Analysis of cell proliferation and growth control during seed development in *Arabidopsis thaliana*

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Manoj Kumar

aus Begusarai; Indien

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Berichterstatter:	Prof. Dr. Martin Hülskamp
	Prof. Dr. Maarten Koornneef
Prüfungsvorsitzender:	Prof. Dr. Siegfried Roth
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Abstract

Endosperm supports the development of embryos and thus, is crucial for the life cycle and reproductive success of flowering plants. In addition, the endosperm of especially grass species is of key importance for human nutrition. Endosperm development relies on rapid free nuclear divisions resulting in formation of large syncytium with many hundred nuclei. Despite of its importance, not much is known about the cell cycle machinery that drives divisions during endosperm development. In the present study, two approaches were followed to get insights into the molecular machinery that controls endosperm proliferation and growth. In the first part, one subfamily of mitotic cyclins, the B1-group comprising five members, was analysed. Expression and localisation studies revealed that B1-type cyclins accumulate during G2/M phase and localise to chromatin and the spindle during mitosis. B1-type cyclins were found to redundantly control mitosis throughout plant development and in particular the nuclear divisions of endosperm were affected in double and triple mutants. This work revealed that B1 type cyclins are responsible to maintain the spindle architecture thereby regulating cell division. To complement the reverse genetics approach, I exploited the existing natural variation in Arabidopsis to identify important regulators of seed growth and nuclear proliferation. The analysis of seed mass trait in three recombinant inbred line populations having one common parent discovered 12 QTLs, 10 main and 2 complex loci influencing seed mass in Arabidopsis. In addition, by using Est-Col RIL population 2 QTLS, 1 main and 1 complex locus that influence the development of autonomous endosperm upon *cdka;1* pollination were found. The loci did not co-localise with B1-type cyclins and thus, represent additional regulators of endosperm proliferation and growth.

Zusammenfassung

Das Endosperm dient der Ernährung des Embryos und ist daher von wesentlicher Bedeutung für das Überleben und den evolutiven Erfolg der Blütenpflanzen. Insbesondere in Gramineen spielt das Endosperm eine entscheidende Rolle für die menschliche Ernährung. Die Entwicklung dieses Gewebes basiert auf der schnellen Abfolge von Kernteilungzyklen, die zur Bildung eines großen Syncytiums mit mehreren hundert Kernen führt. Trotz der eminenten Wichtigkeit des Endosperms ist die dabei treibende Kraft, die Regulation durch die Zellzyklus-Maschinerie während dieses Prozesses, nur wenig verstanden. In der vorliegenden Arbeit wurden zwei Ansätze zur Analyse der molekularen Rolle der Zellzyklusmaschinerie für die Kontrolle des Wachstums und der Proliferation des Endosperms verfolgt. Der erste Abschnitt beschäftigt sich mit einer, fünf Mitglieder zählenden, Unterfamilie mitotischer Zykline vom B1-Typ (B1-Zykline). Die Untersuchung der Expression und Lokalisierung dieser B1-Zykline offenbarte eine Anreicherung während der G2/M-Phase, als auch eine Bindung an Chromatin und den mitotischen Spindelapparat. Diese B1-Zykline regulieren auf redundante Art und Weise die Mitose während des gesamten Lebenszyklus; insbesondere im Endosperm zeigen Doppel- und Tripelmutanten dieser Zykline massive Kernteilungsdefekte. In dieser Arbeit konnte gezeigt werden, dass B1-Zykline essentiell zur Aufrechterhaltung der Architektur des Spindelapparats sind und dadurch die Zellteilung kontrollieren. Zusätzlich zum revers-genetischen Ansatz wurde die Existenz natürlicher Variabilität bei *Arabidopsis* genutzt um wichtige Regulatoren des Samenwachstums und der Endosperm-Proliferation zu identifizieren. Die Analyse der Samenmasse in rekombinanten Populationen dreier Inzucht-Linien (*recombinant imbred lines* - RIL), die aus einer einheitlichen Elterngeneration hervorgehen, ergab 12 QTLs, hierin 10 Haupt- und 2 komplexe loci, die einen wichtigen Einfluss auf die Samenmassenproduktion ausüben. Zusätzlich wurden anhand einer *Est-Col* RIL Population zwei QTLs, ein Haupt- und ein komplexer Locus, identifiziert, die die Entwicklung autonomer Endosperm-Proliferation aufgrund der Befruchtung mit *cdka;1*-mutantem Pollen beinflussen. Die genomische Lokalisierung dieser QTLs stimmt nicht mit der Position der B1-Zykline überein und stellen daher zusätzliche Regulatoren der Endosperm-Proliferation dar.

Publications

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

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

- For this paper, I measured the seed weight and size from different accessions of *Arabidopsis thaliana.*

Abbreviations and gene names

%	percent
°C	degree Celsius
3'	three prime end of a DNA fragment
5'	five prime end of a DNA fragment
C	DNA content of a haploid genome
CDKA;1	*CYCLIN-DEPENDENT KINASE A1*
cDNA	complementary DNA
Col-0	*Arabidopsis thaliana* Columbia-0 accession
DAG	days after germination
DAP	days after pollination
DAPI	4',6-diamidino-2-phenylindole
DNA	desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EDV	endosperm division value
Est-1	*Arabidopsis thaliana* Estland-1 accession
et al.	*et alii* / *et aliae* [Lat.] and others
FACS	Fluorescence-activated cell sorting
F1, F2	first, second, third... filial generation after a cross
FIE	*FERTILIZATION-INDEPENDENT ENDOSPERM*
Fig.	Figure
FIS2	*FERTILIZATION-INDEPENDENT SEED 2*
FIS-class	proteins forming the core of the FIS-PRC2
FIS-PRC2	Arabidopsis PRC2 containing MEA, FIS2, FIE, and MSI1
G1	Gap 1 phase
G2	Gap 2 phase
gene-/-	homozygous mutant of a gene
gene+/-	heterozygous mutant of a gene
GFP	green fluorescent protein
GLC	*GLAUCE*
GUS	*beta-glucuronidase*
i.e.	Id est (Lat.) that is
IKU2	*HAIKU 2*

kb	1000 base pairs
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession
M	Mitosis
MS	Murashige & Skoog media
n	Number
No-0	*Arabidopsis thaliana* Nossen-0 accession
N-terminus	amino-terminus
ORF	OPEN READING FRAME
PCR	polymerase chain reaction
qRT-PCR	quantitative Real-time PCR
QTL	quantitative trait loci
RBR1	*RETINOBLASTOMA RELATED 1*
RNA	ribonucleic acid
RT PCR	reverse transcription followed by a polymerase chain reaction
S phase	synthetic phase of the cell cycle
Sha	*Arabidopsis thaliana* Shahdara accession
TAIR	The Arabidopsis Information Resource
T-DNA	transferred DNA
Tris/HCl	buffer containing 2-amino-e-hydroxymethyl-1,3-propanediol and HCl
UTR	untranslated region
wt	wild-type
x	crossed to (crosses are always indicated in the order: female x male)

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

1 Introduction

1.1 Plant life cycle and seed formation

The life cycle of seed-producing plants begin with the germination of seedlings from seeds. In flowering plants (angiosperms), seeds are the product of a unique double fertilisation event. The general scheme of double fertilisation is that one of the two haploid male gamete fuses to the haploid egg cell resulting in the formation of a diploid embryo, whereas the other haloid male gamete fuses to the homodiploid central cell forming a triploid endosperm (Raghavan, 2006). The developing embryo and the endosperm are surrounded with maternally derived integument, which later develop into the seed coat. The overall structure containing embryo and endosperm along with maternally derived seed coat is called seed. Thus, a tight coordination between these parallelly developing structures is required for seed development (Nowack et al., 2010).

The overall process of seed development differs in dicots and monocots with respect to the development of embryo and endosperm. In dicots, like Arabidopsis, the embryo constitutes the major part of the mature seed and endosperm remains as supportive tissue, whereas in monocots like rice or maize, endosperm covers the majority of seed space with relatively little space left for developing embryo (Raghavan, 2006). Seeds represent almost 60% of the world's direct food supply along with vegetative oils and other nutritive requirements, wherein most of the nutrients being stored in the nourishing endosperm tissues. In addition, due to changing ecological, economical and demographical conditions there exists a huge deficit in the land space for cultivation. Considering these factors, in-depth understanding of seed development and in particular, endosperm development, is essential to meet the ongoing nutrient requirements and to combat environmental challenges in crop cultivation.

1.2 Endosperm development

Following double fertilisation the endosperm development can be divided into three successive phases i.e. syncytial, cellular and maturation (Olsen, 2004).

1.2.1 Syncytial phase

After double fertilisation, the central cell undergoes repeated rounds of nuclear divisions without cytokinesis leading to the formation of multinucleate cell (Figure I-1) (Brown et al., 2003). In absence of cytokinesis, the nuclei divide rapidly and start to settle down on the periphery of the endosperm around the characteristic vacuole in the centre of the cell. The syncytial phase has been further subdivided into stage 1 to 8 in Arabidopsis (Boisnard-Lorig et al., 2001). This classification was based on the spatiotemporal pattern of mitotic cyclins CYCB1;1 and CYCB1;2 accumulation in the syncytium during Arabidopsis endosperm development. The syncytium was organised into three domains in which nuclei divide simultaneously with a specific time course (Boisnard-Lorig et al., 2001).

The syncytial phase nuclei are surrounded by cytoplasm along with dense cortical array of microtubules also known as radial microtubule system (RMS) (Figure I-1). The RMS is responsible to position the nuclei and organising the cytoplasm into nuclear cytoplasmic domain (NCD) and further initiates the formation of phragmoplast at the boundaries of NCDs (Brown and Lemmon, 2001).

1.2.2 Cellular phase

The syncytial phase ends with the beginning of cellularisation of nuclei in the anterior and peripheral domains making individual cells. The study of Arabidopsis recessive mutants like *iku1*, *iku2* and *mini3* which are deficient in either endosperm growth or maternal integument development revealed early cellularisation in the seeds without affecting the cell cycle (Garcia et al., 2003; Luo et al., 2005).

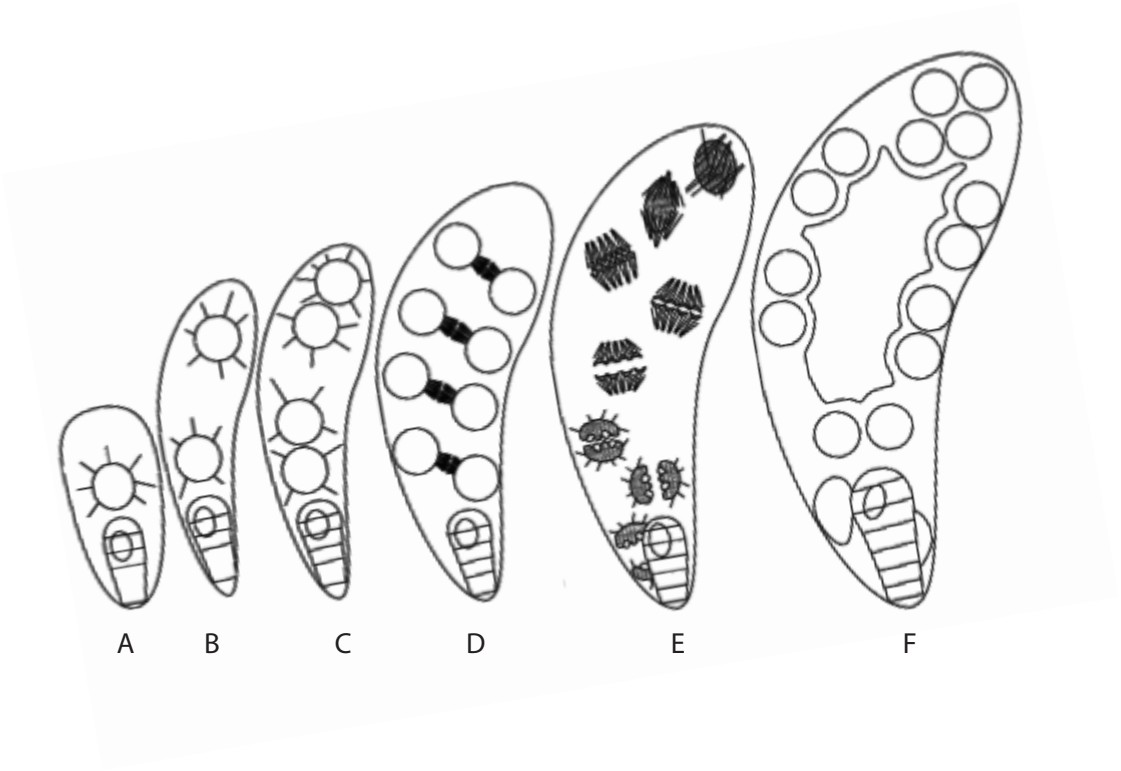

Figure I-1 Schematic representation of development of endosperm (upto 16-nuclei stage) in Arabidopsis thaliana.
(A) Primary endosperm nucleus showing position near zygote. (B) Upon 1st division, two nuclei are formed that get positioned at poles of enlarging central cell. (C) 2nd division produces 4 nuclei, relatively in a filamentous arrangement. (D) Upon 3rd division, 8 nuclei are formed arranged in planar arrangement. These first three rounds of division leading to the 8-nuclei stage are synchronous. (E) Successive 4th division begins at micropylar region and proceeds to the chalazal. (F) Upon 4th division, 16 nuclei are formed that are arranged into three developmental domains within the common cytoplasm. Nuclei around the zygote arranged in the micropylar domain are elongated, those in the central chamber are peripheral to the central vacuole, while nuclei that arranges in the chalaza are clustered in dense cytoplasm (Taken from Brown and Lemmon 2003).

Cellularisation is similar to cytokinesis occurring in usual somatic plant cells involving rearrangement of microtubules, actins and associated proteins and vesicle trafficking with certain differences in cytoskeleton arrangements, for example, during endosperm cellularisation, the future division plate is defined by the RMS surrounding NCDs in absence of preprophase band (Sorensen et al., 2002b; Olsen, 2004).

1.2.3 Maturation phase

Once the cellularisation is over, the maturation phase starts that comprises of three rounds of cell division in Arabidopsis leading to the formation of few more layers of endosperm cells. In Arabidopsis, nutrient reserves are stored in embryo, which grows rapidly consuming the cellular endosperm. In cereals the outer layer of cellular endosperm is known as aleurone layer. The endosperm development ends with the beginning of desiccation of seeds.

1.3 Genetic studies of endosperm development and seed growth

In flowering plants, successful completion of seed formation depends on adequate endosperm development, which further provides nutrient support to the developing embryo. Cooper and Brink provided the first evidence that compromised growth of endosperm leads to early collapse of developing seed (Cooper and Brink, 1942). After double fertilisation event, endosperm nuclei divide much more rapidly compared to embryo or integuments of the ovule nonetheless endosperm development is coordinated with the development of the embryo and seed coat (Garcia et al., 2005). Recent genetic studies suggest that the early growth phase of the endosperm is under the control of the HAIKU (IKU) pathway, in which *IKU1* encodes a VQ motif protein, *IKU2* encodes a leucine-rich repeat kinase and *MINI3* encodes a WRKY family protein, a putative transcription factor regulating endosperm growth (Garcia et al., 2003; Luo et al., 2005; Wang et al., 2010). Similarly, it was also observed that mutation in the *TTG2* gene results in reduction of integument cell elongation, which further reduces endosperm growth (Garcia et al., 2005). On the other hand, mutations in *AP2, ARF2* and *DA1* lead to increase in seed size and mass (Jofuku et al., 2005; Ohto et al., 2005; Schruff et al., 2006; Ohto et al., 2009). Furthermore, the gene dosage also appears to be crucial to determine final seed size considering that increased paternal contribution resulted in larger seeds due to increased number of endosperm nuclei whereas excess of the maternal genome resulted in smaller seeds (Scott et al., 1998).

1.4 Cell cycle regulators during endosperm development

Many of the mutants that affect endosperm development are associated with cell division defects leading to altered endosperm proliferation or cellularisation (Sabelli and Larkins, 2009). A recent study of transcriptome analysis of proliferating endosperm has identified 793 genes that are expressed during early seed development and have endosperm preferred expression profile (Day et al., 2008). Out of the 793 genes, 27 genes were found to be core cell cycle related. The core cell cycle genes included all three classes of cyclins, cyclin dependent kinase B and D along with inhibitors of *CDKs* like *KRPs*. However not much is known about the molecular mechanism of nuclear division in endosperm and how various cell cycle regulators contribute during endosperm development.

Nevertheless, various mutants have been reported with defects in endosperm cellularisation such as *atfh5*, *knolle*, *hinkel*, *pleiade* and *spätzle* (Sorensen et al., 2002a; Ingouff et al., 2005). KNOLLE, HINKEL and ATFH5 localises to the cell plate, whereas PLEIADE is a microtubule-associated protein, which forms cross-bridges among microtubules (Lauber et al., 1997; Muller et al., 2002; Strompen et al., 2002; Ingouff et al., 2005). These mutants are not directly involved in endosperm division instead they regulate endosperm indirectly through cytokinesis.

Some mutants of Arabidopsis like *titan*, *pilz* and *ede1* have been characterised that showed the presence of enlarged nuclei in the endosperm resulting from successive rounds of DNA replication without subsequent separation of the chromosomes and the formation of daughter nuclei (Liu and Meinke, 1998; Steinborn et al., 2002; Tzafrir et al., 2002; Pignocchi et al., 2009). The *PILZ* group of genes encode proteins of the tubulin-folding complex required for microtubule formation whereas *TITAN* genes encode proteins that are involved in chromosome dynamics, and regulation of intracellular vesicle transport (Liu and Meinke, 1998; Steinborn et al., 2002; Tzafrir et al., 2002). The EDE1 is a novel plant-specific protein, which is essential for microtubule function and nuclear proliferation during endosperm development (Pignocchi et

al., 2009). In spite of the fact that these mutants cause defects related to cell division there relationship to core cell cycle genes is not yet understood.

1.5 Cell cycle control in plants

The growth and development of all organisms depends on precise replication and equal distribution of the genetic material, which is accomplished during the cell cycle. The cell cycle is largely divided into two phases: interphase and mitosis (M). The interphase is the period in which cells prepare themselves to undergo mitosis, which is further subdivided into three phases G1 (gap 1), S (Synthesis), G2 (gap 2) (Figure I-2). G1- and G2- phases are also known as growth phase during which cells increase their size and synthesise different proteins and enzymes required for cell division. DNA replication occurs during S-phase. During mitosis, a single cell divides into two daughter cells typically containing equal chromosome numbers and similar genetic material alike the mother cell (Figure I-2). Although the overall cell cycle control machinery in plants is in general very much similar to other eukaryotes, several aspects are unique to plants (Inze and De Veylder, 2006; De Veylder et al., 2007). For instance, there are several cell cycle genes that are unique to plant cells or participate in plant specific processes such as plant specific cyclin-dependent kinases like *CDKBs*, large number of cyclins compared to other organisms or plant specific cell cycle inhibitors e.g. *SIAMESE*/*SIAMESE RELATED* (*SIM*/*SMR*) suggesting the different regulatory network of cell cycle progression in plants compared to other organisms (Churchman et al., 2006; Vandepoele et al., 2006; Peres et al., 2007; Dissmeyer et al., 2010).

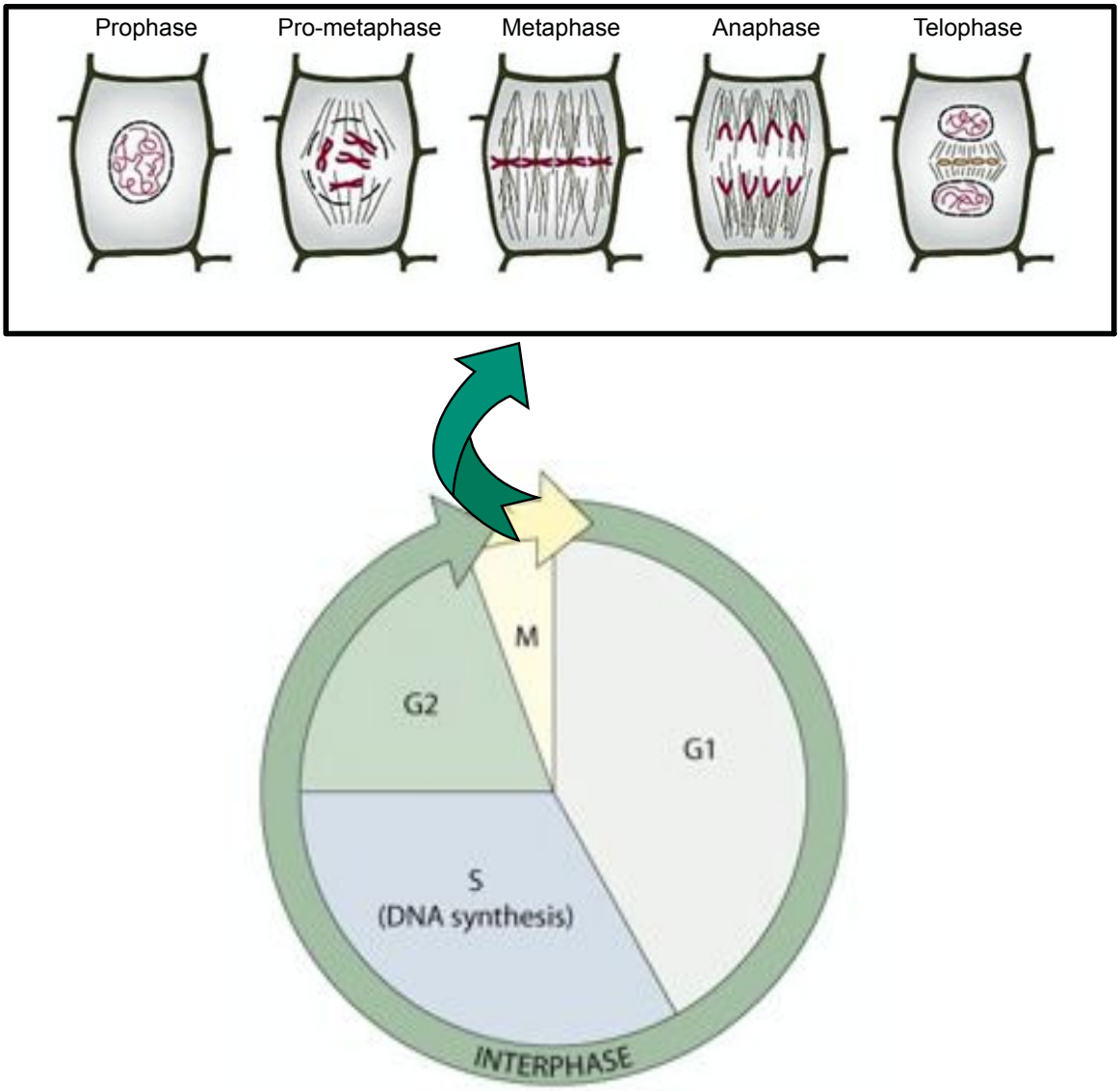

Figure I-2. General scheme of a eukaryotic cell-division cycle.
The cell cycle is largely divided into two phases: interphase and mitosis (M). Interphase starts with the G1 phase, in which the biosynthetic activities of the cells take place; then moves into S phase where the DNA content is doubled and the last phase is G2 phase, which continues until mitosis starts. The mitotic event starts with condensation of chromosome into two sister chromatids (Prophase), break down of nuclear envelope (Prometaphase) and alignment of the chromosomes at metaphase plate (Metaphase), followed by separation of chromatids and movement to spindle poles (Anaphase) and reappearance of nuclear envelope (Telophase). Finally, the two daughter cells are formed upon cytokinesis.

1.5.1 Cyclin-Dependent Kinases

Cyclin-Dependent Kinases (CDKs) are considered to be the central component of cell cycle control machinery in eukaryotes, which regulate the cell cycle checkpoints (Morgan, 1997). These are small proteins (34-40 kDa) belonging to the family of serine-threonine protein kinases and are the essential part of heteromeric enzyme complex also known as mitosis or maturation promoting factor (MPF), consisting of a kinase subunit and a cyclin subunit (Morgan and De Bondt, 1994). The concentration of the kinase subunit is relatively constant in comparison to the cyclin subunit, which oscillates during the progression of cell cycle. The kinase subunit is completely inactive in absence of cyclin subunit, but even after binding of cyclin subunit, it still requires phosphorylation of threonine residues near the kinase active site (Morgan, 2007). CDKs phosphorylate large number of proteins in the cell, thus affecting several cellular activities in various ways (Ubersax et al., 2003). The CDK substrates are phosphorylated either at serine or threonine residue typically found as consensus sequence like [S/T]PX[K/R] where P is proline, X is any amino acid and K/R is basic residue lysine or arginine (Nigg, 1993).

In all eukaryotic organisms, at least one of the CDKs also contains a conserved PSTAIRE motif in the N-terminal cyclin binding domain (Meyerson et al., 1992). *Arabidopsis thaliana* genome consists of 15 CDKs or CDK like kinases, out of which there is only one member which carry PSTAIRE motif and called as CDKA;1, which plays an important role at G1/S and G2/M transition points (Menges and Murray, 2002; Inze and De Veylder, 2006). Over expression of CDKA;1 has no effect in Arabidopsis, but homozygous loss of function mutants could not be generated because of defective second mitosis during male gametophyte development in heterozygous plants (Iwakawa et al., 2006; Nowack et al., 2006). Weak loss of function mutants of CDKA;1 have cells which are significantly larger in size compared to wild type although overall plant size is smaller (Dissmeyer et al., 2007; Dissmeyer et al., 2009).

The non PSTAIRE CDKs have been classified from CDKB to CDKG as well as CDK like kinases (CLKs) (Joubes et al., 2000). CDKBs are plant specific, as they have not been found in any other species yet. CDKBs contain altered PSTAIRE

motif and based on their cyclin binding domains, are further grouped in two classes namely CDKB1 having PPTALRE and CDKB2 having PS/PTTLRE motif instead of PSTAIRE. Both subgroups have two members in each, making four CDKBs in total in Arabidopsis genome (Vandepoele et al., 2002). The transcript accumulation pattern of the *CDKB1* and *CDKB2* members varies during cell cycle as *CDKB1* accumulates during S, G2 and M phase where as *CDKB2* expression is specific to G2 and M phases, suggesting their possible role in mitotic entry as well as in mitosis (Fobert et al., 1996; Segers et al., 1996; Magyar et al., 1997; Umeda et al., 1999; Breyne et al., 2002). Recent reports suggest role of CDKF;1 in post-embryonic development in Arabidopsis by regulating the protein stability of CDKD;2 (Takatsuka et al., 2009). The functions of the remaining CDKs have not yet been understood clearly. However we do not know yet which of the cyclin dependent kinase is important during endosperm development and proliferation.

1.5.2 Regulation of CDK-cyclin activity

The activity of CDK in animals and yeast can be regulated by at least four means - binding of positive cofactors like cyclins, negative regulators like CDK inhibitors and positive and negative phosphorylation events of the T- and P- loop respectively (Pines, 1995). As in other multicellular organisms phosphorylation of the T-loop of the Arabidopsis CDKA;1 is required for proper functioning of CDKA;1 (Dissmeyer et al., 2007; Harashima et al., 2007). The inactivation of CDK/cyclin complexes can be achieved by ubiquitin-mediated degradation of cyclins along with a distinct mode of reversible inactivation triggered by phosphorylating the CDK residues located within the CDK ATP-binding loop (Nurse, 1997; Peters, 2002). The negative phosphorylation event is mediated by Wee1/Mik1/Myt1 protein kinases and the complex remains in an inactive state (Norbury et al., 1991).

Furthermore, CDK inhibitors (CKIs) that often build a small family of low molecular weight proteins, also inhibit CDK activity by tight association with the cyclin/CDK complexes (Denicourt and Dowdy, 2004). In Arabidopsis, seven CDK inhibitors were identified (designated KRPs or Kip-related proteins) and all

except KRP5 seem to interact with CDKA;1 but none with the mitotic CDKB1;1 (Lui et al., 2000; De Veylder et al., 2001).

1.5.3 Cyclins

Cyclins are diverse family of proteins, which regulate the cell cycle by activating CDKs. The concentrations of many cyclins dramatically change during cell cycle, being the primary cause of rising and falling levels of CDK activity, which is the core of cell cycle control system (Morgan, 2007).

Based on their temporal expression and functions in the cell cycle, cyclins has been divided into four classes in yeast and vertebrate somatic cells, G1 cyclins, G1/S cyclins, S cyclins and M cyclins (Morgan, 2007).

The G1 cyclins (Cln3 in budding yeast), are not directly involved in the cell cycle control instead help to coordinate cell growth with entry into a new cell cycle. Studies in yeast suggests that G1 cyclins differ from other cyclins, as their levels do not oscillate during the cell cycle instead increase gradually throughout the cycle in response to cell growth.

The G1/S cyclins (Cln1 and Cln2 in the budding yeast *S. cerevisiae*, cyclin E in vertebrates) concentration rises in late G1 and falls in early S phase (Figure I-3). These form G1/S cyclin–CDK complexes and initiate the DNA replication mechanism.

The S cyclins (Clb5 and Clb6 in budding yeast, cyclin A in vertebrates) concentration goes up in parallel with the rise of G1/S cyclins and form S cyclin–Cdk complexes which are directly responsible for DNA replication. The concentration of S cyclins remains high throughout S- and G2- phase as well as during early mitosis.

In yeast and some of the other organisms, the expression of M cyclins (Clb1, 2, 3 and 4 in budding yeast, cyclin B in vertebrates) start by the end of S phase and their concentrations gradually go up until the end of G2 phase. They form M-CDK complexes, which remain inactive because of inhibitory phosphorylation of the CDK subunit till their entry to mitosis through the G2/M checkpoint. The M

cyclin–CDK complexes are responsible for assembly of the mitotic spindle and the alignment of sister-chromatid pairs on the spindle during metaphase. The destruction of M-cyclin in anaphase leads to mitotic exit and cytokinesis.

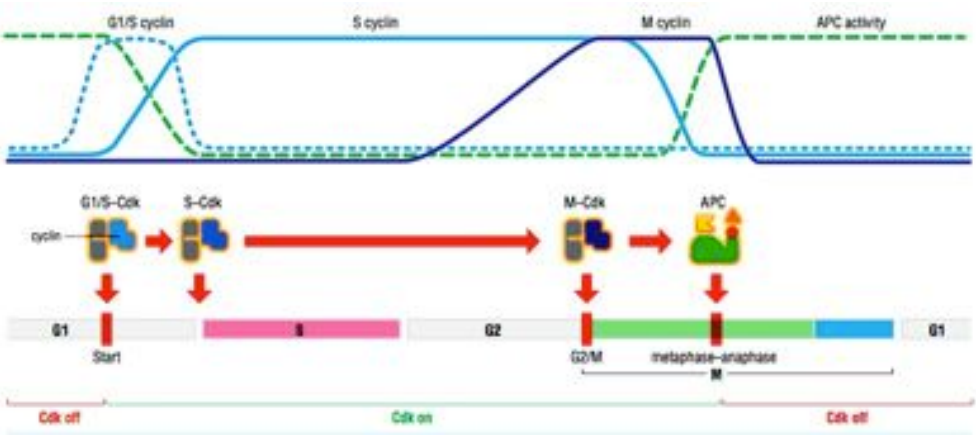

Figure I-3. Cyclins-CDK: cell cycle control system.

The upper part of the figure shows oscillating levels of cyclins namely G1-S, S and M during the cell cycle progression while the level of CDKs remain constant, hence the levels of CDK-cylin complexes very much depend on the levels of oscillating cyclins. Formation of active G1/S-CDK complexes commits the cell to a new division cycle at the Start checkpoint in late G1. G1/S-CDKs then activate the S-CDK complexes that initiate DNA replication at the beginning of S phase. M-CDK activation occurs after the completion of S phase, resulting in progression through the G2/M checkpoint and assembly of the mitotic spindle. APC activation then triggers sister chromatid separation at the metaphase-to-anaphase transition. APC activity also causes the destruction of S and M cyclins and thus the inactivation of CDKs, which promotes the completion of mitosis and cytokinesis (Taken from Morgan 2007).

In yeast and some of the other organisms, the expression of M cyclins (Clb1, 2, 3 and 4 in budding yeast, cyclin B in vertebrates) start by the end of S phase and their concentrations gradually go up until the end of G2 phase. They form M-CDK complexes, which remain inactive because of inhibitory phosphorylation of the CDK subunit till their entry to mitosis through the G2/M checkpoint. The M cyclin–CDK complexes are responsible for assembly of the mitotic spindle and the alignment of sister-chromatid pairs on the spindle during

metaphase. The destruction of M-cyclin in anaphase leads to mitotic exit and cytokinesis.

1.5.3.1 Cyclin domains

Cyclins have ~250-amino acid region known as cyclin core, containing two domains: cyclin N and cyclin C (Nugent et al., 1991; Wang et al., 2004a). The cyclin N domain is also called as cyclin box which is required for CDK binding and activation and is conserved in all cyclins. The cyclin C domain is not so conserved suggesting that it might have a specific role but perhaps not so important. Most of the mitotic cyclins also contain a destruction box (D-box), which is involved in cyclin proteolysis by ubiquitin-dependent proteasome pathway (Glotzer et al., 1991; Wang et al., 2004a). An additional motif known as PEST region, rich in Pro (P), Glu (E), Ser (S), and Thr (T) residues, a marker for unstable proteins is also found in some G1 and S cyclins (Rogers et al., 1986; Rechsteiner and Rogers, 1996). The presence of these motifs is thought to ensure rapid degradation of cyclins to terminate CDK activity at a specific point during the cell cycle.

1.6 Cyclins in Arabidopsis

In Arabidopsis, there are 50 cyclin like genes, which are many more in comparison to yeast or human (Vandepoele et al., 2002; Wang et al., 2004a). It has been reported that out of the 50, at least 32 cyclins could have some role in cell cycle progression (Vandepoele et al., 2002).

The cyclins have been classified into 10 different groups based on their functions and sequence similarity to animal cyclins. The annotated Arabidopsis genome identified 10 A-type, 11 B-type, 10 D-type, and 1 H-type cyclins along with at least 17 other cyclin-related genes classified in types C, P, L, and T (Renaudin et al., 1996; Wang et al., 2004a). The A-type cyclins generally express at the beginning of S-phase (Setiady et al., 1995; Fuerst et al., 1996; Ito et al.,

1997) where as the B-type cyclins are induced in late S to G2 phase, reaching their maximum levels during mitosis. The A and B-type cyclins largely show common regulation in G2/M in Arabidopsis, thus known as mitotic cyclins (Menges et al., 2005).

Putative D-boxes were found to be located in the N-termini of all A-type cyclins except for CYCA3;3 and all B-type cyclins except for CYCB2;5 and CYCB3;1 which mediates the degradation of these proteins prior to anaphase (Genschik et al., 1998; Peters, 1998). Putative PEST regions were also found in 45 out of the 50 Arabidopsis cyclins (Wang et al., 2004b). D-box and PEST motifs mediate irreversible mechanism of CDK downregulation by destruction of cyclin subunits at the G1- to S-phase and metaphase to anaphase transitions through ubiquitin-mediated proteolysis of cyclins (Peters, 2006). Degradation of protein substrates through the ubiquitin-proteasome pathway involves the activity of different E3 ligases, named as anaphase-promoting complex or cyclosome (APC/C). APC/C is a multiprotein complex having at least 11 core subunits and is conserved in eukaryotes. Substrates having a destruction box (D-box) or similar motifs are recognised and ubiquitinated by the APC/C. The degradation of target proteins via this pathway involves tagging of the protein by the covalent attachment of ubiquitin that is followed by its degradation by a multicatalytic protease complex called the 26S proteasome (Genschik et al., 1998). The proteolytic events by the APC/C are required to release sister chromatids during anaphase as well as to exit from mitosis and thus preventing premature entry into S-phase.

1.6.1 B type cyclins

The B-type cyclins have been subdivided into three groups: *CYCB1*, *CYCB2* and *CYCB3*. *CYCB1* and *CYCB2* groups have been reported from both monocotyledonous and dicotyledonous species but the *CYCB3* group has not been found in the monocot species thus far (Wang et al., 2004a; La et al., 2006). In Arabidopsis, both *CYCB1* and *CYCB2* have five members each namely *CYCB1;1* to *CYCB1;5* and *CYCB2;1* to *CYCB2;5* respectively, whereas *CYCB3* has only one member (Wang et al., 2004a; Menges et al., 2005). The typical cyclin B signature

motif 'H/Q)x(K/R/Q)(F/L' is present in all B-type cyclins, along with a conserved destruction box motif and cyclin core domain as well as PEST motif (Wang et al., 2004a). All B-type cyclin members in Arabidopsis also contain mitotic phase-specific activator (MSA) motif (AACGG) in their upstream region, which is responsible for their mitosis-specific expression pattern (Ito et al., 1997; Ito et al., 1998; Ito et al., 2001).

The biochemical activity of plant CYCBs was shown to be complementing animal cyclin B by microinjection of tobacco *CYCB1;1* mRNA into *Xenopus* oocytes, where later was sufficient to overcome the natural G2/M arrest in the oocytes (Qin et al., 1996). In transgenic tobacco expressing a non-degradable version of CYCB1;1, it was demonstrated to interact with and activate both A- and B-type CDKs in vitro (Weingartner et al., 2004). These lines also show alteration of microtubule organisation and dynamics leading to impaired formation of a phragmoplast. On the other hand, *Orysa;CycB1;1*-RNAi transgenic rice plants produced abnormal seeds in which endosperm as well as the embryo had severe defects resulting in seed abortion.

The ectopic expression of CYCB2 protein from alfalfa in tobacco plants leads to earlier mitosis (Weingartner et al., 2003) suggesting its role in controlling progression through mitosis. CYCB2 was also shown to be associated with the mitotic CDKB2;1 in metaphase cells in rice, and then it disappeared after cells pass through metaphase (Lee et al., 2003). The chromosome association and gradual destruction after metaphase was previously reported for two CYCB1 proteins of tobacco as well (Criqui et al., 2001). The B-type cyclins have also been reported to play an important role during endosperm nuclear mitosis and endoreduplication in maize where endoreduplication results by the inhibition of an M-phase- promoting factor (MPF, p34cdc2-cycB complex) alongside induction of S-phase related protein kinases in the endosperm (Grafi and Larkins, 1995). In *Zea mays*, the down-regulation of *Zeama;CycB1;3* (*CycZme1*) has been reported to be involved in the endoreduplication (Sun et al., 1999).

1.7 *CYCB1* gene family in Arabidopsis

In Arabidopsis, the CYCB1 group has five members and all of them carry the typical cyclin B signature, the (H/Q)x(K/R/Q)(F/L) motif (Wang et al., 2004a). They all have a conserved destruction box motif, followed by cyclin N and C domain. CYCB1;2, CYCB1;3 and CYCB1;5 has an additional motif as well called as Barbie box (Roodbarkelari, 2007). Multiple sequence alignment at amino acid level shows overall 30% similarity amongst the family members (Figure I-4). Though the pair-wise alignment between individual cyclin B1 genes show more then 60% similarity to each other. The phylogeny tree constructed from multiple sequence alignment shows that CYCB1;2 and CYCB1;5 are the closest relatives within the family.

Not much is known about B1-type cyclins in Arabidopsis, however ectopic expression of CYCB1;1 under the control of *CDKA;1* promoter in Arabidopsis was shown to stimulate cell division in root, resulting in faster growth rate (Doerner et al., 1996). On the other hand, ectopic expression of a truncated CYCB1;2 was able to induce extra cell division in trichomes of Arabidopsis that normally endoreduplicate resulting in formation of multicellular trichomes, indicating that plant CYCB1s are both functioning in and sufficient to promote the G2/M transition (Schnittger et al., 2002a).

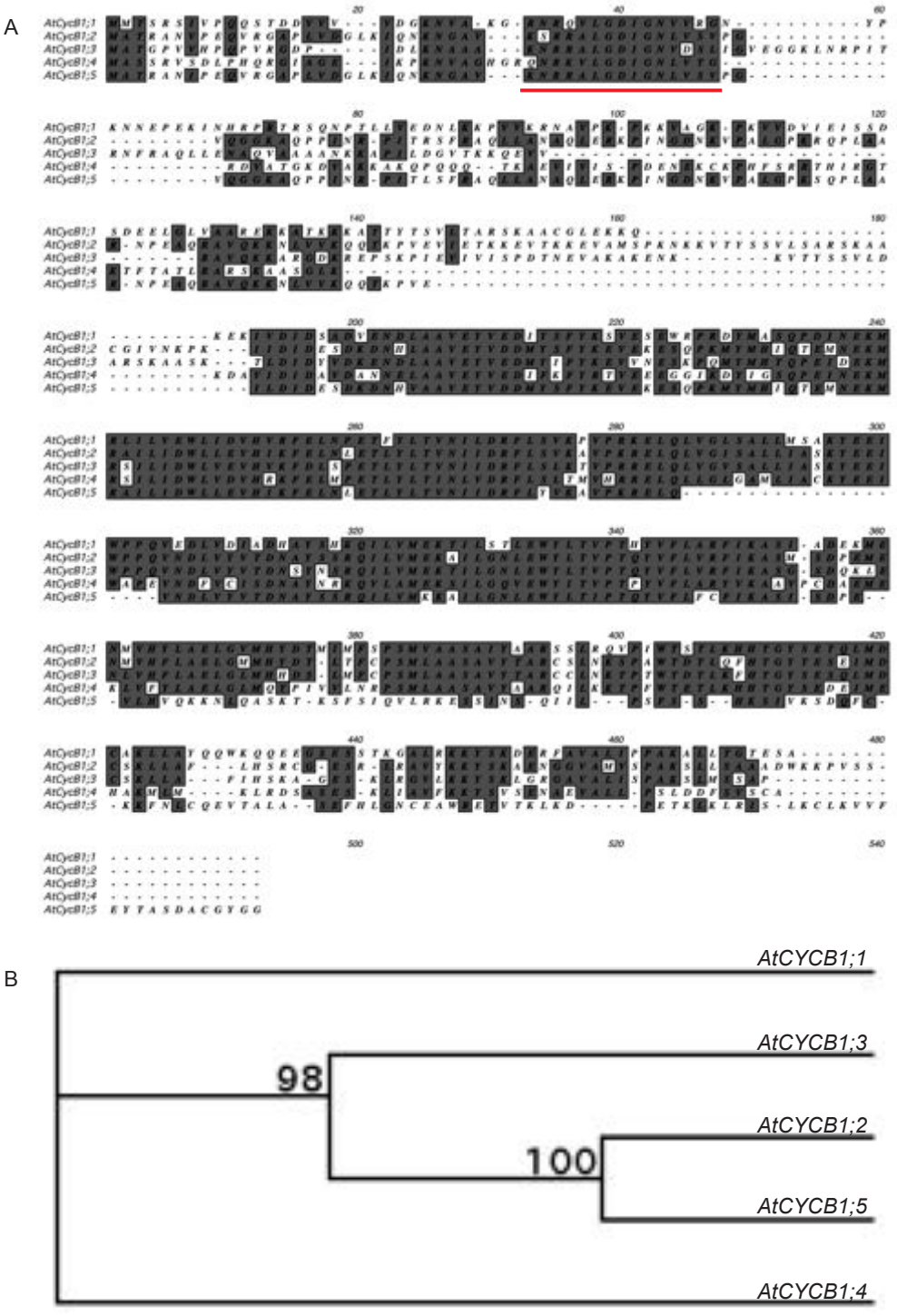

Figure I-4. Sequence homology among B-type cyclins.
(A) Amino acid sequence alignment of *Arabidopsis thaliana* B1-type cyclins. The sequences are presented using the single-letter code. The long conserved region indicates the cyclin box and the sequence of destruction box is underlined. (B) Phylogenetic tree of *Arabidopsis thaliana* B1-type cyclins. The tree was constructed using Macvector® 11.1.1 software.

2 Aim of the study

Previous studies have revealed that many mitotic cyclins are expressed in the developing endosperm of Arabidopsis. However, none of the 11 members of B1-type cyclins was functionally studied and thus their individual functions were not clear at the beginning of this work. Based on a former Ph.D. project, the major aim of this study was to characterise loss of function mutants of B1-type cyclins and to investigate in particular their role for the nuclear divisions during endosperm development. For this T-DNA mutants needed to be isolated and characterized. In addition double and further multiple mutants had to be generated to overcome potentially redundant function of B1-type cyclins. The loss of B1-type cyclin activity was studied at the organismic, at the tissue and at the cellular level.

The second aim of this work was the identification of novel loci that control cell proliferation during seed development using RIL populations. For this, natural variation among different accessions for seed and endosperm specific traits needed to be identified and based on that appropriate RIL populations were selected. Est1- Col-0 RIL population was screened for novel loci controlling cell proliferation during seed development whereas set of three RIL populations having one common parent was screened for trait regulating seed mass.

3 Results

3.1 Expression and localisation analysis of CyclinB1 genes in Arabidopsis

3.1.1 Expression analysis of CyclinB1 genes in Arabidopsis

To examine the expression pattern of CYCB1s, I monitored the expression of promoter reporter lines for CYCB1;1 to CYCB1;4 in root tip cells and endosperm nuclei. The reporter lines contain ~1kb of the promoter region and the N-terminus of respective genes, including the destruction box, fused to GFP. These marker lines were kindly provided by Prof. Peter Doerner.

GFP fluorescence was observed in patchy pattern in roots of all the lines from ProCYCB1;1:CYCB1;1DB-GFP to ProCYCB1;4:CYCB1;4DB-GFP, suggesting that cells which are in the G2-phase and early M-phase are expressing GFP whereas the GFP fusion protein has been degraded in the cells completing mitosis and throughout G1 as well as S-phase (Figure R-1A, D, G and J). In contrast to the root, only CYCB1;1, CYCB1;2 and CYCB1;3 were found to be expressed in the endosperm (Figure R-2A, D and G). The expression of CYCB1;4 was not detected in the endosperm.

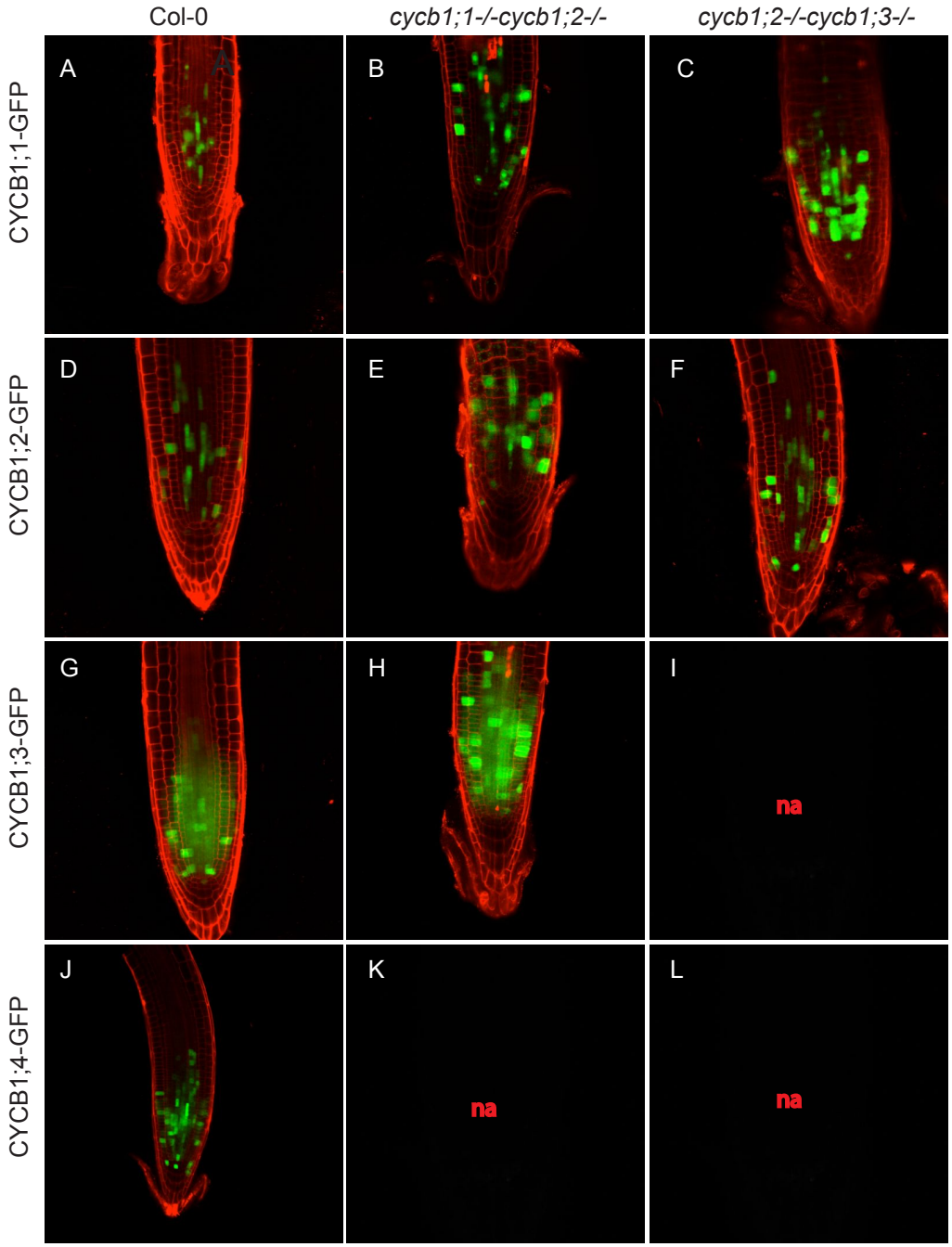

Figure R-1. GFP expression driven by respective promoters of *CYCB1;1* through *CYCB1;4* in roots.

A, D, G and J demonstrate patchy expression of GFP in root tip cells of Col-0 under the control of respective promoters of *CYCB1;1* through *CYCB1;4* indicating that all four CYCB1s are expressed in roots of wild-type plants. B, E and H demonstrate accumulation of GFP signal in roots of *cycb1;1-/-cycb1;2-/-* plants under the control of *CYCB1;1*, *CYCB1;2* and *CYCB1;3* promoters, respectively. *cycb1;1-/-cycb1;2-/-* harbouring ProCYCB1;4:CYCB1;4DB-GFP marker line were not available (na) (K). C and F demonstrate similar accumulation of GFP signal in the roots of *cycb1;2-/-cycb1;3-/-* plants under the control of *CYCB1;1* and *CYCB1;2* promoters, respectively, as was observed in *cycb1;1-/-cycb1;2-/-*. *cycb1;2-/-cycb1;3-/-* harbouring ProCYCB1;3:CYCB1;3DB-GFP and ProCYCB1;4:CYCB1;4DB-GFP marker lines were not available (na) (I and L).

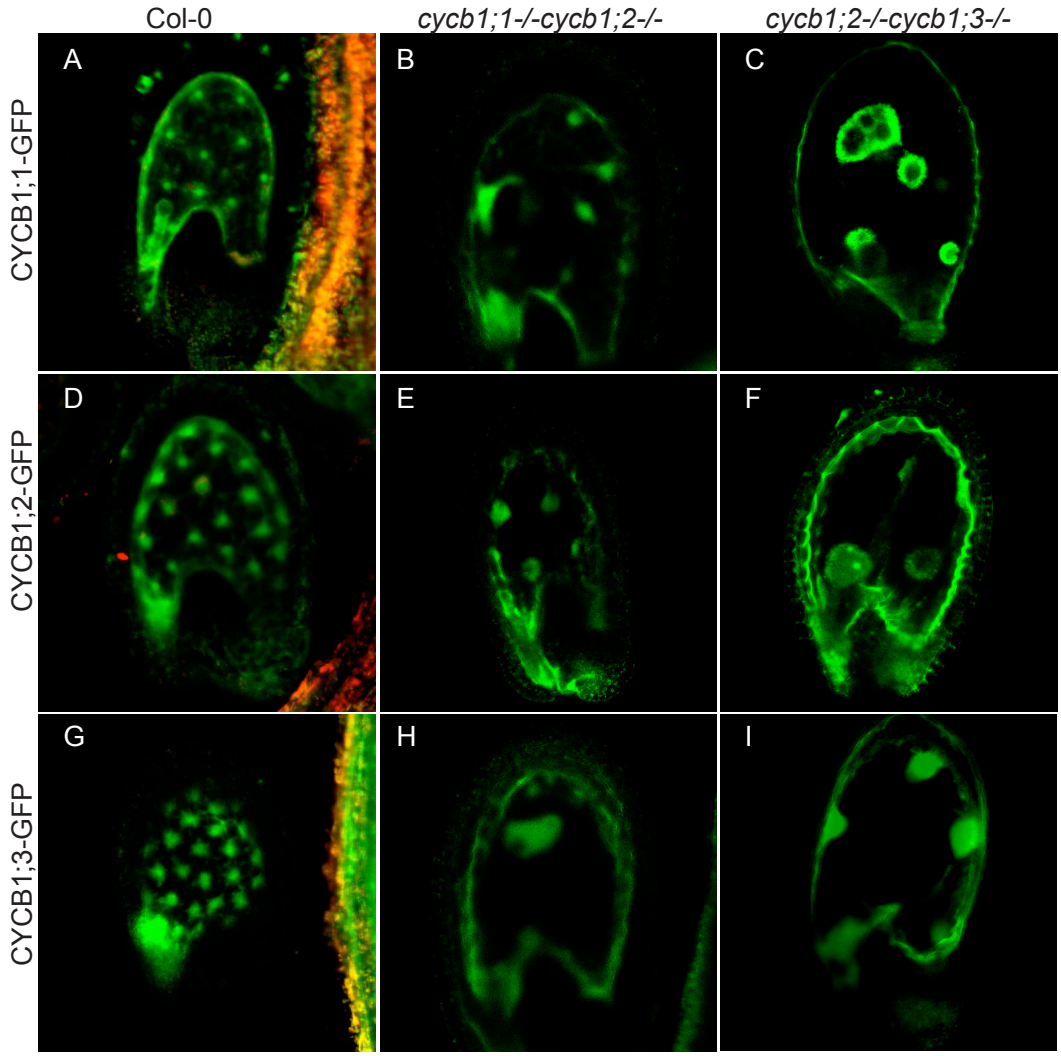

Figure R-2. GFP expression driven by respective promoters of *CYCB1;1* through *CYCB1;3* in endosperm.

A, D and G demonstrate expression of GFP in endosperm nuclei of Col-0 seeds under the control of respective promoters of *CYCB1;1* through *CYCB1;3* indicating that CYCB1;1, CYCB1;2 and CYCB1;3 are expressed in endosperm. However, no GFP expression was detected in the endosperm for ProCYCB1;4:CYCB1;4DB-GFP marker line.

B, E and H demonstrate GFP expression in the endosperm of *cycb1;1-/-cycb1;2-/-* seeds under the control of *CYCB1;1*, *CYCB1;2* and *CYCB1;3* promoters. GFP expression in enlarged endosperm nuclei revealed that they are arrested in G2/M phase.

C, F and I demonstrate GFP expression in the endosperm of *cycb1;2-/-cycb1;3-/-* seeds under the control of *CYCB1;1*, *CYCB1;2* and *CYCB1;3* promoters, respectively. The mutant endosperm of *cycb1;2-/-cycb1;3-/-* showed similar phenotype as observed in *cycb1;1-/-cycb1;2-/-*.

3.1.2 Localisation of B1 type cyclins in mitotic cells

Colleagues from the collaborative laboratory of Dr. Karel Riha performed this work. In order to study intracellular localisation of Arabidopsis B1 cyclins in mitotic cells, the reporter constructs containing entire cyclin genes with promoter sequences and all coding exons of *CYCB1;2*, *CYCB1;3* and *CYCB1;4* were fused to the *GUS* gene at the C-terminus. Localisation of the CYCB1;1 was studied using a reporter line containing promoter and 5' end of the *CYCB1;1* encoding the first 116 amino acids fused to the GUS gene (Colon-Carmona et al., 1999). The fused CYCB1s-GUS proteins were detected in mitotic cells prepared from young anthers by immunolocalisation using an antibody recognising the GUS protein. The structure of the spindle was simultaneously analysed using an anti-alpha tubulin antibody and the chromatin material by DAPI. Immunolocalisation analysis of the lines containing CYCB1;1-GUS and CYCB1;2-GUS revealed similar staining patterns in metaphase, anaphase and telophase (Figure R-3A). A fraction of interphase cells showed very prominent staining in cytoplasm suggesting that these cells are in the G2 phase. The localisation changed dramatically during prophase, where most of the proteins localised to chromatin and were detectable until metaphase. Figure R-3B shows the localisation of CYCB1;1 through CYCB1;4 in metaphase stage of mitosis in young anther cells. The signals for CYCB1;1 and CYCB1;2 decreased during anaphase, mostly detectable in the cytoplasm, and were completely lost in telophase. CYCB1;3 exhibited similar localisation pattern as CYCB1;1 and CYCB1;2, with a higher retention of the signal in the cytoplasm and a slight enrichment at the spindle in metaphase (Figure R-3A). In contrast to the other CYCB1s, CYCB1;4 displayed a very distinct localisation pattern during prophase. While a prominent cytoplasmic staining was visible in G2 as was observed for other cyclins, however, CYCB1;4 remained in the cytoplasm even after the nuclear envelope breakdown in prophase (Figure R-3A). Apparent enrichment of the CYCB1;4 signal could be detected at the spindle in metaphase and also during anaphase; although at these stages its overall intensity was significantly decreased.

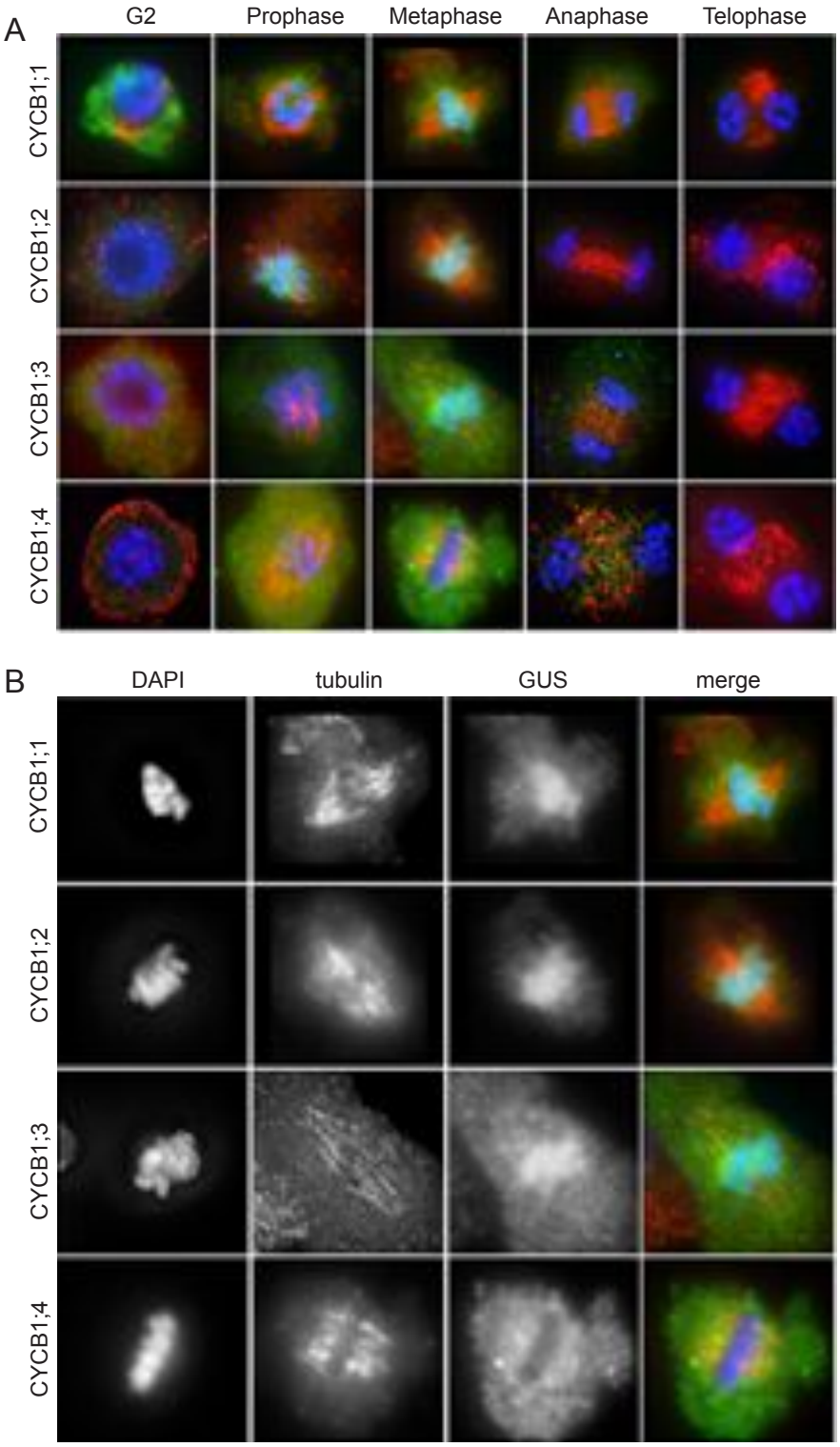

Figure R-3. Localisation of B1 type cyclins during different stages of cell cycle.
(A) Mitotic cells were prepared form anthers and microtubules and CYCB:GUS proteins were detected by immunostaining using α-tubulin (red) and GUS (green) antibodies, respectively. DNA was counterstained with DAPI (blue).
(B) Localisation of B1 type cyclins during metaphase. Pictures are the same as in (A) but signals from DNA, tubulin and CYCB1-GUS proteins are provided separately.

3.2 Isolation of CyclinB1 mutants

Previously, insertion lines for the individual *CYCB1;1* to *CYCB1;4* genes have been obtained and named *cycb1;1-1*, *cycb1;2*, *cycb1;3-1* and *cycb1;4* respectively (Figure R-4A) (Roodbarkelari, 2007). The *cycb1;1-1* and *cycb1;2* were isolated from the Koncz collection (Csaba Koncz, Max Planck Institute for Plant Breeding Research in Cologne, Germany), *cycb1;3-1 (pst15850)* was obtained from the RIKEN collection (Yokohama, Japan) and *cycb1;4 (GK_386C01)* stems from the GABI-Kat collection (Bielefeld, Germany). During subsequent updates of the mutant collections, additional alleles for *CYCB1;1* and *CYCB1;3* were obtained from GABI-Kat collection; and named as *cycb1;1-2 (GK_078G11)* and *cycb1;3-2 (GK_352C11)* respectively. The *cycb1;1-2* carries a T-DNA insertion in sixth exon of *CYCB1;1* where as *cycb1;3-2* harbors a T-DNA insertion in the fifth exon of *CYCB1;3* (Figure R-4A).

Several mutant databases have been screened for insertion in *CYCB1;5* and one mutant line has been identified from SALK collection and named *cycb1;5-1 (*SALK_100844*)*, which carried a T-DNA insertion in first exon according to the TAIR 9 annotation. However, the recently released TAIR 10 annotated the gene differently. Considering the discrepancy, various gene annotation software programs were utilised to reconfirm the annotation. I used Augustus, a web based program (http://augustus.gobics.de/), which is trained for gene prediction in *Arabidopsis thaliana* along with numerous other eukaryotic species (Stanke et al., 2004). The resulting new annotation shifted the transcription start site downstream because of which the T-DNA insertion is now in 5' upstream region (Figure R-4B). In consideration of discrepancies in the annotation two new T-DNA lines were ordered for *CYCB1;5* and name *cycb1;5-2* (SALK_135553.39.20.x) and *cycb1;5-3* (SAIL_587_H11) respectively (Figure R-4B).

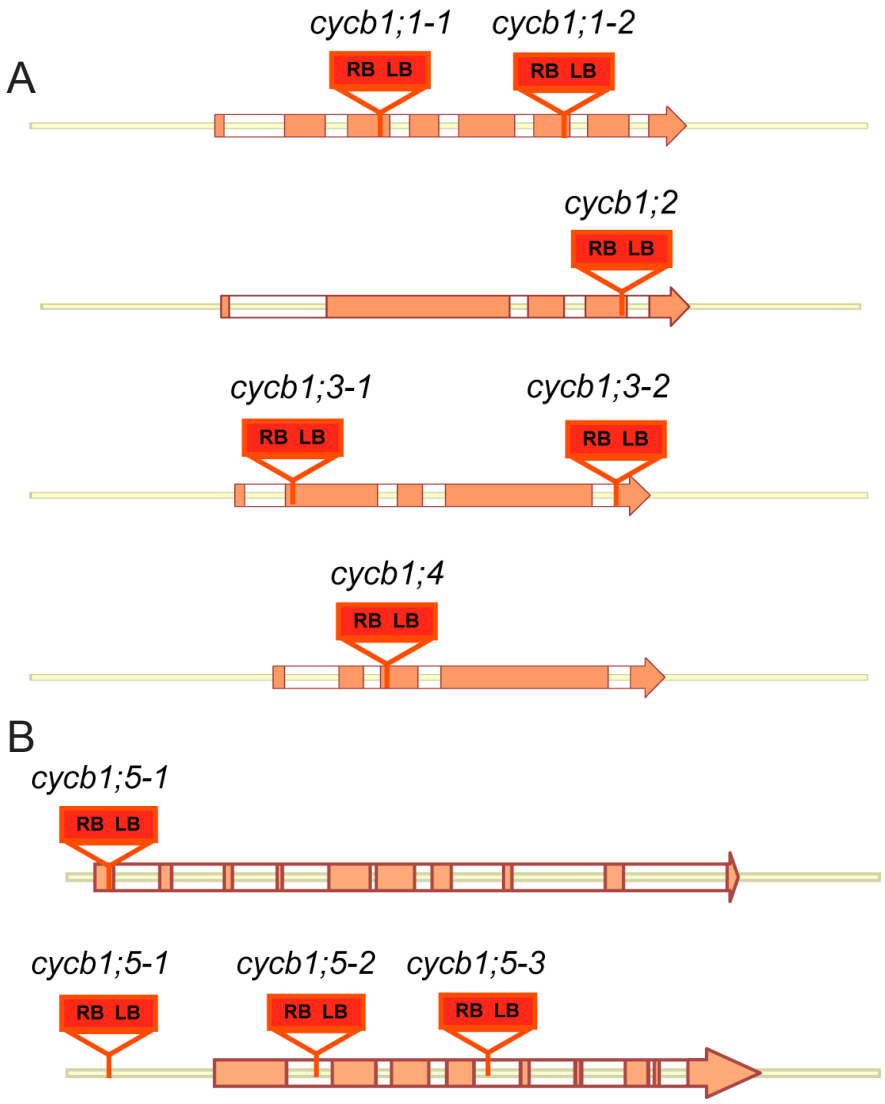

Figure R-4. T-DNA insertion mutants of Arabidopsis CYCB1s.
(A) cycb1;1-1 (*cycb1;1)* and *cycb1;1-2* carries T-DNA insertion in third and sixth exons, respectively. *cycb1;2* has T-DNA insertion in fourth exon, *cycb1;3-1 (cycb1;3)* carry the transposon insertion in second exon whereas *cycb1;3-2* carry the T-DNA insertion in fifth exon. The T-DNA insertion is in third exon in *cycb1;4* (B) Upper drawing depicts exon orientation for *cycb1;5-1* according to TAIR9 annotation whereas the lower illustration depicts the putative exon orientation derived using Augustus gene prediction program, by manual feeding of genomic sequence in the software. The putative annotation shifted the T-DNA insertion position of *cycb1;5-1* which was previously in exon 1 towards *5'* upstream region. The lower illustration also shows two other mutant lines *cycb1;5-2* and *cycb1;5-3* which carries T-DNA insertions in first and fourth introns, respectively. Empty box depicts intron and filled box depicts exon. Abbreviations are: LB and RB – T-DNA Left and Right border sequence.

3.3 Analysis of *CYCB1* mutants

3.3.1 CYCB1 T-DNA mutants are null allele

To test whether the identified *CYCB1* T-DNA mutant plants represent null alleles, the transcript levels of the individual cyclinB1 genes were analysed in floral bud tissue extracts of the wild-type Col-0 and the mutant plants by quantitative PCR using primers flanking the T-DNA insertion site. Actin transcript levels were determined simultaneously as an internal control. None of the respective *CYCB1* transcripts were amplified in the corresponding mutant lines whereas actin transcripts were detected to equal levels as in wild-type. Thus, the *CYCB1* T-DNA mutant lines are null alleles (Figure R-5).

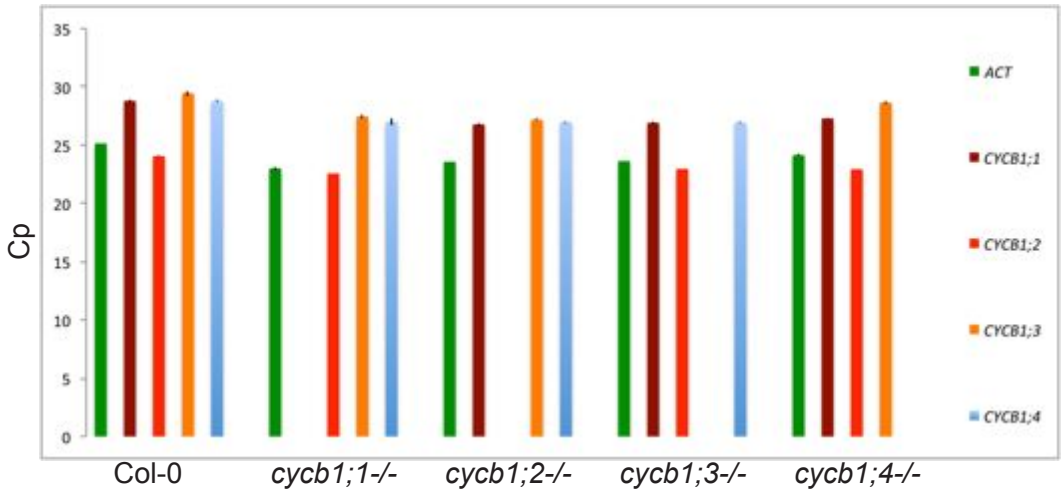

Figure R-5. T-DNA insertion mutant lines for *CYCB1s* are null alleles.
Quantitative real time PCR showed mRNA expression of *CYCB1;1*, *CYCB1;2, CYCB1;3, CYCB1;4* and ACT (actin) in floral bud tissue of Col-0 (wild-type), *cycb1;1-/-, cycb1;2-/-, cycb1;3-/- and cycb1;4-/-* single mutants as evaluated by Cp (crossing point or cycle threshold) values. Wild-type Col-0 showed expression of all *CYCB1s* whereas respective *cycb1* mutants were null for their respective alleles without affecting the gene expression of other B1-type cyclins. Actin gene expression served as internal control.

3.3.2 CYCB1 single mutants show altered endosperm nuclei proliferation

Mutant plants homozygous for each individual B1 cyclin gene were viable and showed no apparent morphological differences compared to wild-type plants. The endosperm nuclei were counted in 3 DAP seeds and classified in three classes: 1-16, 32-64 and 128-512. This classification was based on the spatiotemporal pattern of mitotic CYCB1;1 accumulation in the syncytium during Arabidopsis endosperm development. The syncytium was organised into three domains in which nuclei divide simultaneously with a specific time course (Boisnard-Lorig et al., 2001). Based on this classification, the counted endosperm nuclei from single mutants are presented in table R-1. I found that 15% of *cycb1;1-/-* and 33% of *cycb1;2-/-* seeds have only up to 64 endosperm nuclei in comparison to 7.69% of wild-type plant (Table R-1). The *cycb1;3-/-*, *cycb1;4-/-* and *cycb1;5-/-* mutant seeds did not show any reduction in the number of endosperm nuclei. The mutant seeds having fewer endosperm nuclei remained healthy and germinated normally on soil suggesting that even with relatively fewer endosperm nuclei, seeds can develop normally.

Table R-1 Percentage of seeds showing distribution of endosperm nuclei in different classes in CYCB1 single mutant lines

Parental genotypes (female x male)	seeds (%) with respective endosperm nuclei counts			n
	1 - 16	32 - 64	128 - 512	
Col-0 selfed	0	7.69	92.7	39
cycb1;1-/- selfed	0	15.38	84.62	39
cycb1;2-/- selfed	0	33.33	66.67	39
cycb1;3-/- selfed	0	0	100	40
cycb1;4-/- selfed	0	0	100	39
cycb1;5-/- selfed	0	0	100	45

3.3.3 Certain double mutant combinations of CYCB1s cause seed abortion

To investigate if there exists a functional redundancy in *CYCB1* genes that led to successful seed development in single mutants, I analysed double mutants of *CYCB1s* (provided by Farshad Roodbarkelari). The double homozygous mutants *cycb1;1-/-cycb1;3-/-*, *cycb1;1-/-cycb1;4-/-*, *cycb1;2-/-cycb1;4-/-* and *cycb1;3-/-cycb1;4-/-* were obtained but *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* double homozygous mutants could not be recovered on soil. Instead plants with one homozygous and one heterozygous combination of mutant genes such as *cycb1;1-/-cycb1;2-/+* or *cycb1;2-/-cycb1;3-/+* could be grown in the green house. Similar homozygous/heterozygous combinations were obtained for double mutants of *cycb1;2* when crossed with the second alleles *cycb1;1-2* and *cycb1;3-2*. The work in the rest of this study has been conducted with *cycb1;1-1* and *cycb1;3-1* which are referred in following as *cycb1;1* and *cycb1;3* hereafter. The above-mentioned double homozygous mutant plants for *CYCB1s* did not show any apparent morphological defects and was indistinguishable in rosette leaf growth or inflorescence development compared to wild-type plants. On the other hand, approximately 25% of seeds appeared smaller and shrivelled in the siliques of plants harboring one homozygous and one heterozygous combination of mutant alleles either for *cycb1;1/cycb1;2* or *cycb1;2/cycb1;3* (Table R-2, Figure R-6B and C). The quarter of the total seeds which appeared shrivelled in the siliques of double mutant plants with one homozygous and other heterozygous allele could result either because of recessive sporophytic or less penetrant gametophytic defect, which could be analysed by crossing double heterozygous mutants with wild-type plants in a reciprocal manner. This experiment is still in progress.

Table R-2 Percentage of aborted seeds in CYCB1 double mutant lines

Parental genotypes (female x male)	aborted	n	Percentage Abortion (%)
Col-0 selfed	69	1470	4.69
cycb1;2 -/- cycb1;1-1 -/+ selfed	468	1578	29.65
cycb1;2 -/- cycb1;3-1 -/+ selfed	231	936	26.63
cycb1;2 -/- cycb1;1-2 -/+ selfed	336	1290	26.04
cycb1;2 -/-cycb1;3-2 -/+ selfed	295	1114	26.48
Col-0 selfed	36	895	4.02
cycb1;1 -/- cycb1;5-/+ selfed	293	1037	28.25
cycb1;2 -/- cycb1;5-/- selfed	23	542	4.24
cycb1;3 -/- cycb1;5-/- selfed	27	648	4.16

Further, I also generated respective double mutants of *CYCB1;5* by crossing *cycb1;5* with *cycb1;1, cycb1;2* and *cycb1;3*. I obtained double homozygous *cycb1;2-/-cycb1;5-/-* and *cycb1;3-/-cycb1;5-/-* plants but only *cycb1;1-/-cycb1;5-/+* plants could be obtained. There was no seed abortion in *cycb1;2-/-cycb1;5-/-* and *cycb1;3-/-cycb1;5-/-* but I found approximately 25% of seeds collapsing in the siliques of *cycb1;1-/-cycb1;5-/+* (Table R-2). This result suggests that *CYCB1;5* might be involved in seed development. I decided not to proceed with this mutant line considering the putative annotation of the gene and the resulting changes in the coding sequences.

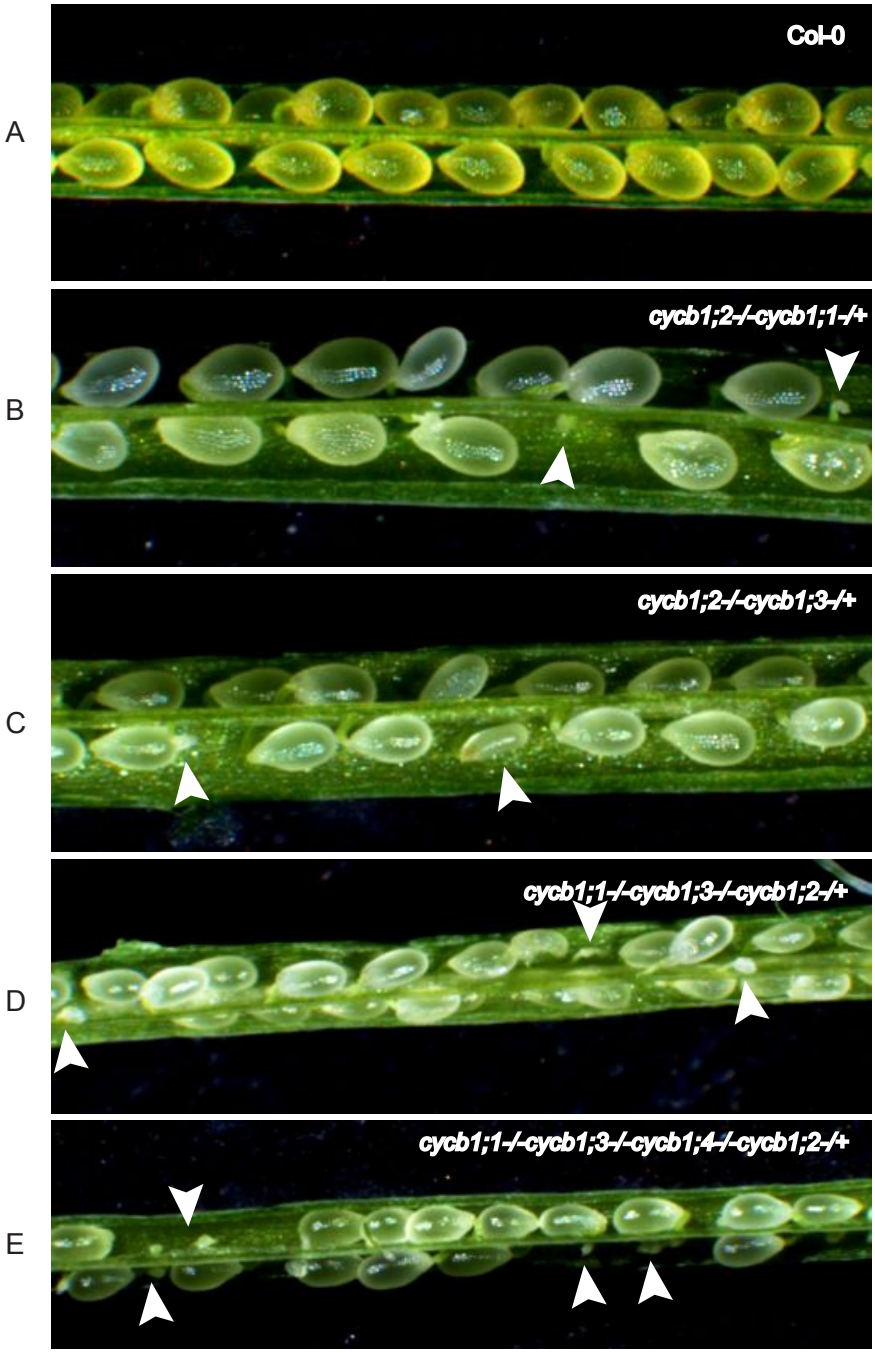

Figure R-6. Seed defect in homozygous/heterozygous double, triple and quadruple mutants of *CYCB1s*.

Representative picture of siliques of *cycb1;2-/-cycb1;1-/+* (B) and *cycb1;2-/-cycb1;3-/+* (C) homozygous/heterozygous double mutant plants showed desiccated seed (white arrowheads). The frequency of seed abortion increased with mutation of an additional allele as shown in *cycb1;1-/-cycb1;3-/-cycb1;2-/+* triple mutants (D). The quadruple mutant *cycb1;1-/- cycb1;3-/-cycb1;4-/- cycb1;2-/+* showed ~50% seed abortion as depicted in (E). The first panel shows healthy seeds in wild-type Col-0 siliques (A).

3.3.4 Endosperm nuclei proliferation defect in CYCB1 double mutants

To investigate the effect of double mutation on endosperm proliferation, I analysed 3 DAP seeds that have been pollinated using their own pollen and counted the endosperm nuclei from all the double mutants of *CYCB1s* (Table R-3). The distribution of number of endosperm nuclei differed strikingly in *cycb1;1-/-cycb1;2-/+* and *cycb1;1-/+cycb1;2-/-*. The 40% of seeds from *cycb1;1-/+cycb1;2-/-* plants contained endosperm nuclei in the range of 1-16 whereas only 17% seeds from *cycb1;1-/-cycb1;2-/+* belong to this range. On the other hand 53% of total endosperm nuclei from *cycb1;1-/+cycb1;2-/-* were in the range of 32-64 compared to 13% of *cycb1;1-/-cycb1;2-/+*. There was slight difference in the distribution pattern of endosperm nuclei obtained from *cycb1;2-/-cycb1;3-/+* and *cycb1;2-/+cycb1;3-/-* seeds. Almost 25% of the total seeds from both the genotypes carried endosperm nuclei in the range of 1-16. Notably 31% seeds carried 32-64 endosperm nuclei in *cycb1;2-/-cycb1;3-/+* compared to only 19% in *cycb1;2-/+cycb1;3-/-*.

Table R-3 Percentage of seeds showing distribution of endosperm nuclei in different classes in CYCB1 double mutant lines

Parental genotypes (female x male)	seeds (%) with respective endosperm nuclei counts			n
	1 - 16	32 - 64	128 - 512	
Col-0 selfed	0	7.69	92.71	39
cycb1;1-/-cycb1;3-/- selfed	0	1.92	98.08	52
cycb1;1-/-cycb1;4-/- selfed	0	19.23	80.77	26
cycb1;2-/-cycb1;4-/- selfed	0	30	70	50
cycb1;3-/- x *cycb1;4-/-* selfed	0	0	100	50
cycb1;1-/-cycb1;2-/+ selfed	17.39	13.76	68.85	138
cycb1;1-/+cycb1;2-/- selfed	40.22	53.63	6.15	179
cycb1;2-/-cycb1;3-/+ selfed	25.49	31.37	43.14	102
cycb1;2-/+cycb1;3-/- selfed	26.92	19.23	53.85	104

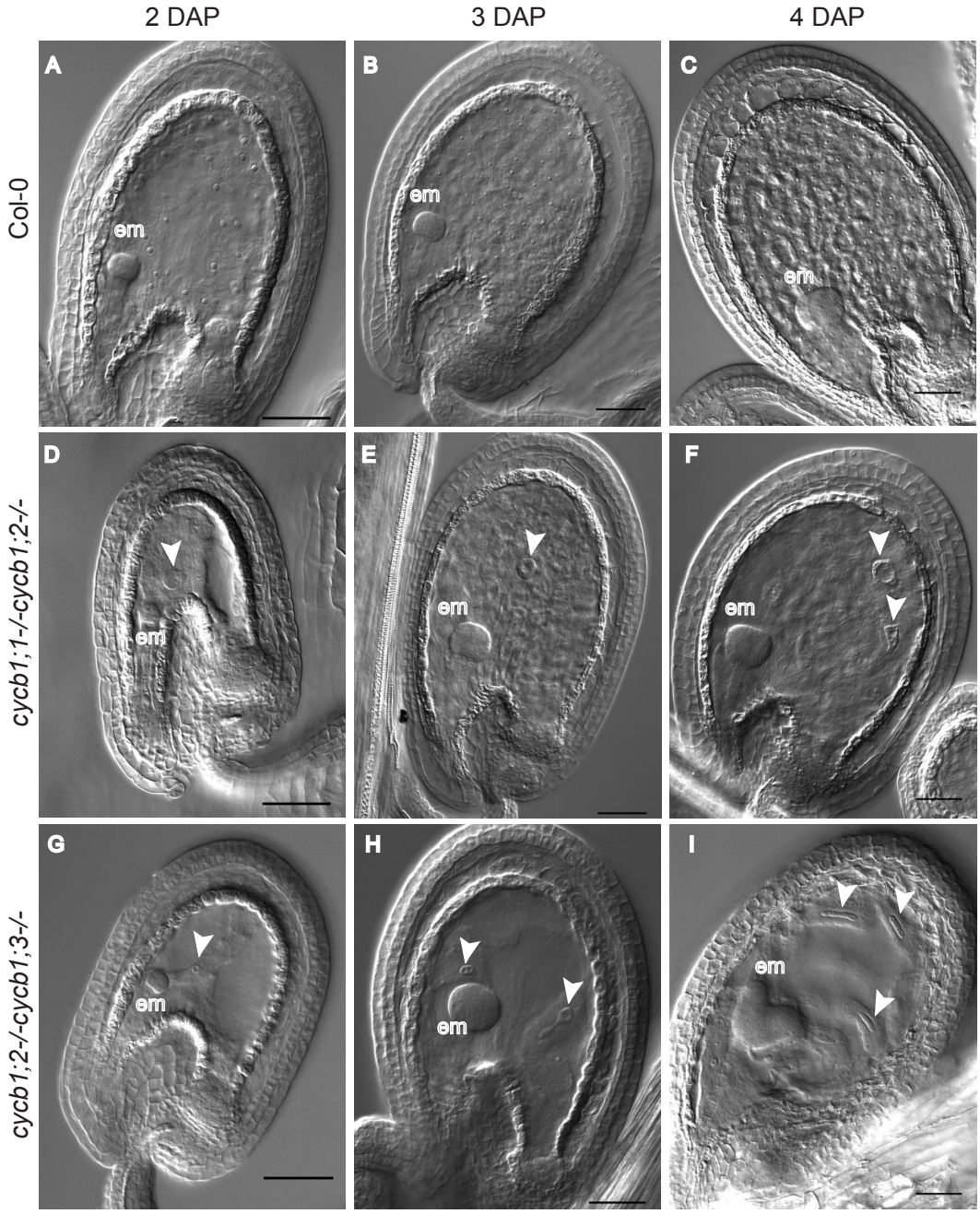

Figure R-7. Endosperm nuclei defects in *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-*
DIC light micrographs of wild-type Col-0 seeds (A-C), double homozygous mutants *cycb1;1-/-cycb1;2-/-* (D-F) and *cycb1;2-/-cycb1;3-/-* (G-I) seeds showing embryo and endosperm development at 2, 3 and 4 DAP are represented here. In Col-0, the number of endosperm nuclei successively increases and are of regular shape. In mutant seeds, fewer numbers of endosperm nuclei were visible with varying shapes (white arrowpoints) ranging from very large rounded (E), multipolar and spoon shaped (F) to tubular structure (H and I). Embryo (em) of the double homozygous mutants appeared to be normal at different developmental stages as in wild-type. All pictures are placed such that the chalazal pole of the seed points to the right and the micropylar pole with the developing embryo to the left. Scale bars: 50 µm

The double mutants *cycb1;1-/-cycb1;4-/-* contained 19% seeds having endosperm nuclei in the range of 32 to 64 where as 30% of seeds from *cycb1;2-/-cycb1;4-/-* carried 32-64 nuclei. The endosperm nuclei proliferation remained unaltered in the seeds obtained from *cycb1;1-/-cycb1;3-/-* and *cycb1;3-/-cycb1;4-/-* compared to wild-type. The data suggests that almost quarter of seeds or more contained 1-16 endosperm nuclei in the homozygous/heterozygous combination of *cycb1;1/cycb1;2* and *cycb1;2/cycb1;3* indicating that endosperm nuclear division is severely affected in double homozygous seeds.

The endosperm nuclei in the seeds which were either *cycb1;1-/-cycb1;2-/-* or *cycb1;2-/-cycb1;3-/-* were of varying shape and size. The shape of mutant seeds varied from round to tubular structure but were typically enlarged compared to wild-type (Figure R-7F and I). I did not see any defect in embryo development in these seeds. However, at instances, it was observed that the double homozygous seeds *cycb1;1-/- cycb1;2-/- or cycb1;2-/- cycb1;3-/-* were of smaller size compared to wild-type (Figure R-7A, D and G). The one important event in the endosperm development is the cellularisation of endosperm nuclei in due course of seed development. The 6 DAP seeds of *cycb1;1-/- cycb1;2-/- or cycb1;2-/- cycb1;3-/-* were stained with feulgen reagent to visualise the cellularisation of endosperm nuclei. The result revealed that the mutant endosperm nuclei failed to cellularise (Figure R-8).

Considering the previous observations that *cycb1;2-/-* seeds showed reduction in endosperm nuclei proliferation and further severe additive effects in conjugation with *cycb1;1* and *cycb1;3,* suggest that *CYCB1;2* is the most important B1-type cyclin during endosperm development. In addition, the analysis of distribution of proliferating endosperm nuclei in double mutants revealed that having *cycb1;2-/-* allele in the seeds severely affected the endosperm nuclei proliferation compared to *cycb1;2-/+* raising the possibility of *CYCB1;2* having any maternal effect of its own.

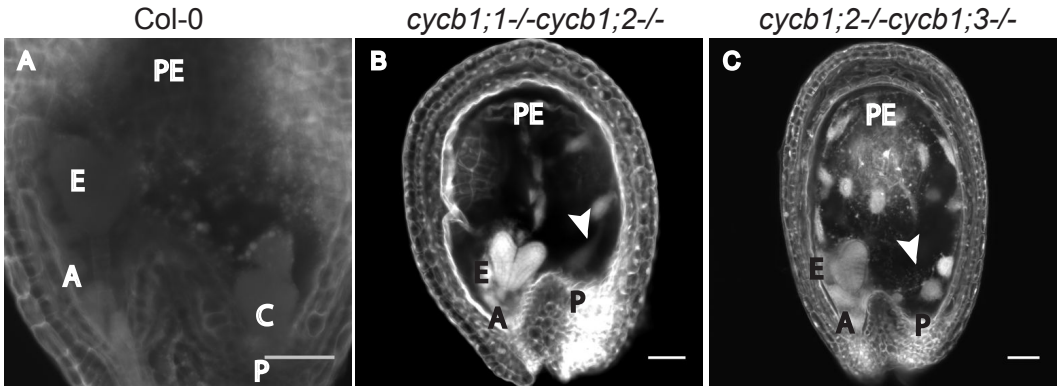

Figure R-8. Cellularisation defect in *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* endosperm

A-C show confocal microscopic pictures of feulgen-stained seeds from wild-type Col-0 (A), *cycb1;1-/-cycb1;2-/-* (B) and *cycb1;2-/-cycb1;3-/-* (C) plants at 6 DAP stage. Wild-type seed showed typical cellularised endosperm and normal chalazal cyst, which was not visible in double homozygous mutants, although one or two endosperm nuclei were observed near posterior pole. Abbreviations are: A, anterior pole; C, chalazal cyst; E, embryo; P, posterior pole; PE, peripheral endosperm. Scale bars: 50µm

3.3.5 cycb1;2-/- shows maternal effect in endosperm proliferation

To test whether there is any maternal effect responsible for decrease in the number of endosperm nuclei, I performed reciprocal cross of *cycb1;2-/-* with wild-type Col-0 and counted the endosperm nuclei 3 DAP (Table R-4). 37% of seeds obtained from *cycb1;2-/-* x Col-0 had up to 64 endosperm nuclei whereas in the progeny of the cross Col-0 x *cycb1;2-/-* all seeds contained more then 64 endosperm nuclei. Only 5% of the seeds of self fertilised Col-0 had up to 64 endosperm nuclei compared to ~40% seeds of self fertilised *cycb1;2-/-*. The reduced number of endosperm nuclei in the seeds obtained from *cycb1;2-/-* x Col-0 demonstrated a maternal effect of *cycb1;2* mutant on to endosperm proliferation. Now, it has to be distinguished whether this maternal effect is gametophytic or sporophytic and I have performed reciprocal crosses of *cycb1;2-/+* with Col-0 to distinguish between these two possibilities.

Table R-4 Percentage of seeds showing distribution of endosperm nuclei in different classes upon reciprocal cross of cycb1;2-/-

Parental genotypes (female x male)	seeds (%) with respective endosperm nuclei counts			n
	1 - 16	32 - 64	128 - 512	
Col-0 selfed	0	7.69	92.31	39
cycb1;2-/- selfed	0	40	60	35
Col-0 x *cycb1;2-/-*	0	0	100	30
cycb1;2-/- x Col-0	0	36.84	63.16	38

3.3.6 The cycb1;1-/-cycb1;2-/- and cycb1;2-/-cycb1;3-/- plants show sporophyte defects

While analysing the *cycb1;1-/-cycb1;2-/- or cycb1;2-/-cycb1;3-/-* seeds, I could observe that many of these seeds have an embryo, which reached at least up-to late heart stage (Figure R-9). To test whether these embryos can germinate, the seeds from *cycb1;2-/-cycb1;1-/+* and *cycb1;2-/-cycb1;3-/+* plants were seeded on MS plate. Some plantlets with distinct morphology compared to wild-type were observed and genotyping of these seedlings revealed that these were indeed double homozygous for both the genes. The frequency of occurrence of the double homozygous mutants was 13% in case of *cycb1;2-/-cycb1;1-/+* but and 5.40% in *cycb1;2-/-cycb1;3-/+* (Table R-5).

Table R-5 Percentage frequency of double homozygous plants of CYCB1;1 and CYCB1;2 that survived on 1/2MS plates

Parental genotypes (female x male)	No. of double homozygous	Total number of plants/seed	% double homozygous
cycb1;2-/-cycb1;1-/+ selfed	87	649	13.4
cycb1;2-/+cycb1;3-/- selfed	40	740	5.4

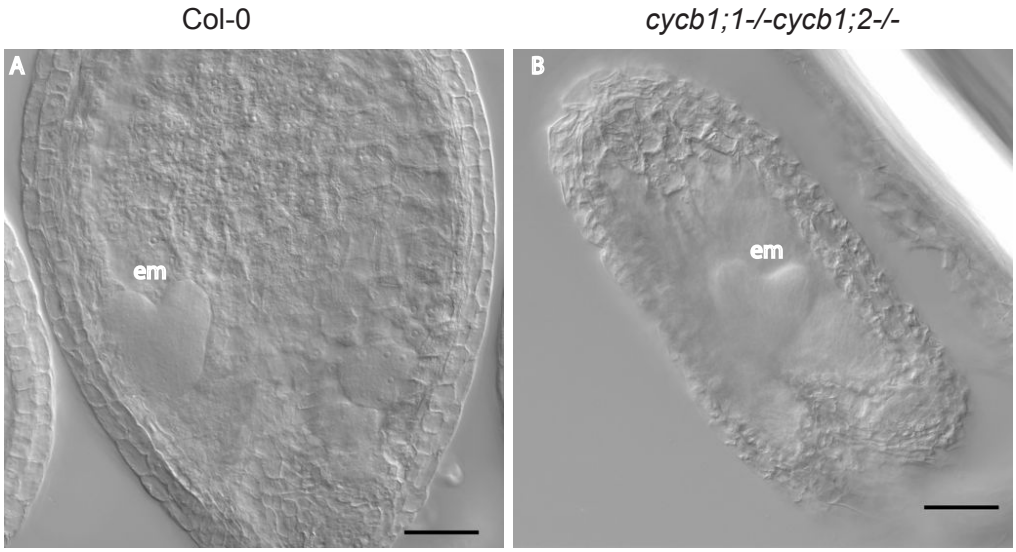

Figure R-9. No embryo defect in *cycb1;1-/-cycb1;2-/-* seeds
Representative pictures of Col-0 (A) and *cycb1;1-/-cycb1;2-/-* (B) seeds showing heart stage embryo. It is postulated that such heart stage embryo can grow further with shrivelled seed coat and might germinate under controlled conditions giving rise to double homozygous plants. Scale bars: 50µm

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The *cycb1;1-/-cycb1;2-/-* seedlings appeared sick and smaller compared to wild-type. The leaves of *cycb1;1-/-cycb1;2-/-* plants were also different with conical tips compared to oval wild-type (Figure R-10A,B). The *cycb1;2-/-cycb1;3-/-* seedlings appeared very sick compared to wild-type and were smaller in size with dark brown-pigmented cotyledons in contrast to green cotyledons of wild-type. In addition they showed various degree of phenotypes in developing root and cotyledons with few seedlings lacking primary root and even cotyledons (Figure R-10E,F and G). The *cycb1;2-/-cycb1;3-/-* seedlings did not grow beyond cotyledon stage where as *cycb1;1-/-cycb1;2-/-* could survive on soil although appeared smaller and bushy compared to wild-type (Figure R-10C). The *cycb1;1-/-cycb1;2-/-* plants could flower but almost 90% of their seeds aborted.

The compromised growth of these double mutants under controlled conditions suggests that these cyclins are not only important for endosperm development but also for sporophytic growth and development of the Arabidopsis. This also indicates that probably these cyclins are required for progression of cell differentiation during later stages.

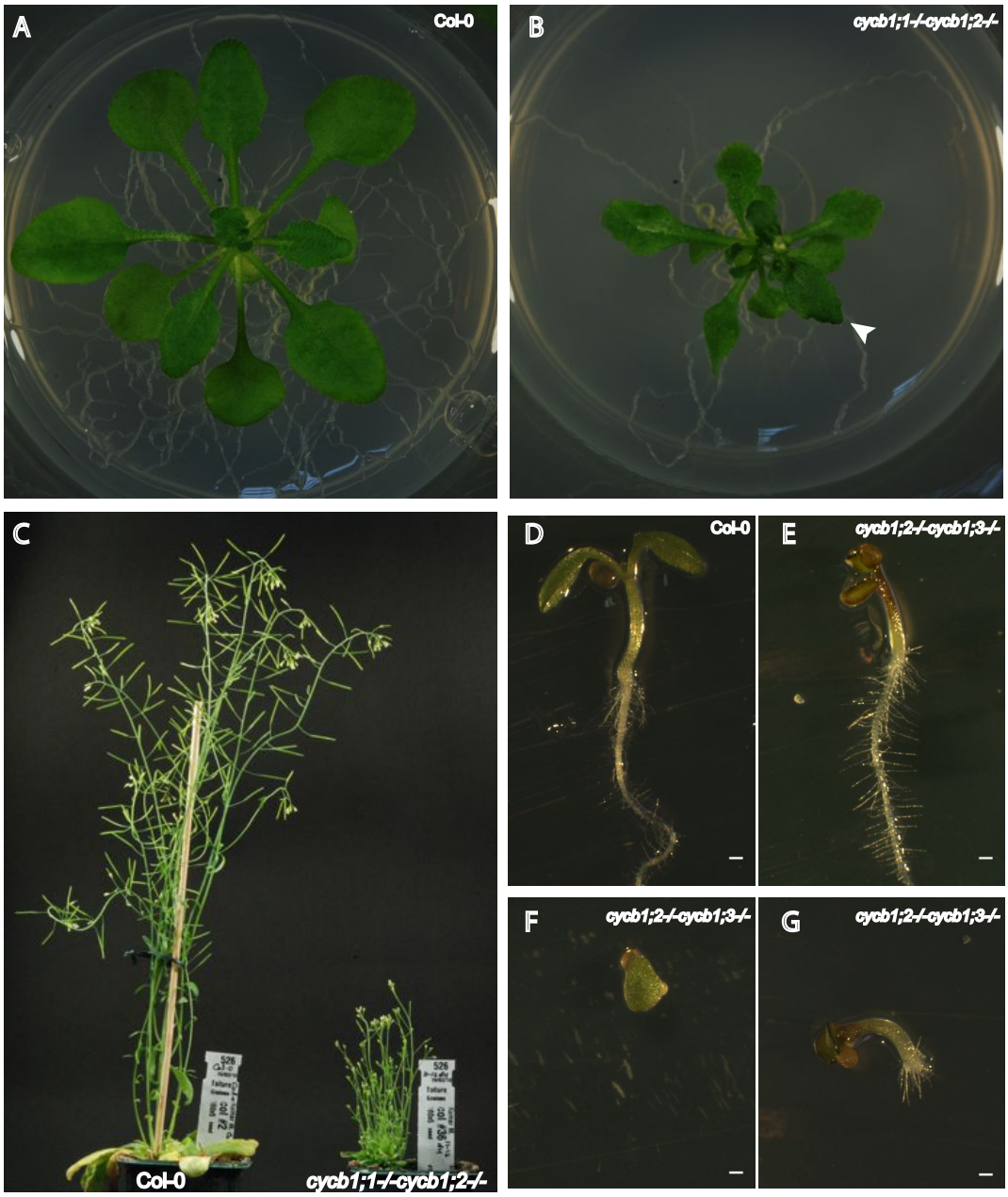

Figure R-10. Phenotype of *cycb1;1-/-cycb1;2-/- and cycb1;2-/-cycb1;3-/-* plants
A & B show 20 days old seedlings of Col-0 and *cycb1;1-/-cycb1;2-/-* growing on ½ MS plate. White arrowheads show conical leaf tip (B). 5 weeks old Col-0 and *cycb1;1-/-cycb1;2-/-* plants (C). 5 days old Col-0 (D) and *cycb1;2-/-cycb1;3-/-* seedlings (E, F and G) showed range of phenotypes like pigmented cotyledon stage (E), seedlings without primary roots (F) and cotyledons (G).

3.3.7 Growth rate defect in primary roots of cycb1;1-/-cycb1;2-/-

The existence of *cycb1;1-/-cycb1;2-/-* plants allowed to investigate mitotic defects in other tissues in these plants. Considering the root as a model to study cell growth and division, I opted to examine the root development in the double homozygous plants. My colleague Annika Weimer helped me by performing the experiment in which she measured the root length of double mutant plants grown on MS plate in comparison to wild-type and found that the primary root of double mutant plants grew slower compared to wild-type (Figure R-11). One probable reason for this retarded root growth could be that mitotic efficiency of the root cells in the mutant plant is hampered, which might result in reduced root length in mutant plants.

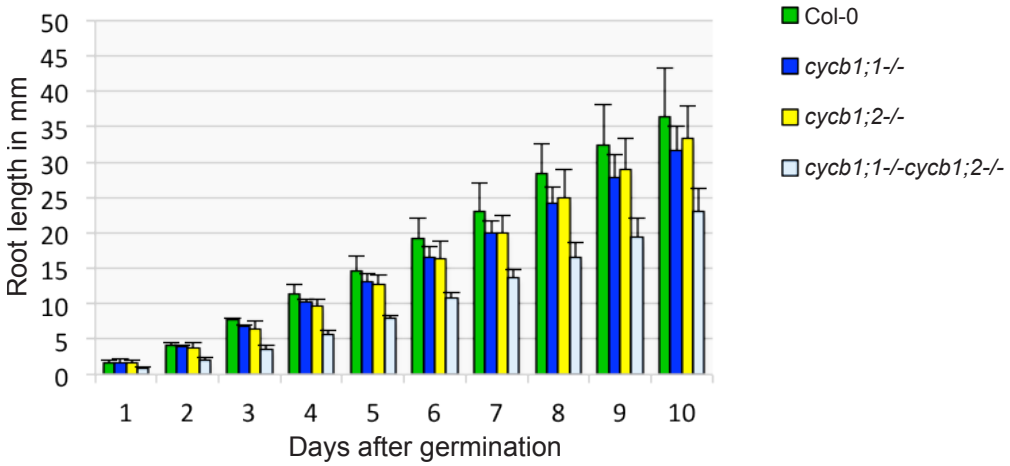

Figure R-11. *cycb1;1-/-cycb1;2-/-* plants show retarded root growth.
Root lengths (in mm) measured for Col-0 (wild-type), *cycb1;1-/-*, *cycb1;2-/-* and *cycb1;1-/-cycb1;2-/-* mutant plants grown on ½ MS plates for 10 days are depicted in the bar graph. *cycb1;1-/-cycb1;2-/-* mutants showed smallest roots in comparison to single mutants and wild-type plants.

3.3.8 cycb1;1-/-cycb1;2-/- roots show mitotic defects

To investigate any mitotic defect in root cells of the mutant plants, I analysed the microtubules in the mutant cells undergoing mitosis by immunostaining the five days old roots. Immunostaining with antibody recognising alpha tubulin revealed highly distorted microtubule orientation in the mutant cells undergoing mitosis. I could observe oblique cells in prophase, atypically condensed chromosome in prometaphase, multipolar microtubule orientation in metaphase, fused nuclei in telophase and untimely appearance of the preprophase band (Figure R-12). Based on the microtubule defect observation, it appears that the dividing cells probably passed the G2-phase but arrested in the M-phase.

3.3.9 Mitotic defects in cycb1;1-/-cycb1;2-/- endosperm

The microtubule cytoskeleton during endosperm development in *cycb1;1-/-cycb1;2-/-* double mutants was analysed by immunostaining (Figure R-13). The mutant endosperm appeared very different compared to wild-type having enlarged nuclei with much more DNA compared to wild-type, which was stained using DAPI (Figure R-13B and D). The large endosperm nuclei from mutant plants lacked the radial microtubules and appeared to have cortical microtubules network surrounding them suggesting that they are still in the interphase whereas wild-type nuclei is surrounded with radial microtubules (Figure R-13A and C). The larger size and accumulated DNA in mutant nuclei along with microtubule orientation suggests that probably they are arrested after G2-phase.

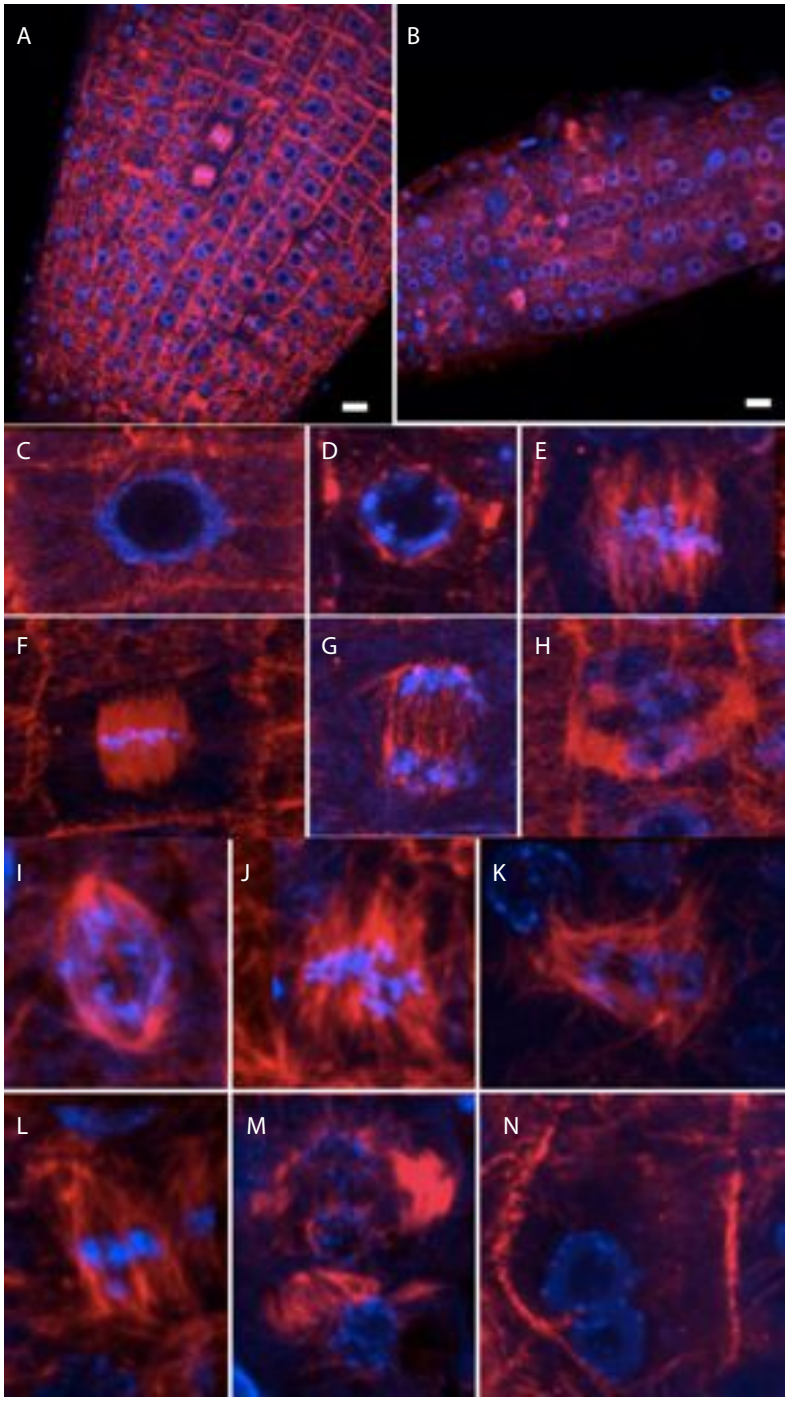

Figure R-12. *cycb1;1-/-cycb1;2-/-* roots show mitotic defects
Immunolabelling of Arabidopsis roots with anti-tubulin antibodies (red) and DAPI staining (blue). (A) Col-0 root. (B) *cycb1;1-/-cycb1;2-/-* root. The roots of *cycb1;1-/-cycb1;2-/-* showed distorted microtubule orientation compared to well organised microtubules in Col-0. C-H and I-N depicts early G2, late G2, prometaphase, metaphase, anaphase and telophase, respectively. The mutant roots showed oblique prophase (I), atypically condensed chromosomes in prometaphase (J), abnormal 3 to 4 polar mitosis (K), 3 polar metaphase (L), abnormal telophase with fused nuclei in M and N. Abnormal microtublar organization suggests that the cells are arrested in G2/M phase. Scale bars: 50µm

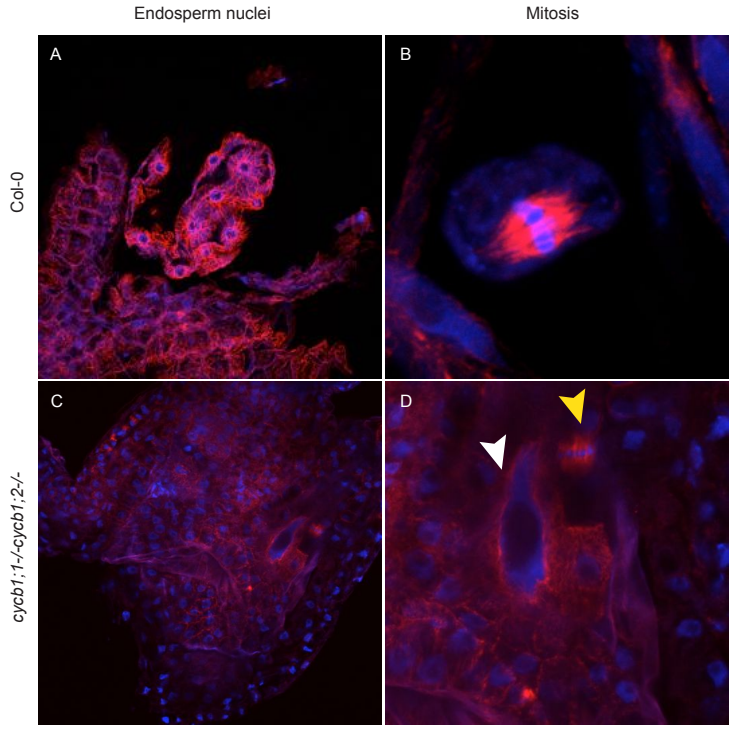

Figure R-13. *cycb1;1-/-cycb1;2-/-* endosperm show mitotic defects
Immunolabelling of Arabidopsis seeds from Col-0 (A & B) and *cycb1;1-/-cycb1;2-/-* (C & D) with anti-tubulin antibodies (red) and DAPI (blue) is depicted. DAPI staining showed large (D, white arrowhead) and multipolar (D, yellow arrowhead) nuclei whereas tubulin staining showed absence of radial microtubules in the mutant endosperm nuclei compared to normally dividing nucleus (B) in wild-type.

3.3.10 Ploidy analysis reveals the accumulation of cells in G2 phase

Considering the large size of mutant nuclei in *cycb1;1-/-cycb1;2-/-*, I investigated the effect of these mutants on cellular ploidy. I analysed the ploidy distribution in cotyledons (10 DAG), roots (10 DAG), and seeds (4 DAP) using flow cytometry (Figure R-14). The ablation of *CYCB1;1* and *CYCB1;2* genes resulted in increase of the proportion of cells with ploidy levels of 4C in cotyledons and roots (Figure R-14B and D). The ploidy analysis of cotyledons and roots further indicate that the cells are not able to complete mitosis instead they undergo endoreplication, which leads to accumulation of cells with 4C ploidy level. The double homozygous *cycb1;1-/- cycb1;2-/-* cotyledons had more number of cells with 8C which could be the result of endoreplication in the 4C cells. In view of large sized endosperm nuclei and accumulated DNA, an increase in 6C ploidy in the

endosperm was expected. However, the ploidy levels did not change much in the *cycb1;1-/-cycb1;2-/-* seeds however a small increase in 6C peak was observed compared to wild-type (Figure R-14E and F). The FACS analyses of double homozygous *cycb1;1-/-cycb1;2-/-* indicates that cells are arrested in G2/M transition phase in roots as well as cotyledons.

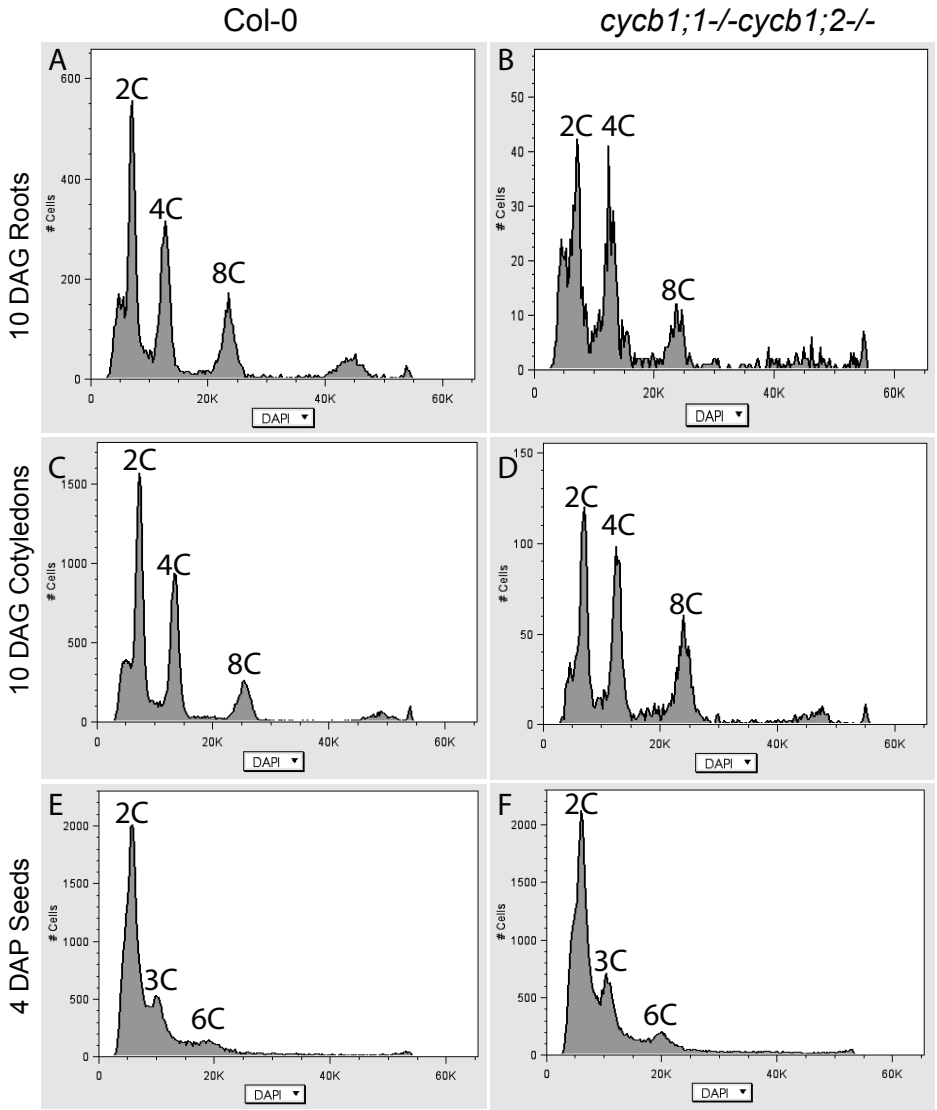

Figure R-14. *cycb1;1-/-cycb1;2-/-* endosperm show accumulation of cells in G2 phase
FACS analysis of roots (10 days after germination) (A & B), cotyledons (10 days after germination) (C & D) and seeds (4 days after pollination) (E & F) from Col-0 and *cycb1;1-/-cycb1;2-/-* plants revealed increased accumulation of 4C ploidy in roots and cotyledon, 8C ploidy in cotyledon and only a slight increase of 6C in seeds obtained from *cycb1;1-/-cycb1;2-/-* mutant plant compared to wild-type Col-0.

3.3.11 Marker lines confirm mitotic arrest of cells in G2-M in double mutants

Flow cytometry analysis demonstrated accumulation of cells with increased ploidy levels in double mutant roots and cotyledons, suggesting a mitotic defect at G2-M transition stage. To confirm the mitotic cell arrest in G2-M phase in *cycb1;1-/-cycb1;2-/-* plants *invivo*, ProCYCB1;1:CYCB1;1DB-GFP, ProCYCB1;2:CYCB1;2DB-GFP and ProCYCB1;3:CYCB1;3DB-GFP marker lines were crossed with double mutants *cycb1;1-/-cycb1;2-/+* and *cycb1;2-/-cycb1;3-/+* and were selected in subsequent generations in the respective double mutant background. As discussed earlier, expression of these marker lines in the cells under G2 or M phase would express GFP whereas no GFP expression would be visible in cells, which have already completed mitosis. The GFP expression of all three marker lines was observed in *cycb1;1-/-cycb1;2-/-* roots (Figure R-1B, E and H). Apparently, the number of GFP positive cells were increased in *cycb1;1-/-cycb1;2-/-* roots compared to wild type suggesting the accumulation of cells in G2/M transition phase. A similar GFP expression pattern was observed for ProCYCB1;1:CYCB1;1DB-GFP, ProCYCB1;2:CYCB1;2DB-GFP in the *cycb1;2-/-cycb1;3-/-* roots (Figure R-1C and F). The *cycb1;2-/- cycb1;3-/-* seedlings harbouring ProCYCB1;3:CYCB1;3DB-GFP could not be obtained. The analysis of ProCYCB1;4:CYCB1;4DB-GFP in the mutant background is under progress.

The analysis of ProCYCB1;1:CYCB1;1DB-GFP, ProCYCB1;2:CYCB1;2DB-GFP and ProCYCB1;3:CYCB1;3DB-GFP marker lines in the *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* seeds revealed intense GFP expression in the giant endosperm nuclei (Figure R-2B,E,H,C,F and I). Considering the fact that these double homozygous seeds contain only upto 16 endosperm nuclei, its interesting to observe the large nuclei expressing GFP, suggesting these are in G2/M transition phase and there is a either a block or delay in endosperm nuclei division. Figure R-2C is a representative picture showing large endosperm nuclei expressing GFP with several dark spots within, which could be the number of nucleoli after several rounds of endoreplication.

3.3.12 Stomata development remains unaltered in cycb1;1-/- cycb1;2-/-

In general, stomata undergo a regulated cell division, which might be altered in *cycb1;1-/-cycb1;2-/-* mutants. To investigate any such potential stomata defects, I examined the leaves from two weeks old seedlings from the mutant plants and compared them with the wild-type. No obvious differences were observed in the mutant stomata compared to wild-type with respect to size, number and structure (Figure R-15). This indicates that *CYCB1;1* and *CYCB1;2* are dispensable for stomatal development in Arabidopsis.

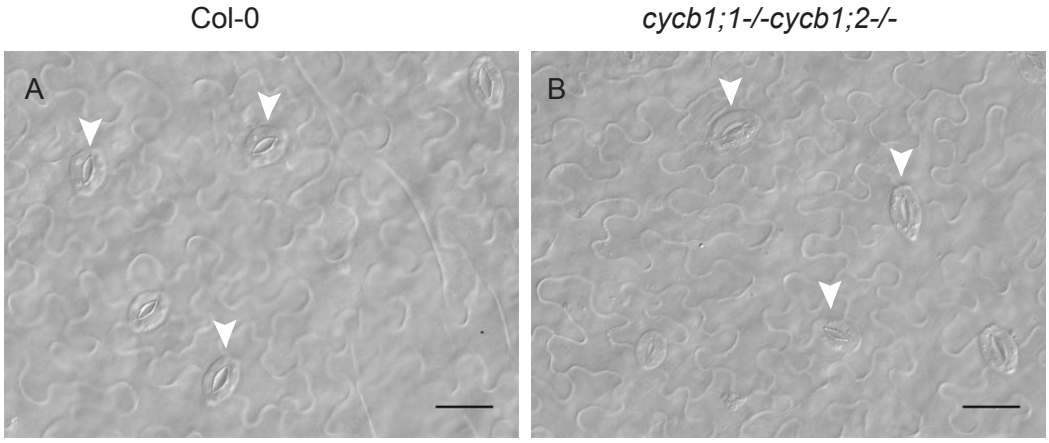

Figure R-15. *cycb1;1-/-cycb1;2-/-* show unaltered stomata development
No differences were visible in stomata (white arrowheads) development in Col-0 (A) and *cycb1;1-/-cycb1;2-/-* cotyledons 14days old (B).

3.4 The seed abortion phenotype of *cycb1;1* and *cycb1;2* double mutants is significantly enhanced by additional loss of *CYCB1;3*

Inspite of knocking down *CYCB1;1 and CYCB1;2*, the endosperm nuclei division was not completely abolished, although it lead to seed growth arrest, raising the probability of an additive or dosage effect of other *CYCB1s* during seed development and endosperm nuclear division. To examine this possibility, I crossed *cycb1;1-/-cycb1;2-/+* and *cycb1;2-/-cycb1;3-/+* plants and obtained *cycb1;1-/+cycb1;2-/+cycb1;3-/+* in F1 generation and *cycb1;1-/-cycb1;3-/-*

cycb1;2-/+ in subsequent F2 generation. I observed the seeds in the siliques of self pollinated *cycb1;1-/-cycb1;3-/-cycb1;2-/+* plants and found 34% shrivelled seeds (Figure R-6D, Table R-6).

Table R-6 Percentage of aborted seeds in CYCB1 triple and quadruple mutant lines

Parental genotypes (female x male)	aborted	n	Percentage Abortion (%)
Col-0 selfed	18	1020	1.76
cycb1;1-/-cycb1;3 -/- cycb1;2 -/+ selfed	452	1323	34.16
cycb1;1-/-cycb1;3 -/-cycb1;4 -/- cycb1;2 -/+ selfed	332	613	54.15

As the proportion of shrivelled seeds was higher than 25%, it was likely that the triple mutation caused failure in the development of one of the gametophytes. I performed the reciprocal crosses between *cycb1;1-/+cycb1;2-/+b1;3-/+* mutants and wild-type plants, which revealed only ~40% transmission of each of the mutant allele instead of normal 50% through the male side however the transmission through female side appeared normal suggesting the defect in male gametophyte (Table R-7).

Table R-7 Transmission rates of the cycb1;1, cycb1;2 and cycb1;3 alleles in triple mutants

Parental genotypes (female x male)	Genotypes of F1 plants			n
	cycb1;1-/+	*cycb1;2-/+*	*cycb1;3-/+*	
cycb1;1-/+cycb1;2-/+cycb1;3-/+ x Col-0	50%	51.50%	54.60%	192
Col-0 x *cycb1;1-/+cycb1;2-/+cycb1;3-/+*	43%	39.44%	40.55%	360

3.5 Analysis of quadruple mutants of *CYCB1;1, CYCB1;2, CYCB1;3 and CYCB1;4*

The loss of *CYCB1;4* slightly enhanced the endosperm phenotype by increasing the number of seeds with 32-64 nuclei in conjugation with *cycb1;1-/-* and *cycb1;2-/-* (Table R-3). Considering this fact, I further generated quadruple mutant *cycb1;1-/-cycb1;3-/-cycb1;4-/-cycb1;2-/+.* The mutant plants did not show any morphological difference in leaves or inflorescence compared to wild-type, although 54% of the seeds in the siliques were aborted (Figure R-6E, Table R-6). The 50% seed abortion with one of the heterozygous allele clearly indicated towards the gametophytic defect which was further confirmed by reciprocal cross of *cycb1;1-/-b1;3-/-cycb1;4-/-cycb1;2-/+* mutant with wild-type plants. The transmission rate of heterozygous *cycb1;2* allele was only 9.38% through female gametophyte and 36% through male gametophyte instead of normal 50% (Table R-8). The homozygous *cycb1;1* allele did not show any transmission defect and transmitted 100% as expected. The reduced transmission of *cycb1;2* allele through both the parents suggests that in *CYCB1s* are required for both male and female gametophyte development.

Table R-8 Transmission rate of the cycb1;2 allele in quadrupule mutant

Parental genotypes (female x male)	Genotypes of F1 plants		n
	cycb1;1-/+	*cycb1;2-/+*	
*cycb1;1-/-cycb1;3-/-cycb1;4-/-*cycb1;2-/+ x Col-0	100%	9.38%	96
Col-0 x *cycb1;1-/-cycb1;3-/-cycb1;4-/-cycb1;2-/+*	100%	36%	61

3.6 EDE1, a possible substrate for CYCB1/CDK complex?

In a recent study, ENDOSPERM DEFECTIVE1 (EDE1) has been characterised in Arabidopsis, which functions as a microtubule binding protein and is essential for seed development (Pignocchi et al., 2009). The *ede1* mutant came to our attention owing to its similar large endosperm nuclei phenotype as observed in

cycb1;1-/-cycb1;2-/- and *cycb1;2-/-cycb1;3-/-* double mutants. Interestingly, amino acid sequence analysis of EDE1 revealed presence of seven consensus CDK phosphorylation sites within the EDE1 sequence, leading to the hypothesis that EDE1 could be a possible substrate for CYCB1/CDK complexes. To get a first hint if EDE1 is a possible substrate for CYCB1/CDK complex, the obvious question was whether EDE1 co-localises with CYCB1s. As it has already been shown that EDE1 co-localises with microtubules during metaphase, I performed co-immunofluorescence staining with antibodies recognising alpha tubulin and EDE1 (provided by Dr. John Doonan) in the five days old roots of *cycb1;1-/-cycb1;2-/-* and wild-type Col-0. The EDE1 signal was not detected along the spindle microtubules in the dividing cells (Figure R-16). This result suggests that probably the EDE1 expression level is too low or the antibody is not specific enough to detect EDE1 *in vivo*. To genetically validate the hypothesis, I have crossed *ede1* mutant with *cycb1s* mutants and F2 generation analysis is awaited.

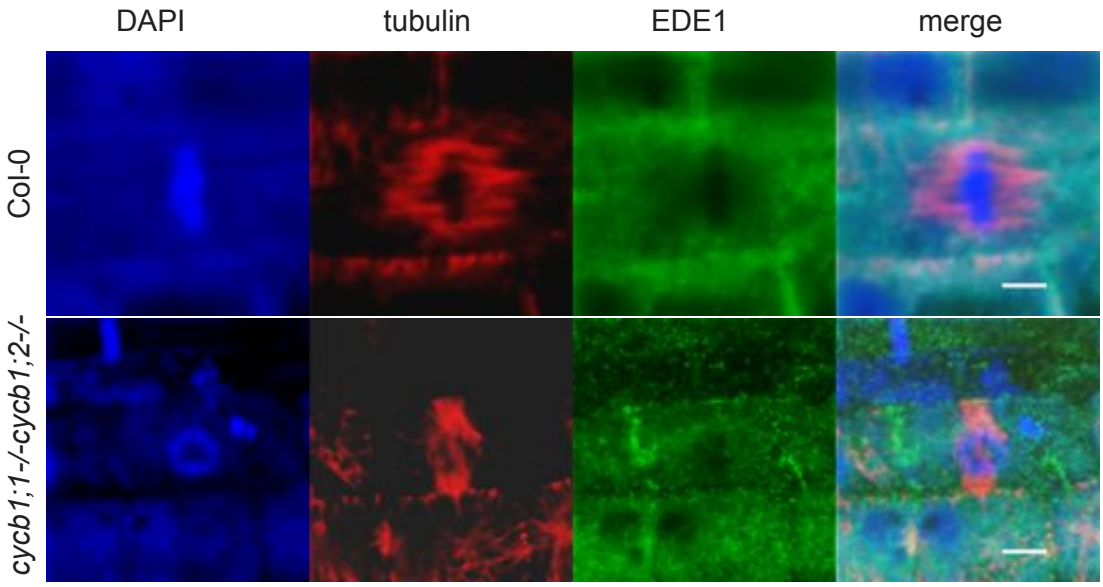

Figure R-16. Co- immuno-flouroscence staining of EDE1 with alpha-tubulin in Col-0 and *cycb1;1-/-cycb1;2-/-*

Immunolabelling of roots with antibodies recognising EDE1 (green), tubulin (red) and nuclear staining with DAPI staining (blue) was performed on Col0 control root (A) and *cycb1;1-/-cycb1;2-/-* double mutant (B). Co-localisation of EDE1 with tubulin was not detected in the dividing cells.

3.7 QTL analysis of seed weight using three sets of RIL populations

To complement the reverse genetics approach to dissect seed development, I exploited the existing natural variation in Arabidopsis to identify important regulators of seed growth and development. The natural variation has been observed in the seed weight and seed size among different accessions of Arabidopsis (Ungru et al., 2008). In an earlier study involving the RIL population Ler and Cvi, six QTLs affecting seed size were identified (Alonso-Blanco et al., 1999). In the present study I have used three RIL populations An1-Cvi, An1-Ler and Ler-Cvi. The choice of these RIL populations was based on the fact that An-1 seeds are smaller as well as lighter where as Cvi produces heavier and bigger seeds. The seeds obtained from Ler are bigger and heavier compared to An-1 but smaller and lighter compared to Cvi. The analysis of seed mass trait using these three RIL populations having one common parent allowed direct comparison of the loci segregating in the three accessions. For QTL analysis, 100 seeds were counted and weighed (mg) in triplicates for each RIL of three populations. With this quantitative data set, QTL analysis was performed for each RIL population and QTLs were identified and localised by Composite Interval Mapping (CIM). The QTL analysis of three RIL populations revealed detection of several loci, which is presented in the table with the respective variance for individual locus (Table R-8). In addition two complex QTLs were detected as well. The first interaction was between chromosome III linked to marker GD.160C and IV linked to marker g4539 in Cvi-Ler, where as the second was in Ler-An1 between chromosome I linked to marker nga 128 and III linked to marker SNP81. The data analysis suggests that the obtained QTLS can potentially regulate seed mass in Arabidopsis.

Table R-8 QTL analysis for seed weight

QTL number	RIL population	Chromosome no.	marker	variance
QTL1	An-1 X Cvi	1	nga59	9.60%
QTL2	An-1 X Cvi	3	MSAT3.5	7.30%
QTL3	An-1 X Cvi	4	CIW7	16.80%
QTL4	Cvi X Ler	1	PVV4	15.00%
QTL5	Cvi X Ler	1	EC.66C	15.80%
QTL6	Cvi X Ler	3	FD.111L-Col/136C	4.70%
QTL7	Cvi X Ler	4	GB.490C	12.70%
QTL8	Cvi X Ler	5	BF.168	4.80%
QTL9	Ler X An-1	3	SNP 105	11%
QTL10	Ler X An-1	5	snp236	6.10%

3.8 QTL analysis of autonomous endosperm development upon pollination with *cdka;1* pollen

The mutation in the core cell cycle regulator CDKA;1 in Arabidopsis, results in the defect in pollen mitosis II, and the seeds formed after pollinating with the mutant *cdka;1* pollen found to have diploid endosperm nuclei instead of normal triploid, suggesting their autonomous proliferation without proper double fertilisation. There was natural variation observed in the number of diploid endosperm nuclei upon *cdka;1* pollination in different accessions (Ungru et al., 2008). The observed natural variation in autonomous endosperm proliferation can be explained by communication of presumptive signal through mutant pollen that triggers autonomous endosperm division upon fertilisation of the egg cell but failing karyogamy of the second sperm cell with the central cell (Nowack et al., 2006; Aw et al., 2010). The observed natural variation can also be explained by the efficiency of the transmission of the signal from paternal side or

the efficiency to perceive the signal by maternal central cell. It has been observed that *fis*-class mutants that show autonomous endosperm proliferation without fertilisation, could mutually rescue the seeds obtained upon pollination with mutant *cdka;1* pollen (Nowack et al., 2007). In this situation one hypothesis is that the natural variation in the expression or function of genes, which cause maternal gametophyte defect e.g. FIS-class genes might be responsible for the observed variation in autonomous endosperm proliferation.

The autonomously proliferating endosperm nuclei upon *cdka;1* pollination has been estimated keeping in mind that during early seed development they divide synchronously and all the numbers have been converted into endosperm division value (EDV), which is defined as the number of divisions necessary to reach the corresponding nuclei numbers within the respective seed; *e.g.*, 28 nuclei were scored as being in the fifth cycle ($28 \sim 2^5$) (Ungru et al., 2008). Based on the number of EDV a quantification approach was chosen to identify possible loci on the chromosomes that might relate to the trait by using an established Recombinant Inbred Line (RIL) population. The two ecotypes forming the RIL population were Estland (Est-1), and *Columbia* (Col-0) differed significantly in their EDV, Est-1 having fewer EDV compared to Col-0 (Ungru et al., 2008). Out of 283 RILs of the core collection, 211 were grown and each line was pollinated with pollen from *cdka;1* mutant plants. The seeds from 5 siliques were cleared after 3 DAP and the numbers of endosperm nuclei were counted from the aborting seeds. The large variation of the EDVs among the RIL population differed substantially from the Est-1 and Col-0 parents. The QTL analysis revealed the detection of one QTL located on top of chromosome III linked with the marker *112* (40.88cM or 6.63 Mb) (Figure R-17). This QTL explained roughly 12 percent of the total variance in EDVs with a heritability of 0.63. The detected LOD of this QTL was 2.68. The alleles of Col-0 at this position increased the number of endosperm divisions. Upon comparing the QTL analysis from this study with the previously performed study using Bay-Sha RIL, it was revealed that this QTL colocalises with *KALYPSO* (*KAL*) QTL found in Bay-Sha RIL population (Ungru et al., 2008). In addition to main effect QTL data analysis revealed one complex QTL between chromosome III, marker M146 and chromosome IV, marker M176 as well (Figure R-17). The appearance of the QTL

at same position in two different RIL populations suggest that indeed there could be some genes which might be involved in the perception of paternal signal or have some maternal gametophytic defect. I found one gene RBR1, which cause female gametophyte defect in the spanning region of QTL (Ebel et al., 2004).

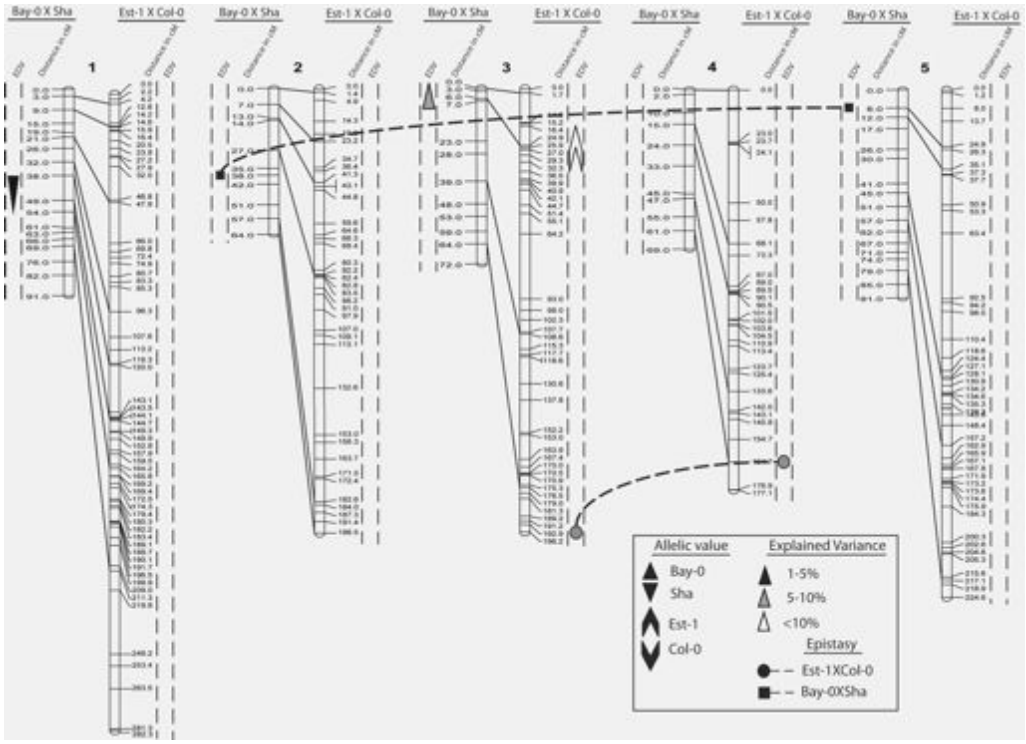

Figure R-17. QTL associated with endosperm division value (EDV) for Est-1-Col-0 (EC) and Bay-Sha (BS) RIL populations. Linkage maps are shown for each chromosome with BS on the left and EC on the right. QTL identified using CIM are shown by arrows and complex QTLs are represented by the dotted lines.

Considering that RBR1 cause female gametophytic defect which is in accordance with our hypothesis, I need to validate it by making genetic cross with *cdka;1* to confirm the mutual rescue or increased transmission of mutant *cdka;1* allele.

4 Discussion

The key regulator of G2–M transition of the cell cycle is M-phase promoting factor (MPF), a complex composed of B-type cyclins and Cyclin Dependent Kinases. In Arabidopsis, 11 B-type cyclins are known, which are sub-grouped in three classes: B1, B2 and B3. Previous studies in Arabidopsis suggest that all B-type cyclins show a common pattern of regulation during both cell cycle re-entry and progression, with a peak in early mitosis (Menges et al., 2005). *CYCB1;1*, *CYCB1;2* and *CYCB1;3* along with several other core cell cycle genes including mitotic CDKBs and other cyclins were reported to be upregulated in proliferating endosperm nuclei in Arabidopsis (Day et al., 2008) suggesting their potential role in seed development. In this study, I have investigated the role of B1 type cyclins in seed development of Arabidopsis.

4.1 B1-type cyclins accumulate in G2/M and localise to chromatin and spindle during mitosis

The expression of CYCB1;1 through CYCB1;4 was investigated using marker lines ProCYCB1;1:CYCB1;1DB-GFP to ProCYCB1;4:CYCB1;4DB-GFP. The analysis of GFP expression in roots revealed patchy expression pattern for all CYCB1 marker lines. Similar expression pattern has been reported earlier for CYCB1;1 marker line displaying patchy pattern of GUS expression in cells which are in G2/M transition phase in Arabidopsis (Doerner et al., 1996; Shaul et al., 1996; Colon-Carmona et al., 1999). Further, ProCYCB1;2:GUS line has also been reported as mitotic marker line for cells in G2/M transition phase (Schnittger et al., 2002b). In conformity with these previous reports, the expression of ProCYCB1;1:CYCB1;1DB-GFP to ProCYCB1;4:CYCB1;4DB-GFP in root cells signifies that these cells are in G2/M transition phase. The lack of GFP expression in the remaining cells indicates that GFP fusion protein has been degraded in the cells completing mitosis and throughout G1 as well as S-phase. This suggests that CYCB1;3 and CYCB1;4 follow similar temporal expression during mitosis in roots as has been reported earlier for CYCB1;1 and CYCB1;2.

I found similar oscillating expression of CYCB1;1 to CYCB1;3, in proliferating endosperm nuclei. In contrast, no CYCB1;4 driven GFP expression was observed in the proliferating endosperm. This suggests that CYCB1;4 exhibits a distinctive tissue specific expression pattern in comparison to other CYCB1s and is probably not required during nuclear divisions in endosperm.

The localisation study of CYCB1s in Arabidopsis revealed that CYCB1;1 and CYCB1;2 exclusively localised to the chromatin during metaphase whereas CYCB1;3 was mainly associated with chromatin although a large fraction was also in the cytoplasm and at the spindle. Interestingly, CYCB1;4 mainly localised at the spindle and in the cytoplasm with no detectable association with the chromatin during metaphase. As a truncated version of the CYCB1;1 was used for the reporter construct, we can not fully exclude a possibility that the full length protein localises to other cellular structures, besides chromatin. The localisation studies of mammalian B type cyclins B1, B2 and B3 showed differences in their intracellular localisation pattern. Among the three B type cyclins, only CYCB1 is essential in mammals, which is mainly cytoplasmic during the interphase and rapidly translocates to the nucleus at the end of prophase and associates with mitotic apparatus in the course of mitosis. It localises to chromatin, microtubules, kinetochores and centrosomes (Bentley et al., 2007; Chen et al., 2008). In contrast, CYCB2 localises mostly to Golgi apparatus in both interphase and metaphase (Draviam et al., 2001). CYCB3 is only poorly expressed in mitotic cells, but its mRNA is readily observed in both male and female meiosis (Nguyen et al., 2002). On the other hand, Drosophila has two B-type cyclins, CYCB and CYCB3 (Nieduszynski et al., 2002). It has been reported that CYCB localises to the cytoplasm during interphase and early prophase before entering to the nuclei during prophase where it associates with the spindle after nuclear envelope breakdown. In contrast, CYCB3 was reported to localise in the nuclei throughout the whole cell cycle (Huang and Raff, 1999). The onset of degradation of B and B3 cyclins in Drosophila also differs as CYCB is degraded at metaphase/anaphase transition, while CYCB3 is degraded during anaphase (Parry and O'Farrell, 2001). None of the CYCB1s in Arabidopsis showed a expression and degradation pattern similar to CYCB3 in Drosophila. Taken together, the cell cycle dependent localisation of Arabidopsis CYCB1s resembles behaviour of mammalian CYCB1 and *Drosophila* CYCB but not CYCB3.

4.2 *cycb1;2-/-* represents a maternal effect mutant

To investigate the functional relevance of *CYCB1s* in Arabidopsis, T-DNA mutant lines for *CYCB1;1* through to *CYCB1;5* were analysed. Compromising the function of individual *CYCB1s* neither showed an obvious morphological alteration nor affected the overall plant growth compared to wild-type suggesting that none of the individual *CYCB1s* are essential by its own for vegetative growth of Arabidopsis plant. However, *cycb1;2-/-* seeds displayed fewer nuclei in endosperm compared to wild-type seeds without affecting their viability.

One third of 3DAP seeds of *cycb1;2-/-* contained up-to 64 nuclei compared to 7% of 3DAP seeds of wild-type endosperm nuclei. Interestingly, the number of endosperm nuclei in *cycb1;2-/-* showed ~25% reduction in comparison to wild-type which pointed to a potential sporophytic recessive defect. The reciprocal cross of *cycb1;2-/-* with wild-type plants confirmed that indeed there is a maternal effect of *CYCB1;2* and the cross of *cycb1;2-/+* with wild-type can distinguish between sporophytic and gametophytic effect. The analysis of segregation of number of endosperm nuclei in the seeds obtained from the reciprocal cross of *cycb1;2-/+* with wild-type would help in distinguishing between sporophytic and gametophytic defect. In case of gametophytic defect, I would expect quarter of total seeds having fewer number of nuclei as what was observed in *cycb1;2-/-* reciprocal cross whereas sporophytic defects should not cause any reduction in number of endosperm nuclei until unless it is dominant. The quarter number of the seeds showing mutant phenotype in case of gametophytic defect can be explained by the less penetrance of the mutant allele.

The T-DNA mutant for CYCB1;5 did not show any obvious defects. However, during the course of study, certain discrepancies emerged with respect to *CYCB1;5* gene annotation. In one of the previous reports, the gene annotation of *CYCB1;5* suggested that CYCB1;5 lacked Cyclin C domain and was speculated as a pseudogene, most likely a product of duplication event in CYCB1;2 (Wang et al., 2004a). Meanwhile, in one of our current studies, my colleague Daniel Bouyer found upregulation in the expression of *CYCB1;5* in *fie* mutants along with gain in H3K4me3, indicating that *CYCB1;5* is probably not a pseudogene (data not shown). Recent release of TAIR 10 database changed the previous annotation of *CYCB1;5* which led me to investigate the alternative annotation for *CYCB1;5*. I used the

program 'Augustus' (http://augustus.gobics.de/), which is trained for the gene prediction in *Arabidopsis thaliana,* to cross-check the annotation of *CYCB1;5*. Augustus annotated *CYCB1;5* differently compared to TAIR 9 and TAIR 10 and shifted the transcription start site downstream by skipping first four small exons, whereas the annotation of other CYCB1s did not change on this server (Stanke et al., 2004). This annotation of *CYCB1;5* also resulted in change of the insertion site of T-DNA from first exon to ~1.2 Kb upstream of the gene. Further, no ESTs spanning the first four exons could be found in the databases whereas two ESTs traversing cyclin box were found in the database. Considering these new findings, new T-DNA lines for *CYCB1;5* were ordered for further analysis.

4.3 *CYCB1s* are required for nuclear division of endosperm and seed development in Arabidopsis

Analysis of specific double mutants *cycb1;1-/-cycb1;2-/+, cycb1;1-/+cycb1;2-/-*, *cycb1;2-/-cycb1;3-/+* and *cycb1;2-/+cycb1;3-/-* revealed the occurrence of 25% shrivelled seeds in the siliques. A quarter of seed abortion in these double mutants suggests that *CYCB1;1, CYCB1;2* and *CYCB1;3* are required during seed development and act redundantly. According to classical genetics, the selfing of homozygous heterozygous mutant plants would result in 25% double homozygous progeny thus the phenotype of the shrivelled seed in these double mutant combinations might originate from a sporophytic recessive trait. The endosperm nuclei number in the shrivelled seeds also showed a distribution pattern which supports the sporophytic recessive trait in the double mutant combinations as almost 25-40% such seeds contained only up to 16 nuclei. This result suggests that *CYCB1;1*, *CYCB1;2* and *CYCB1;3* are essential for nuclear division in endosperm. The analysis of shrivelled seeds revealed the presence of enlarged nuclei of different shapes and sizes suggesting that these nuclei failed to undergo mitosis efficiently and most likely endoreplicated, resulting in enlarged nuclei.

The seeds from double mutants *cycb1;2-/-cycb1;3-/+* contained more number of nuclei in the range of 128-512 compared to *cycb1;2-/-cycb1;1-/+*, although the percentage of shrivelled seeds remained similar in both genotype combinations. This difference in endosperm nuclei number could be a result of the Noessen ecotype

background of *cycb1;3-/-* which shows faster nuclei proliferation and larger seed size compared to Col-0.

The double homozygous mutants *cycb1;1-/- cycb1;3-/-*, *cycb1;1-/- cycb1;4-/-*, *cycb1;2-/-cycb1;4-/-* and *cycb1;3-/-cycb1;4-/-* did not show any apparent morphological defects or shrivelled seeds, although there were some seeds with reduced number of endosperm nuclei compared to wild type in *cycb1;1-/-cycb1;4-/-* and *cycb1;2-/-cycb1;4-/-,* suggesting that other B1 type cyclins might be compensating for the mutated *CYCB1s* in these plants.

The *cycb1;1-/-cycb1;5-/+* plants also contained 25% shrivelled seeds whereas *cycb1;2-/-cycb1;5-/-* and *cycb1;3-/-cycb1;5-/-* plants did not show any seed defect. The appearance of shrivelled seed phenotype in *cycb1;1-/-cycb1;5-/+* suggests a potential role of *CYCB1;5* during seed development which is plausible considering that CYCB1;5 shares high sequence homology with CYCB1;2. Although as the mutation in *CYCB1;5* is neither in the coding region nor in the UTR, it is difficult to make a conclusion on the basis of the data obtained with this mutant line.

4.4 Embryo development in spite of compromised endosperm proliferation in *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* seeds

The presence of only upto 16 enlarged endosperm nuclei in the mutant seeds did not appear to influence the embryo growth and proliferation. In some of the shrivelled seeds, embryo was observed in late heart stage with only upto 16 endosperm nuclei. However, it has been reported in Arabidopsis that at the time of first zygotic division, 12 to 16 endosperm nuclei are already present, suggesting a interrelationship between embryo development and endosperm proliferation (Boisnard-Lorig et al., 2001). Nevertheless, the present data suggests that embryo development continues even with fewer number of endosperm nuclei in consistence with the recently reported *cdka;1* and *glauce* mutants which show development of embryo upto globular stage with fewer number of autonomously proliferating endosperm nuclei and single central cell nucleus, respectively (Ngo et al., 2007; Ungru et al., 2008). This raised the question to what extent embryo development depends on endosperm proliferation as well as whether the fewer endosperm nuclei could cellularise at later stages of seed development.

Cooper and Brink postulated that compromised endosperm proliferation leads to early collapse of developing seeds (Cooper and Brink, 1942). However in the present study the embryo proliferation does not appear to be dependent on number of endosperm nuclei. Nevertheless, the size of the endosperm nuclei appears very large and might be able to store the optimum amount of nutrition required for developing embryo. In such circumstances, the appearance of shrivelled phenotype of mutant seeds could be explained by the collapse of empty endosperm cavity because of continuous growth of maternal integuments to support developing embryo. This might further lead to arrest of the embryo growth. However, *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* mutant seeds obtained from homozygous heterozygous parents could germinate with 13% and 5% efficiency, respectively, if grown in a controlled environment on ½ MS plate, whereas in normal situation the 25% double homozygous seeds should be obtained. The existence of *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* clearly indicates that embryo growth and development does not depend on number of endosperm nuclei. However, the low germination efficiency of *cycb1;1-/-cycb1;2-/-* could be because of shrivelled seed coat that could desiccate early, thus leading to embryo growth arrest. The same argument can explain the less efficiency of germination of *cycb1;2-/-cycb1;3-/-* seeds considering the fact that because of Noessen ecotype the seeds tend to grow larger but due to lack of endosperm nuclei results in shrivelling of the seed coat, early desiccation and arrest of embryo development.

4.4.1 The endosperm fail to cellularise in cycb1;1-/-cycb1;2-/- and cycb1;2-/-cycb1;3-/- seeds

The *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* seeds could develop with as few as upto 16 endosperm nuclei or less. However the endosperm nuclei did not cellularise in these mutants. The previous studies have suggested that in Arabidopsis the endosperm nuclei start cellularising once the embryo reach upto globular stage (Mansfield and Briarty, 1990). However another study suggested that cellularisation in endosperm is coupled to mitosis and it only starts at globular stage of embryogenesis in conjugation with 8th mitotic cycle in endosperm i.e. with more then 100 nuclei present in the endosperm (Sorensen et al., 2002b). In contrast to these two

reports, the present study suggests that embryo can grow beyond globular stage without depending on cellularisation in the endosperm nuclei however it appears that 4 division of endosperm nuclei is not sufficient for the cellularisation to take place.

4.4.2 The cycb1;1-/-cycb1;2-/- and cycb1;2-/-cycb1;3-/- show sporophyte growth defects

The *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* mutants fail to grow on soil but were found to germinate on ½ MS plates. The viability of these plants under controlled conditions suggests that there might be redundancy among the cyclin B1 family members and other *CYCB1s* take over their function up to some extent. The *cycb1;1-/-cycb1;2-/-* plants appeared smaller, bushy and almost sterile compared to wild-type. The slow growth rate of primary roots of *cycb1;1-/-cycb1;2-/-* plants compared to wild-type could be explained by compromised mitosis in the dividing cells of roots resulting in overall smaller roots.

On the other hand, *cycb1;2-/-cycb1;3-/-* showed range of phenotypes. These seedlings could not go beyond cotyledon stage. Interestingly, the phenotype appeared to be similar to *hobbit* mutant of Arabidopsis (Willemsen et al., 1998). Arabidopsis *HOBBIT* encodes a homolog of the CDC27 subunit of the anaphase-promoting complex (APC/C) and its transcription is cell cycle regulated (Blilou et al., 2002). Further, *hobbit* mutants show a reduction in *DR5 : GUS* auxin reporter gene expression and accumulate the AXR3/IAA17 repressor of auxin responses. The overlapping similarity in the mutant phenotypes of *hobbit* and *cycb1;2-/-cycb1;3-/-* indicates that CYCB1;2 and CYCB1;3 may involve in APC/C pathway.

4.4.3 The cycb1;1-/-cycb1;2-/- and cycb1;2-/-cycb1;3-/- show G2/M arrest

The accumulation of cells in G2 phase compared to wild type in cotyledons (10 DAG) and roots (10 DAG) of *cycb1;1-/-cycb1;2-/-* suggests that in absence of CYCB1;1 and CYCB1;2 the cells can not undergo mitosis efficiently and are arrested in G2/M phase. However the FACS analysis of double homozygous seeds did not show similar pattern. According to our hypothesis, we expected similar

peaks with 3C and 6C in mutant seeds, however the data showed prominent peak of 2C and very small peaks of 3C and 6C. This deviation could be because of very few number of endosperm nuclei in the mutant seeds compared to overall large number of somatic cells, which is sorted as 2C peak.

The GFP expression of marker lines ProCYCB1;1:CYCB1;1DB-GFP, ProCYCB1;2:CYCB1;2DB-GFP and ProCYCB1;3:CYCB1;3DB-GFP in the double mutant background confirmed the arrest of endosperm nuclei in G2/M phase in *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* seeds. The mutant nuclei were visible throughout the seed, which indicates that the mitotic arrest did not happen from the beginning; instead the central cell could undergo few rounds of division covering all the mitotic domains of the seeds.

The GFP expression of marker lines ProCYCB1;1:CYCB1;1DB-GFP, ProCYCB1;2:CYCB1;2DB-GFP and ProCYCB1;3:CYCB1;3DB-GFP in the double homozygous roots showed more number of cells expressing GFP, confirming the G2/M phase arrest.

4.4.4 The mitotic defects in cycb1;1-/-cycb1;2-/- roots

The immunostaining analysis of *cycb1;1-/-cycb1;2-/-* roots revealed various defects related to chromatin material and microtubule organisation during mitosis. In the mutant roots we observed untimely appearance of pre-prophase band, which eventually hampered the cytokinesis in the roots. The formation of a cortical preprophase band (PPB) of microtubules during prophase is a characteristic feature of plant cell division, which decides the future plane of division. Once the cell enters into mitosis the microtubule PPB is disassembled and the actin component of the PPB also disappears, leaving behind an actin depleted zone in the cell cortex, which further marks the division site throughout mitosis and cytokinesis. After completion of mitosis, phragmoplast appears between daughter nuclei, which are formed of microtubules and actin filaments.

Although the formation of PPB is unique to plants, the microtubule is universally involved during cell division in different forms. In a recent study in mammals showed that CYCB1 localises to unattached kinetochores during prometaphase and it has also been reported that CYCB1 may play a role in the

attachment of microtubules to kinetochores and maintenance of the spindle checkpoint that controlling the timing of anaphase (Bentley et al., 2007; Chen et al., 2008). This suggests that the activity of cyclin B1-CDK molecules localised to kinetochores may contribute to the attachment of microtubules to kinetochores and chromosome alignment, presumably through phosphorylation of co-localised substrates (Chen et al., 2008). Considering the precedence of mammalian CYCB1 and its function during mitosis, our data suggests that *CYCB1;1* and *CYCB1;2* are required for proper microtubule orientation during mitosis in roots.

4.4.5 The mitotic defects in cycb1;1-/-cycb1;2-/- endosperm

The presence of more than one enlarged endosperm nuclei in mutant seeds suggests that initial rounds of mitosis might result in few nuclei but later on mutant nuclei continue to grow in size, without cytokinesis, generating large nuclei with several nucleoli. This could be explained by several rounds of failed mitosis or by fusion of multiple post mitotic nuclei, or by endoreduplication. However, the large mutant endosperm nuclei did not show any chromosomal condensation or aberrant mitotic structures suggesting that the enlarged nuclei might arise by endoreduplication.

The microtubule organisation is different in endosperm compared to root as cortical microtubules or preprophase bands are absent, whereas a branched phragmoplast spreads along the interzone formed by the overlap of radial microtubule systems emanating from each nuclear-cytoplasmic domain. However no radial microtubules could be observed in *cycb1;1-/-cycb1;2-/-* endosperm nuclei suggesting a spatial role of B1-type mitotic cyclins in microtubule organisation and orientation in Arabidopsis.

As discussed earlier, the enlarged *cycb1;1-/-cycb1;2-/-* endosperm nuclei fail to cellularise. Similar enlarged endosperm nuclei phenotype have previously been reported in the *ttn3 and ede1* mutant endosperm (Liu and Meinke, 1998; Pignocchi et al., 2009). The cellularisation occurred in *ttn3* seeds despite the presence of giant nuclei and viable seeds were produced, however *ede1* mutant endosperm could never cellularised and showed aborted seeds.

The embryo did not appear to be affected by cytokinesis defects in the aborting seeds of cyclin B1 mutants. It is possible that the functional redundancy among the

members of CYCB1s or may be other B type cyclins could account for no embryo defect.

4.4.6 The triple mutants of CYCB1;1, CYCB1;2 and CYCB1;3 indicate gametophytic defect

The triple mutant *cycb1;1-/-cycb1;3-/-cycb1;2-/+* plants showed 34% shrivelled seed which is higher than 25% compared to double mutant plants suggesting that these mutants might have developed gametophytic defect. The reciprocal cross between *cycb1;1-/+cycb1;2-/+cycb1;3-/+* and wild-type Col-0 revealed that transmission of each of the mutant alleles was slightly reduced through the male side however the transmission through female side appeared normal suggesting a male gametophytic defect in triple mutants. Although, there exists a possibility that carry over of maternal transcripts of *CYCB1s* could maintain the development of female gametophyte in these mutants, as it is known in Maize that unfertilised egg cells stocks large quantities of ribosomal proteins, histones and cyclin mRNAs (Dresselhaus et al., 1999). Under normal conditions, the gametophytic defects should ideally lead to lethality of 50% of total seeds, which does not occur in these mutants. In addition, 10% less transmission of mutant pollen can not lead to 34% seed abortion alone. Instead, I would expect that almost 16% of seeds might undergo one or more divisions of mitosis in endosperm nuclei considering that *cycb1;3* allele is present in homozygous situation resulting in only 34% shrivelled seeds. Nonetheless, it can also be postulated that the additive effect of triple mutation can lead to a defect in developing embryo, which might in turn lead to seed growth arrest.

4.5 The quadruple mutants of *CYCB1;1*, *CYCB1;2*, *CYCB1;3* and *CYCB1;4* cause gametophytic defect

The quadruple mutant *cycb1;1-/-cycb1;3-/-cycb1;4-/- cycb1;2-/+* showed 54% seed abortion and reciprocal cross of this line with wild-type suggested that transmission of *cycb1;2* allele was reduced to 9% through female side and 36% through male side compared to expected 50% in ideal condition, confirming the defect in both male as

well as female gametophyte development. This suggests that mitotic cyclins *CYCB1;1, CYCB1;2, CYCB1;3* and *CYCB1;4* are required for male as well as female gametophyte development. However, as the transmission is still not completely blocked, it raises a possibility that *CYCB1;5* might also be required during gametophytic development and in absence of first four *CYCB1s,* try to compensate the effect. It is apparent that 41% of female gametophyte is defective suggesting that additional 13% seeds might abort due to defective pollen homozygous for all four *CYCB1s*.

The female gametophyte appears to be severely affected compared to pollen, which can be explained by the fact that megaspore cells are larger in size compared to microspores and they require progression through three full mitotic cycles, for which cyclins could have become limited. At the same time pollens are produced in large number and can cover up defective pollen resulting in more transmission rate.

Recent reports of *duo1* mutants in Arabidopsis has described failure of mitotic entry in *duo1* germ cells due to lack of germline expression of G2/M regulator *CYCB1;1*, suggesting a DUO1 dependent CYCB1;1 activity during male germline development (Brownfield et al., 2009). Further, it has also been reported that APC/C stimulates production of miR159, which down regulates *DUO1* expression, leading to reduced *CYCB1;1* transcription in male germline of Arabidopsis (Zheng et al., 2011). In coherence with these reports our data confirms that B1 type cyclins indeed are involved in male germline development. Further genetic analysis of *duo1* and *miR159* mutant with *CYCB1* mutants needs to confirm the hypothesis.

4.6 Identification of potential substrates for CYCB1/CDK complex?

Recently characterised *ede1* mutant showed similar endosperm phenotype as was observed in *cycb1;1-/-cycb1;2-/-* and *cycb1;2-/-cycb1;3-/-* seeds. The EDE1 protein also contains seven consensus CDK phosphorylating sites and was expressed in the microtubules during mitosis. This led to the hypothesis that EDE1 could be a potential substrate for CYCB1/CDK complex. The immunostaining of roots with EDE1 antibody did not recognise any signal, which could be because of low expression of EDE1 *invivo* or low sensitivity of the antibody. To further investigate this hypothesis,

in vitro kinase assay has been planned. The *CYCB1* mutants have also been crossed with *ede1* to identify the potential genetic interactions.

Additionally other proteins that are expressed in microtubules during mitosis could also be the potential substrate for CYCB1s. Recently, the transcriptome analysis of endosperm revealed several seed specific endosperm preferred cytoskeleton-associated genes listed in Table D-1 (Day et al., 2009). Many of these genes contain putative CDK phosphorylation site and genetic studies with *CYCB1* mutants could identify novel substrates for *CYCB1*s.

Table D-1 Early seed specific endosperm preferred cytoskeleton-associated genes[a]

Locus	Name	Description	No. of CDK phosphorylation sites	CDK phosphorylation site
Spindle assembly				
At2g28620	AtKRP125c	Kinesin-5	1	TPRK
At2g36200	AtKRP125b	Kinesin-5	1	TPTK
At3g10310	ATK4 like	Kinesin-14	2	TPPR, TPVK
Phragmoplast formation				
At1g18370	HIK	Kinesin-14	3	TPER, TPQK, TPAR
At3g23670	PAKRP1L	Kinesin-12	2	SPAK, SPSK
At4g14330	PAKRP2	Kinesin-12	2	TPNK, SPDK
Uncharacterised kinesins				
At5g02370		Kinesin-13	2	TPAK, SPTK
At5g23910		Kinesin-13	4	TPRK, SPFK, TPEK, SPWK
At1g63640		Kinesin-14	1	SPRR
At1g55550		Kinesin-4	1	TPKK

[a] Data derived and modified from (Day et al., 2009).

4.7 Identification of QTL loci regulating seed mass

The rate of endosperm proliferation during early stages of seed development has been identified as an important factor to decide final seed mass/ size (Scott et al., 1998). The role of the endosperm in determining seed mass/size was also

revealed by characterisation of three mutants *HAIKU1* (*IKU1*), *IKU2* and *MINISEED3* (*MINI3*) (Garcia et al., 2003; Luo et al., 2005). The small seed size of these mutants is caused because of reduced growth and early cellularization of the endosperm. These observations along with the previous study of Alonso-Blanco et al. led the idea to utilise natural variation in seed mass from different *Arabidopsis* accessions, by choosing RIL population of parents with large differences in seed mass. I found 10 main effects QTL, 3 in An1-Cvi, 5 in Cvi-Ler and 2 in Ler-An1 along with 2 complex QTLs, one each in Cvi-Ler and Ler-An1 respectively. The QTLs obtained from An1-Cvi RIL population were also found in QTLs obtained from Cvi-Ler, which can be explained by a possible dominant effect of Cvi regulating the increase in seed mass. The obtained QTLS explained ~70% of the variation in seed mass. The QTL with the maximum effect (explaining 16.8% variation) was located on chromosome 4 in An1-Cvi RIL and the trait was influenced by the presence of Cvi. Noteworthy, Cvi-Ler RIL also produced one QTL in the similar position linked to marker g4539 but in our study it has appeared as a complex QTL, which is interacting with chromosome III marker GD.160C. A known seed size-affecting gene *AINTEGUMENTA* or *APETALA2* from chromosome 4 is not covered in this QTL region (Mizukami and Fischer, 2000; Jofuku et al., 2005). Nonetheless, the second major QTL obtained from our analysis is from Ler-Cvi on chromosome I linked with marker PVV4 and explains 15% variance for the seed mass trait, and interestingly *MINI3* gene encompasses this QTL region. However to validate the QTL we need to do classical mapping approach.

The QTL analysis for seed weight using Ler-Cvi RIL population has been performed earlier as well and 10 QTL was obtained compared to six from present study (Alonso-Blanco et al., 1999). The six QTL explain 68.5% variance for the seed mass trait. The loss of four QTL could be because of environmental changes or some other external factors, which might have influenced seed mass at the time of plant growth.

4.8 Identification of one locus controlling the autonomous endosperm division upon pollination with *cdka;1* pollen

The natural variation tool has further been utilised for a very specific trait associated with autonomous proliferation of central cell without proper double fertilisation upon pollination with *cdka;1* pollen (Nowack et al., 2006; Ungru et al., 2008; Aw et al., 2010). The *cdka;1* mutant pollen cause almost 50% seed arrest due to lack of second fertilisation, however these pollen can mutually rescue the abortion of seeds in maternal gametophytic FIS class mutants showing over proliferating of endosperm nuclei without fertilisation. This led to the hypothesis that the natural variation in the expression or function of genes, which cause maternal gametophyte defect e.g. FIS-class genes, might be responsible for the observed variation in autonomous endosperm proliferation. The QTL analysis based on the EDV obtained from individual RIL of Est-Col population revealed one QTL on top of chromosome III and coincidently this QTL colocalised with previously described *KALYPSO* (*KAL*) QTL found in Bay-Sha RIL population (Ungru et al., 2008). The colocalisation with *KAL* QTL allowed comparing the genomic position of identified QTL in two RIL populations. The comparative analysis of marker positions from both RIL populations decreased the QTL size upto 260MB containing ~800 genes. However, we can not rule out that the QTL might be completely independent of each other and this can be checked by various reciprocal crosses between the parents of these two RIL populations. Nonetheless, the obvious question was whether there is any known maternal effect gene is present or not in the list of 800 genes, and indeed we found RBR1 in the middle of this list. RBR1 is only homologue of retinoblastoma related gene in Arabidopsis, which is expressed, in female gametophyte and the mutation in RBR1 initiated endosperm division in absence of fertilisation (Ebel et al., 2004). This much information makes RBR1 a potential candidate which can probably rescue the *cdka;1* seed abortion. However, in our lab, even the strong alleles of RBR did not show the autonomous endosperm proliferation without fertilisation. Thus the classical QTL mapping approach is required to identify the associated locus for the concerned trait of interest.

5 Materials & Methods

5.1 Materials

5.1.1 Chemicals and antibiotics

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

5.1.2 Enzymes, primers and kits

Taq Polymerase used was produced in lab. Primers were purchased from Invitrogen (Karlsruhe, Germany. Kits were supplied from Sigma (Deisenhofen, Germany), Roche (Mannheim, Germany) and QIAGEN (Hilden, Germany).

5.1.3 Plant material

Plants were germinated on soil or 1/2 MS medium and either grown under standard greenhouse conditions or in a growth room. Arabidopsis mutant plants used in this study were as follows: the *cycb1;1-1* and *cycb1;2* from the Koncz collection (Csaba Koncz, Max Planck Institute for Plant Breeding Research in Cologne, Germany), *cycb1;3-1 (pst15850)* from the RIKEN collection (Yokohama, Japan), *cycb1;1-2 (*GK_078G11), *cycb1;3-2 (*GK_352C11) and *cycb1;4 (GK_386C01*) from the GABI-Kat collection (Bielefeld, Germany) and *cycb1;5-1 (*SALK_100844*)* and *cdka;1-1* (SALK_106809.34.90.X) were obtained from SiGNAL collection (SALK institute in La Jolla, California, USA).

A core set of 283 recombinant inbred lines (RIL) derived from a cross between Est-1 and Col-0 was used (Schwartz et al., 2009). The RIL population Ler-Cvi, Cvi-An1 and Ler-An1 were kindly provided by Prof. Maarten Koornneef (Max Planck Institute for Plant Breeding Research in Cologne, Germany).

The ProCYCB1;1:CYCB1;1DB-GFP to ProCYCB1;4:CYCB1;4DB-GFP reporter lines were kindly provided by Prof. Peter Doerner.

5.2 Methods

5.2.1 Plant growth conditions

Arabidopsis thaliana seeds were germinated either on soil or on 1/2 MS medium containing 0.5% sucrose and 0.8% agarose. The seeds were sterilised inside a small vacuum container, which contained 20 ml of bleach (DanKlorix by Colgate-Palmolive, Hamburg) and 2 ml of concentrated hydrogen chloride. The evaporating chlorine sterilised the seeds within approximately 4 to 12 hours. Mutant plants resistant to sulfadiazine, kanamycin or hygromycin were selected on ½ MS- agar plates by using 5.25 µg/ml sulfadiazine, 50 µg/ml kanamycin or 25 µg/ml hygromycin, respectively. Plants were grown under standard greenhouse conditions with the temperature conditions ranging from 18 to 25°C or in culture rooms under long-day conditions with a 16/8 h light/dark cycle at 18°C or 20°C, respectively.

5.2.2 Genomic DNA preparation from plant tissue

For high-throughput PCR based genotyping, genomic plant DNA was isolated using a quick and alternative preparation method (Berendzen et al., 2005). One or two young leaves were harvested and put into a 2 ml deep well of a 96 well titer plate (Part Number: 267001, Beckman or similar product from VWR). The plant samples were treated directly with 500 µl of DNA extraction buffer (5 ml 1 M Tris/HCl pH 7.2 + 6 ml 5 M NaCl + 10 g sucrose and adjusted to the final volume of 100 ml with water) and a small metal bead was added to facilitate homogenisation. After closing the titer plate with collection microtube caps (Qiagen, Mat. No.1051163), the plant tissue was homogenised at a high frequency in the MixerMill MM 301 (Retsch, Haan, Germany) for 2-3 min. The plates were centrifuged for 10 s to spin down the liquid from the microtube caps and thus to avoid cross contamination. 1.5 µl of this solution was used as template for PCR using standard Taq-Polymerase and 10 times PCR buffer

(Tris/HCl pH 8.7: 200 mM / KCl: 500 mM / MgCl2: 20 mM). The extracted DNA was either directly used for genotyping or stored at –20°C for further use.

5.2.3 Identification of mutant alleles by PCR

To determine a wild type or a mutant allele, allele-specific primer combinations were generated as illustrated in Table MM-1. The gene specific forward and reverse primer was used to amplify a wild type allele whereas mutant allele was amplified using gene specific forward primer and T-DNA vector specific reverse primer, respectively.

Table MM1: Primers used to amplify a wild-type or mutant allele

Primer name	Primer sequence (5' - 3')	Gene
cyclin b11 f	CGTGGCTTTGACTGGAATTT	*CYCB1;1*
cyclin b11 r	TAATCTCGTGGCCTCCATTC	
cyclin b11 gk-078g11 f	CTGATCCTGGTGGAGTGGTT	*CYCB1;1-2*
cyclin b11 gk-078g11 r	AAGCGTTCGTCCTTGGAGTA	
cyclin b12 f	GAAGAAATCTGGCCACCTCA	*CYCB1;2*
cyclin b12 r	GGAAACTAATTGCACGCATCT	
cyclin b13 f	GGCGTGAATCTTCACAATCC	*CYCB1;3*
cyclin b13 r	CGTATTCCACAGCAGCAAGA	
cyclin b13 gk-352C11 f	TTTTGGGGAATCTTGAATGG	*CYCB1;3-2*
cyclin b13 gk-352C11 r	ACGTCGTCGTTTGGTGTTTT	
cyclin b14 f	GTGGAATCGCAGGTTTTTGT	*CYCB1;4*
cyclin b14 r	CGTCTCTGGCATCAACTCAA	
cyclin b15 844 f	GGAGAGACAAGGTGGCTTTG	*CYCB1;5*
cyclin b15 844 r	TGCCCGATATCTTCCAGTTC	
K204-FISH2	CAGTCATAGCCGAATAGCCTCTCCA	*KONCZ*
K54-Salk-LB	GCGTGGACCGCTTGCTGCAACTCTCTCAGG	*SALK*
F156-Ds5-2a	TCCGTTCCGTTTTCGTTTTTTAC	*RIKEN*
K59-GabiLB1	ATATTGACCATCATACTCATTGC	*GABI*
N-034	CCAGATTCTCCGTGGAATTGCG	*CDKA;1*

5.2.4 Quantitative PCR

Total RNA was isolated from plant tissues using the RNeasy Plant Mini Kit (Qiagen). A DNase (TaKaRa) treatment was also performed to avoid DNA contamination. The RNA concentration was measured using a Nanodrop ND-1000 instrument. 3.5 µg of total RNA was reverse transcribed using SuperScript® III reverse transcriptase kit (Invitrogen). An additional step of RNase H treatment at 37°C for 20 minutes was performed to eliminate remaining RNA. The cDNA was further purified and concentrated by using QIAquick PCR Purification Kit (Qiagen) and the concentration was determined by Nanodrop ND-1000 instrument. Finally, using cDNA as the template, qPCR was performed on a Light-cycler LC480 instrument (Roche) as per the manufacturer's instructions. To avoid amplification of unspecific PCR fragments or primer dimers, lightCycler 480 SYBR Green I Master Kit (Roche) was used, and correct melting points were monitored. The primers used for quantitative PCR are listed in Table MM2.

Table MM2: Primers for quantitative real time PCR

Primer name	Primer sequence (5' - 3')	Gene
cycb11qfp1	TTGCTGCTCGAGAGAAGAAGGCTA	*CYCB1;1*
cycb11qrp1	ACATATTCCACAGCTGCGAGGTCA	
cycb12qfp1	TACATTGCAGTTCCACACCGGCTA	*CYCB1;2*
cycb12qrp1	TAGCAACACCTCCATTCTCTGCCT	
cycb13qfp3	AGTCGAAGGAGGAAAGCTTAACCG	*CYCB1;3*
cycb13qrp3	GCTCACGCTTATCACCTCTTGCTT	
cycb14qfp1	CCGAAGAATGTTGCAGGACATGGA	*CYCB1;4*
cycb14qrp1	TTGTCTGCTGCTGTGGTTGTTTCG	
ACTF	GTTGCCATTCAGGCCGTTCTTTC	*ACT7*
ACTR	CAGAATCGAGCACAATACCGGTTG	

5.2.5 Crossing of plants

The recipient flowers were emasculated when they were still closed and the pollen of the anthers was not ripe. The anthers were removed completely using very fine forceps and rest of the older and younger flowers were removed to avoid cross-pollination. Three day after emasculation stigma of the carpels was pollinated with pollen from the donor plant.

5.2.6 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 min, 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 h at 4°C before inspection. Light microscopy was performed with a Zeiss Axiophot microscope using Differential Interference Contrast (DIC) optics and AxioVs40 V 4.5.0.0 software.

5.2.7 Stomata staining

To perform stomata analysis, fully differentiated leaves, e.g. 14 days old cotyledons, were kept overnight in 100% ethanol, washed once in lactic acid and mounted on microscopy slides in lactic acid. The processed leaves could either be analysed directly or stored for several weeks on slides at room temperature.

5.2.8 Flow cytometry for ploidy analysis

Flow cytometry analysis was performed on roots, cotyledons, and seeds of Arabidoposis. Tissue samples were prepared for staining as follows. Cotyledons and roots were finely cut with a razor blade whereas seeds were lightly squashed with a plastic pistil in nuclear staining solution (CyStain UVprecise kit by Partec GmbH, Muenster, Germany) containing 4',6-Diamidino-2-phenylindole (DAPI). The samples were filtered through a 30 µm nylon mesh and were analysed with the CyFlow® Ploidy Analyser (Partec GmbH, Muenster, Germany). 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 of Arabidopsis. The obtained data were presented using Flowjo analysis software (Tree Star, Inc., OR, USA).

5.2.9 Feulgen staining

To fix the seeds to be stained, both ends of silique was cut with a razor blade and incubated with 1 ml of fixative (3:1, ethanol:acetic acid) overnight at 4°C. The tissue was washed with deionised water for 15 min x 3 and hydrolysed with 5N HCl for 60 min and washed 3x with deionised water for 10 min each and stained the samples with Schiff's reagent (Sigma S5133) for 2-3 hours in the dark. Once the pink staining was visible the samples were washed 3x with cold tap water for 10 min each. Dehydrated the stained tissue by increasing ethanol concentrations from 70% ethanol for 10 min, 95% ethanol for 10 min and two rounds of 100% ethanol for 10 min each and left over night in 100% ethanol. Next day, 100% ethanol solution was changed every hour till decolourisation stops. The samples were incubated in 1:1 ethanol:LR white resin (London resin co.) for 1 h with intermittent shaking and then with pure LR white for 1 h. The tissue was stored overnight with fresh LR pure white. To mount the sample, the stained siliques were placed on the accelerated resin blob onto the middle of microscope slide and the siliques were gently opened to coax the seeds out into the resin, avoiding puncturing of the seeds. Upon polymerisation of the resin, the stained samples

were analysed under confocal microscope LSM 700 META (Zeiss, Heidelberg, Germany) with argon ion laser at 488 nm excitation, >515/=nm emission.

5.2.10 Immunostaining

Immunofluorescence staining was performed on roots or siliques of plants. The young seedling or root was fixed in 1x PEM with the additives of 1.5% Paraformaldehyde, 0.5% Glutaraldehyde and 0.05% Triton X-100 for 30 min followed by 3x washing using 1x PEMT buffer before final washing with 1x PBS for 30 min. The young seeds were fixed in 1x PHEM with the additives of 5% DMSO, 4% PFA and 0.05% Triton X-100 for 2 min x 3 under vacuum. Thereafter, the tissue was washed three times with PHEM DMSO for 10 min each followed by methanol fixation for 15 min at -20°C. The tissue was washed again with 1x PBS for 30 min. Hereafter the protocol remain same for both kind of tissues. The tissue was washed twice with NaBH4 (0.1% in 1x PBS) for 10 min each and left in NaBH4 overnight. The seeds were treated with 100% ethanol for 1 h, followed by 1x PBS washing. The fixed root or siliques were placed on poly lysine (1mg/ml) coated slides and incubated with enzyme mixture (Cellulase 0.3%, macerozyme 0.1% to 1%, pectolyase 0.02% in MES 25mM, pH 5.5 $CaCl_2$ 8mM, Mannitol 600mM), 10 times diluted in enzyme buffer for 30 min followed by 3x PBSG wash for 5 min each. The tissue was treated with anti-alpha tubulin antibodies raised in mouse (Molecular Probes) (1:500 in 1x PBS) for overnight at 4°C, followed by 3x 10 min PBSG washes. Respective secondary antibodiy (Molecular Probes) in a dilution of 1:300 in 1x PBS, coupled to fluorescent Alexa-dyes 488 or 568, were applied for 1 h at room temperature. Nuclear staining was performed by using 4, 9, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (l mg/ml). After 3x washing with PBSG, tissue was mounted with mowiol containing DAPCO.

5.2.11 QTL mapping

QTL analysis was done using the software MapQTL 5.0 (Van Ooijen, 2004). A permutation test using 1000 permutations of the original data resulted in a genome-wide 95% LOD threshold of ~2.4. The automatic cofactor selection procedure was applied per chromosome to select markers to be used as cofactors for the composite interval mapping procedure (CIM). Markers most closely linked to QTL that appeared only after each round of CIM mapping were also selected as cofactors. The results of CIM mapping provided the variance explained by each and by all detected QTL as well as their additive allelic effect. The heritability was calculated by dividing the genetic variance by the sum of the genetic and the environmental variance.

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7 Appendix

ERKLÄRUNG

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die von mir vorgelegte Dissertation ist von Prof. Dr. Martin Hülskamp betreut worden.

Manoj Kumar

Lebenslauf

Manoj Kumar

Institut de Biologie Moleculaire des Plantes (IBMP)
12, Rue du General Zimmer
67000, Strasbourg, Frankreich
Geburtsdatum: 09. 02. 1980
Geburtsort: Begusarai, Indien
Staatsangehörigkeit: Indisch
E-mail: kmanojt@gmail.com

Schulausbildung

1994	Raj High School, Darbhanga, Indien
1994-1996	I.Sc. (Biology), C. M. Sc. College, Darbhanga, Indien

Hochschulausbildung

1996-1999	B.Sc. in Zoologie (Hons.), L.N. Mithila University, Darbhanga, Indien
2000-2001	Post Graduate Diploma (Hauptstudium) in Biochemischer Technologie, Sri Venkateshwara College, University of Delhi, Neu Delhi, Indien
2002-2004	M.Sc. in Life Sciences, School of Life Sciences, Devi Ahilya University, Indore, Indien; Note: 8,89/10
2006-2011	Doktorarbeit in Botanik, Universität zu Köln, Köln, Deutschland; Titel der Doktorarbeit: Analysis of cell proliferation and growth control during seed development in *Arabidopsis thaliana*; Betreuer: PD Dr. Arp Schnittger and Prof. Dr. Martin Hülskamp

Publikationen

1. Pareek, A, Singh, A, **Kumar, M**, Kushwaha, HR, Lynn, AM, and Singla-Pareek, SL (2006). Whole-genome analysis of *Oryza sativa* reveals similar architecture of two-component signaling machinery with Arabidopsis. **Plant Physiol**, 142: 380–397
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Buchkapitel

1. **Kumar M** and Pareek A (2004) Molecular basis of plant microbe interaction. In: Basic Research and Biotechnological Applications: Microbes. Ed. Verma A, IK International Publishers

Teilnahme an wissenschaftlichen Konferenzen

11/2004	Internationaler Workshop über "Analysis of stress responsive plant genes" bei International Centre for Genetic Engineering and Biotechnology (ICGEB), Neu Delhi, Indien
06/2007	3rd Internationale Konferenz über APOMIXIS, Wernigerode, Deutschland Poster-Präsentation: A new genetic screen to identify signaling pathways during seed growth and development
08/2008	XXth Internationale Konferenz über Sexual Plant Reproduction, Brasilia, Brasilien Poster-Präsentation: persephone – a sporophytic maternal effect mutant controlling seed development
09/2008	2nd Seeds for growth meeting ERA-NET Plant Genomics, Oslo, Norwegen Vortrag: persephone – a maternal effect mutant controlling seed development
07/2009	8th SFBV Meeting, Strasbourg, Frankreich Poster-Präsentation: B1 type cyclins play an important role in seed growth and development in *Arabidopsis thaliana*
12/2009	3rd Seeds for growth meeting ERA-NET Plant Genomics, Venedig, Italien Oralen-Präsentation: B1 type cyclins play an important role in seed growth and development in *Arabidopsis thaliana*

Stipendien und Auszeichnungen

06/2002	Auszeichnung "All India Entrance Test for Master of Science in Life Sciences" Devi Ahilya University, Indore, Indien
06/2004	Auszeichnung National Eligibility Test (NET), CSIR-UGC, Neu Delhi, Indien
09/2006	NRW International Graduate Fellowship, Universität zu Köln, Köln, Deutschland