, G. (1978). "Cell cycle mutants." Annu Rev Genet **12**: 161-91.

Sorensen, M. B., U. Mayer, W. Lukowitz, H. Robert, P. Chambrier, G. Jurgens, C. Somerville, L. Lepiniec and F. Berger (2002). "Cellularisation in the endosperm of Arabidopsis thaliana is coupled to mitosis and shares multiple components with cytokinesis." Development **129**(24): 5567-76.

Sorrell, D. A., M. Menges, J. M. Healy, Y. Deveaux, C. Amano, Y. Su, H. Nakagami, A. Shinmyo, J. H. Doonan, M. Sekine and J. A. Murray (2001). "Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar Bright Yellow-2 cells." Plant Physiol **126**(3): 1214-23.

Spillane, C., C. MacDougall, C. Stock, C. Kohler, J. P. Vielle-Calzada, S. M. Nunes, U. Grossniklaus and J. Goodrich (2000). "Interaction of the Arabidopsis polycomb group proteins FIE and MEA mediates their common phenotypes." Curr Biol **10**(23): 1535-8.

Sprunck, S., U. Baumann, K. Edwards, P. Langridge and T. Dresselhaus (2005). "The transcript composition of egg cells changes significantly following fertilization in wheat (Triticum aestivum L.)." Plant J **41**(5): 660-72.

Stern, B., G. Ried, N. J. Clegg, T. A. Grigliatti and C. F. Lehner (1993). "Genetic analysis of the Drosophila cdc2 homolog." Development **117**(1): 219-32.

Strasburger, E. (1900). "Einige Bemerkungen zur Frage nach der ‘‘Doppelten Befruchtung’’ bei Angiospermen." Bot. Z. **58**: 293–316.

Surani, M. A., S. C. Barton and M. L. Norris (1984). "Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis." Nature **308**(5959): 548-50.

Thomas, E. N. (1907). "Some aspects of "double fertilization" in plants." Sci. Prog. **1**: 420-426.

Tian, H. Q. (2005). "Relationship between double fertilization and the cell cycle in male and female gametes of tobacco." Sex Plant Reprod **17**: 243-252.

Ubersax, J. A., E. L. Woodbury, P. N. Quang, M. Paraz, J. D. Blethrow, K. Shah, K. M. Shokat and D. O. Morgan (2003). "Targets of the cyclin-dependent kinase Cdk1." Nature **425**(6960): 859-64.

Vandepoele, K., J. Raes, L. De Veylder, P. Rouze, S. Rombauts and D. Inze (2002). "Genome-wide analysis of core cell cycle genes in Arabidopsis." Plant Cell **14**(4): 903-16.

Verkest, A., C. L. Manes, S. Vercruysse, S. Maes, E. Van Der Schueren, T. Beeckman, P. Genschik, M. Kuiper, D. Inze and L. De Veylder (2005a). "The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf development through inhibition of mitotic CDKA;1 kinase complexes." Plant Cell **17**(6): 1723-36.

Verkest, A., C. Weinl, D. Inze, L. De Veylder and A. Schnittger (2005b). "Switching the cell cycle. Kip-related proteins in plant cell cycle control." Plant Physiol **139**(3): 1099-106.

Vielle-Calzada, J. P., R. Baskar and U. Grossniklaus (2000). "Delayed activation of the paternal genome during seed development." Nature **404**(6773): 91-4.

Vielle-Calzada, J. P., J. Thomas, C. Spillane, A. Coluccio, M. A. Hoeppner and U. Grossniklaus (1999). "Maintenance of genomic imprinting at the Arabidopsis medea locus requires zygotic DDM1 activity." Genes Dev **13**(22): 2971-82.

Vinkenoog, R., M. Spielman, S. Adams, R. L. Fischer, H. G. Dickinson and R. J. Scott (2000). "Hypomethylation promotes autonomous endosperm development and rescues postfertilization lethality in fie mutants." Plant Cell **12**(11): 2271-82.

Virchow, R. L. K. (1855). "Cellular-Pathologie." Archiv für Pathologische Anatomie und Physiologie **8**: 3-39.

Vivian-Smith, A. and A. M. Koltunow (1999). "Genetic analysis of growth-regulator-induced parthenocarpy in Arabidopsis." Plant Physiol **121**(2): 437-51.

Wang, H., Y. Zhou, S. Gilmer, S. Whitwill and L. C. Fowke (2000). "Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology." Plant J **24**(5): 613-23.

Wang, J., J. Mager, Y. Chen, E. Schneider, J. C. Cross, A. Nagy and T. Magnuson (2001). "Imprinted X inactivation maintained by a mouse Polycomb group gene." Nat Genet **28**(4): 371-5.

Weijers, D., N. Geldner, R. Offringa and G. Jurgens (2001). "Seed development: Early paternal gene activity in Arabidopsis." Nature **414**(6865): 709-10.

Weinl, C., S. Marquardt, S. J. Kuijt, M. K. Nowack, M. J. Jakoby, M. Hulskamp and A. Schnittger (2005). "Novel Functions of Plant Cyclin-Dependent Kinase Inhibitors, ICK1/KRP1, Can Act Non-Cell-Autonomously and Inhibit Entry into Mitosis." Plant Cell **17**(6): 1704-22.

Wersto, R. P., F. J. Chrest, J. F. Leary, C. Morris, M. A. Stetler-Stevenson and E. Gabrielson (2001). "Doublet discrimination in DNA cell-cycle analysis." Cytometry **46**(5): 296-306.

Weterings, K. and S. D. Russell (2004). "Experimental analysis of the fertilization process." Plant Cell **16 Suppl**: S107-18.

Xiao, W., M. Gehring, Y. Choi, L. Margossian, H. Pu, J. J. Harada, R. B. Goldberg, R. I. Pennell and R. L. Fischer (2003). "Imprinting of the MEA Polycomb gene is controlled by antagonism between MET1 methyltransferase and DME glycosylase." Dev Cell **5**(6): 891-901.

Yadegari, R. and G. N. Drews (2004). "Female gametophyte development." Plant Cell **16 Suppl**: S133-41.

Yadegari, R., T. Kinoshita, O. Lotan, G. Cohen, A. Katz, Y. Choi, K. Nakashima, J. J. Harada, R. B. Goldberg, R. L. Fischer and N. Ohad (2000). "Mutations in the FIE and MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms." Plant Cell **12**(12): 2367-2382.

Zhou, Y., H. Wang, S. Gilmer, S. Whitwill, W. Keller and L. C. Fowke (2002). "Control of petal and pollen development by the plant cyclin-dependent kinase inhibitor ICK1 in transgenic Brassica plants." Planta **215**(2): 248-57.

ERKLARUNG

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Novel Functions of Plant Cyclin-Dependent Kinase Inhibitors, ICK1/KRP1, Can Act Non-Cell-Autonomously and Inhibit Entry into Mitosis

Weinl, C., S. Marquardt, S. J. Kuijt, M. K. Nowack, M. J. Jakoby, M. Hulskamp and A. Schnittger. Plant Cell 17(6): 1704-22 (2005).

A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis

Nowack, M. K., P. E. Grini, M. J. Jakoby, M. Lafos, C. Koncz and A. Schnittger. Nat Genet 38(1): 63-7 (2006).

T-Loop Phosphorylation of Arabidopsis CDKA;1 Is Required for Its Function and Can Be Partially Substituted by an Aspartate Residue

Dissmeyer, N., M. K. Nowack, S. Pusch, H. Stals, D. Inzé, P. E. Grini and A. Schnittger. Plant Cell (in print)

Bypassing genomic imprinting allows seed development

Nowack, M. K., R. Shirzadi, N. Dissmeyer, A. Dolf, E. Endl, P. E. Grini and A. Schnittger. (Manuscript under review)

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